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ANTIBODY RESPONSE INDUCED AFTER INTRAOCULAR VIRAL GENE ADDITION THERAPY USING ADENO-ASSOCIATED VIRUS, LENTIVIRUS, AND ADENOVIRUS VECTORS WITH THE GFP TRANSGENE IN DOGS

presented by

Jennifer Vander Kooi Den Houter

has been accepted towards fulfillment
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M.S. degree in Comparative Medicine and Integrative Biology

Major Professor's Signature

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Date

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Jennifer Vander Kooi Den Houter

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ABSTRACT

ANTIBODY RESPONSE INDUCED AFTER INTRAOCULAR VIRAL GENE ADDITION THERAPY USING ADENO-ASSOCIATED VIRUS, LENTIVIRUS, AND ADENOVIRUS VECTORS WITH THE GFP TRANSGENE IN DOGS

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A humoral immune response induced after ocular gene addition therapy using viral vectors, to either the transgene or the viral vector, can impact therapy safety and efficacy. Three vectors with the green fluorescent protein transgene (GFP): adeno-associated virus (AAV), lentivirus, and adenovirus (Av-1), were studied in order to monitor the circulatory antibody response after intraocular treatment in dogs. Aqueous samples were taken both pre- and post-treatment from AAV treated dogs to monitor the local humoral response. In order to monitor the antibody response, two dog-specific enzyme-linked immunosorbent assays (ELISAs) were designed: a GFP ELISA and an AAV ELISA. In dogs treated with AAV-GFP, a high level of circulating anti-GFP antibodies were detected in 4 out of 22 dogs. The anti-GFP antibody response was observed to be influenced by using the chicken-beta actin promoter (CBA) and treatment age of 3 weeks. Anti-GFP antibodies were also found in aqueous samples up to 96 weeks post-treatment, and a significant anti-AAV antibody response was also detected. Anti-GFP antibodies were also found for lentivirus-GFP and Av-1-GFP treated dogs. These results show that despite the immune-privileged nature of the eye, an antibody response to both the transgene protein and the viral vector occurs and should remain a concern in future intraocular gene therapy studies.

Dedicated to my father, Leonard H. Den Houter

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My family and friends, thank you for your support as well as a listening ear through this experience. Mom, thank you for your continued persistence. Jon, Jessica, and Ben, thank you for being the greatest siblings anyone could ever have. Last but not least, thank you to my father, who always believed in me and challenged me until the end.

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LIST OF ABBREVIATIONS

AAV = adeno-associated virus

AAV2= adeno-associated virus serotype 2

AAV5 = adeno-associated virus serotype 5

AAV-CBA-GFP = adeno-assoaciated2/5 vector with CBA and GFP

AAV-MOPS-GFP = adeno-assoaciated2/5 vector with mOPS and GFP

APC = antigen presenting cells

Av-1 = adenovirus

CAR = coxsackie-adenovirus receptor

CBA = chicken beta-actin promoter

cGMP-PDE = cyclic GMP phosphodiesterase holoenzyme

CMV = cytomegalovirus

dsDNA = double-stranded DNA

EAM = encapsidated adenovirus mini-chromosomes

ELISA = enzyme-linked immunosorbent assay

ERG = electroretinography

GC = guanylate cyclase

GFP = green fluorescent protein

HIV = human immunodeficiency virus

IU = infectious unit

LacZ = β-galactosidase

LCA = leber congenital amaurosis

LGN = lateral geniculate nucleus

LSM = least squares mean

LTR = long terminal repeats

mOPS = mouse opsin promoter

NK cells = natural killer cells

OD = optical density

OTC = ornithine transcarboxylase

PBS = phosphate-buffered saline

PDE= phosphodiesterase

qPCR = quantitative PCR

rAAV2/5 = recombinant AAV serotype 2/5

rAAV = recombinant AAV

RAC = NIH Recombinant DNA Advisory Committee

Rh= rhodopsin

rHIV = recombinant HIV

RK= rhodopsin kinase

RP = retinitis pigmentosa

RPE = retinal pigment epithelium

SAS = Statistical Analysis Software

SCID-X1 = severe combined immunodeficiency disease

SEM= standard error of the mean

T= transducing

VSV-G = vesicular stomatitis virus

Chapter 1

INTRODUCTION

1.1. Somatic Cell Gene Addition Therapy for Inherited Retinal Degenerations

Gene therapy has been at the forefront of research since the 1980's and continues to draw considerable attention as a novel therapy. Currently, treatments are progressing for diseases that are difficult or even impossible to treat with contemporary medicine, such as hemophilia B (Chao and Walsh, 2004), severe combined immune deficiency (Cavazzana-Calvo et al., 2000; Cavazzana-Calvo et al., 2005), and many forms of cancer.

Gene therapy has been considered only for fatal, chronic, or severely debilitating diseases in the past. Recently though, diseases of the special sense organs, such as the ear and eye, have been considered for gene therapy. The eye is a particularly interesting organ for gene therapy research since it is a relatively isolated part of the body and also an immune-privileged site and thus gene therapies would theoretically allow for long-term treatment success with less risk of adverse immune responses.

Examples of gene therapy in the eye include somatic cell gene addition therapies for autosomal recessive diseases in which the disease results from a lack of gene product (Auricchio and Rolling, 2005); antisense RNA and ribozyme

treatments for autosomal dominant diseases in which the disease results from accumulation of the mutated gene product (Lewin et al., 1998); and generic treatments such as introduction of neurotrophic factors to protect and sustain the photoreceptors as long as possible (Bessant et al., 2001). Unfortunately, each approach has its own challenges. For instance, when using gene addition therapy in an attempt to produce the missing protein for an individual with a null mutation, the vector may be expressing a protein for which the body has not had previous exposure, thus increasing the potential for a host immune response against the therapeutic gene's protein (Tripathy et al., 1996).

Since gene therapy is a relatively new field, much research needs to be done to determine the most efficacious and safest methods for treatment of a variety of inherited, debilitating eye diseases. Currently, little research has been performed to investigate in detail the immune response, either within the eye or systemically, after intraocular injection with a vector that expresses a foreign transgene. With the potential for gene therapy currently under scrutiny, the need to learn more about the immune response after intraocular gene therapy is necessary to determine the safety and efficacy of this form of treatment for future research and clinical trials.

1.2. Literature Review

1.2.1. Overview of Canine Ocular Anatomy

The anatomy of the eye is quite complex with each region or tissue having its own specific function. Sequentially, from the cornea to the optic nerve, a brief description will be given.

Overall, there are three important layers of the eye as shown in Figure 1.1: 1) the fibrous, outermost layer consists of the cornea and sclera, deemed the corneoscleral layer with the junction between cornea and sclera termed the limbus, 2) the middle layer consists of the uveal tract which is composed of the iris, the ciliary body, and the choroid, and 3) the innermost retinal layer which consists of the retinal pigment epithelium (RPE) and neurosensory retina which are continuous with the ciliary epithelium anteriorly.

The cornea is the roughly circular, transparent area at the front of the eye that allows light to enter the globe. The majority of light refraction occurs at the corneal surface. Light then passes through the anterior chamber, which is filled with aqueous, and then continues through the lens where it is further refracted. The lens is a transparent, crystalin structure that is held in place by the lens zonules and its shape is modified by the action of the ciliary body musculature. Just in front of the lens is the iris which acts as a diaphragm to control the amount of light that enters the lens. Along the periphery of the iris lies the

iridocorneal drainage apparatus that allows fluid drainage from the anterior chamber to the blood stream and thus controls the pressure in the globe.

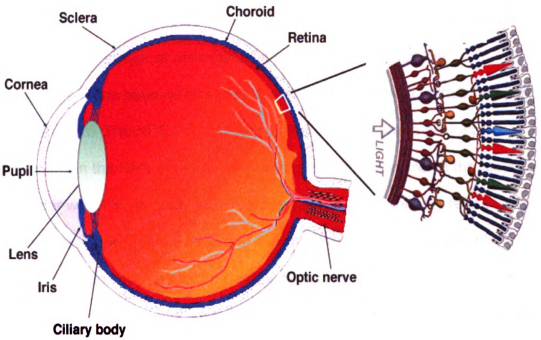

Figure 1.1. Image of the vertebrate eye with an expanded view of the retina. (http://www.webvision.med.utah.edu, 2-24-07).

Light continues through the lens and through the vitreous to be focused onto the retina. The retina, which lines the back of the eye, is composed of several layers, and acts to convert light into an electric signal which is modified within the inner retina and then travels to the brain initially via the optic nerve and allows the organism to visualize its environment.

1.2.2. The Retina

The retina lines the back of the eye globe as shown in Figure 1.2. It ends anteriorly at the ora ciliaris retinae which is positioned just posterior of the ciliary body. The outer layer of the retina is known as the retinal pigment epithelium (RPE) and is a monolayer of cells that is in intimate contact with the photoreceptors of the neurosensory retina and plays a vital role in their nutrition and maintenance. The RPE lies adjacent to Bruch's membrane posteriorly. Tight junctions in the RPE and the retinal vessel endothelium create the blood-retinal barrier which limits access of molecules to the neurosensory retina. The sub-retinal space between the RPE and the photoreceptor outer segments, a potential space into which gene therapy injections are given, has been shown specifically to be immune-privileged due to the action of the blood-retinal barrier (Hoffman et al., 1997; Wenkel and Streilein, 1998; Anand et al., 2002; Isenmann et al., 2001). With this in mind, gene therapy of the eye may be influenced by this deviant immune response due to this immune-privileged environment.

The three main neuronal cells of the retina are the photoreceptors, the bipolar cells, and the ganglion cells. Photoreceptors are slender cells responsible for the conversion of light into an electrical signal. There are two main types of photoreceptors: cones and rods. Approximately 95% of photoreceptors are rods in the canine eye which is comparable to the human eye in which approximately 95% are rods and 5% are cones (Koch and Rubin, 1972; Forrester et al., 1996). In contrast, the distribution of rod and cones across the retina varies between the

human and canine eye. In the human eye, the macula, which is superotemporal to the optic disc, contains a clustering of cone photoreceptors while in the canine there is a region of high cone density in the *area centralis* which forms a streak above the optic disc.

Rod photoreceptors as shown in Figure 1.3 are responsible for vision in dim light while cone photoreceptors provide for color, bright light vision. The number of cone types vary between species in mammals. Two or three types of cones are typically present: long and short wavelength or blue, green, and red (short, medium, and long wavelength), respectively.

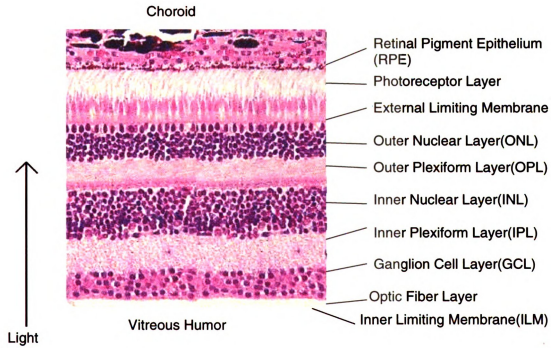

Figure 1.2. Schematic of the retinal layers.
(http://webvision.med.utah.edu/2003olb.pdf#How%20the%20Retina%20Works, 2-8-07)

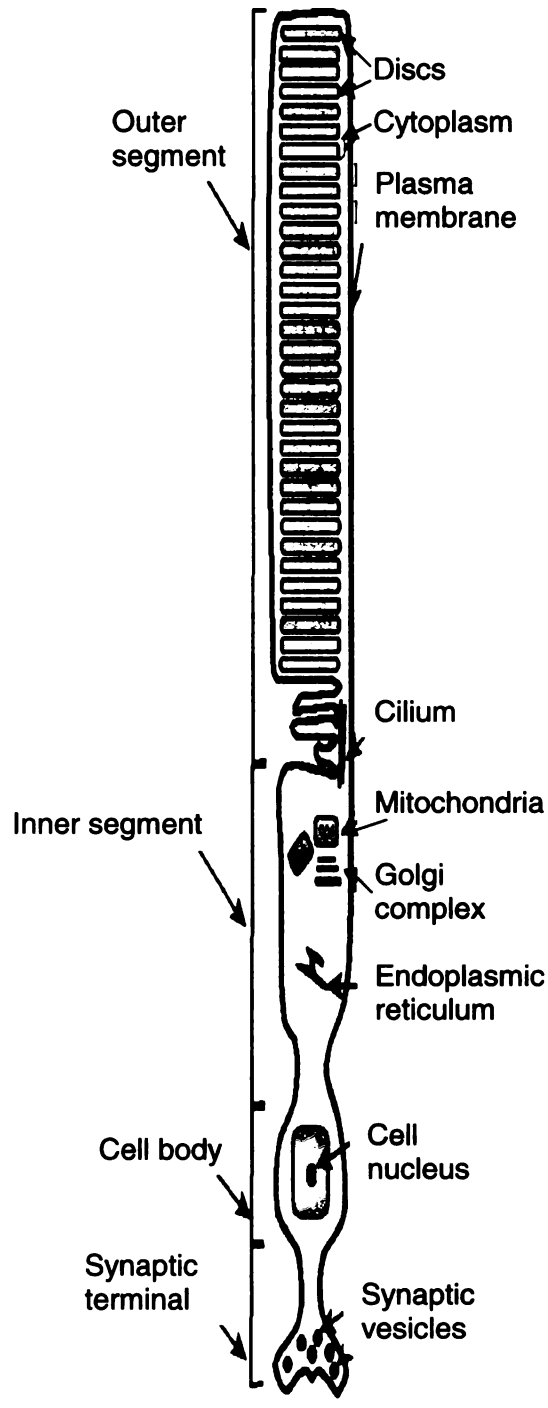

Figure 1.3. Diagram of a rod photoreceptor from the vertebrate retina. The rod photoreceptor consists of four main regions: the outer segment complete with discs where phototransduction occurs, the inner segment which is connected to the outer segment via the cilium and creates the energy for the cell, the cell body which holds the nucleus of the cell, and finally the synaptic terminal which contains synaptic vesicles that transmit the message to the next neuron (http://www.csulb.edu/~cwallis/482/visualsystem/cwrod.jpg , 7-20-06).

The photoreceptor layer is made up of the inner and outer segments of the photoreceptors, the outer nuclear layer is made up of the nuclei of the photoreceptors, and the outer plexiform layer is made up of the photoreceptor termini. The outer segment of the photoreceptor, made up of stacks of discs for the rods and a comb-like structure for the cones, is connected to the inner segment via a cilium. Within the inner segments of the photoreceptor, metabolic processes occur to keep the cell alive (Baldridge and Kurennvi., 1998). The outer and inner segments are made distinct by the thin outer-limiting membrane which creates a layer across the retina at this level (Buntmilam et al., 1985).

The termini of the photoreceptors contain synaptic vesicles with the neurotransmitter glutamate and form synapses with both bipolar and horizontal cells of the inner nuclear layer. Bipolar cells are second order neurons that bridge between 20-60 photoreceptors to the ganglion cells. Rod ON-bipolar cells connect to the ganglion cells via amacrine cells but may also contact them via cone OFF-bipolar cells (Soucy et al., 1998; Hack et al., 1999). Horizontal cells, which are responsible for horizontal signaling across the retina, also reside in the inner nuclear layer and send dendrites out to synapse with the termini of the photoreceptors.

The inner plexiform layer is formed by the connections of the inner nuclear layer cells, such as amacrine cells and bipolar cells, with ganglion cells and others.

Amacrine cells help the communication between the inner and outer retina through dopaminergic pathways between the bipolar cells and ganglion cells.

The ganglion cell layer contains the cell bodies of the ganglion cells as well as those of some amacrine and astroglial cells. Ganglion cells receive input from many photoreceptors within their receptive field and then send this electrical signal in the form of action potentials through the unmyelinated nerve fiber layer to the myelinated optic nerve. There are two classes of ganglion cells: 1) ON-center which depolarize and 2) OFF-center which hyperpolarize in response to light stimulation.

The innermost layer of the retina is the inner limiting membrane that is composed of Müller cell end feet and astrocytes. Müller cells are retinal glial cells that originate from hemopoietic cells. Their cell body is in the inner nuclear layer while their cell processes spread out to form the outer and inner limiting membranes.

1.2.3. Phototransduction and the Visual Cycle

Phototransduction (Figure 1.4.) is the process by which the photoreceptors convert photons of light into an electrical signal which is then transmitted through inner retina to the ganglion cell and via ganglion cell axons to the brain. Rod

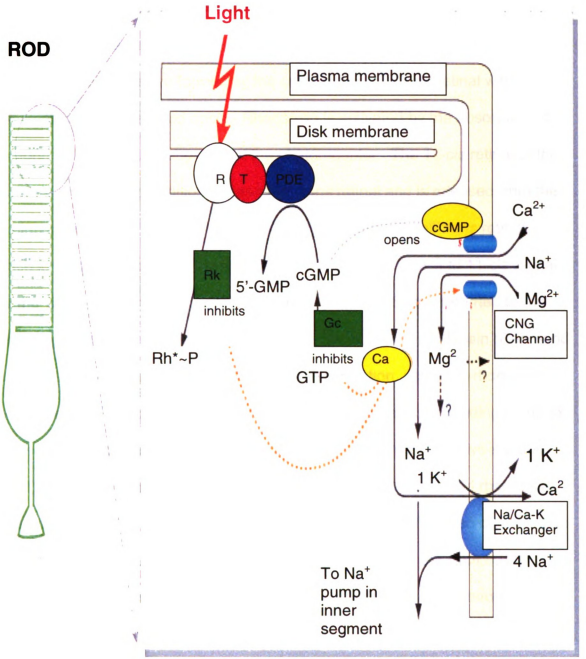

Figure 1.4. Phototransduction within a rod photoreceptor. (http://www.biol.tsukuba.ac.jp/~nakatani/phototransduction-scheme-1.jpg 2-7-07) light to be converted to an electrical message through the stimulation of the photopigment rhodopsin. R= rhodopsin, T= transducin, PDE= phosphodiesterase, Gc= guanylate cyclase, and Rk= rhodopsin kinase.

phototransduction has been studied extensively, and it initiates with the activation of the photopigment rhodopsin (see Pugh and Lamb, 2005 for a review). Rhodopsin molecules are formed by the combination of 11-cis retinal with the transmembrane protein rod opsin. Rhodopsin is activated by the absorption of photons within the outer segment of the photoreceptor. The 11-*cis* retinal of the rhodopsin molecule photoisomerizes to all-*trans* retinal and is released from the rhodopsin complex. This all-*trans* retinal is cycled back into all-*trans* retinol which is then transported to the RPE where it is converted back to 11-cis retinal via the visual cycle involving rpe65 isomerase (Figure 1.5) and returned to the outer segments to once again recombine with opsin to form rhodopsin (Redmond et al., 1998; Mata et al., 2004). Following light-activation, activated-rhodopsin then forms a complex with transducin, the rhodopsin-transducin binding complex. Transducin is a trimeric G-protein consisting of: Tα which is the active subunit, and Tβ and Tγ which are the inhibitory subunits. The light-activated rhodopsin changes the GDP of the transducin complex to a GTP and Tβγ then dissociate to release Tα and GTP. The active transducin then stimulates the cyclic GMP phosphodiesterase holoenzyme (cGMP-PDE). cGMP-PDE is a heterotrimeric complex with two catalytic subunits, PDEα and PDEβ that are inhibited by the two PDEγ subunits (see Stryer, 1991 for a review). Activated Tα removes the inhibitory gamma subunits from this complex leaving the active PDEα/β complex. PDEα/β hydrolyzes cGMP to GMP resulting in closure of the cGMP-gated channels due to the decreased concentration of cytosolic cGMP. The closure of

these channels results in the hyperpolarization of the photoreceptor cell membrane.

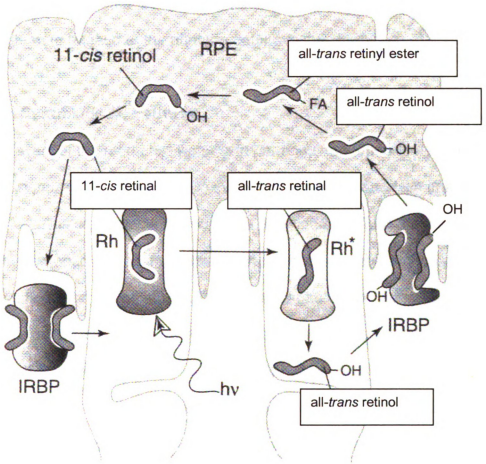

Figure 1.5. Diagram of the visual cycle. The cycling of the compounds all-*trans* retinal to 11-*cis* retinol occurs through a series of steps with various isomerases and through transport via IRBP (interphotoreceptor binding protein) between the RPE and photoreceptor layers. Rh is the molecule rhodopsin which is the photopigment involved in phototransduction (http://www.bumc.bu.edu/www/busm/by/images/carter.jpg, 2-7-07)

The closed cGMP-gated channels consist of two subunits and one glutamate residue which block the cation flow into the cell when cytosolic cGMP is in low concentrations. Consequently, cations such as calcium are not able to flow into

the cell and at the same time the Na/Ca-K ion pumps are continuously pumping Ca^{2+} out of the cell which then results in hyperpolarization of the cell (Haase et al., 1990). This results in a discontinuation of the 'dark current' and consequently inhibits glutamate release at the photoreceptor synaptic terminus.

1.2.4. The Central Visual Pathway

When the layers of the retina are working in harmony, the retina turns light into an electric signal which is then sent to the brain for perception of the image (Figure 1.6.). This signal is sent through the ganglion cell axons via the optic nerve. The two optic nerves, from the left and right eyes, meet at the optic chiasm and fibers either continue to the ipsilateral optic tract or decussate to the contralateral optic tract. If the impulse originated from the nasal portion of the retina, then the signal travels to the opposite side of the brain. Those from the temporal side of the retina project to the ipsilateral optic tract. Fibers travel to the lateral geniculate nucleus (LGN) for conscious perception of vision, the pretectum, and the superior colliculus for reflex pathways. Ganglion cell axons synapse in the LGN, and then axons pass via the optic radiation to the visual cortex. The visual cortex is divided into regions specific, also known as retinotopic, to the many areas of the visual field (Vanduffel et al., 2002).

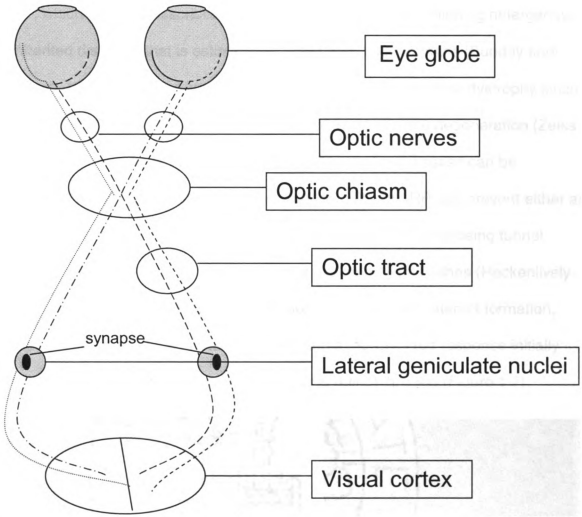

Figure 1.6. An illustration of the central visual pathway. This picture shows the visual field in relationship to the path that the information portraying the image follows along the optic nerve, through the optic chiasm, and via a synapse in the lateral geniculate nucleus to the visual cortex (Forrester et al., 1996).

1.2.5. Inherited Retinal Dystrophies

At the time of writing, 185 different genes have been linked to retinal dystrophies (summarized in RetNet, http://www.sph.uth. tmc.edu/Retnet/sum-dis.htm#A-genes, 2007). Retinal dystrophies are divided into many forms including Leber Congenital Amaurosis and retinitis pigmentosa (RP).

RP, which was first described in 1857, is a subset of these blinding hetergenous, inherited diseases that is estimated to affect 1 in 3,000 humans (Bundey and Crews, 1984). This bilateral disease is described as a rod-cone dystrophy since the rods degenerate initially and are often followed by cone degeneration (Zeiss et al., 2004; Huang et al., 1995). The inheritance of the disease can be autosomal dominant, autosomal recessive, or X-linked. RP can present either as a slow or fast onset disease with the most common symptoms being tunnel vision, night blindness, headaches, and unexplained light flashes (Heckenlively et al., 1988). Secondary to these common symptoms are cataract formation, pigment changes in the retinal layers, a decreased ERG rod response initially followed by a decreased cone response, and retinal thinning (Figure 1.7).

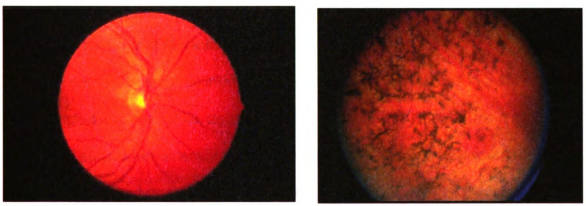

Figure 1.7. Fundus pictures from two different human patients. The retina on the left is normal while the retina on the right is affected with retinitis pigmentosa. The diseased fundus has bone-spicule pigmentation as well as vascular attenuation.

There are four distinct categories into which most of the RP mutations fall: 1) phototransduction cascade proteins, 2) photoreceptor structural proteins, 3) proteins involved in photoreceptor and retinal pigment epithelial metabolism, and 4) proteins that regulate gene expression (Bessant et al., 2001).

Leber Congenital Amaurosis (LCA) is a severe early-onset condition that causes blindness in children (Morimura et al., 1998). Causal mutations have been identified in several genes that are either expressed in the RPE or retinal cells. One example is the *RPE65* gene which codes for an isomerase in the RPE and plays an important role in the visual cycle; a mutation in this gene results in a severe vision loss but only slow degeneration of the retina and has shown promise for gene therapy treatments (Cremers, et al., 2002). LCA is characterized by early onset blindness and typically include a decreased electroretinographic (ERG) rod response initially followed by a decreased cone response. Nystagmus, cataract formation, and impairment of the pupillary light reflex are also features of the condition.

1.2.5.a. Small Animal Models of Retinal Degenerations

There are numerous small animal models of retinal degenerations in a variety of species including Drosophila, zebra fish, and many rodent models. Most of the models are autosomal recessive while a few are inherited in an autosomal dominant or X-linked manner. The most widely used species are the laboratory rodents: rats and mice. Rat models include the Royal College of Surgeons rat (*RCS* rat), the *Rho* knockout rat, and the *P23H* transgenic rat while the mouse models include the spontaneously occurring *rd1* mouse, the peripherin 2 (*Prph2)* mouse (also known as *rd2)*, and a knockout *RPE65* mouse.

The *RCS* rat is an inbred line with an autosomal recessive mutation in the *Mertk* gene which normally serves a role in RPE phagocytosis of the outer segments of photoreceptors. In the RCS rat, there is a build up of photoreceptor outer segment debris in the subretinal space and the photoreceptors eventually degenerate as a consequence (for a review see Dejneka et al., 2003). This rat serves as a model for the humans with *Mertk* gene mutation (Gal et al., 2000; D'Cruz et al., 2000).

The *P23H* rat is a transgenic rat that was created to have an autosomal dominant mutation in the rhodopsin gene due to the alteration of proline-23 to histidine (Rho^{P23H}) in one allele. This mutation leads to eventual blindness, and is one of the most common rhodopsin mutations of humans (for a review see Dejneka et al., 2003; Chader, 2002).

The *rd1* mouse has an autosomal recessive mutation in the phoshodiesterase beta subunit (*PDEB)* gene which normally functions as a component of the rod phototransduction cascade. This mutation leads to a fast retinal degeneration and results in blindness. This was the first mouse model of retinal degeneration discovered and has been widely used as a model to mimic phototransduction-related disease in humans (Chader, 2002; Pittler et al., 1991).
The knockout *RPE65* mouse was developed as a model for LCA. This mouse has been used extensively for gene therapy trials since it has a similar disease phenotype to the human LCA patient (for a review see Dejneka et al., 2003).

1.2.5.b. Large Animal Models of Retinal Degenerations

Progressive Retinal Atrophy is the canine equivalent of RP and LCA and similarly shows genetic heterogeneity. These spontaneous canine models are useful because the canine eye is similar in size to the human eye.

Rod cone dysplasia type 1 (*rcd1*) affecting the Irish Setter breed is a model of early onset autosomal recessive RP and has a nonsense mutation in the *PDE6B* gene in exon 21 (Suber et al., 1993). *Rcd1* has a fast onset with night blindness occurring within 25 days after birth. The *rcd1* Irish Setter is used as a model for fast onset, phototransduction-related RP.

The Cardigan Welsh Corgi has an autosomal recessive PRA due to a 1 base pair deletion mutation in exon 16 of the phosphodiesterase six alpha subunit (*PDE6A)* gene which results in a frameshift and downstream premature stop codon (Petersen-Jones et al., 1999). The disease onset is comparable to the *rcd1* Irish Setter and has been named 'rod-cone dysplasia type 3' (*rcd3).* This disease was described in detail by Tuntivanich (2006).

The Briard, with a 4-bp deletion mutation in the *RPE65* gene that leads to a frameshift and downstream premature stop codon, is a canine model of LCA (Veske et al., 1999; Aguirre et al., 1998; Wrigstad, 1994). The absence of the rpe65 protein in the Briard results in a severe loss of vision and markedly reduced ERG responses. However despite the early onset of vision loss,

structural deterioration of the photoreceptors is slow which provides a wide opportunity to test possible gene therapy treatments.

1.2.6. Gene Therapy Viral Vectors

Viral vectors are currently used extensively for the transport of a transgene into a target cell. This method has been the basis for many gene addition therapy trials, and has shown success in many studies. Three commonly used viral vectors are adeno-associated virus, lentivirus, and adenovirus vectors. These vectors have demonstrated beneficial treatment effects but also unexpected side effects. Most viral vectors are chosen based on the disease that is to be treated. Disease factors such as degree of transgene expression required, duration of treatment, and target cell determine the viral vector that would be optimal. The ultimate goals for a gene therapy vector include, but are not limited to: 1) low immunogenicity, 2) regulated vs. unregulated expression, 3) duration of transgene expression, 4) amount of vector needed for transduction, 5) integration vs. no integration of the viral genome, and 6) tissue specificity (Kay et al., 2001).

1.2.6.a. Recombinant Adeno-Associated Virus (AAV)

One of the first successful gene therapy trials utilized a recombinant adeno-associated virus vector to treat hemophilia. This disease was treated using a serotype 2 recombinant AAV vector that contained the cDNA sequence for the coagulation factor IX gene. Not only has this vector been shown to be safe as a gene therapy vector in a wide variety of studies, it has also been shown to be

efficacious in gene delivery and long-term transgene expression (Chao and Walsh, 2004; Flannery et al., 2005).

Adeno-associated viruses are in the family *Parvoviridae* and are 'helper-dependent' viruses. Wild type AAV are typically associated with subclinical or latent infections, and typical viral gene expression in the cell occurs during the 'S' phase of the cell cycle which can be activated by an adenovirus or herpesvirus infection, for example. Adeno-associated viruses are small particles 26 nm in diameter with an icosahedral capsid made up of viral particles VP1 and VP2. The genome is single-stranded DNA with flanking terminal repeat regions, the normal genes of the virus being *rep* and *cap,* and is approximately 5,000 bases in size which significantly limits the space available, to approximately 2500bp, for the transgene of interest (Figure 1.8) (Vihinen-Ranta et al., 2004).

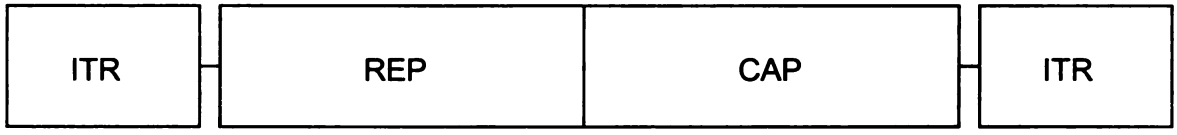

Figure 1.8. A representation of the adeno-associated virus genome. The viral genome consists of the Rep and Cap genes with flanking inverted terminal repeat regions (ITR).

Two of the many AAV serotypes are more commonly used for gene therapy trials: serotype 2 (AAV2) and serotype 5 (AAV5). The major difference between the two serotypes is mainly the viruses' method of cell entry. AAV2 binds the heparin-sulfate proteoglycan on the cell surface while AAV5 binds α2,3 or α2,6 sialic acid on the cell surface for entry through receptor-mediated endocytosis (Vihinen-Ranta et al., 2004). Upon entry into the cell, the endosome undergoes

acidification which results in capsid degradation and viral escape into the cytoplasm. Once in the cytoplasm, the virus is shuttled on microtubules to the nuclear pore. When the virus contacts the nuclear pore at the 5-fold axis of the virus capsid, the DNA is made into double-stranded DNA (dsDNA) by a DNA polymerase that lies at this axis. The dsDNA is then transported into the nucleus, through the pore at the 5-fold axis, where it is free to be transcribed into RNA through the host cell transcription process (Figure 1.9).

AAV2 has been the primary rAAV for gene therapy studies while AAV5 also has been studied in retinal gene therapy research in recent years. AAV2 was efficacious in retinal gene therapy studies; it had the ability to infect ganglion cells, photoreceptors, and the RPE of dogs (Bainbridge et al., 2003), mice (Grant et al., 1997; Ali et al. 1998), and non-human primates (Bennett et al., 1999). AAV5 has been shown to be up to 1,000 times more efficient than AAV2 when infecting retinal cells, such as photoreceptors, in the mouse and non-human primate eye, with the average transgene activation time being between 2-4 weeks (Yang et al., 2002; Lotery et al., 2003). A further study pseudotyped AAV2 with the capsid of AAV5 (AAV2/5) and this vector was found to be just as efficient at infecting photoreceptors as AAV5 (Auricchio et al., 2001).

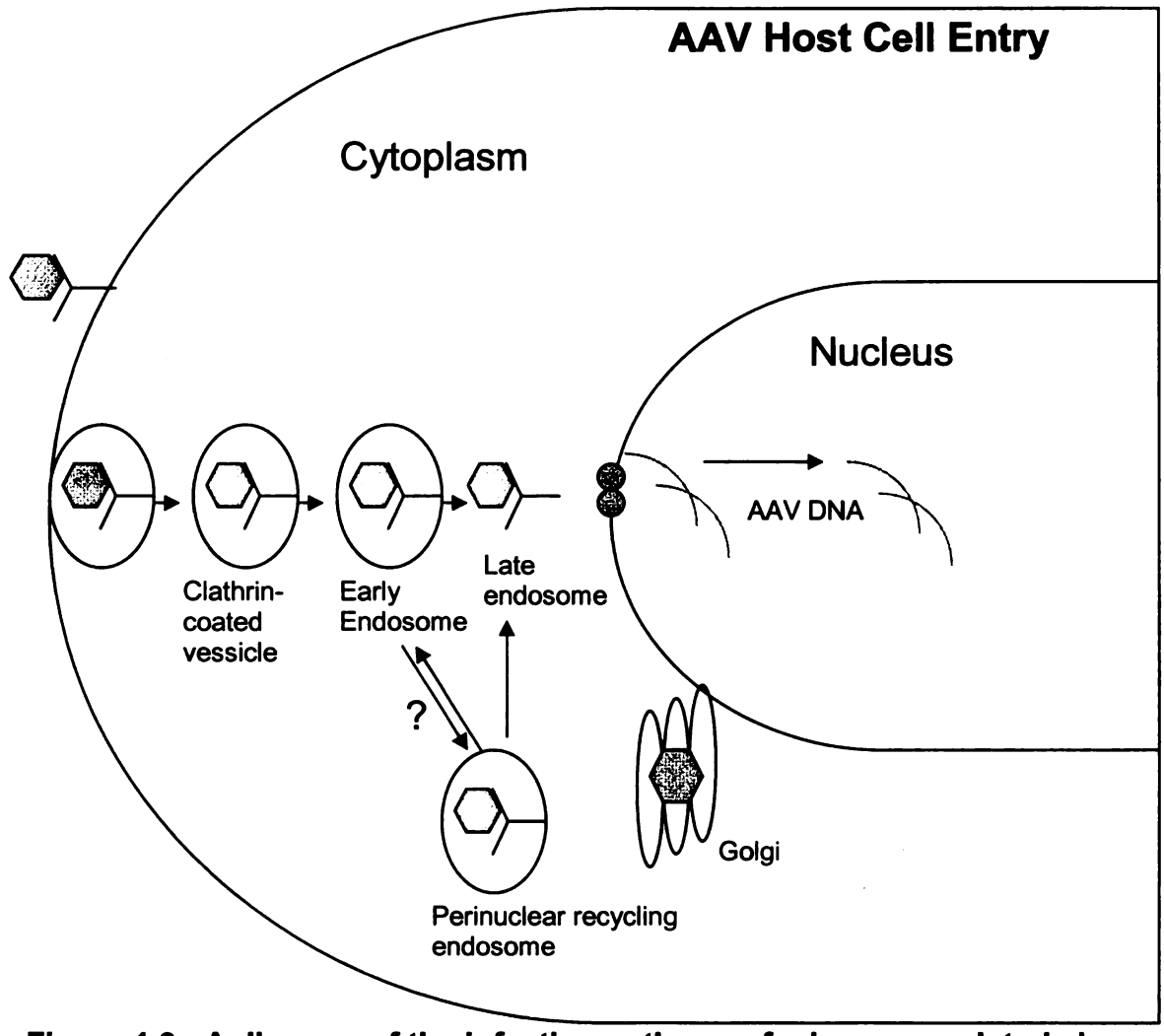

Figure 1.9. A diagram of the infection pathway of adeno-associated virus (Modified from Vihinen-Ranta et al., 2004). The AAV enters the host cell via receptor-mediated endocytosis. After escape from the endosome, the virus makes a dsDNA version of its ssDNA genome at the 5-fold axis of the capsid. The dsDNA then travels through the nuclear pore into the nucleus where it is then transcribed into RNA.

1.2.6.b. Recombinant Lentivirus

Lentiviruses, such as the human immunodeficiency virus (HIV) are a kind of retrovirus. Retroviruses, which were some of the first vectors used to transfer cellular DNA, consist of a single stranded RNA genome, reverse transcriptase, a viral envelope, and a complex genome (Figure 1.10.).

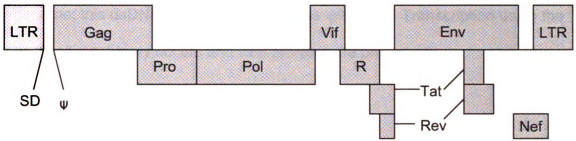

Figure 1.10. Diagram of the HIV provirus. The provirus is the dsDNA genome that has been altered from a dsRNA genome via reverse transcriptase within the host cell and is ready to be inserted into the host cell genome (modified from Naldini, 1996). All genes of the provirus are shown in this diagram including the accessory viral proteins: Env=viral envelope, Rev=reverse transcriptase, Pol=viral polymerase, SD=splice donor site, ψ=packaging signal, Gag=structural polyprotein, Vif, Tat, Nef=regulatory proteins, and Pro and R=accessory proteins.

The two essential components needed to make a recombinant HIV (rHIV) vector in cell culture are: 1) a modified genome with packaging signals (Ψ), a primer-binding site, a polyurine tract, the flanking LTRs, and the transgene in *cis*-acting sequence and 2) a helper virus that can provide all the packaging materials to create the vector (Figure 1.11.) (Lever, 1999).

Wild-type HIV are able to infect T-cells by binding helper T cell surface receptors CD4 and CXCr4. Recombinant viruses have been pseudotyped with the Vesicular Stomatitis Virus Glycoprotein (VSV-G) which is related to the rabies virus and consequently allows the recombinant virus to infect neuronal cells (Cronin et al., 2005). Once the virus has bound to the cellular receptor, the viral envelope fuses with the host cell membrane. The viral core then travels into the cell's cytoplasm where reverse transcription takes place. The now double-stranded DNA genome enters the nucleus where it integrates with the host cell

genome; this dsDNA genome is termed the 'provirus'. Transcription using the host cell machinery occurs and viral proteins are produced.

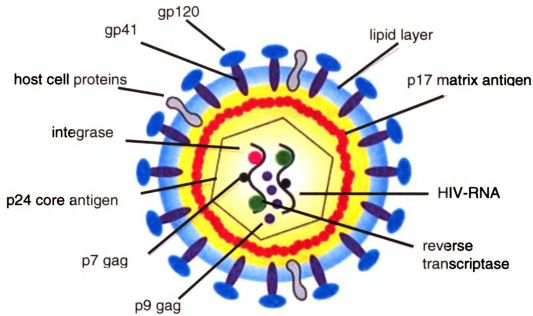

Figure 1.11. A representation of an HIV particle. (http://www.hivmedicine.com/textbook/images/image65.jpg, 2/8/07). The HIV particle has both a protein capsid as well as a lipid outer membrane. Within the viral capsid, the reverse transcriptase, integrase, and dsRNA genome reside.

One desirable trait of rHIV with respect to gene therapy applications is its potential to infect non-dividing cells. Retinal cells do not divide after differentiation, thus rHIV may prove beneficial as a vector for retinal gene therapy. In retinal research, it has been found that the rHIV pseudotyped with VSV-G is able to enter and integrate into maturing photoreceptors within the first 15 days of a mouse's life (Pang et al., 2006a). rHIV was shown to be able to transfect both photoreceptors and RPE with the most efficient transfection occurring from postnatal day 1 to 3. Use of enzymes has been shown to

increase photoreceptor transduction in the eye, as access of lentivirus vectors to photoreceptors may be limited by a physical barrier within the adult retina (Gruter et al., 2005).

1.2.6.c. Recombinant Adenoviruses (Av-1)

Recombinant adenoviruses have been used successfully as vectors for gene therapy ranging from management of cancer to treatment of inherited systemic diseases.

Adenoviruses are non-enveloped viruses that consist of an icosahedral, spiked capsid that is 70-90nm in diameter. The viral genome is linear, double-stranded DNA and is approximately 36KB in size (Figure 1.12) (Connelly, 1999). This virus is in the family *Adenoviridae* and consists of at least 50 distinct serotypes of which group 'C' serotype 5 is the most commonly used for gene therapy purposes. Adenoviruses infect many different cell types including liver, intestine, and even cells of the retina. Wild-type serotype 5 adenovirus can cause respiratory infections, gastroenteritis, and even conjunctivitis, but typically little inflammation is induced from the virus alone (Connelly, 1999).

Three main recombinant adenoviruses are available: Av-1, Av-2. and Av-3. Av-1 is the first generation recombinant adenovirus that was created by deleting the entire region for the E1A gene and 60% of the E1B gene which was then replaced with the transgene of interest. Av-1 uses the cytomegalovirus (CMV)

promoter, a strong viral promoter that has been known to induce a strong inflammatory response (Connelly, 1999). Av-2 vectors have one or more genes deleted in any combination of the following genes: E1, E2, E3, and E4. The transgene insert site is still located in the E1 gene deleted region, but these vectors use either the CMV promoter or can use another promoter of choice (Connelly, 1999).

Av-3, also known as 'Helper-Dependent Ads', are the most recent adenovirus vectors produced. In Av-3 vectors, the entire viral genome has been deleted and therefore the vector must be grown in the presence of 'helper' viruses. The DNA within the virus is the promoter and transgene DNA along with more 'filler' DNA to stabilize the structural integrity of the virus (Kumar-Singh et al., 1998; Connelly, 1999).

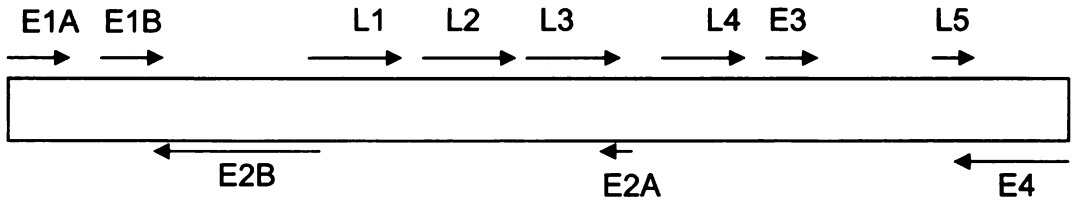

Figure 1.12. A representation of the adenovirus genome (Modified from http://www-ermm.cbcu.cam.ac.uk/fig005jfo.gif, 2-24-07). The adenovirus genome is a linear dsDNA genome that is transcribed from both 5' ends since viral genes can be oriented in both directions. 'E' genes represent the early transcribed genes whereas the 'L' genes represent the later transcribed genes.

Adenoviruses enter the host cell by binding a cell surface receptor, of which two are of importance to retinal gene therapy: CAR (Coxsackie-adenovirus receptor), which are present on most cells but not photoreceptors, and CD46, which has

been found on photoreceptors and binds to a fiber, F35, on the adenovirus capsid (Mallam et al., 2004; Von Seggern et al., 2003). Integrins are also involved in the entry of the virus and aid the cell in the process of endocytosis. The virus can tolerate the acidic conditions within the vesicle and escapes into the cytoplasm with the viral capsid fully intact. Once in the cytoplasm, the virus is transported along microtubules and directed toward the nuclear pores. The adenovirus attaches to the nuclear pore and the viral genome is transported into the nucleus where it is then transcribed (Connelly, 1999).

When using recombinant adenoviruses for gene therapy, it has been found that there is typically a waning of transgene expression approximately 2 weeks post transfection. There are many theories to explain this phenomenon. One theory suggests that antibodies generated by the humoral immune system eliminate the transgene and virus. Another theory suggests that there is CMV-promoter shutdown. This was eloquently demonstrated by Everett in a study which compared the same vector in both normal mice with an intact immune system and nude mice which lack an intact immune system (Everett et al., 2004). Both of the mice, despite the lack of an immune response in the nude mice, showed a decrease in transgene expression over time, suggesting promoter shutdown rather than immune response was the cause of loss of transgene expression.

1.2.7. Summary of Successful Retinal Gene Therapy Treatments

1.2.7.a. AAV Preliminary Studies in the Eye

Recombinant AAV vectors have been studied extensively for gene therapy purposes. AAV vectors are easy to create in large quantities, have low immunogenicity, and can transduce many different cell types. Several studies have looked at the ability and efficiency of these vectors to infect retinal cells in various species. Initially mouse models were studied followed later by dog and primate models. AAV vectors have been shown to be effective and safe with little inflammation or toxicity and to produce stable long term transgene expression (Auricchio and Rolling, 2005; Ali et al., 1998; Bainbridge et al., 2003; Bennett et al., 1999).

The kinetics of the AAV5 vectors was studied in the mouse eye in order to understand the transduction and gene expression variables. Mice were given injections of between $2x10^6$ and $2x10^{10}$ IU/mL into the subretinal space where the vector transduced retinal cells as soon as 3 days after injection. The time lag between transduction of retinal cells and gene expression is believed to be due to the requirement for dsDNA synthesis from the ssDNA viral genome. The retinal cells that were the main targets of the vector were located at the injection site, but some vector diffused past the borders of the injection site for unknown reasons. It was also determined that increasingly higher titers were able to transduce cells more effectively and were able to yield higher gene expression rates in retinal cells (Sarra et al., 2002).

1.2.7.b. Successful AAV Gene Addition Therapy for Retinal Degenerations

Attempts to treat mouse models of RP and LCA using AAV vectors have been at least partially successful in several mouse models. Both the *RPE65* mutant mouse and the *rd1* mouse have shown improvements after AAV gene addition therapy (Pang et al., 2006b; Lai et al., 2004; Jomary et al., 1997). Further work into the treatment of the *RPE65* null mutation model of LCA has utilized a large animal model, the Briard dog. Using an AAV vector transporting the normal *RPE65* transgene, subretinal injection was performed in an attempt to treat a portion of the retina by gene addition therapy. These treated dogs showed significant improvement in vision with dose-dependent, long-term results for several years (Acland et al., 2001; Narfstrom et al., 2003a; Ford et al., 2003; Narfstrom et al., 2003b; Acland et al., 2005; Jacobson et al., 2006).

There have also been other successful retinal treatments using an AAV vector; the *Prph2* mutant mouse showed rescue when an AAV vector with a rhodopsin promoter driving expression of peripherin was used (Ali et al., 2000), and the *RCS* rat with a mutation in the *Mertk* gene was rescued using an AAV vector to deliver a normal copy of the *Mertk* gene as well (Smith et al., 2003).

1.2.7.c. Lentivirus Preliminary Studies in the Eye

HIV vectors pseudotyped with the VSV glycoprotein were created with the aim of transducing differentiated, non-dividing cells such as retinal cells (Naldini et al., 1996; Bemelmans et al., 2005). Work using these vectors in rodent models

showed some successful results but with limitations. In one study, subretinal injections of the vector in rat eyes showed sustained transgene expression 12 weeks after injection with no signs of decrease in expression (Miyoshi et al., 1997).

1.2.7.d. Successful Lentivirus Gene Addition Therapy for Retinal Degenerations

Continued efforts have shown the efficiency and promise of recombinant lentivirus vectors in delivering the missing gene responsible for fast onset retinal degenerations. The *rd1* mouse, the *RPE65* knockout mouse, and the *RCS* rat have been treated using a rHIV vector. Early treatment of the *rd1* mouse has shown promise in slowing photoreceptor degeneration for up to 24 weeks (Takahashi et al., 1999). In the *RPE65* knockout mouse, early treatment of the retina led to increased cone photoreceptor survival for up to 4 months while adults treated with the same vector did not show increased cone photoreceptor survival (Bemelmans et al., 2006). Finally, lentivirus vector treatment of the RCS rat by subretinal injection at 10 days of age resulted in survival of photoreceptors up to 7 months (Tschernutter et al., 2005;). Unfortunately, for unknown reasons the rescue was only temporary.

1.2.7.e. Adenovirus Preliminary Studies in the Eye

Adenoviruses were among the first viral vectors that were studied for gene addition therapy in the retina. Today, this vector is still being researched as a potential vector for treatment of many types of ocular disease.

As stated earlier, adenoviruses enter the host cell by binding a cell surface receptor, either CAR or CD46, depending on the viral capsid (Von Seggern et al., 2003). Importantly, CAR receptors do not exist in large numbers on the surface of photoreceptors and therefore these cells are not well transduced by the commonly used AdV5 vectors for gene therapy research (Mallam et al., 2004).

The first study using adenoviruses for retinal gene therapy were done using Av-1 vectors with the beta-galactosidase (LacZ) transgene in mice. Within 24-48 hours after subretinal injection, transgene expression was evident in the retinal layer (Bennett et al., 1994). Transduction by the recombinant virus and also transgene expression was found to be dose-dependent in that study. Two weeks after the injection, the transgene expression had waned completely.
Another study looking to determine the length of transgene expression used the same vector type as the above experiment and found that the adenovirus-delivered transgene could be expressed for almost 16 months in rat retinas, with most of the infected cells being RPE with only a few cells of the neurosensory retina infected (Loewen et al., 2004).

1.2.7.f. Successful Adenovirus Gene Addition Therapy for Retinal Degenerations

One of the first successes using Av-1 for treatment of a retinal disease involved treatment of *rd1* mice. This study demonstrated photoreceptor survival up to six weeks post injection. The reporter transgene slowly waned for a period of two weeks and then became completely undetectable thereafter (Bennett et al., 1996). This study established the potential usefulness of this vector, so other researchers have attempted to use this vector for other models of retinal degeneration. (See Figure 1.13. for an image of a dog retina transduced by an Av-2 vector.)

Since the RPE is transduced well by Av-1 vectors, several retinal degeneration models were suitable for treatment using this vector. For instance, the *RCS* rat showed retinal saving after subretinal injection of the vector carrying the *Mertk* transgene (Vollrath et al., 2001). Treatment of the *RPE65* knockout mouse resulted in the presence of functional rpe65 and preservation of photoreceptors (Chen et al., 2006).

Av-3 vectors have also been investigated for retinal degeneration treatments. An EAM with a β-pde transgene and β-pde promoter was injected into the subretinal space of the *rd1* mouse eye. This vector resulted in improved photoreceptor

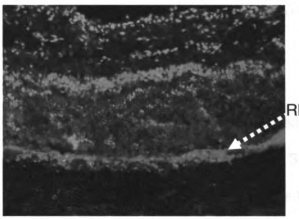

Figure 1.13. Immunohistochemistry of the dog retina transduced by an Av-1 vector after subretinal injection. The red fluorescence indicates the green fluorescent protein (GFP) within the RPE layer of the retina and the blue fluorescence indicates the nuclei of individual cells.

survival compared with the results using an Av-1 vector (Kumar-Singh and Farber., 1998).

1.2.8. Safety Concerns that Arise from Viral Gene Therapy

On September 17th, 1999, Jesse Gelsinger, an 18 year old ornithine transcarboxylase (OTC) deficient patient who was a voluntary participant in a gene therapy trial died 4 days after the treatment due to multi-organ-system failure (Stolberg, 1999; Hartogs, 1999; Recombinant DNA Advisory Committee, 2002). Adenovirus vectors were used in the trial in an attempt to treat the OTC deficiency. Gelsinger received the highest dose used in the trial of 3.8×10^{13} viral particles in an effort to determine the dose-dependent nature of the treatment. It was determined on autopsy that the extreme reaction to the vector was to the viral capsid. In response to this unfortunate event, the NIH Recombinant DNA Advisory Committee (RAC) made recommendations for the

protocol and monitoring of human clinical trials in order to lessen the chance of future adverse events (RAC, 2002).

Another serious adverse event occurred during clinical trials using gene therapy to treat X-linked severe combined immunodeficiency disease (SCID-X1). In this study, retroviruses were used to infect T cells *ex vivo* that were then injected into the blood stream. Two of the youngest patients contracted leukemia due to insertional oncogenesis of the retrovirus near the LMO2 promoter, which is a proto-oncogene (Hacein-Bey-Abina et al., 2003; ASGT, 2002). The NIH RAC has produced statements in regards to these concerns and has warned researchers of this rare but serious consequence that is possible when using retroviral vectors for gene therapy (Sadelain, 2004).

The safety and efficacy of gene addition therapy is a serious and legitimate concern. Issues encompass several areas including: the concern of viral biodistribution, insertional oncogenesis, host immune responses to both the transgene and viral vector, and the potential for genotoxicity of the transduced cells.

1.2.8.a. Host Immune Response

The host immune system is stimulated to eradicate foreign invaders as quickly and as efficiently as possible (Figure 1.14). Initially, the host tries to eradicate the invader through the innate immune response such as through phagocytosis,

neutralization, and complement (Janeway et al., 2005). If the innate immune system is unable to inhibit or destroy the virus, host cells are then infected. This is where the life cycle of the virus begins and the eventual death of the cell ensues. The host's immune cells, such as antigen presenting cells (APCs); macrophages, neutrophils, and dendritic cells, and also natural killer cells (NK cells), recognize the infected cells and thus the antigen. The host also mounts a second attack against the antigen known as the adaptive immune response. This response takes 4-7 days for elimination of the antigen after infection, which involves both the cell-mediated and humoral pathways, separately and collaboratively (Janeway et al., 2005).

The cell-mediated immune response is through activation of T cells by antigen presentation by APCs within lymph nodes. Cytotoxic T cells, also known as CD8+ T cells, and helper T cells, CD4+ Th1 and CD4+ Th2 cells, are activated by antigens presented by APCs. CD8+ T cells, are the effector cells that destroy infected cells by way of a pathway that induces apoptosis (Janeway et al., 2005). CD4+ Th1 and Th2 cells each have their own unique role. Th1 cells leave the lymph node after activation to stimulate cells, such as macrophages, into action. Th2 cells activate B cells within the lymph node (Janeway et al., 2005).

The humoral immune response is activated immediately to any antigen upon entry into the host. The humoral immune response is initiated by Th2 cell activation of B cells and also B cell presentation of antigens to Th2 cells through

the MHCII receptor. These immature B cells produce antibodies and they are dispersed into the blood. IgM antibodies are the first antibodies produced by immature B cells within the first few days of an infection (Janeway et al., 2005). Some of the B cells travel to the germinal centers within the lymph nodes where they mature through isotype switching, which allows for a variety of antibodies to be produced such as IgG, thus creating plasma cells. The peak IgG production occurs between 7-10 days post-infection. The antibodies bind to the antigens and allow for neutralization of the antigen, induction of complement, or promotion of phagocytosis (Janeway et al., 2005).

Memory T and B cells are formed after an initial exposure to an antigen. Both memory T and B cells are thought to be sustained through constant cytokine stimulation. Upon a repeat exposure to the same antigen, such as a viral vector, B cells undergo clonal expansion and are able to produce high levels of IgG within a few days after infection. T cells also undergo proliferation after encountering the same antigen and create effector cells that travel throughout the host killing infected cells.

For the viruses that some of the recombinant vectors are derived from, the details of the host response have not been elucidated, but for others, such as adenoviruses, extensive work has been done in order to understand the immune response (for a review see Schaack, 2005).

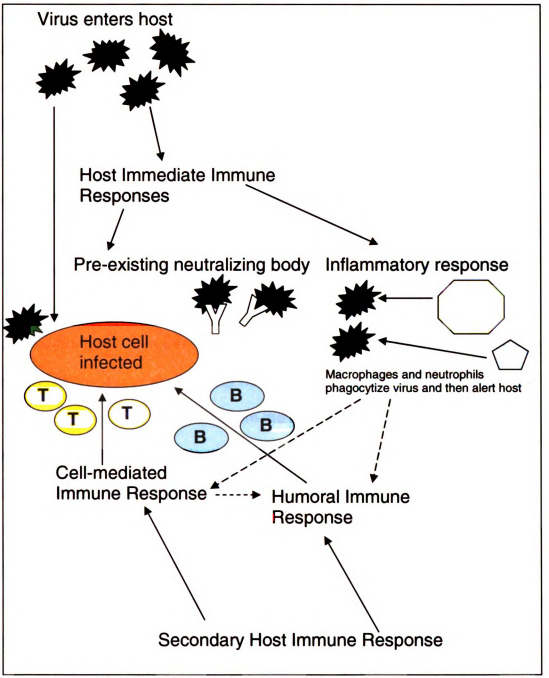

Figure 1.14. An overview of the host immune response to a foreign invader.
NK= natural killer cells, T= T cell, and B= B cell.

1.2.8.b. Adeno-Associated Vectors- Safety and Host Immune Response

Wild-type adeno-associated viruses are known for their low immunogenicity during infections. Because of this trait, they have proven useful as gene therapy vectors with the potential for repeated injection without adverse effects (Anand et al. 2002, Anand et al., 2000). Studies to determine the safety of AAV vectors have included the systemic safety, the possibility of germline transmission, the immune response to the viral capsid after intraocular injection, and the effect of the immune response on the repeated administration of the vector.

AAV vectors have been found to be safe and efficacious for systemic use (Chao and Walsh, 2004). While this may not be a major concern for ocular therapy, the potential for spread of the vector from the eye to other parts of the body does remain a concern. In order to determine the overall safety of the AAV vector systemically, intramuscular injections of an AAV vector were performed on nonhuman primates (Favre et al., 2001). Within 48-72 hours after the injection, the viral genome was detected in the serum and up to 6 days post-injection the vector was detected in various body fluids. No tissue abnormalities were found at time of necropsy performed 8-18 months post-injection, but the vector was found in the lymph nodes and liver (Favre et al., 2001).

Germline transmission has been a concern after an AAV vector was used to treat hemophilia in human patients and the AAV vector DNA was found in the semen of treated males (Manno et al., 2003; Arruda et al., 2001). Schuettrumpf et al.

designed a study to determine if the germ cells were infected or if the viral vector was only in the semen transiently. Using rabbits to study the germline transmission after a systemic injection of AAV, vector sequences were found in the semen in a dose-dependent manner and disappeared also in a dose-dependent manner. No infection of the germ cells was evident when monitored over a period of 18 months when analyzed using a quantitative real-time polymerase chain reaction (qPCR) (Schuettrumpf et al., 2006).

One issue of remaining controversy over this vector is whether or not rAAV has the ability to insert itself into the host cell's genome. Random insertion into the genome could potentially create insertional oncogenesis. In humans, it has been shown that the wild-type virus has the ability to integrate into the genome at a specific place on chromosome 19 (Kotin et al., 1990). Others contest that the viral DNA, once inside the nucleus, does not integrate into the host genome (Song et al., 2004). As this continues to be debated, the possibility of integration remains a concern for the safety of gene therapy trials using AAV vectors.

Immune responses to the AAV capsid have been previously studied. After intraocular injection of the vector, mild to moderate inflammation has been observed in Briard dogs, but this resolved over a period of 3 months post-injection (Jacobson et al., 2006). An adaptive immune response has been detected after intraocular injection of the vector. A slight qualitative anti-AAV humoral response was detected in mice and dogs after intravitreal and subretinal

injection in both pre- and post-injection (time not specified) serum and aqueous samples (Dudus et al., 1999). Further work into the adaptive immune response was studied in mice; subretinal injections were performed in mice and a deviant immune response was found in the eye. A footpad thickness test performed after intraocular injection (which demonstrates the cell-mediated response) the results of which indicated there was a minimal response against the viral capsid (Anand et al., 2002). Bennett et al. detected a Th2-dependent humoral response to the viral capsid (by ELISA) and found that individual primates had up to a 16-fold increase in IgG and Dudus et al. showed a statistically significant increase in mice and dogs of IgG antibodies to the viral capsid (Bennett et al, 1999; Dudus et al., 1999).

Readministration studies have been done in order to determine if a systemic humoral response would interfere with transgene expression in the eye. In a study by Bennett et al., they found that after initial injection neutralizing antibodies were formed (Bennett et al., 1999). When the same AAV-GFP vector was injected into the contralateral eye 7 months after the initial injection, no inflammatory response was detected and transgene expression was evident, despite the presence of neutralizing antibodies and a 128-fold increase in serum anti-AAV antibody after readministration (Bennett et al., 1999). In a similar study, mouse eyes were given subretinal injections of the vector, at repeat administration transgene expression was still observed even though there was a significant anti-AAV humoral response present in the mice (Anand et al., 2000).

1.2.8.c. Safety of Lentivirus Vectors

The two main concerns when using lentiviruses for gene therapy are: 1) the potential for insertional oncogenesis and 2) spontaneous recombination occurring creating a wild-type virus (Lever, 1999).

The phenomenon of insertional oncogenesis is pertinent, though rare, because the random insertion of the viral genome next to active genes of the host cell genome can interfere with the normal cellular functioning (Recchia et al., 2006). If near a promoter of a tumor-suppressor gene or oncogene, oncogenesis and consequential disease could result for the patient. New vectors are being designed that will help alleviate this issue by directing viral genome insertion to a consistent and safe location within the genome. Non-integrating HIV vectors are also being designed that will remain in the nucleus to allow transcription to proceed but will not undergo genome insertion (Yanez-Munoz, 2006).

The issue of recombination occurring and leading to self-sufficient wild-type virus regeneration can be easily overcome by designing a plasmid system that plans for this potential hazard. Several methods have been created that are specifically designed to inhibit insertional complications (Lever, 1999; Lu et al., 2004).

1.2.8.d. Adenovirus Vectors in the Eye- Safety and Host Immune Response

Although adenovirus vectors have been shown to be efficient for gene therapy, the recombinant adenovirus vectors have induced inflammatory and other immune responses. It is theorized that inflammation is induced for several reasons: size of the virus, capsid structure, and transgenes such as LacZ and GFP.

Three distinct phases in recombinant adenovirus induced inflammation have been described (Figure 1.15). The first phase is a short-lived response to the capsid, which induces cytokine release. The second phase occurs after internalization of the virus and is thought to be due to the adenovirus genes. The third phase is immune recognition of the adenovirus gene products and also possibly the transgene product. Antibodies are then formed which might result in elimination of the transgene and viral vector (Schaak, 2005).

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While the first and second generation adenoviruses show a substantial immune response complete with inflammation and a secondary immune response, the Av-3, also known as encapsidated adenovirus mini-chromosomes (EAMs), has shown a significantly lower secondary immune response since they lack all viral genes (Muruve, 2004).

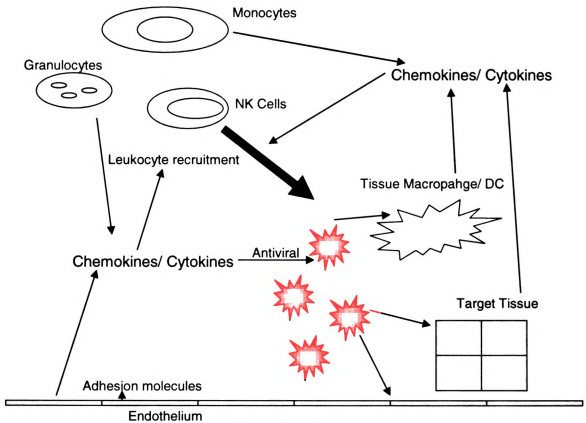


Figure 1.15. The typical host immune response to recombinant adenovirus vectors.
NK= natural killer cells, DC= dendritic cells,

In the eye after subretinal injection of an adenovirus (Av-2) vector, there seems to be a deviant immune response (Hoffman et al., 1997). Subretinal injection of the vector has shown to induce a systemic humoral response, yet after readministration of an adenovirus vector, transgene expression did not appear to be inhibited by the host immune response (Isenmann et al., 2001). Interestingly, in another study, vaccination of the animals in order to create an immune response and then followed by adenovirus vector intraocular administration every two weeks thereafter showed to have little if any effect on transgene expression within the eye (Hamilton et al., 2006).

1.2.8.e. Host Immune Response to Expressed Transgene in the Eye

Few studies have focused on the immune response to the transgene after intraocular injection. In a study done by Isenmann et al., there was a difference in immune response based on injection site. Following intravitreal injections, an immune response to the transgene was detectable where as following subretinal injection an immune response was not detected (Isenmann et al., 2001).

Two studies have looked specifically at the humoral immune response to GFP after intraocular injection of a viral vector carrying the GFP transgene. Both of the studies qualitatively analyzed the presence of anti-GFP antibodies via Western Blot. Dudus et al. used pre- and 7 week post-injection serum samples and found that 1 out of 3 mice had an antibody response to GFP (Dudus et al., 1999). Another study showed similar results with a high circulating anti-GFP antibody level detected in 2 out of 4 mice after subretinal injection of an AAV-GFP vector (Bennett et al., 1999).

1.2.8.f. Biodistribution of the Viral Vector within the Host after Intraocular Treatment

AAV delivered by subretinal or intravitreal injection has been shown to spread along the optic nerve to the optic tract in several species: dog, primate, and mouse (LeMeur et al., 2005; Jacobson et al 2006; Dudus et al.1999; Shen et al., 2003; Provost et al., 2005). Both gross investigation using reporter genes as well

as quantitative PCR (qPCR) have been used in order to demonstrate the distribution of the vector in the host after intraocular injection.

In the mouse, GFP fluorescence has been observed in the layers of the neuroretina and also along the optic nerve after subretinal injection. GFP was observed in the retinal ganglion cells, the optic nerve fibers, and the optic disc. Along the visual pathway, GFP was observed in the optic nerve, lateral geniculate body, optic radiation all the way to the occipital lobe (Shen et al., 2003). Interestingly, the type of injection performed had an effect on the distribution of the vector. Comparing intravitreal and subretinal injections in both dogs and mice, GFP fluorescence was detected along the visual pathway to a greater extent after intravitreal injection than after subretinal injection as might be expected (Dudus et al., 1999).

PCR has also been used to determine the relative amount and location of the vector after spread from an intraocular injection in the rat, dog, and nonhuman primate. Two studies have worked to address this issue, but with different results. Provost et al. injected, via intravitreal or subretinal routes, an AAV-GFP vector into the eyes of rats, dogs, and nonhuman primates; PCR for the GFP transgene sequence was performed to detect the sequence within many body tissues including retina, optic nerve, optic chiasm, optic tract, lateral geniculate nuclei, optic radiation, visual cortex, superior colliculus, inferior colliculus, temporal lobe, thalamus, cerebellum, spinal cord, cerebrospinal fluid, pituitary

gland, lachrymal gland, amygdala, salivary gland, submandibular lymph node, preauricular lymph node, thymus, axillary lymph nodes, gonads, liver, and peripheral blood mononuclear cells (Provost et al., 2005). Sequence was detected occasionally in peripheral blood mononuclear cells. As expected, vector sequence was found in the optic nerve, and along the visual pathway after intravitreal injection, but interestingly the vector sequence was also found in the optic nerve after subretinal injection (Provost et al., 2005).

In another study done by Jacobson, subretinal injection of a vector carrying the *RPE65* transgene led to distribution and thus viral sequence detection by qPCR in various organs. Tissues that were analyzed included: optic nerve, optic chiasm, optic tract, latertal geniculate nucleus, optic radiation, visual cortex, superior colliculus, mandibular lymph node, parotid lymph node, heart, lung, diaphragm, liver, pancreas, spleen, kidney, jejunum, gonad, and skeletal muscle. The spread of the vector was found not to be dose-dependent (Jacobson et al., 2006). Vector sequence assayed by amplifying a portion of the CMV enhancer/ CBA promoter was found at a level of 100 copies/μg or higher of DNA in the mandibular lymph nodes, heart, diaphragm, optic nerve, and optic chiasm.

1.2.8.g. Genotoxicity

Issues of genotoxocity remain a concern when introducing a vector that has a ubiquitous promoter or when a foreign protein such as GFP, LacZ, or in gene addition therapy if the transgenic protein is introduced into an animal that is

deficient for the particular protein. For example, very high doses of the AAV vector carrying the *RPE65* transgene has shown to produce toxic effects in the retina when the transgene is under the control of a ubiquitous promoter (Jacobson et al., 2006). Thus, dosage toxicity studies are necessary before clinical trials begin to determine the safe dosage range.

In most studies, there is little systemic toxicity and retinal damage after intraocular injection of a vector (Jacobson et al., 2006; LeMeur et al., 2005). For example, subretinal injection of AAV-2, -4, and -5 with the GFP transgene in 14 beagles and 9 macaques showed normal retinal angiography images and ERG amplitudes (Le Meur et al., 2005). In another study, AAV-GFP vectors were injected into the eye and high levels of GFP in retinal cells up to 270μM showed no toxic effect and did not seem to affect normal photoreceptor function based on ERG results (Rex et al. 2005; Shen et al., 2003).

One way to reduce the risk of genotoxicity is to use a tissue-specific promoter. For the retina, a mouse opsin promoter has been developed that only expresses the transgene in rods and cones (Glushakova et. al., 2006). Another way to solve this issue is by using inducible promoters such as tetracycline- or erythropoietin-inducible promoters that are activated upon systemic administration of the appropriate drug. These methods have even been shown to work in the eye despite the blood-ocular barriers (Auricchio et al., 2002; Blau et al., 1999).

1.3. Objectives, Hypotheses, and Experimental Design

1.3.1. Objectives

The purpose of this work was to investigate in detail the humoral immune response to GFP after treatment with viral vectors carrying the GFP transgene administered intraocularly in dogs. We focused primarily on AAV constructs since these are the most widely used vector types in retinal gene therapy. The systemic immune response was investigated for both GFP and AAV capsid antibodies while the local immune was investigated for GFP antibodies. The systemic immune response to GFP after intraocular treatment with adenovirus and lentivirus vectors was also performed.

1.3.2. Hypotheses

In previous studies, intraocular administration of AAV-GFP in mice and dogs was reported to induce a minor, individual systemic and intraocular humoral immune response to both the viral capsid and GFP. We hypothesize that the same response will be observed and we wish to perform a more detailed analysis of the timing of the humoral immune response in dogs. The systemic AAV antibody response should be detectable at 2 weeks post-treatment while the GFP antibody response should be detected systemically 4 weeks post-treatment due to slow expression of the transgene that is typical for AAV vectors. The local GFP antibody response should be detectable at approximately 4 weeks post-treatment for similar reasons. Variables such as the promoter used in the

construct and the age of the animal at treatment are expected to influence the immune response.

We anticipate that GFP expression resulting after intraocular treatment with lentivirus and Av-1 vectors will also result in a detectable systemic humoral immune response.

1.3.3. Experimental Design

Intraocular injections with vectors carrying the GFP transgene were performed: AAV vectors were used in 22 dogs, lentivirus was used in 3 dogs, and Av-1 was used in 1 dog.

For the AAV-treated pups, one of two AAV constructs were used: AAV-CBA-GFP or AAV-mOPS-GFP and injections performed at either ~3 days of age or ~3 weeks of age (figure 1.16). For lentivirus- and Av-1- treated dogs, subretinal injections were performed. Aqueous samples were collected from AAV-treated dogs pre-injection and then at the time of euthanasia. Sera samples were collected from each dog pre- and post-treatment. Sera samples were collected from AAV- and lentivirus-treated dogs every two weeks up to 8 weeks for most dogs. The Av-1-treated dog sera samples were collected at 2 and 3 weeks post-treatment. These serum and aqueous samples were then analyzed using canine-specific IgG ELISAs (GFP and AAV) in order to understand the systemic and local humoral responses.

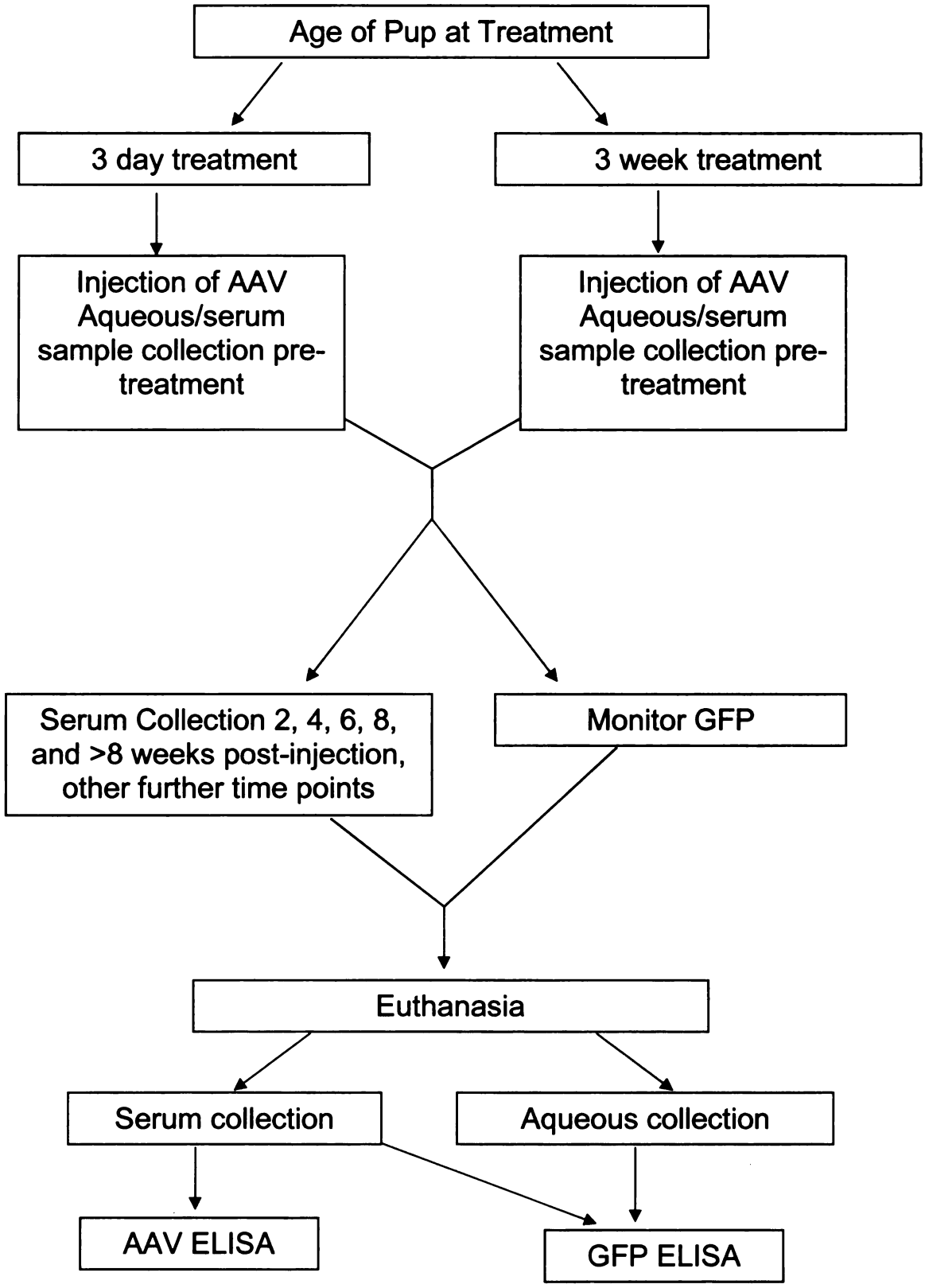


Figure 1.16. Flow chart of experimental design for the AAV–treated dogs.

Sera from AAV-treated dogs were analyzed using the AAV and GFP ELISAs while the aqueous samples were analyzed using the GFP ELISA. Sera from the lentivirus- and Av-1-treated dogs were analyzed using the GFP ELISA. The optical density (OD) values from these ELISAs were then analyzed using SAS.

Chapter 2.

DEVELOPMENT OF ELISA ASSAYS

2.1. Introduction

Two canine-specific enzyme-linked immunosorbent assays (ELISAs) were developed in order to analyze the humoral immune response after intraocular injection of viral vectors carrying the GFP transgene : rAAV2/5, Av-1, and lentivirus. The first ELISA designed was the GFP ELISA to detect anti-GFP antibodies and the second was the AAV ELISA to detect the anti-AAV capsid antibodies. Previous studies have analyzed the humoral immune response in mice and dogs based on human serum controls which could create bias and distorted results. By creating canine- specific ELISAs, the humoral immune response can be more accurately assessed.

2.2. Materials and Methods

2.2.1. Immunization of Dogs

Colony dogs were immunized with the intent of using serum samples as controls for the GFP ELISA and AAV ELISA. Each dog was injected using a multi-channel LectraJet injector (D'Antonio Consultants International, East Syracuse, NY) with a vector preparation, described below, that was intended to create an immune response to both the vector capsid as well as the GFP transgene (Carter and Kerr, 2003).

The pHR'-CMV-GFP vector was created in the laboratory of Vincente Planelles (University of Utah) and was used for immunizations because it induced a high level of circulating anti-GFP antibodies. The protocol for creation of these vectors has been published (Zhu et al., 2001: Planelles, 2003). The lentivirus vector (pHR-CMV-GFP) was prepared for injection by diluting the virus to ~10^6 infectious units of the vector in sterile saline.

The AAV-GFP vector was created in the laboratory of William Hauswirth at the University of Florida. Another member of our research group, Danielle Eifler, prepared the UF 12 type 5 AAV vector to a concentration of ~10^13 vp/mL in sterile saline.

A total of 4 dogs were injected with the appropriate virus and 2 negative control dogs were injected with sterile saline as outlined in Table 2.1 below. Each dog was sedated with 0.03-0.05 mg/kg of acepromazine (Boehringer Ingelheim Vermdica, Inc., St. Joseph, MO) and 0.2mg/kg of butorphanol (Fort Dodge Animal Health, Fort Dodge, Iowa). The area on the medial thigh was then shaved and scrubbed. Topical lidocaine was applied to area to be injected. Two separate injection sites on the medial thigh of each leg were used with a total of four sites per injection day. A vector preparation volume of 0.5 mL was injected per site. After, the injection protocol was finished, each dog received 100mL saline subcutaneously following the injection procedure. Immunizations were first performed for all dogs on 7/21/04 and then repeated on 8/8/04 and 8/18/04. Dogs were re-immunized on 4/20/05.

Mica	Negative control dog-0.5ml of saline
Snap	Negative control dog-0.5ml of saline
Pluto	Lentivirus-GFP-0.5ml of virus solution
Spruce	Lentivirus-GFP-0.5ml of virus solution
Red	AAV-GFP- 0.5ml of virus solution
Roscoe	AAV-GFP- 0.5ml of virus solution

Table 2.1. Immunized dogs. The vector and amount of virus injected per dog is described.

Sixty milliliters of blood was collected from each dog prior to immunization and then serum was collected every week for the following six weeks. After the six weeks, blood was collected every two weeks. The blood was placed on ice following collection, allowed to sit at 4°C for twenty-four hours, and then serum was collected and stored at -20°C. After dogs were re-innoculated on 4/20/05, serum was collected 1 and 2 weeks after innoculation.

2.2.2. Development of GFP ELISA

During the initial phases of development, GFP and antibodies were used at varying concentrations in order to create optimal assay conditions. GFP was used to coat the plate (BD Falcon, ref 353279, Bedford, MA) using dilutions of 1:100, 1:200, 1:500, 1:1,000, 1:10,000, and 1:30,000 in a bicarbonate solution (see methods) with the initial concentration GFP at 1mg/mL (Clontech, Mountain View, CA), resulting in working concentrations of 0.01 mg/mL, 0.005mg/mL, 0.002mg/mL, 0.001mg/mL, 0.0001mg/mL, and 0.000033mg/ml, respectively

(Figure 2.2). The optimal GFP concentration range was found by using GFP hyperimmune rabbit serum (HIRS) as a positive control (Abcam, Cambridge, MA), normal rabbit serum as a negative control, and anti-rabbit-specific HRP-conjugated IgG as the secondary antibody (Caltag. L42007, Carlsbad, CA). Varying dilutions of the normal and HIRS were tested: 1:500, 1:1,000, and 1:2,500, with the secondary antibody at a dilution of 1:20,000. The immunized dog serum was then used to create a canine-specific assay (Figure 2.3.). The positive control dog sera collected on 8/25 was chosen because it produced the highest OD values and was then used to finally optimize the assay for dog sera samples. After analyzing serum dilutions of 1:5, 1:10, 1:25, and 1:50, the optimal positive control sera with the highest OD being ~1.2 was a 1:1 mixture of a both positive control dog sera at a 1:25 dilution using PBS-T. The same 1:1 mixture of negative control dog sera was used at a 1:25 dilution using PBS-T which gave an OD of ~0.1 (Figure 2.4.). Positive and negative control sera were used for each plate. Upon completion of the assay design, the test dog serum samples were then analyzed using the finalized protocol described next.

2.2.2.a. Reagents

(see Appendix A for detailed instructions)

- Phosphate-buffered solution (PBS)
- 0.05% PBS-Tween wash buffer
- Bicarbonate solution (Pierce, Rockford, IL)

- GFP antigen solution for coating wells at 0.002µg/mL (Clontech, Mountain View, CA)
- HRP-anti-Dog IgG (H+L) (Bethyl, Montgomery, TX) was made fresh for each day of use from stock solution
- 1-Step ABTS used as substrate (Pierce, Rockford, IL)

2.2.2.b. Detailed GFP ELISA Protocol

The GFP ELISA (Figure 2.1.) was designed in order to determine if a systemic antibody (IgG) response was induced following intraocular injection of viral vectors [AAV2/5, Av-1, lentivirus] with a GFP transgene. (see Appendix A for laboratory protocol).

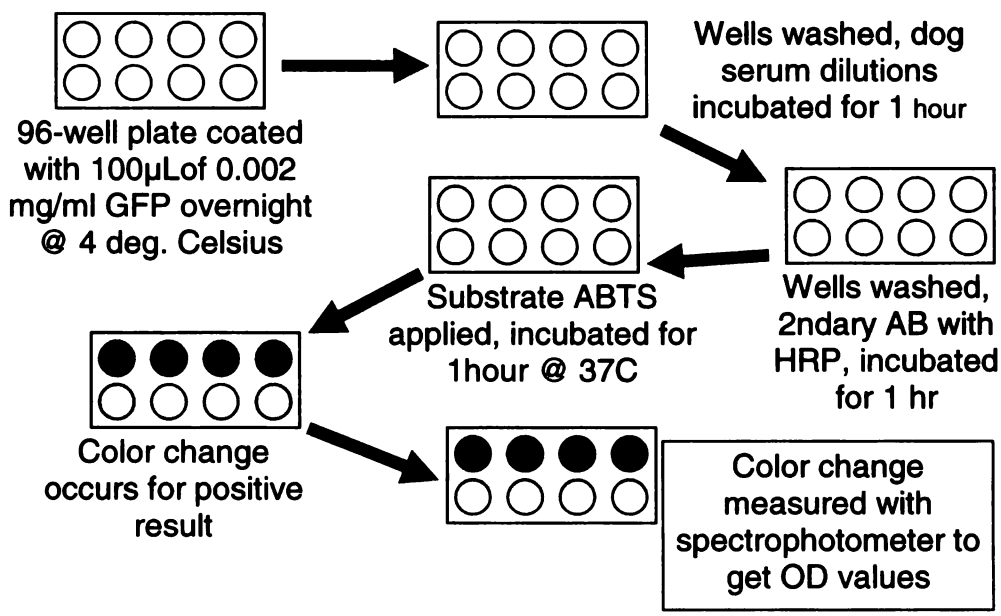


Figure 2.1. GFP ELISA Protocol Flow Chart

- 96-well flat- bottom ELISA plates (BD Falcon, Bedford, MA) were coated with 100µL per well of GFP antigen solution, covered with clear adhesive slips (Nunc, Denmark) and left for 15 hours at 4°C.
- The wells were then washed and emptied three times with 200µL PBS-T solution
- Serum dilutions were prepared and applied to the wells at 100µL per well
    - Serum dilutions were prepared using a serial doubling dilution that ranged from 1:5 to 1:320 in PBS-T down the plate column and each dilution was run in duplicate
    - Aqueous dilutions in PBS-T were done at 1:20 and were run only once due to sample size limitations
    - Positive and negative control sera samples at 1:25 in PBS-T were used for every plate
- Plate re-covered with plastic slip and placed on shaker, setting #5 (Wellmix, Thermo LabSystems), and left at room temperature for one hour
- The plates was removed from the shaker and the wells were then washed and emptied three times with 200µL PBS-T solution
- The secondary antibody was added at a dilution of 1:20,000, the plate re-covered with the slip, and left at room temperature on a shaker (setting #5) for one hour
- The plate was removed from the shaker and the wells were then washed and emptied three times with 200µL PBS-T solution

- 125µL of 1-Step ABTS was added to each well, the plate was re-covered with the slip and was left at 37°C for one hour without shaking
- The plate was read at dual wavelengths of 405nm and 450nm using a plate reader (Bio-rad microplate reader, Hercules, CA) to determine optical density (OD) values

2.2.2.c. Results of GFP ELISA Development

The GFP ELISA was optimized through a series of steps. First, the optimal GFP concentration was determined using anti-GFP rabbit serum (Figure 2.2). The three best GFP dilutions for rabbit samples were found to be 1:10, 1:100, and 1:1000. Next, GFP dilutions in the range determined above were tested at 1:100, 1:200, 1:500, and 1:1000 and ELISA conditions optimized for dog samples using immunized dog serum at dilutions of 1:10, 1:100, 1:500, and 1:1000 (Figure 2.3). The GFP dilution of 1:500 was chosen from this trial for the canine GFP ELISA. The next step involved determining the optimal canine control sera dilutions (Figure 2.4). Sera from positive and negative control dogs as well a pool of the control dog sera were tested at dilutions of 1:5,1:10,1:25,1:50, 1:250, 1:500, 1:750, and 1:1000. The 1:25 dilution provided the best dilution tested since it produced the highest OD for the positive control samples with little the least amount of background from the negative control samples.

2.2.3. Development of AAV ELISA

During the initial phases of development, the AAV2/5 vector (Hauswirth Laboratory, University of Florida, UF11 T5 #E894) was tested at several concentrations in order to create an optimal assay. Using flat-bottom plates (BD Falcon, ref 353279, Bedford, MA) and sterile saline (Butler, Columbus, OH) as the diluent, the AAV2/5 virus (5.6E14vp/ml stock) was tested at concentrations of 1,000vp/mL, 5,000vp/mL, 10,000vp/mL, 20,000vp/mL, 40,000vp/mL, 100,000vp/Ml, and at several dilutions of the virus of 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600. Sera from dogs that had been vaccinated with an AAV2/5 virus were used as positive controls for the development of the assay only. Varying dilutions of the control dog sera were used at: 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. The greatest OD values were observed at the highest virus concentrations, but were affected by the prozone effect (concentration of antibody exceeds the concentration of antigen resulting in a false negative result) at the highest concentrations of sera. A virus concentration of 100,000vp/mL was chosen for the assay since there was a limited amount of virus available and the OD values were comparatively high with no prozone influence (Figure 2.5). A serum dilution of 1:5 was run in duplicate for test samples since this provided the highest OD value per virus used. The negative control serum was not run due to background interference observed at the 1:5 dilution, but a negative control without sera was run per plate.

2.2.3.a. Reagents

- Phosphate-buffered solution (PBS)
- 0.05% PBS-Tween wash buffer
- A sterile saline solution (Butler, Columbus, OH) was used for the virus solution preparation
- The recombinant virus (made by the Hauswirth Lab at the University of Florida, UF11 AAV Type 5, 5.60E+13 vp/mL).
- The negative control consisted of wells with all reagents except serum
- HRP-anti-Dog IgG (H+L) (Bethyl, Montgomery, TX)
- 1-Step ABTS used as substrate (Pierce, Rockford, IL)

2.2.3.b. Detailed AAV ELISA Protocol

The AAV ELISA was designed in order to determine the systemic antibody (IgG) response created after intraocular injection of the AAV5 vectors into the eye. (see Appendix B for laboratory protocol)

- 96-well flat- bottom ELISA plates (BD Falcon, Bedford, MA) were coated with 100µL per well of virus solution, covered with clear adhesive slips (Nunc, Denmark) and left for 15 hours at 4°C.
- The wells were then washed with 200µL PBS-T solution and emptied three times
- Serum dilutions were prepared and applied to the wells at 100µL per well
    - Serum dilutions of 1:5, 1:10, and 1:20 in PBS-T and were run
    - Positive control serum was run on each plate

- Negative control consisted of all reagents except sera to account for plate variability

- Plate re-covered with plastic slip and placed on shaker, setting #5 (Wellmix, Thermo LabSystems), and left at room temperature for one hour
- The plate was removed and washed with PBS-T and then emptied three times as before
- The secondary antibody was added at a dilution of 1:20,000, plate re-covered with the slip, and left at room temperature on a shaker (setting #5) for one hour without shaking
- The plate was removed and washed with PBS-T and then emptied three times as before
- 125µL of 1-Step ABTS was added to each well, re-covered with the slip, and left at 37°C for one hour
- The plate was read at dual wavelengths of 405nm and 450nm using a plate reader (Bio-rad microplate reader, Hercules, CA) to determine optical density (OD) values

2.2.3.c. Results of AAV ELISA Development

The AAV ELISA was optimized using a range of control dog sera and virus concentrations (Figure 2.5). The optimal virus concentration was determined using the AAV-GFP immunized dogs. The optimal virus concentration ranged between $2.8x10^{11}$vp/mL and 100,000vp/mL. A range of positive control serum dilutions were used in order to determine the optimal serum dilution for the test

sera. The final assay design utilized 100,000vp/mL and a 1:5 serum dilution since this used the least amount of virus per well while resulting in high enough OD values to analyze the anti-AAV antibody level with little worry of a prozone effect occurring.

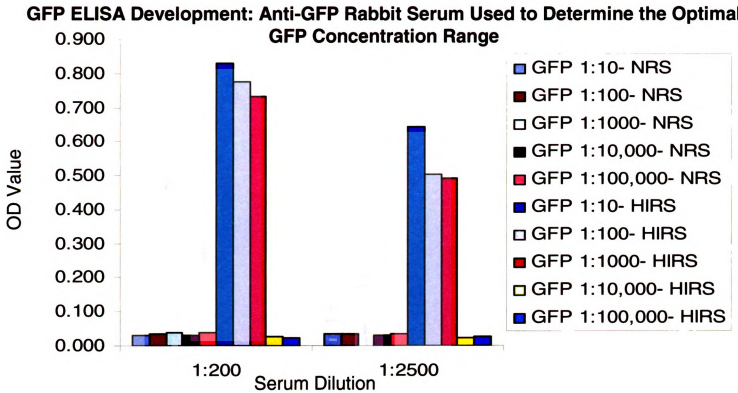


Figure 2.2. This graph depicts the use of anti-GFP rabbit serum in order to determine the range of GFP concentration that would be optimal for the dog-specific ELISA.
NRS= normal rabbit serum, HIRS=hyper-immune rabbit serum.

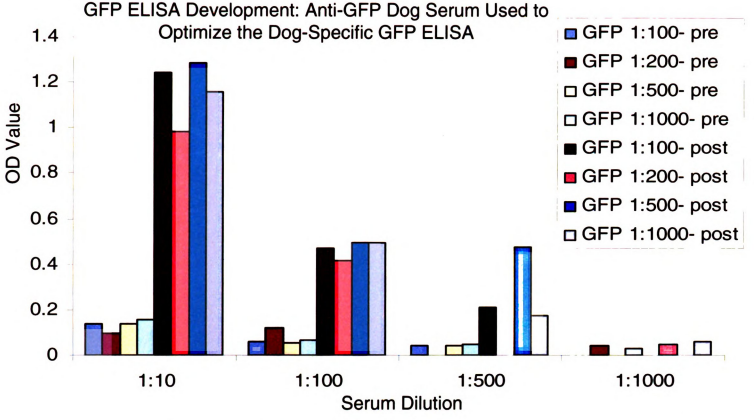


Figure 2.3. This graph shows the optimal GFP concentration based on sera from dogs immunized with GFP expressing lentivirus. pre= negative control sera , post= positive control sera

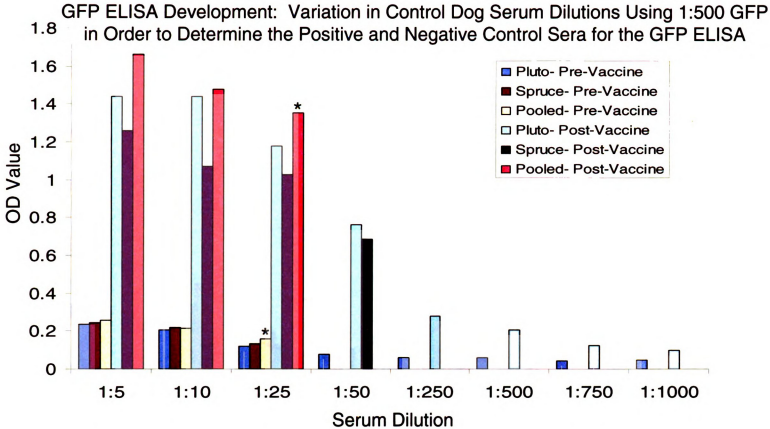


Figure 2.4. This graph shows the positive control sera dilutions used to determine the optimal concentration to serve as a plate control. Pooled= Spruce + Pluto mixed 1:1. * = dilution chosen

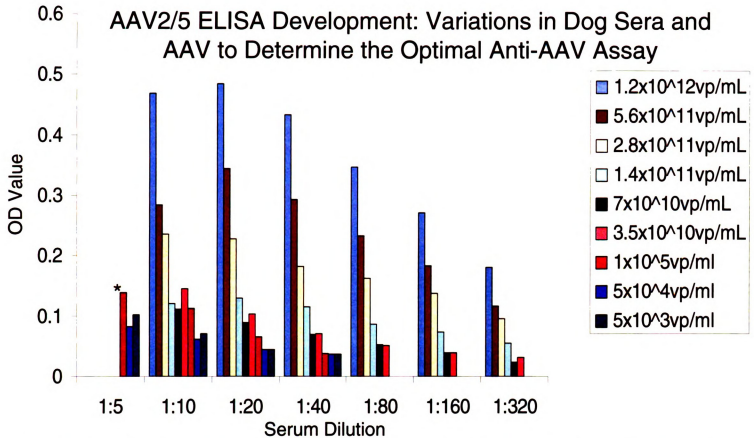


Figure 2.5. This graph shows the sera and virus dilutions used to optimize the AAV ELISA. * = viral concentration and serum dilution that was found to be optimal based on virus availability and OD value.

Chapter 3

HUMORAL IMMUNE RESPONSES DETECTED IN DOGS GIVEN INTRAOCULAR TREATMENT OF VIRAL VECTOR

3.1. Introduction

The humoral immune response induced from three different viral vectors after intraocular administration was investigated. AAV-GFP vectors were studied extensively since the AAV vectors have been most commonly used for intraocular gene therapy. Systemic and intraocular immune responses to both GFP and the AAV capsid were investigated. Lentivirus and Av-1 vectors were being assessed as potential vectors for ocular gene therapy by using the GFP reporter transgene. The anti-GFP response was monitored in dogs tested with these vectors.

3.1.1. Intraocular Injections

In puppies after eyelid opening a subretinal injection was made under direct visualization using an operating microscope via a transvitreal approach. Subretinal injections were performed on puppies approximately 3 weeks of age or older while transscleral injections were performed on neonatal pups 3 to 8 days of age. In neonates the eyelids are fused shut and the cornea is not yet clear so injection under direct visualization is not possible. The neonatal pups have immature retinas and the aim of the injection was to reach the developing neurosensory retina to introduce the transgene at an early stage of retinal development. This timing may be required for very early-onset retinal

dystrophies. The precise site of injection, subretinal, intraretinal, or intravitreal, cannot be visualized when transscleral injections are performed prior to eyelid opening.

3.1.2. Anesthesia

Anesthesia was induced in three day old puppies with isoflurane (Abbott Laboratories, North Chicago, IL) delivered by oxygen mask and anesthesia was maintained in the same manner. For three week old puppies, induction was by masking with isoflurane; they were then intubated and maintained at a surgical level of anesthesia with isoflurane (Abbott Laboratories, North Chicago, IL) delivered in oxygen. For dogs older than 9 weeks of age, intravenous thiopental sodium (Pentothal®, 6-12 mg/kg) was used to induce anesthesia; dogs were then intubated and maintained using isoflurane (Abbott Laboratories, North Chicago, IL) delivered in oxygen for the rest of the procedure. A pulse-oximeter (Vet/Ox 4400, Heska Corporation, Fort Collins, CO) was used to monitor the animal while under anesthesia, recording both blood oxygenation and pulse rate. A heating pad was used to maintain the dog's body temperature. Pulse rate, oxygen saturation, and body temperature were recorded every 5 minutes during the injection procedure.

3.1.3. rAAV2/5, Lentivirus, and Av-1

rAAV2/5 vectors with one of two different promoters driving the GFP transgene expression were used; one promoter was the chicken-beta actin promoter (AAV-

CBA-GFP) which results in ubiquitous expression of the transgene and the other was the mouse opsin promoter (AAV-mOPS-GFP) which results in photoreceptor-specific expression of the transgene. Both vectors were used in both neonate and 3 week old puppies. These rAAV vectors were created in the laboratory of William Hauswirth (University of Florida). The protocol for creation of these vectors has been published (Zolotukhin et al., 2002).

The Av-1 vector was a serotype 5 vector that was used at a concentration of 3x10^12 vp/mL (Amalfitano Laboratory, Michigan State University).

The lentivirus vector (pHR'-CMV-GFP, Planelles, University of Utah) was used at a concentration of 10^7 to 5x10^7 infectious units`(Zhu et al., 2001: Planelles, 2003).

3.1.4. Injection Technique Used in Puppies Where the Eyelids Have Opened

Subretinal injections were performed by Simon Petersen-Jones under aseptic surgical conditions. A subretinal injector as seen in Figure 3.1. (RetinaJect Injector, Surmodics, Irvine, CA), with Hamilton syringe (Hamilton Company, Reno, Nevada) and loaded with the vector solution, was used to enter the eye through the sclera (Figure 3.2.). The injector was positioned just anterior to the retina in the tapetal region dorsal to the optic disc.

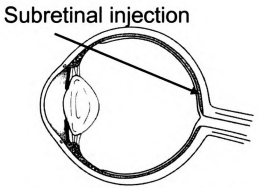


Figure 3.1. Path taken by the injector for the subretinal injection procedure. The arrow indicates the area of injection, the subretinal space between the RPE and photoreceptor layers of the retina.

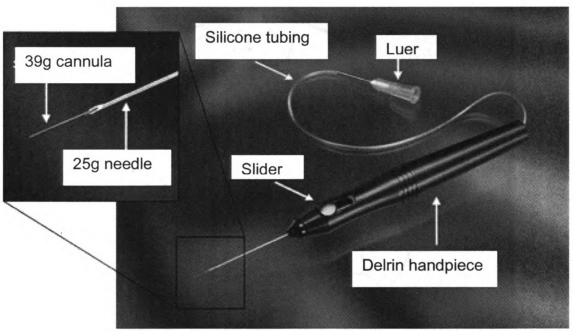


Figure 3.2. Subretinal injector (RetinaJect Injector, Surmodics, Irvine, CA) used to perform the subretinal injections.
(http://www.surmodics.com/pageDetail.aspx?pageId=93&menuId=95, 3/6/07)

The small internal cannula inside the injector was then advanced so that it was pressed against the retinal surface. The vector was then injected and the force of the injected fluid created a retinotomy allowing the fluid, ~50µL, to pass into the subretinal space resulting in a retinal detachment (see Figure 3.3. for fundus

photographs showing before and after injection). The cannula was withdrawn into the needle and the injector withdrawn from the eye.

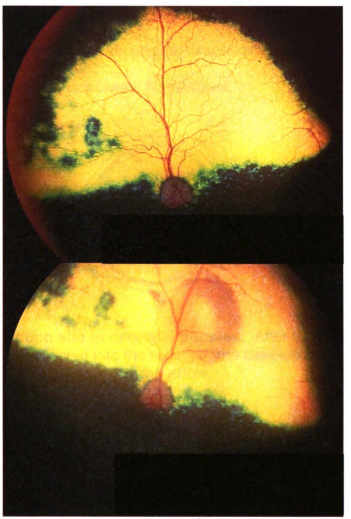


Figure 3.3. Before and after subretinal injection in a dog's eye. The top image is prior to injection. In the bottom image, a retinal detachment is evident in the tapetal fundus dorsal to the optic disc. This is the site of subretinal injection.

3.1.5. Injection Technique Used in Neonates

These injections were performed by Simon Petersen-Jones on pups prior to eyelid opening. A small area on the upper eyelid was cleaned with diluted betadine, and a small skin incision was then made. Blunt dissection of the area

was performed using tenotomy scissors until the sclera of the eye was identified. A small incision was made through the sclera posterior to the ciliary body region to expose the choroid. A 30g blunt cannula was introduced through the choroid and an injection was made, On average, a 25µLvolume of vector was injected into the eye (Figure 3.4.). The subcuticular tissues and skin incision were closed using 6-0 vicryl (Ethicon, Piscataway, NJ) sutures.

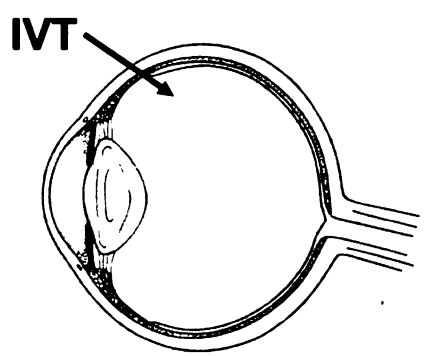


Figure 3.4. Injection site in neonatal puppies. After incision into the eye globe, the vector was injected into the region of the developing retina as is shown by the arrow.

3.1.6. Dogs Used in Study and Sample Collection

The dogs used in this study were from a purpose-bred colony of mixed and purebred (Cardigan Welsh Corgis and Briards) dogs at Michigan State University. All animals were treated with care under IACUC specifications. See Tables 3.1 and 3.2 for a list of dogs in the AAV anti-GFP study and in the anti-AAV study, respectively. Adult dogs that underwent intraocular lentivirus (Planelles, University of Utah) treatments included Haley, Comet, and Jackie. All dogs were treated with 50µL of the vector through subretinal injection.

One dog underwent intraocular treatment with 50µL of an Av-1 through subretinal injection.

3 Weeks of Age Injections (0-8 week serum samples)				
Dog	*ID Number*	*Eye Injected*	*Vector*	*Injection Type*
Kumquat	8	Left	AAV-CBA-GFP	Subretinal
Lazarus	12	Left	AAV-CBA-GFP	Subretinal
Martha	13	Left	AAV-CBA-GFP	Subretinal*
Magdalene	14	Left	AAV-CBA-GFP	Subretinal*
Mary	15	Left	AAV-CBA-GFP	Subretinal
Andrew	16	Left	AAV-CBA-GFP	Subretinal
Moses	28	Right	AAV-mOPS-GFP	Subretinal
Barbie	29	Left	AAV-mOPS-GFP	Subretinal
3 Days of Age Injections (0-8 week serum samples)				
Dog	*ID Number*	*Eye Injected*	*Vector*	
Jude	10	Right	AAV-CBA-GFP	Transscleral
Bartholomew	11	Right	AAV-CBA-GFP	Transscleral
Steve	26	Right	AAV-mOPS-GFP	Transscleral
Kelly	27	Right	AAV-mOPS-GFP	Transscleral
3 Weeks of Age Injections (0-4 weeks serum samples)				
Dog	*ID Number*	*Eye Injected*	*Vector*	
Chinook	1	Left	AAV-CBA-GFP	Subretinal
Hyde	2	Right	AAV-CBA-GFP	Subretinal
Aramis	3	Left	AAV-CBA-GFP	Subretinal
Frenchy	4	Right	AAV-CBA-GFP	Subretinal
Penny	5	Left	AAV-CBA-GFP	Subretinal
Shaila	6	Left	AAV-mOPS-GFP	Subretinal
Merry	7	Left	AAV-CBA-GFP	Subretinal
Lucy	17	Right	AAV-mOPS-GFP	Subretinal
Marty	19	Left	AAV-CBA-GFP	Subretinal
Tywyn	30	Right	AAV-CBA-GFP	Subretinal

Table 3.1. Colony dogs injected with AAV2/5-CBA-GFP or AAV2/5-mOPS-GFP vectors for the GFP ELISA protocol.
Subretinal* = some vector went intravitreal.

Anti-AAV Dog Serum Samples for Anti-AAV ELISA				
Dog	Number	Eye Injected	Vector	*Injection Type*
Chinook	1	Left	AAV-CBA-GFP	Subretinal
Aramis	3	Left	AAV-CBA-GFP	Subretinal
Frenchy	4	Left	AAV-CBA-GFP	Subretinal
Penny	5	Left	AAV-CBA-GFP	Subretinal
Shaila	6	Left	AAV-mOPS-GFP	Subretinal
Merry	7	Left	AAV-CBA-GFP	Subretinal
Bartholomew	11	Right	AAV-CBA-GFP	Transscleral
Lazarus	12	Left	AAV-CBA-GFP	Subretinal
Martha	13	Left	AAV-CBA-GFP	Subretinal*
Magdalene	14	Left	AAV-CBA-GFP	Subretinal*
Mary	15	Left	AAV-CBA-GFP	Subretinal
Andrew	16	Left	AAV-CBA-GFP	Subretinal
Marty	19	Left	AAV-CBA-GFP	Subretinal
Steve	26	Right	AAV-mOPS-GFP	Transscleral
Kelly	27	Right	AAV-mOPS-GFP	Transscleral
Moses	28	Right	AAV-mOPS-GFP	Subretinal
Barbie	29	Left	AAV-mOPS-GFP	Subretinal
Tywyn	30	Right	AAV-CBA-GFP	Subretinal
Braedon	31	Both	Both	Subretinal

Table 3.2. Colony dogs injected with AAV2/5 vectors that were used for the AAV ELISA protocol. Subretinal* = some vector went intravitreal.

Both aqueous and serum samples were taken from all dogs injected with the GFP vectors. Aqueous samples were collected pre-injection on the day of surgery and then post-injection at the time of euthanasia. Serum samples were collected in two different groups of sampling. The first group of dogs had serum collected on the day of injection and then every two weeks until four weeks post-injection and at the time of euthanasia. The second group of dogs had serum samples taken the day of injection and then every two weeks until eight weeks post-injection and at the time of euthanasia. Several dogs had samples taken

past the eight week limit that were not 'day of euthanasia' serum samples. Serum samples were centrifuged for 30 minutes in at 3500 rpm at room temperature and then stored in 1 mL aliquots at -80°C.

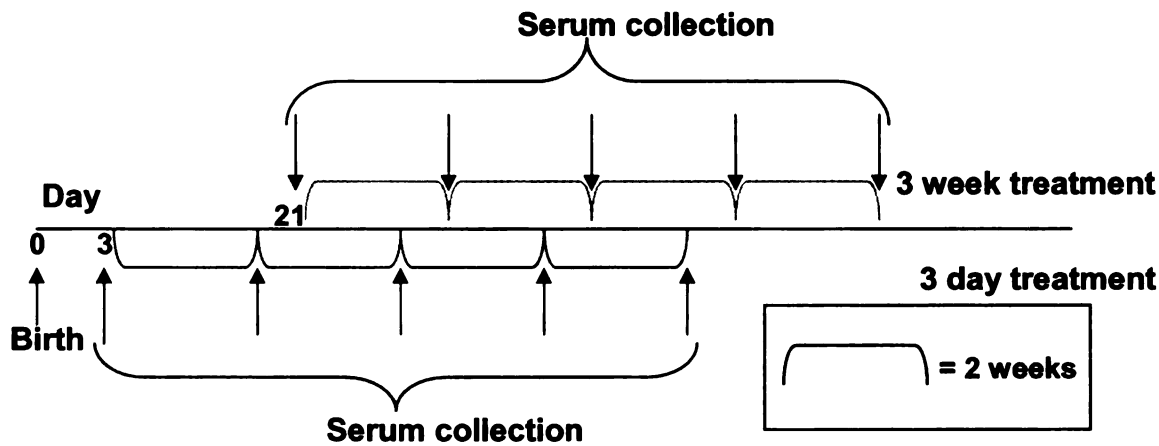


Figure 3.5. Serum and Aqueous Sampling Time Line.

3.1.7. Monitoring GFP Expression

GFP expression in the eye was monitored using a RetCam (Clarity Medical Systems, Inc., Pleasanton, CA). Every two weeks post-injection until sacrifice, all of the injected dog eye(s) were examined under regular white light and then using the fluorescein angiography setting. This setting provides light of a wavelength to cause fluorescein to fluoresce and a barrier filter in the viewing pathway to enhance the visualization of the fluorescence. The images were used to observe the GFP fluorescence after intraocular injection and were stored for further assessment at a later date (Figure 3.6.).

Figure 3.6. GFP expression was monitored using a RetCam. In the top image, a white light image of the fundus in which the retina appears normal with a small trace of a subretinal injection scar. In the bottom image, the fluorescein angiography capabilities of the RetCam are used to view GFP expression.

3.1.8. Data Analysis using SAS

Statistical Analysis Software (SAS) was used to analyze data sets. All data sets (AAV-GFP, Av-1, and lentivirus) were analyzed separately.

The anti-GFP AAV-GFP serum and aqueous data sets were not normally distributed but were still analyzed using a 'mixed' model analysis. The statistical model to analyze this data set included fixed effects of injection age, injection method, and promoter. The dog (based on ID number) was the random effect for the model. The positive and negative control sera were used as covariates in the analysis in order to account for individual plate variations. The least squares mean (LSM) antibody level was reported for pre- and post-treatment, time points collected, injection method, injection age, and promoter. All 3 week serum samples were included in the 4 week serum sample category for this analysis.

The anti-AAV data set was not normally distributed but was still analyzed using a 'mixed' model analysis. The statistical model to analyze this data set included fixed effects of injection age and injection method. The dog (based on ID number) was the random effect for the model. The assay date was used to account for plate variation. The LSM antibody level was reported for pre- and post-treatment, time points collected, injection method, and injection age.

The anti-GFP lentivirus data set was normally distributed and was analyzed using a 'mixed' model analysis. The dog (based on ID number) was the random effect for the model. The positive and negative control sera were used as covariates in the analysis in order to account for individual plate variations. The LSM antibody level was reported for pre- and post-treatment and time points collected.

The anti-GFP Av-1 data set included only one dog for analysis and thus was not analyzed using SAS. All original values were reported.

A 'PROC CORR' analysis was used to compare the OD values of two data sets: anti-AAV and anti-GFP AAV-GFP. The correlation value was reported.

A 'PROC CORR' analysis was used to compare the OD values of two data sets: Anti-GFP AAV-GFP aqueous OD values and anti-GFP AAV-GFP serum ODs. The correlation value was reported.

A p-value of <0.05 was considered significant for all statistical analyses performed.

3.2. Results

Two ELISAs were developed in this work and used to determine if gene therapy administered in the canine eye induced local and/or systemic antibody responses either to the therapy gene and/or the virus vector. In this study, GFP was used as a reporter gene, and AAV, Av-1, and lentivirus were the vectors for subretinal and intravitreal injection methods. Thus, ELISAs developed utilize either GFP or AAV viral capsid as antigens. The GFP ELISA was used for the samples collected from each dog in order to detect a systemic humoral response to GFP after treatment with AAV-GFP, Av-1, and lentivirus. The AAV ELISA was used for the serum samples in order to detect a systemic humoral response to the AAV viral capsid.

3.2.1. Presence of Circulating Anti-GFP Antibodies after Intraocular Treatment with AAV-GFP

Three serum dilutions were tested using the GFP ELISA previously described (Figure 3.7.). All dilutions followed a parallel pattern and therefore serum dilution 1:5 is reported. The least squares mean (LSM) was reported for each dilution; therefore, a negative value may be reported for the pre-treatment sera since the mean has been calculated using the positive and negative covariates from each plate.

The anti-GFP antibody response was found to vary between individual dogs. A high anti-GFP level, comparatively, was observed in 4 out of the 22 dogs tested

(Figure 3.8.). Overall, there was a statistically significant systemic humoral response detected to GFP after intraocular injection of the AAV-GFP viral vectors with a p-value of 0.0029 for the comparison of pre- versus all post-treatment time points (Figure 3.9.).

Time points analyzed in this assay were: 0, 2, 4, 6, 8, and >8 weeks post-injection (Figure 3.10.). Using paired t-tests, antibody levels for time points 4 weeks post-injection showed a significant difference from zero (p= 0.0409). The results from samples collected at 6, 8, and >8 weeks post-injection were not significant (p= 0.1038, p= .190, and p= 0.115, respectively) based on the LSM compared to zero. Overall, the trend observed in dogs was a detectable immune response at 4 weeks post-treatment and was found to decrease starting at 6 weeks post-treatment. One dog that was monitored long-term had an antibody response that was maintained up to 26 weeks post-injection.

Additional independent variables included in the statistical model were: promoter (CBA vs. mOPS) and injection age (3 days vs. 3 weeks). The animals treated with the AAV-CBA-GFP vector had a significant antibody response while those treated with the AAV-MOPS-GFP construct did not. Dogs treated at 3 weeks of age had a significant anti-GFP antibody response while the group at 3 days of age did not (see Table 3.3).

3.2.2. Presence of Anti-GFP Antibodies in Aqueous after Intraocular Treatment with AAV-GFP

An aqueous dilution of 1:20 was used for assays for two reasons: 1) limited sample volume and 2) higher aqueous concentrations did not result in an increased antibody observation capability based on sample trials (not shown).

The anti-GFP antibody response was found to vary between individual dogs with some dogs having an aqueous anti-GFP response while others did not. Overall, there was not a significant local humoral response detected to GFP after intraocular injection of the AAV-GFP viral vectors with a p-value of 0.0667 for the comparison of pre- versus post-treatment samples (Figure 3.11.). Based on euthanasia schedules, a range of 13 to 96 weeks post-injection aqueous samples were analyzed with an equally wide range of individual antibody responses observed (Figure 3.12.).

Two independent variables were analyzed including: promoter (CBA vs. mOPS) and injection age (3 days or 3 weeks). Unlike the case for serum anti-GFP antibody responses, none of these variables were shown to have a significant effect on the local immune response in the eye (Table 3.4.).

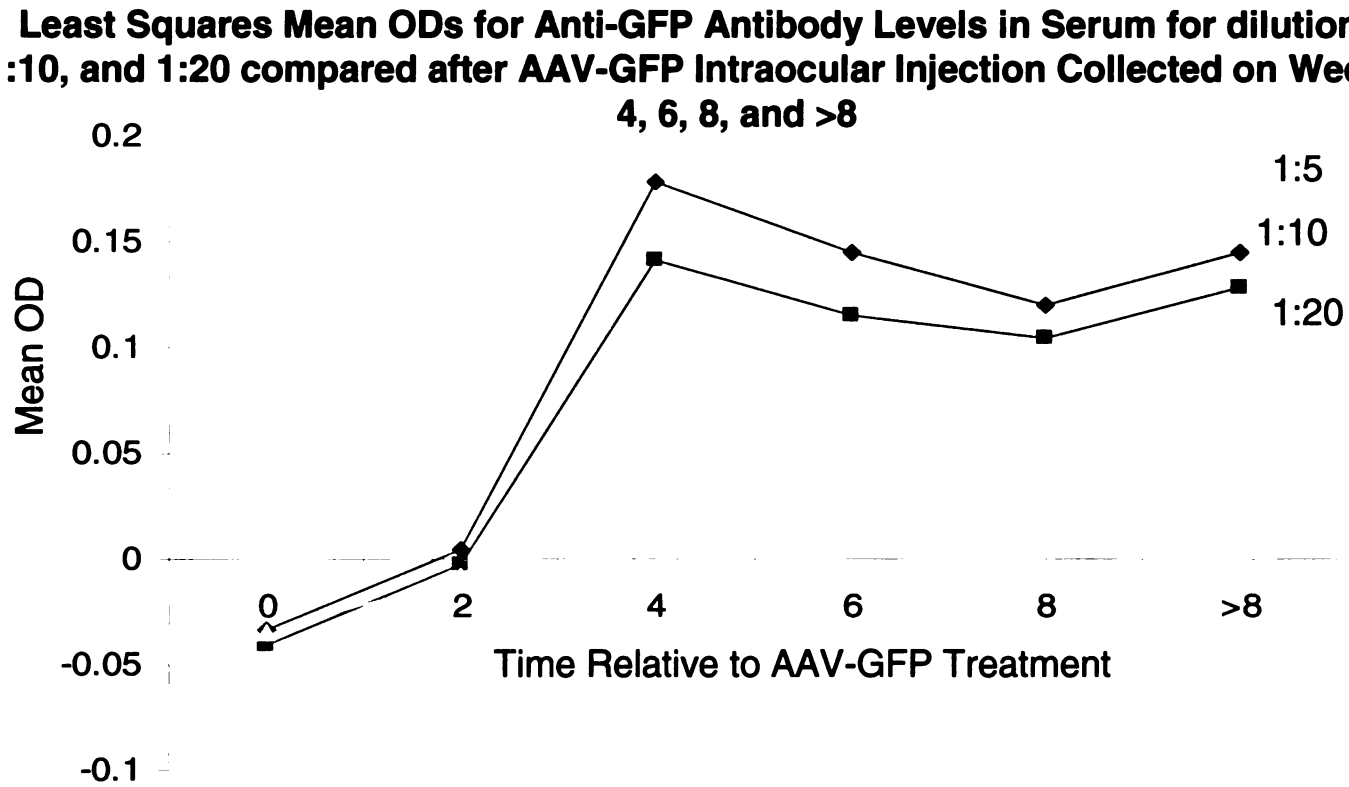


Figure 3.7. This graph illustrates the three sera dilutions used for the GFP ELISA from AAV-treated dogs to demonstrate that there is a parallel relationship between the dilutions. For further analysis, only the 1:5 dilution was utilized.

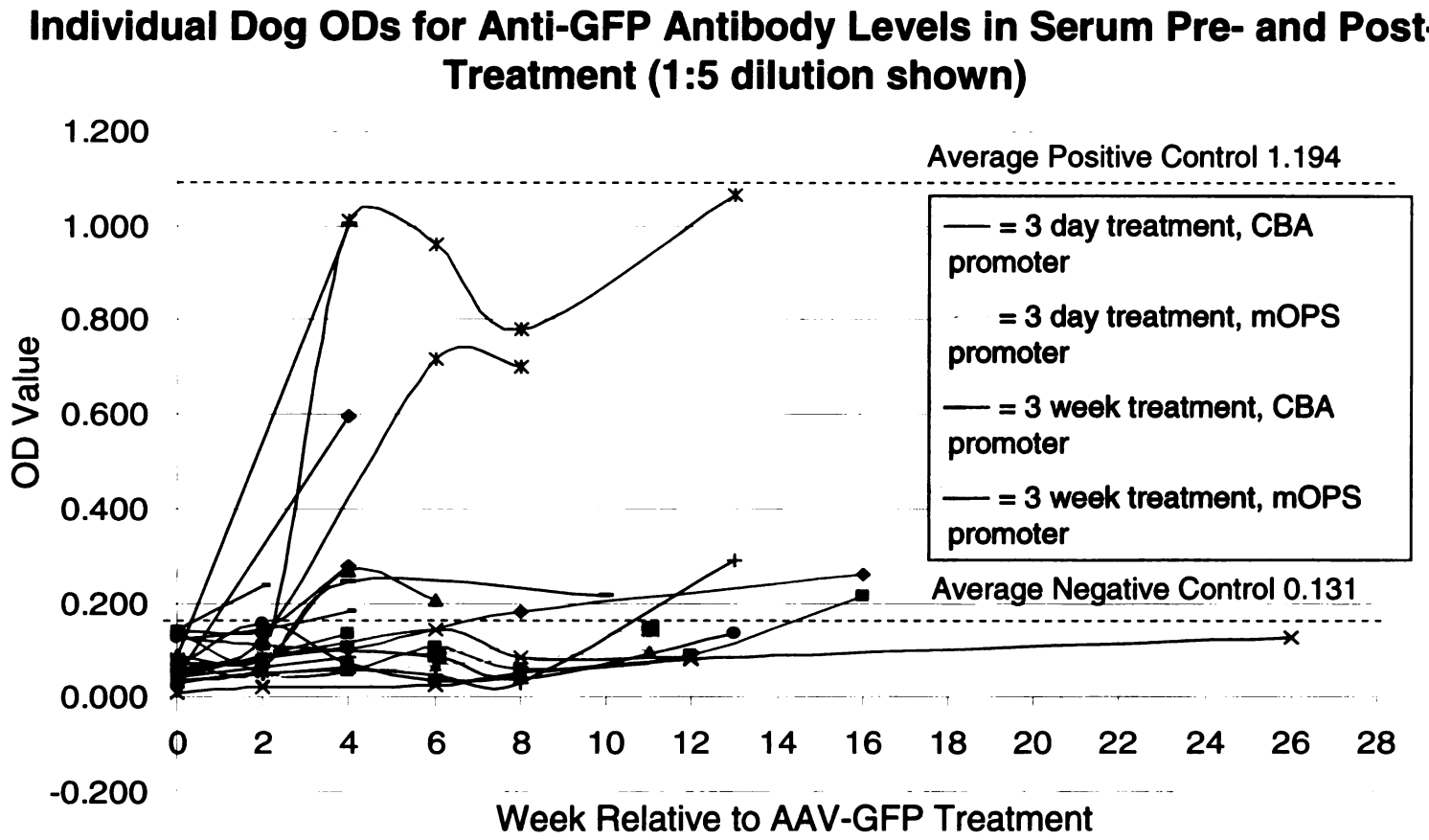


Figure 3.8. This graph shows the raw OD values for sera from the GFP ELISA for each individual dog after intraocular treatment with AAV vector.

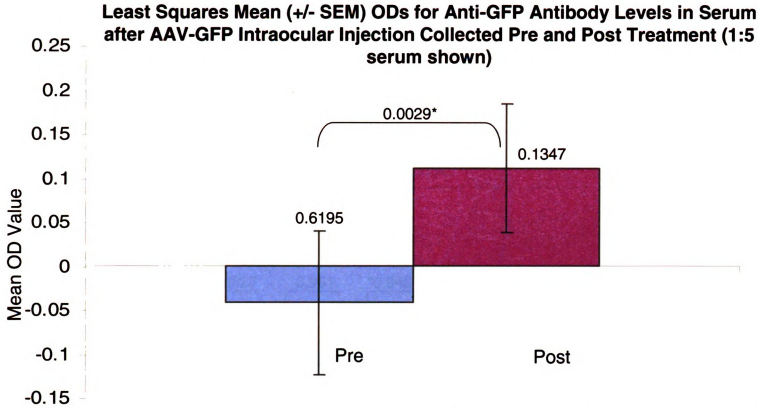


Figure 3.9. The p-value for each bar is labeled and indicates the significance based on comparison of the LSM to zero. The overall p-value based on comparison of pre- and post-treatment OD values is labeled above with a bracket for bars shown . *= statistically significant.

Least Squares Mean (+/- SEM) ODs for Anti-GFP Antibody Levels in Serum after AAV-GFP Intraocular Injection Collected on Weeks 0, 2, 4, 6, 8, and >8 (1:5 serum shown)

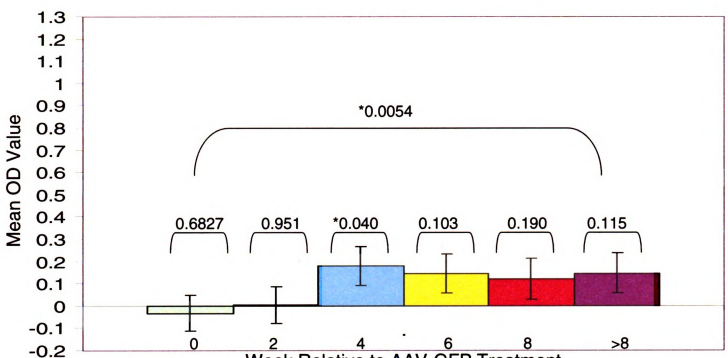


Figure 3.10. The p-value for each bar is labeled and indicates the significance based on comparison of the LSM to zero. The overall p-value based on the independent variable of time is labeled above with a bracket for bars shown . *= statistically significant.

Table 3.3.

Least Squares Means of GFP ELISA OD Values from Dog Serum Samples (1:5) for Independent Variables				
*=stat. sig.	Promoter (p=0.1683)		Injection Age (p=0.2775)	
	CBA	MOPS	3 days	3 weeks
LS Mean	0.169	0.01805	-0.06482	0.2518
Standard Error	0.05866	0.1137	0.2509	0.09886
p- value (mean different from zero)	0.0058*	0.8745	0.7542	0.0139*

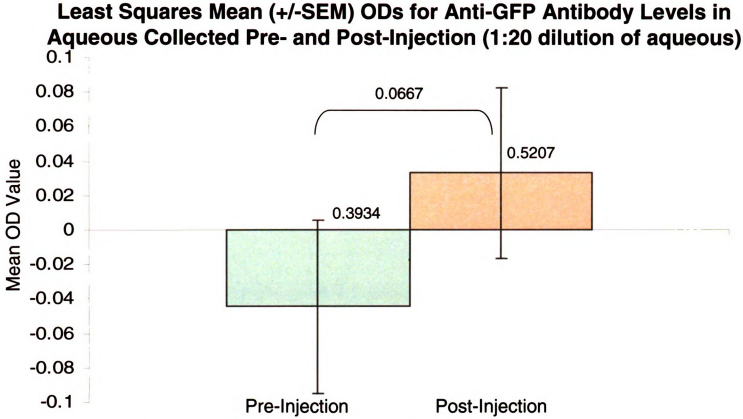


Figure 3.11. The p-value for each bar is labeled and indicates the significance based on comparison of the LSM to zero. The overall p-value based on comparison of pre- and post-treatment OD values and is labeled above with a bracket for bars shown . *= statistically significant.

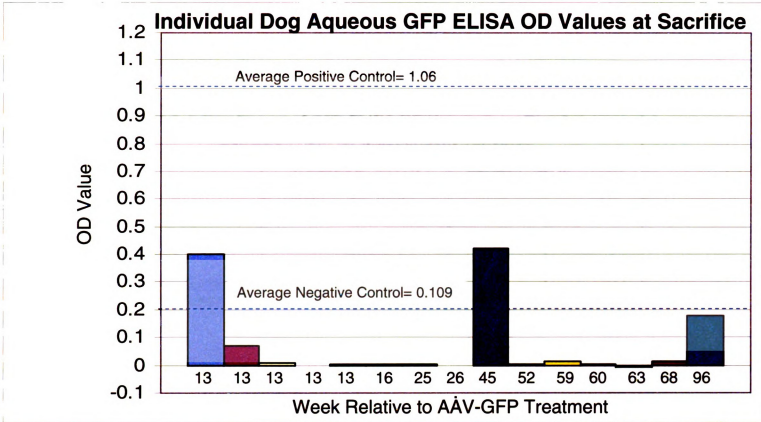


Figure 3.12. This graph shows the raw OD values from aqueous (GFP ELISA) for each individual dog after intraocular treatment with AAV vector.

Table 3.4.

Least Squares Means of GFP ELISA OD Values from Dog Aqueous Samples (1:20) for Independent Variables				
	Promoter (p=0.5534)		Injection Age (p=0.9648)	
	CBA	MOPS	3 weeks	3 days
LS Mean	0.02215	-0.03401	-0.00803	-0.00383
Standard Error	0.04706	0.07957	0.07581	0.05388
p- value (mean different from zero)	0.6463	0.6766	0.9174	0.9444

3.2.3. Correlation between Anti-GFP Antibody Responses in Serum and Aqueous Samples

A correlation analysis (PROC CORR) was performed in SAS for the GFP ELISA results between the serum and aqueous samples for 17 dogs (Figure 3.13.). A correlation (R^2) of 0.30062 was observed based on this analysis with a p-value of 0.2255; therefore, a relationship between the systemic and local antibody responses against the GFP reporter gene was not determined from this sample of dogs. One dog had a high level of both aqueous and circulating anti-GFP antibodies at euthanasia (seen in Figure 3.13.). The data were analyzed with and without this dog's data, which was removed for the final analysis since they were found to significantly bias the analysis.

3.2.4. Presence of Circulating Anti-AAV Antibodies after Intraocular Treatment with AAV-GFP

A single dog out of the 19 dogs tested had a high anti-AAV OD value. The data were analyzed both with and without this dog included, and the dog was found to bias the data set and was therefore removed. Overall, there was a statistically significant systemic anti-AAV antibody level after intraocular injection of the AAV-GFP viral vectors with a p-value 0f 0.0157 for the comparison of pre- versus all post-treatment time points (Figure 3.14). Time points analyzed were: 0, 2, and 4 weeks post-treatment (Figure 3.15). Overall, the trend observed started with a significant immune response detectable at 2 weeks post-treatment which then tapered off slightly at 4 weeks post-treatment.

Additional independent variables were included in this statistical analysis which included: promoter and injection age (3 days vs. 3 weeks). Neither of the variables was observed to significantly influence the anti-AAV antibody response (Table 3.5.).

3.2.5. Correlation between Anti-AAV and Anti-GFP Antibodies in the Serum

A correlation analysis (PROC CORR) was performed in SAS for the GFP ELISA and AAV ELISA results for serum samples at time points 0, 2, and 4 weeks post-treatment (Figure 3.16.). A correlation (R^2) of 0.07859 was observed based on this analysis with a p-value of 0.6390; therefore, no relationship between the systemic anti-AAV and anti-GFP was detected in this sample of dogs.

3.2.6. Presence of Circulating Anti-GFP Antibodies after Intraocular Treatment with Lentivirus

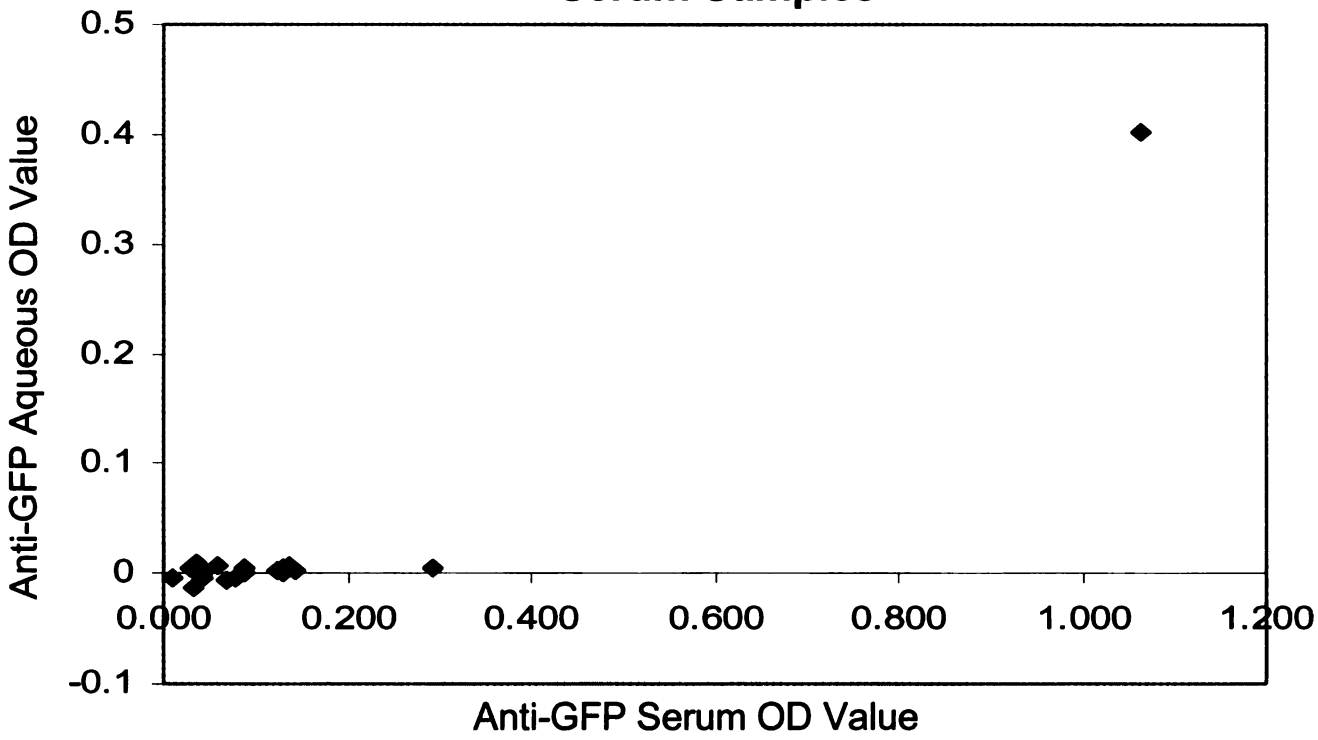
Overall, there was a systemic humoral response detected to GFP after intraocular injection of the lentivirus viral vectors with a p-value of 0.0200 for the comparison of pre- versus all post-treatment time points (Figure 3.17.).

Individual time points analyzed in this assay were: 0, 2, 4, 6, and 8 weeks post-injection (Figure 3.18.). Using paired t-tests, antibody levels for time points 2, 4, 6, and 8 weeks post-injection showed a significant difference. Overall, the trend observed starts with the largest peak at 2 weeks which then tapers off slowly until 8 weeks post-treatment.

3.2.7. Presence of Circulating Anti-GFP Antibodies after Intraocular Treatment with Av-1

One dog was treated with Av-1 in order to study the potential systemic antibody response to GFP. Time points analyzed in this assay were 0, 2, and 3 weeks post-injection using a 1:20 serum dilution since the 1:5 and 1:10 serum dilution ODs were influenced by a prozone. At 0 weeks, the OD value was 0.116 while at 2 and 3 weeks post-treatment the OD values were 1.055 and 1.078, respectively. Overall, the antibody response observed was high at 2 weeks post-treatment and increased further at 3 weeks post-treatment (at which time the trial was terminated).

Figure 3.13. Correlation of Anti-GFP ELISA OD Values in Aqueous and Serum Samples



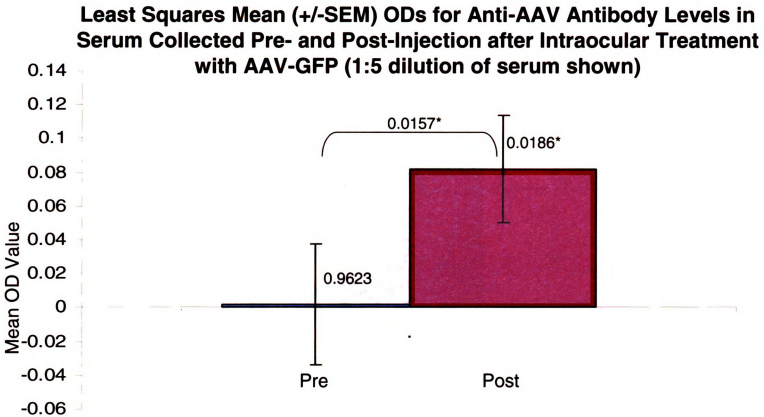


Figure 3.14. The p-value for each bar is labeled and indicates the significance based on comparison of the LSM to zero. The overall p-value based on the comparison of pre-and post-treatment is labeled above with a bracket for bars shown . *= statistically significant.

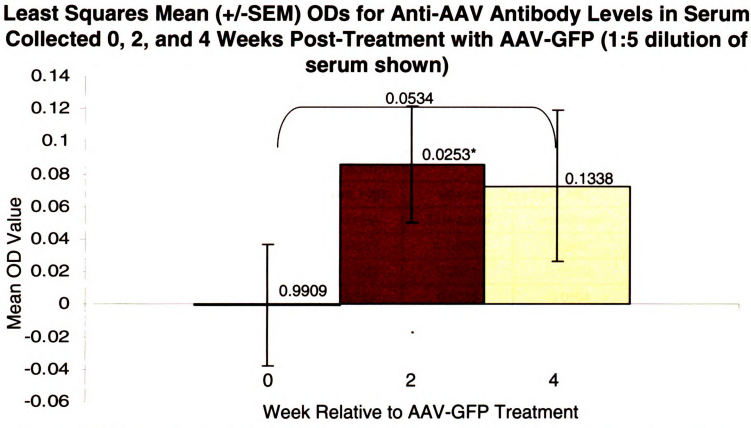
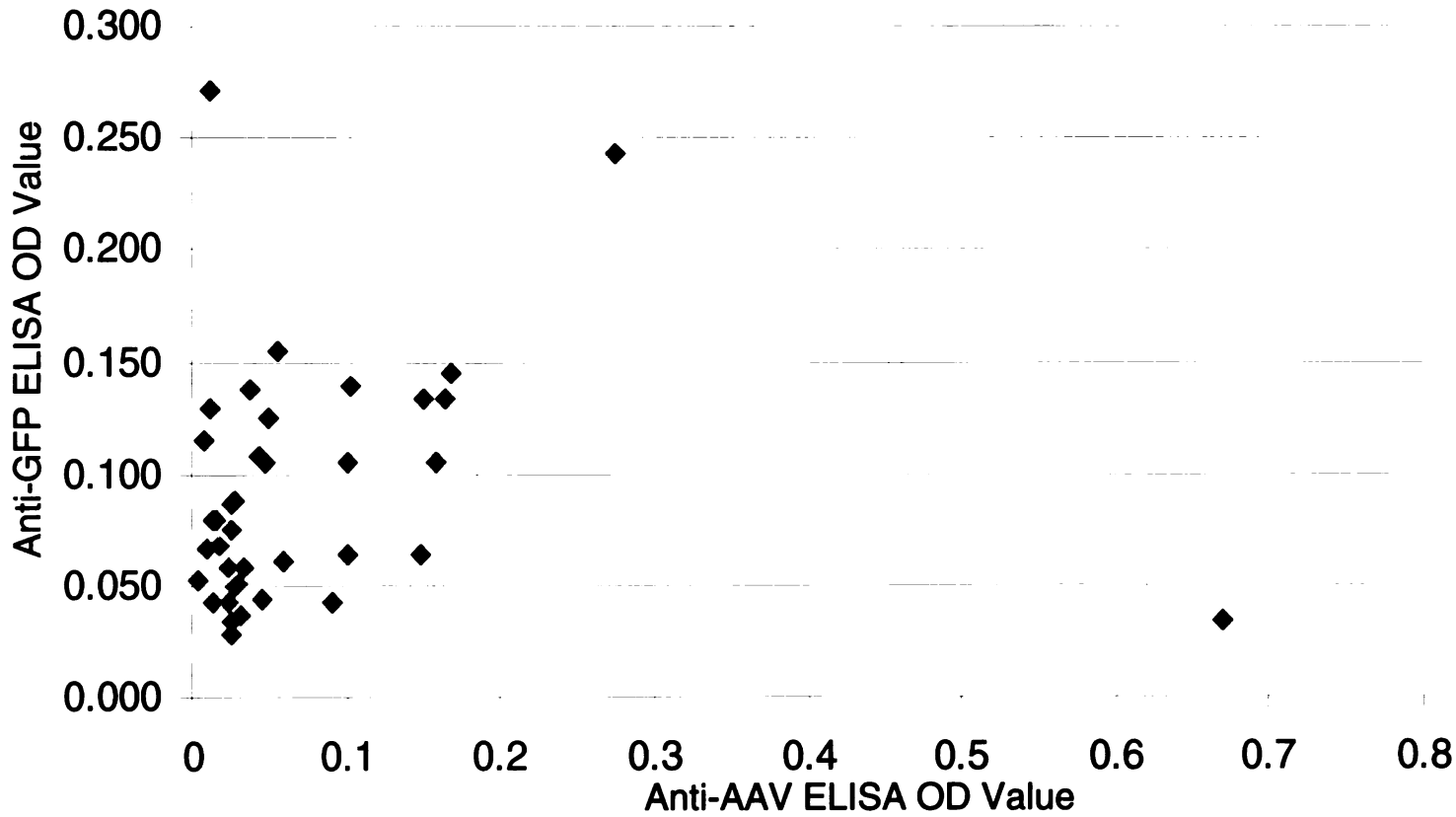


Figure 3.15. The p-value for each bar is labeled and indicates the significance based on comparison of the LSM to zero. The overall p-value based on the independent variable of time is labeled above with a bracket for bars shown . *= statistically significant.

Table 3.5.

Least Squares Mean of AAV ELISA OD Values for Dog Serum Samples				
	Injection Time (p=0.7053)		Injection Type (p=0.367)	
	3 days	3 weeks	Subretinal	Intravitreal
LS Mean	0.02263	0.08289	0.03683	0.0687
SEM	0.04392	0.04692	0.0661	0.03288
P-value	0.6123	0.0934	0.5839	0.0504

Figure 3.16. **Correlation of Anti-AAV and Anti-GFP ELISA OD Values from Serum Samples**



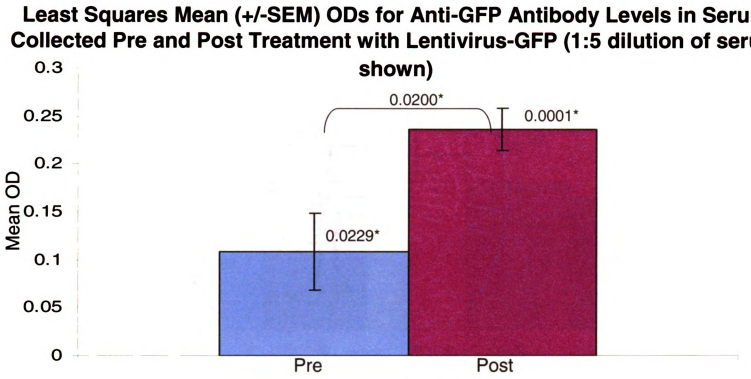


Figure 3.17. The p-value for each bar is labeled and indicates the significance based on comparison of the LSM to zero. The overall p-value based on the comparison of pre- and post-treatment LSM is labeled above with a bracket for bars shown . *= statistically significant.

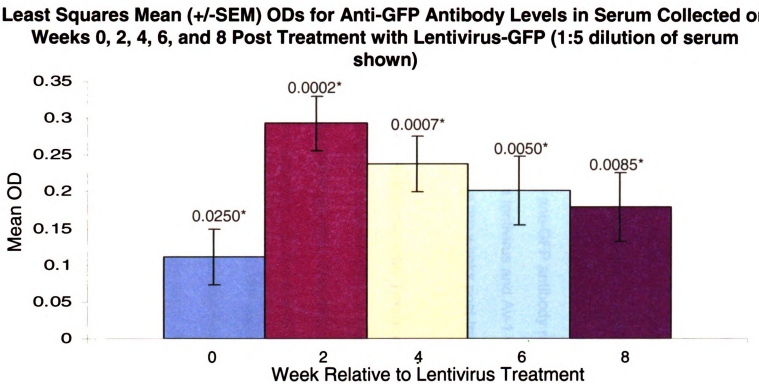


Figure 3.18. The p-value for each bar is labeled and indicates the significance based on comparison of the LSM to zero. The overall p-value based on the independent variable of time is labeled above with a bracket for bars shown . *= statistically significant.

3.3. Discussion and Future Work

3.3.1. Discussion and Summary of Results

Circulatory anti-GFP and anti-AAV antibodies were observed in some but not all of the dogs treated with the AAV-GFP vector. The single dog treated with the Av-1 vector had a high immune response to GFP and the lentivirus treated dogs all maintained a circulatory antibody response. Variations in antibody response differed between vectors based on time of onset and level of antibodies detected. The AAV-GFP vectors induced an anti-GFP antibody response approximately 4 weeks post-treatment whereas the lentivirus and Av-1 vectors induced an anti-GFP antibody response as soon as two weeks post-treatment.

3.3.1.a. Anti-GFP Antibody Response after Treatment with AAV-GFP

As we hypothesized, an anti-GFP humoral immune response was detectable in serum as early as 4 weeks post-treatment. This delay in development of a humoral immune response correlates with the relatively slow onset of transgene expression typical of AAV vectors. The anti-GFP antibody response was also detectable up to 26 weeks post-treatment (the last time point that was collected). An anti-GFP antibody response could be detected in post-treatment aqueous samples as late as 96 weeks post AAV-GFP treatment.

3.3.1.b. Anti-AAV Antibody Response after Treatment with AAV-GFP

The AAV ELISA detected an anti-AAV humoral immune response in serum samples from some of the treated dogs, and this response was first detected 2

weeks post-injection. The antibody response was detected two weeks sooner than for GFP, as would be expected since the antigen is present at injection and does not require transgene expression to be occur. The antibody response was low compared to the anti-GFP response but was still significant over the time points analyzed.

3.3.1.c. Discussion of Results from AAV Treated Dogs

Unlike other studies, we were able to study the antibody response to GFP in enough detail to understand the approximate onset of the anti-GFP antibody response and also duration of up to 26 weeks for serum and 96 weeks for aqueous samples. One area of interest in this study was the variation in anti-GFP antibody responses between individual dogs. Previous studies have observed an antibody response to GFP in both serum and aqueous post-treatment samples after intraocular treatment of AAV-GFP (Bennett et al., 1999; Dudus et al., 1999). In those studies, 2 out of 4 and 1 out 3 mice, respectively, had a similar circulating high level of anti-GFP antibodies after intraocular treatment with AAV-GFP (Bennett et al., 1999; Dudus et al., 1999). Specific for the AAV-GFP treated dogs in this study, two independent variables in the study were found to significantly influence the anti-GFP response: the CBA promoter led to a greater immune response compared to the mOPS promoter and injections given at 3 weeks of age resulted in a greater response than those given at 3 days of age.

Since the eye is an immune-privileged environment, antibodies would be unable to cross the blood-retinal barrier due to the size of the molecule; therefore, an intraocular immune response has been explored. Recently, the iris was found to produce a homing effect which led monocytic bone marrow cells to the iris which then participated in a local immune response (Li et al., 2006). Furthermore, the iris monocytic cells may participate in the systemic induction of regulatory T cells; thus, systemic viral transduction may not need to occur to induce a systemic humoral response.

Viral escape from the eye could potentially allow for viral systemic transduction of host organs and tissues. Virus escape could occur through many avenues which include escape through the drainage apparatus as well as escape through a degraded blood-retinal barrier. Upon aqueous sampling from the eye, breakdown of the blood-retinal barrier occur this could allow virus to escape into the bloodstream (Strubbe and Gelatt, 1998). Intravitreal or subretinal injection can also allow viral vectors to reach the central visual pathway. The highly significant influence of the CBA promoter on the anti-GFP response could be because the mOPS promoter limits expression of GFP to the photoreceptor; thus, if the viral vector construct reached other tissues, GFP expression would not have occurred in those tissues, whereas with the CBA promoter it would. The CBA promoter is a ubiquitously expressed promoter whereas the mOPS promoter is photoreceptor-specific.

The influence of the age of 3 weeks at the time of treatment is also fascinating since the pup's immune system at 3 days and 3 weeks of age are different. At 3 days of age, the pup's immune system is less mature and is more dependent on the passive immunity from mother's colostrum and is in the process of recognizing "self" (Tizard, 2004). By three weeks of age, the pup is starting to lose maternal antibodies and therefore its immune system is more able to recognize and produce antibodies to foreign antigens. Also to consider, since only an IgG antibody response was analyzed, genetic variability between dogs may account as well for the individual response observed between dogs.

3.3.1.d Correlation Analyses

No correlation was observed between the anti-AAV antibody response and the anti-GFP response after intraocular treatment of AAV-GFP. The other correlation, between the anti-GFP antibody responses in serum and aqueous samples, also showed no significant relationship. A small number of euthanasia serum and aqueous samples were collected from a large range of time points post-treatment and therefore could account for the lack of correlation observed.

3.3.1.e. Anti-GFP Antibody Response after Treatment with Lentivirus

The serum samples from dogs treated with lentivirus underwent analysis using the GFP ELISA. As we hypothesized, an anti-GFP systemic humoral immune response was detectable in serum from all dogs as early as 2 weeks post-treatment up to 8 weeks post-treatment (the last time point at which samples

were collected). These results showed the highest antibody level at 2 weeks post-treatment with a slow decrease over the 8 week time period. These results are limited since the serum samples were collected every two weeks, and thus an antibody response may be detectable sooner than 2 weeks post-treatment due to fast expression of the GFP transgene. Further work should be done in order to understand in more detail the antibody response produced against the virus as well as the transgene.

3.3.1.f. Anti-GFP Antibody Response after Treatment with Av-1

The serum sample from the dog treated with Av-1 underwent analysis using the GFP ELISA. This vector results in rapid, strong GFP expression within 24 hours post-treatment and also induces some degree intraocular inflammation. As we hypothesized, an anti-GFP systemic humoral immune response was detectable in serum at 2 weeks post-treatment and remained high at 3 weeks post-treatment (the last time point at which samples were collected). More work should be done using this vector in order to understand the antibody response in dogs and it should also be studied over a longer period of time.

3.3.1.g. AAV-GFP, Lentivirus, and Av-1 Antibody Responses Compared

Each of the vectors induced a different anti-GFP response after intraocular treatment (Figure 3.19.). The AAV vectors, overall, seemed to produce a low antibody level, but this response did vary between the dogs. The lentivirus vector produced a slightly higher antibody level in the three dogs studied, and the

antibody levels did seem to decrease over time. Finally, one dog was studied after intraocular treatment of Av-1 which induced the highest antibody level. These results are important for continued research using viral vectors for gene addition therapy within the eye. A humoral immune response could limit the therapeutic effectiveness over time and could also inhibit success of repeated dosing.

3.3.2. Future Research

The AAV vector ELISA results allow for the continuation of research in several connected areas. The potential for genotoxicity and other adverse long-term effects such as waning of the transgene or limited therapy potential remain important issues for the feasibility and success of ocular gene addition therapy.

- The spread of the vector within the host may be correlated with the systemic antibody response. Real-Time PCR analysis should be performed in order to detect the spread of the vector from the treatment site. Many filtering organs, such as kidney, liver, and lung, as well as tissues from along the central visual pathway, should be included in this analysis.

- Since, from previous studies, no inflammation or decreased transgene expression was observed upon repeat administration of an AAV vector into the eye, tolerance may be occurring (Bennett et al., 1999; Anand et

al., 2000; Isenmann et al., 2001). Using a constant dose of vector for intraocular injections, the level of GFP expression after one or several injections could be quantified for individual photoreceptors (using a photon-counting confocal laser scanning microscope as described in Rex et al., 2005) and averaged together at each injection time point for individual animals. This would allow for a more detailed analysis of the neutralizing antibody effects on the transgene expression over time and after repeat administration.

- As mentioned previously, an antibody response may not significantly affect the transgene expression in the eye. Since ubiquitous promoters are used, the concern about genotoxicity within organs remains a concern. Further studies to observe the possible cytotoxic effect of either single or repeated administration over time would create a more thorough analysis of the effects of using ubiquitous promoters as opposed to tissue-specific promoters for gene therapy.

Further work into the lentivirus and Av-1 vectors could also be done in order to further understand the antibody response in the host after intraocular treatment.

- An ELISA could be developed to study the antibody response to the lentivirus packaging.

- An ELISA could be developed to study the antibody response to the Av-1 capsid.

3.3.3. Conclusion

A variable degree of humoral immune response to the transgene and AAV capsid was observed after intraocular injection of AAV vectors. For lentivirus and adenovirus vectors, a humoral immune response was observed after intraocular injection. This information is imperative for the safety of intraocular gene addition therapy trials since every patient will have a unique response to the foreign transgene.

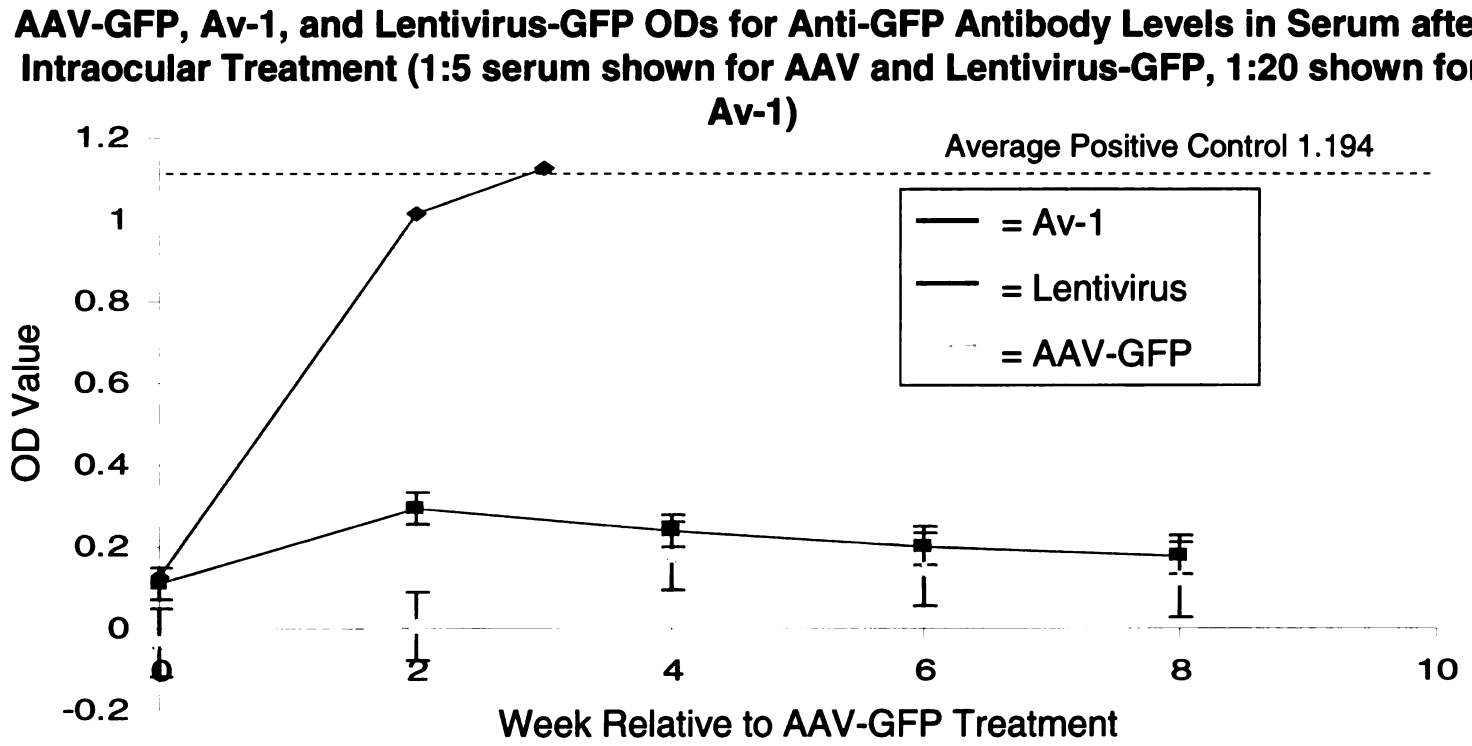


Figure 3.19. LSM value with SEM reported per week for the AAV and lentivirus. Raw OD values for the Av-1 dog reported as well.

APPENDICES

Appendix A. ELISA GFP Laboratory Protocol

MATERIALS NEEDED:

For solutions:

-Na2HPO4
-KH2PO4
-KCL
-NaCl
-Tween (Polyoxyethylene- Sorbitan Monolauvate)
-gelatin-
-bicarbonate packets- from Pierce
-access to milli-Q water
-pH meter
-Probind ELISA plates

For ELISA protocol:

-GFP from Clonetech
-secondary antibody-
    -HRP- anti-dog IgG
    -HRP- anti-rabbit IgG
-ABTS (acts as the substrate)

PREPARATION OF EQUIPMENT AND SOLUTIONS:

Cleaning of Conboy:

Rinse conboy thoroughly with tap water five times, distilled water five times, and with milli-Q water five times.

Preparation of PBS:

- In a 1000ml beaker:
    - Na_2HPO_4 – 23g
    - KH_2PO_4 – 4g
    - KCl – 4g
    - NaCl – 160g
    - Added 800ml milli-Q water to mix
    - Added ~ 200ml to make 1L
- Add solution to large conboy
- Add milli-Q water until 20L
- Calibrate pH meter
    - Want pH to be 7.2 – add drops of HCl or NaOH solutions until desired pH attained

Preparation of Bicarbonate solution for dilution of GFP:

- Bicarb will last a long time in cabinet- but need to watch out for contamination!
- Add 500mL nanopure water to one packet of powdered buffer

Preparation of GFP for coating ELISA plates:

GFP concentration (from Clonetech) = 1mg/mL = 1000ug/mL

- Aliquoted into 20µL/ per tube → 1 tube/ 96-well plate

To dilute GFP to coat ELISA plates:

Need 100µLper well at a concentration of 1µg/mL

20µLof GFP and add Bicarb to a total volume of 10,000uL(10mL)

-Only thaw and dilute GFP to be used that day

-Do NOT pre-dilute GFP

Control serum samples

Dog serum was tested for the best results on varying concentrations of GFP. The serum from 8/25 was combined from both dogs (either + or – control dogs) in a 1:1 mixture and then diluted to 1:25 using wash buffer. The mixture and 1:25 control dilutions were then stored in the freezer.

+ control = Spruce and Pluto

- control = Mica and Snap

WASH/ DILUENT solution = 0.05% PBS-T

10L BULK-add 5mL of Tween-20 to 10L of PBS

1 500mL bottle- add .25mL Tween to 500 mL bottle of PBS

ELISA Procedure:

1- **Coating Plate with GFP: DO THIS 15 HOURS BEFORE OTHER STEPS!**
   a. 100µl/well of GFP- bicarbonate solution
   b. Place clear, adhesive coverslip on plate
   c. Incubate overnight at 4°C (in fridge) on a flat surface, do not tip plate
2- **Wash Plate:**
   a. Following overnight incubation at 4°C, dump solution in wells and wash plate with PBS-T
      i. Pipette 200µl/well of PBS-T – repeat this for a total of 3 rinses- empty wells of solution
3- **"Whack" Plate:**
   a. Slam plate upside down onto a stack of paper-towels until paper-towels do not have any moisture on them (plate is essentially dry)
4- **Serum Dilutions:**
   a. During 1 hour block, prepare serum dilutions:
      i. Using a 96 round bottom well plate, pipette 115µl/well for doubling dilutions into second thru last rows.
      ii. Pipette 230µl/well of the starting dilution of serum in the first row of wells.

iii. Pipette 115µl out of the first row and pipette this into the second row, remembering to mix well before pipetting 115µl into the next row.
iv. Continue in this manner to the end of the plate.
v. From the last row, pipette 115µl out and discard so that all rows end up with 115µl volume.

****Start with 1:5 dilution****

230µLin first well= 46µLserum and 184 µLwash buffer

- If using pooled normal dog serum for a negative control, dilution used is 1:25. Use wash buffer to dilute.
- If using pooled hyperimmune serum, dilution used is 1:25. Use wash buffer to dilute.
- If using rabbit Hyperimmune serum (rabbit anti-GFP from abcam), dilution used is: 1:2500. Use PBS-T to dilute.
- If using normal rabbit serum for a negative control, dilution used is 1: 2,500. Use PBS-T to dilute.

5- Add Serum Dilutions:

a. Pipette 100µl of serum dilution from round well plate into corresponding well of ELISA plate

6- Incubate:

a. Leave for 1 hour at room temperature with vigorous shaking (setting #5 on shaker)

7- Preparation of Secondary Antibodies :

Prepare secondary detection antibodies while plate is incubating with serum samples

HRP-Anti-Dog:

- 100µl/well
  - A dilution of 1/20,000 is used- use PBS-T to dilute
  - 1 uL/20ml PBS-T

HRP-Anti-Rabbit:

- 100µl/well
  - A dilution of 1/20,000 is used- use was buffer to dilute.
  - 1 uL/20ml wash buffer.

8- Wash and 'Whack' plate:

a. Following incubation, wash plate with 200µl/well of PBS-T (3X) and 'wack' dry on a stack of paper towels

9- Addition of secondary detection antibody:

a. Add 100µl/well of the appropriate (dog or rabbit) HRP-conjugated secondary detection antibody

10- Incubation:

a. Incubate plate with secondary antibodies for 1 hour at room temperature with vigorous shaking (setting #5 on shaker)

11- Wash and 'Whack' plate:

a. Following incubation, wash plate with 200µl/well of PBS-T (3X) and 'wack' dry on a stack of paper towels.

12-Addition of ABTS:

a. Add 125µl/well 1-step ABTS and incubate for 1 hour at 37°C without shaking

13-Use Plate Reader to determine absorbance (set filter at 405nm and 450nm)

Storage of antibodies/substrates etc:

Goat Anti-Rabbit IgG (H+L)

a. Caltag – L42007
b. Store at 2-8 °C, dilute only what is needed at a time

HRP-Anti-Dog IgG (H+L)

c. Bethyl – A40-123P
d. Store at 2-8 °C

Rabbit Anti-GFP (Rabbit Polyclonal to GFP)

e. Store at 4 °C short term
f. Long term: -20 or -80°C - add 1:1 glycerol for stability
    i. Discussed with Dr. Burton –at 4 °C

GFP – 1mg/mL

g. Clontech #632373
h. Aliquots of 20µl at -80 °C

1-STEP ABTS for ELISA from Pierce Biotechnology

i. Store at 4 °C

Serum samples from Subretinal Injection Dogs and Positive and Negative Control from Immunized Dogs:

j. Stored at -80 °C

- **Plates- BD Falcon 353279 sold through VWR**

Appendix B. Anti-AAV ELISA Protocol and Summary of References

MATERIALS NEEDED:

For solutions:

-Na2HPO4
-KH2PO4
-KCL
-NaCl
-Tween (Polyoxyethylene- Sorbitan Monolauvate)
-access to milli-Q water
-pH meter
-Sterile saline
-Probind ELISA plates from VWR

For ELISA protocol:

-recombinant UF11 virus from Hauswirth Lab, FL
-secondary antibody-
    -HRP- anti-dog IgG
-ABTS (acts as the substrate)

PREPARATION OF EQUIPMENT AND SOLUTIONS:

Cleaning of Conboy:

Rinse conboy thoroughly with tap water five times, distilled water five times, and with milli-Q water five times.

Preparation of PBS:

- In a 1000ml beaker:
    - Na_2HPO_4 – 23g
    - KH_2PO_4 – 4g
    - KCl – 4g
    - NaCl – 160g
    - Added 800ml milli-Q water to mix
    - Added ~ 200ml to make 1L
- Add solution to large conboy
- Add milli-Q water until 20L
- Calibrate pH meter
    - Want pH to be **7.2** – add drops of HCl or NaOH solutions until desired pH attained

<u>Virus concentration math→ 100,000 vp/ml per 100uL/well</u>

Use saline for dilutions

5.6E+13 → 1:1000 = 5.6E+10 → 1:1000 = 5.6E+7
[Virus] dilute dilute
vortex between dilutions

(5.6E+7) X = (4.5ml)x(100,000vp/ml)
X= 8.03 µLvirus in 4.5 ml saline solution

Add 100µLper each well used for Anti-AAV ELISA

WASH/ DILUENT solution* = *0.05% PBS-T

BULK-add 5mL of Tween-20 to 10L of PBS
1 500mL bottle at a time- add .25mL Tween to 500 mL bottle of PBS

ELISA Procedure:

1- **Coating Plate with virus: DO THIS 15 HOURS BEFORE OTHER STEPS!**
   a. 100µl/well of virus-saline solution
   b. Place clear, adhesive coverslip on plate
   c. Incubate overnight at 4°C (in fridge) on a flat surface, do not tip plate
2- **Wash Plate:**
   a. Following overnight incubation at 4°C, pour off solution from wells and wash plate with PBS-T
      i. Pipette 200µl/well of PBS-T – repeat this for a total of 3 rinses- empty wells of solution
3- **"Whack" Plate:**
   a. Slam plate upside down onto a stack of paper-towels until paper-towels do not have any moisture on them (plate is essentially dry)
4- **Serum Dilutions:**
   a. During 1 hour block, prepare serum dilutions- use 1:5 dilutions for all samples:
      i. 115/5= 23 µL
      ii. 23µLserum and 92µLwash buffer per well in round bottom plate
      iii. Aliquot 100µLinto each well

Control Dog Serum

- If using pooled normal dog serum for a negative control, dilution used is 1:25. Use wash buffer to dilute.
- If using pooled hyperimmune serum, dilution used is 1:25. Use wash buffer to dilute.

5- **Add Serum Dilutions:**
   a. Pipette 100µl of serum from round well plate into corresponding well of ELISA plate
6- **Incubate:**
   a. Leave for 1 hour at room temperature with vigorous shaking (setting #5 on shaker)
7- **Preparation of Secondary Antibodies :**
   Prepare secondary detection antibodies while plate is incubating with serum samples

HRP-Anti-Dog:

- 100µl/well
  - A dilution of 1/20,000 is used- use PBS-T to dilute
  - 1 µL/ 20ml PBS-T

8- Wash and 'Whack' plate:

a. Following incubation, wash plate with 200µl/well of PBS-T (3X) and 'wack' dry on a stack of paper towels

9- Addition of secondary detection antibody:

a. Add 100µl/well of the appropriate HRP-conjugated secondary detection antibody

10- Incubation:

a. Incubate plate with secondary antibodies for 1 hour at room temperature with vigorous shaking (setting #5 on shaker)

11-Wash and 'Whack' plate:

a. Following incubation, wash plate with 200µl/well of PBS-T (3X) and 'wack' dry on a stack of paper towels.

12-Addition of ABTS:

a. Add 125µl/well 1-step ABTS and incubate for 1 hour at 37°C without shaking

13-Use Plate Reader to determine absorbance (set filter at 405nm and 450nm)

Appendix C. AAV2/5-GFP Vector Construction

AAV2/5 Plasmid Construction

The AAV2 vector pseudotyped with the capsid from AAV5 was produced in the laboratory of Dr. Hauswirth at the University of Florida. An adenovirus helper plasmid was used to pseudotype the vector with the AAV5 capsid.

Adenovirus Helper Plasmid Production

The creation of the adenovirus helper plasmid with AAV5 capsid was performed in a series of steps. The open reading frame (ORF) of AAV5 was amplified with PCR and was then subcloned into the plasmid pACG2 resulting in the hybrid plasmid pACG2R5C which contains the ORF for the AAV2 Rep proteins and the ORF for the AAV5 capsid proteins. A fragment from the pACG2R5C plasmid was subcloned into the pXYZ adenovirus helper plasmid to create the pXYZ5 plasmid complete with the AAV5 capsid gene (Zolotukhin et al., 2002).

AAV Vector Plasmid Production

The AAV vector plasmid was constructed using the pTR-UF backbone and consists of AAV2 flanking inverted terminal repeat (ITR) regions (Zolotukhin et al., 2002). Two different AAV vector plasmids were used in this study to create two different AAV2/5 vectors. The first AAV vector consisted of the chicken beta actin promoter, CMV enhancer regions, and GFP transgene. The second AAV vector consisted of the mouse opsin promoter and GFP transgene (Glushakova et al., 2006).

Promoters

Chicken Beta Actin (CBA)

The CBA promoter is a ubiquitous promoter that allows a constant and powerful expression of the transgene. This promoter is active in any host cell and thus transcribes the transgene in any cell of the body.

```
  1 catggtcgag gtgagcccca cgttctgctt cactctcccc atctcccccc
 51 cctccccacc cccaattttg tatttattta ttttttaatt attttgtgca
101 gcgatggggg cgggggggggg gggggggcgc gcgccaggcg gggcggggcg
151 gggcgagggg cggggcgggg cgaggcggag aggtgcggcg gcagccaatc
201 agagcggcgc gctccgaaag tttcctttta tggcgaggcg gcggcggcgg
251 cggccctata aaaagcgaag cgcgcggcgg gcg
```

Figure A. The CBA promoter sequence (provided by the Hauswirth Lab). This sequence was Blasted (RID: 1171314962-8045-14060912295.BLASTQ3, 2/12/07) with hits found only in the chicken genome.

Mouse Opsin (mOPS500)

The mOPS500 promoter is a tissue-specific promoter present in some of the AAV vectors that was characterized by the Hauswirth laboratory (Glushakova et al., 2006). The promoter was shown to be specific for photoreceptors and thus the likelihood that the transgene would be expressed outside the eye is low.

```
  1 cgtcaagtga gccattgtca gggcttgggg actggataag tcaggggtc
 51 tcctgggaag agatgggata ggtgagttca ggaggagaca ttgtcaactg
101 gagccattgt ggagaagtga atttagggcc caagggttcc agtcgcagcc
151 tgaggccacc agactgacat ggggaggaaa tcccagagga ctctggggca
201 gacaagatga gacacccttt cctttcttta cctaagggcc tccacccgat
251 gtcaccttgg cccctctgca agccaattag gccccggtgg cagcagtggg
301 attagcgtta gtatgatatc tcgcggatgc tgaatcagcc tctggcttag
351 ggagagaagg tcactttata agggtctggg gggggtcagt gcctggagtt
401 gcgctgtggg agccgtcagt ggctgagctc gccaagcagc cttggtctct
451 gtctacgaag agcccgtggg gcagcct
```

Figure B. The mOPS500 promoter sequence (provided by the Hauswirth Lab). This sequence was blasted (RID: 1171314867-26111-185748596015.BLASTQ3, 2/12/07) with hits only found in the mouse genome on chromosome 6.

The Green Fluorescent Protein (GFP) Transgene

The GFP transgene is a reporter gene commonly used to determine the efficacy of the gene transfer event. GFP originates from the jellyfish, *Aequorea victoria,* and fluoresces a bright green color when under a UV or blue light and has been shown to be effective in many species and systems.

```
   1 caccatgagc aagggcgagg aactgttcac tggcgtggtc ccaattctcg
  51 tggaactgga tggcgatgtg aatgggcaca aattttctgt cagcggagag
 101 ggtgaaggtg atgccacata cggaaagctc accctgaaat tcatctgcac
 151 cactggaaag ctccctgtgc catggccaac actggtcact accctgacct
 201 atggcgtgca gtgcttttcc agatacccag accatatgaa gcagcatgac
 251 tttttcaaga gcgccatgcc cgagggctat gtgcaggaga gaaccatctt
 301 tttcaaagat gacgggaact acaagacccg cgctgaagtc aagttcgaag
 351 gtgacaccct ggtgaataga atcgagctga agggcattga ctttaaggag
 401 gatggaaaca ttctcggcca caagctggaa tacaactata actcccacaa
 451 tgtgtacatc atggccgaca agcaaaagaa tggcatcaag gtcaacttca
 501 agatcagaca caacattgag gatggatccg tgcagctggc cgaccattat
 551 caacagaaca ctccaatcgg cgacggccct gtgctcctcc cagacaacca
 601 ttacctgtcc acccagtctg ccctgtctaa agatcccaac gaaaagagag
 651 accacatggt cctgctggag tttgtgaccg ctgctgggat cacacatggc
 701 atggacgagc tgtacaagtg agc
```

Figure C. The GFP cDNA sequence used in the AAV viral vector constructs (provided by the Hauswirth Lab).

Protocol for AAV2/5 vector production

Cell transfection

293 cells (ATCC, Manassas, VA) were used for the transfection with the above plasmid constructs. The cells were cultured with Dulbecco's modified Eagle's medium (DMEM) which was supplemented with 5% fetal bovine serum (FBS). Cells were split 1:3 one day prior to transfection and were allowed to reach ~75-80% cell confluency. The plasmid is CsCl-purified and was then transfected using a $CaPO_4$ precipitate. 1.8mg of pXYZ5 and 0.6mg of AAV vector plasmid in a total volume of 50mL of 0.25M $CaCl_2$ followed by the addition of 50mL of 2x

HBS. pH7.05, to the DNA/$CaCl_2$ (Zolotukhin et al., 2002). After 1-2 minutes of room temperature incubation, 1100mL of warm DMEM with 5% FBS was added.

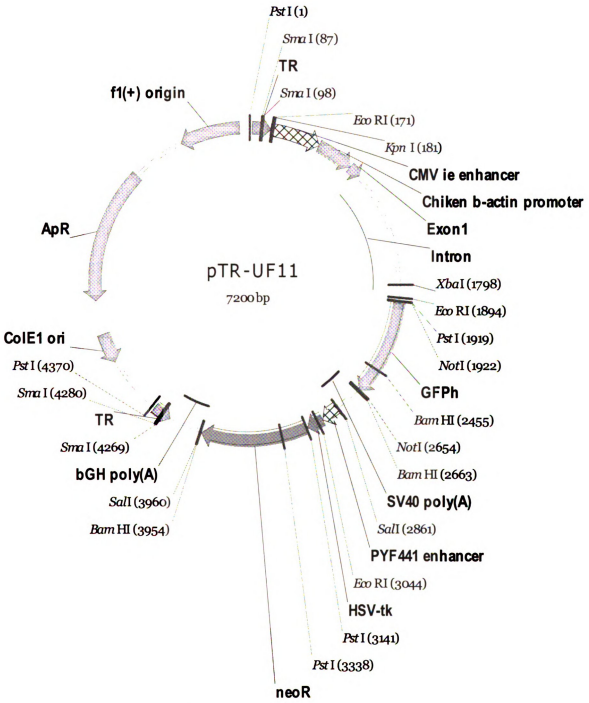


Figure D. The pTR-UF11 plasmid with CMV enhancer, CBA promoter, and GFP transgene (Vince Chiodo, Hauswirth Lab, University of Florida).

The medium was removed from the cells and then the plasmid solution was added to the cells and allowed to incubate for 60h at 37°C at 5% CO_2. Cells were then washed with PBS and then harvested. The cells then underwent centrifugation for 10 minutes at 1000g, were resuspended in 60mL of lysis solution (150mM NaCl, 50mM Tris, pH 8.4), and then stored at -20°C (Zolotukhin et al., 2002).

AAV2/5 Purification

Purification of the AAV5 vector occurred through a series of steps: cell lysis, iodixanol gradients, and column chromatography. Cells were lysed using a freeze/thaw method that involved dry ice in ethanol and a 37°C water bath. Benzonase (Sigma, St. Louis, MO) was then added to the cell lysate and incubated for 30 minutes (Zolotukhin et al., 2002). The lysate was then centrifuged for 30 minutes at 4000g in order to clarify the crude lysate. The supernatant was then divided 4 ways into iodixanol gradients.

Iodixanol gradients are created by using quick-seal tubes (25 x 89 mm, Beckman) and iodixanol (5,5'-[(2-hydroxy-1,3-propanediyl)bisacetyl-amino)] bis[N,N'-bis (2,3dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide] using a 60% sterile solution of OptiPrep (Nycomed) and PBS-MK buffer (1 x PBS containing 1mM $MgCl_2$ and 2.5mM KCl). The cell lysate solution was added then centrifuged for 1 hour at 18C at 69,000rpm (Zolotukhin et al., 2002). 5mL of the

60-40% step interface was aspirated with an 18g needle, and the bands of the gradient were stored either in the freezer or refrigerator (Zolotukhin et al., 2002). Column chromatography of the iodixanol gradient further purifies the vector. A 4-mL HiTrap Q column (Pharmacia) was used. First, the column was equilibrated at 5mL/min with 5 column volumes of Buffer A (20mM Tris, 15mM NaCl, pH 8.5) followed by 25mL of Buffer A using the Pharmacia ATKA FPLC system (Zolotukhin et al., 2002). The iodixanol fraction was diluted 1:1 with Buffer A and then added to the column at a rate of 3-5 ml/minute. The column was then washed with 10 column volumes (50mL) of Buffer A. The vector was eluted with Buffer B (Zolotukhin et al., 2002).

AAV2/5 Concentration

The vector was concentrated through a three cycle-centrifugation method involving a Biomax 100 K concentrator (Millipore, Bedford, MA). The virus is concentrated to 1 mL and is then stored at -80C in 1 x PBS (Zolotukhin et al., 2002).

AAV2/5 Quality Control Assays

Quality control measures are taken using an assay to determine the protein purity of the AAV2/5, another assay for the infectiousness, and then a dot-blot assay for the physical particle titer (Zolotukhin et al., 2002). The protein purity of the AAV2/5 vector was determined by running a 10% SDS-polyacrylamide gel with silver staining. A western blot was then done using an anti-AAV2 capsid

monoclonal antibody B1 (American Research Products) that recognizes AAV5 capsids at a dilution of 1:2000. A horseradish peroxidase-conjugated sheep anti-mouse (Amersham) was used for detection at a dilution of 1:5000 and Super Signal (Pierce, Rockford, IL) was used as the substrate (Zolotukhin et al., 2002).

The infectious center assay (ICA) was used to determine the infectiousness of the vector. 96-well plates were coated with 2 x 10^4 C12 cells and infected 16 hours after coating using 10-fold dilutions of the AAV and also infected with adenovirus 5 at a multiplicity of infection of 10 (Zolotukhin et al., 2002). The C12 cells were then harvested and suspended in 5 mL of 1 x PBS. The cells are then vacuum filtered onto nylon membranes (0.45µm) which were then transferred to filter paper moistened with 0.5 N NaOH/1.5 M NaCl and 1M Tris-Cl. The filter paper was then probed for the transgene. Titer was calculated by using the dilution factor from the infectious centers (spots) observed on the filter paper, accurate in the range of 10-200 spots (Zolotukhin et al., 2002).

Another assay was used to determine the infectiousness of the AAV2/5 vectors that have the GFP transgene. The single-cell fluorescence assay (SCFA) determined the vector titer based on the expression of GFP. 2 x 10^4 293 or C12 cells are seeded into a 96 well plate. Serial dilutions of the AAV2/5 and Ad5 (MOI of 10) were added to wells. After thirty hours, cells infected with the GFP vector were visually scored using a fluorescent microscope. Titer is calculated based on the dilution factor (Zolotukhin et al., 2002).

The last quality assurance assay used is the dot-blot assay. This assay determines the number of vectors with a viral genome. DNase I (Roche) was used to digest all plasmid and unpackaged vector DNA for 1 hour at 37°C in a volume of 200µL containing 5U of the enzyme, 10mM Tris-Cl, pH 7.5, and mM $MgCl_2$. Then, in order to free the viral genomes from the capsids, an equal volume of proteinase K buffer (20mM Tris-Cl, pH 8.0, 20mM EDTA, pH 8.0, 1% SDS) was added as well as proteinase K (30µg). This solution was incubated for 1 hour at 37°C. The DNA was then extracted using phenol and precipitated using ethanol and a glycogen carrier (Zolotukhin et al., 2002). DNA was then dissolved in 40µL of distilled water. 10µL of this was diluted in 400µL 0.4 N NaOH/10mM EDTA. A twofold dilution series was prepared from the plasmid DNA as above. The viral DNA was immobilized onto a nylon membrane. The plasmid DNA was also immobilized onto the membrane using a dot-blot apparatus (Bio-Rad, Hercules, CA). The membrane was then probed for the transgene and exposed to film. Comparing the plasmid DNA standard curve to the vector DNA allows for extrapolation of the viral titer.

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