Safety and Biodistribution Evaluation in Cynomolgus Macaques of rAAV2tYF-PR1.7-hCNGB3, a Recombinant AAV Vector for Treatment of Achromatopsia

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Applied Genetic Technologies Corporation (AGTC) is developing rAAV2tYF-PR1.7-hCNGB3, a recombinant adeno-associated viral (rAAV) vector expressing the human *CNGB3* gene, for treatment of achromatopsia, an inherited retinal disorder characterized by markedly reduced visual acuity, extreme light sensitivity, and absence of color discrimination. We report here results of a study evaluating the safety and biodistribution of rAAV2tYF-PR1.7-hCNGB3 in cynomolgus macaques. Three groups of animals ($n = 2$ males and 2 females per group) received a subretinal injection in one eye of 300 μ l containing either vehicle or rAAV2tYF-PR1.7-hCNGB3 at one of two concentrations (4×10^{11} or 4×10^{12} vector genomes/ml) and were evaluated over a 3-month period before being euthanized. Administration of rAAV2tYF-PR1.7-hCNGB3 was associated with a dose-related anterior and posterior segment inflammatory response that was greater than that observed in eyes injected with the vehicle control. Most manifestations of inflammation improved over time except that vitreous cells persisted in vector-treated eyes until the end of the study. One animal in the lower vector dose group was euthanized on study day 5, based on a clinical diagnosis of endophthalmitis. There were no test article-related effects on intraocular pressure, visual evoked potential responses, hematology or clinical chemistry parameters, or gross necropsy observations. Histopathological examination demonstrated minimal mononuclear infiltrates in all vector-injected eyes. Serum anti-AAV antibodies developed in all vector-injected animals. No animals developed antibodies to CNGB3. Biodistribution studies demonstrated high levels of vector DNA in the injected eye but minimal or no vector DNA in any other tissue. These results support the use of rAAV2tYF-PR1.7-hCNGB3 in clinical studies in patients with achromatopsia caused by *CNGB3* mutations.

INTRODUCTION

ACHROMATOPSIA IS AN INHERITED retinal disorder characterized by markedly reduced visual acuity, nystagmus, photoaversion (intolerance of bright light), a small central scotoma, eccentric viewing, and complete or severe loss of color discrimination.¹ Unlike the common forms of inherited “color blindness,” in which there is difficulty distinguishing color differences but normal visual acuity, individuals with achromatopsia have markedly impaired visual function. Best visual acuity even under nonbright light conditions is usually about 20/200. Nystagmus develops during the first few weeks after

birth and extreme light sensitivity with daytime blindness is a major feature of the disease. People with achromatopsia report that being colorblind is the least troublesome manifestation of this vision disorder.² Poor visual acuity (especially for distance) and hypersensitivity to bright light cause much more disability.

The genetic basis of achromatopsia can be established in the majority of individuals. Approximately 50% of cases in the United States and Europe are caused by mutations in the cone photoreceptor-specific cyclic nucleotide-gated channel β -subunit (*CNGB3*) gene³ and 25% are caused by mutations in

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the cone-specific cyclic nucleotide-gated channel α -subunit (*CNGA3*) gene,⁴ and a small percentage are caused by mutations in other genes.^{5–9} Most *CNGB3* mutations result in a premature stop codon, and some missense mutations result in an abnormal *CNGB3* protein that interacts with the α -subunit (*CNGA3*) protein to form channels with increased affinity for cGMP compared with wild-type channels.¹⁰ As a result there is a loss of cone photoreceptor function in individuals with *CNGB3* mutations and in animals with mutations in the homologous gene.^{11,12} Studies in dog and mouse models of achromatopsia caused by mutations in the *CNGB3* gene indicate that gene therapy using a recombinant adeno-associated viral (rAAV) vector expressing a normal human *CNGB3* protein can restore cone photoreceptor function.^{13,14}

Applied Genetic Technologies Corporation (AGTC; Alachua, FL) is developing an rAAV vector expressing human *CNGB3* as a potential product for treatment of achromatopsia caused by *CNGB3* mutations. The product, rAAV2tYF-PR1.7-h*CNGB3*, contains a cone-specific PR1.7 promoter,¹⁵ a codon-optimized human *CNGB3* cDNA, and a simian virus 40 (SV40) polyadenylation sequence, and is packaged in an AAV2 capsid containing three tyrosine-to-phenylalanine (YF) mutations.¹⁶ As part of our efforts to develop this product, we conducted a toxicology and biodistribution study in normal cynomolgus macaques with a single subretinal administration of the vector at two dose levels.

RESEARCH DESIGN AND METHODS

Promoter Selection

The expression cassette in the vector used in proof-of-concept studies in cone photoreceptor-specific cyclic nucleotide-gated channel β -subunit (*CNGB3*) mutant dogs¹⁷ contains a 2.1-kb human L-opsin promoter and a 2.43-kb human *CNGB3* cDNA and has a total size of 5.23 kb, which exceeds the optimal packaging capacity of adeno-associated virus (AAV).¹⁸ We therefore evaluated shorter promoters in a study using AAV vectors expressing green fluorescent protein (GFP) driven by different promoters administered by subretinal injection in cynomolgus macaques. Results of that study showed that a vector containing a shortened version of the PR2.1 promoter, termed PR1.7, directed robust and specific GFP expression in all three types of primate cone photoreceptors, and that it was better than the vector containing the PR2.1 promoter.¹⁹

Capsid Selection

Capsids with mutations in surface-exposed tyrosine residues have been shown to enhance the

efficiency of transduction when administered by subretinal or intravitreal injection in mice. AAV vectors expressing GFP and packaged in capsids with single tyrosine-to-phenylalanine (YF) mutations achieved stronger and more widespread transgene expression in many retinal cells compared with their wild-type capsid counterparts,²⁰ and AAV vectors expressing GFP packaged in capsids with multiple YF mutations achieved GFP expression in the ganglion, Müller, and inner retinal cells.²¹ We used AAV vectors expressing GFP driven by the PR2.1 or PR1.7 promoter to compare an AAV2 capsid containing three Y-to-F mutations (AAV2tYF) with AAV5 and AAV9 capsids for their ability to target cone photoreceptors after subretinal injection in rhesus macaques. The AAV2tYF and AAV9 capsids were similarly efficient, and more efficient than the AAV5 capsid, in targeting GFP expression to nonhuman primate cone photoreceptors (data not shown).

Choice of cDNA

Studies with AAV vectors expressing cDNA for a variety of genes have shown that codon optimization may increase the efficiency of gene expression. We modified the normal human *CNGB3* (h*CNGB3*) cDNA by means of algorithms that replace rare codons based on human codon usage, optimized the sequence near the start codon to a Kozak consensus sequence, and changed other aspects of mRNA structure to improve the stability of transcripts and gene expression. Subretinal injection of vectors expressing either normal or codon-optimized h*CNGB3* cDNA, driven by the PR1.7 promoter, restored cone function in dog and mouse models of achromatopsia.^{22,23}

Vector Production

The rAAV2tYF-PR1.7-h*CNGB3* vector was produced using a recombinant herpes simplex virus (rHSV) complementation system in suspension-cultured baby hamster kidney (sBHK) cells.²⁴ Two rHSV helper viruses, one containing the AAV2 *rep* and AAV2tYF *cap* genes and the other containing the h*CNGB3* expression cassette, were used to co-infect sBHK cells grown in serum-free medium. One day later the cells were lysed with Triton X-100 detergent, treated with Benzonase (Merck, Whitehouse Station, NJ), clarified by filtration, purified by AVB Sepharose (GE Healthcare Life Sciences, Piscataway, NJ) affinity chromatography followed by CIM (convective interaction media) SO₃[−] (BIA Separations, Ajdovščina, Slovenia) cation-exchange chromatography, and eluted in 2.6× balanced salt solution containing 0.014% (v/v)

Tween 20 (BSST). The purified bulk was concentrated and buffer exchanged to 1× BSST (drug substance) and sterile filtered (pore size, 0.2 μm) to generate drug product. The drug product was further concentrated, as needed, using a 100-kDa molecular weight cutoff (MWCO) ultracentrifugal filter unit (EMD Millipore, Billerica, MA), and refiltered (pore size, 0.2 μm) to generate drug product sublots of specific concentrations, which were stored at $\leq -65^\circ\text{C}$.

Vector Characterization

Vector concentration (vector genomes [VG]/ml), purity (silver-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS–PAGE] analysis), and concentrations of endotoxin and HSV protein were measured as previously described.²⁵ Concentrations of BHK protein, bovine serum albumin, Benzonase, and AVB ligand were measured by ELISA, using commercially available kits. HSV and BHK DNAs were measured by quantitative PCR (qPCR). Testing for mycoplasma, bacteria, and fungi was performed according to standard microbiological methods. Testing for infectious HSV was by serial passage in V27 cells.

Device Compatibility Testing

Product stability before and after exposure to an extendible 41G subretinal injection needle (Cat. No. 1270.EXT; DORC, Exeter, NH) used to administer the vector by subretinal injection was determined by measuring the vector concentration (VG/ml). Vials of vector were thawed, diluted to a concentration of 4×10^{12} or 4×10^{11} VG/ml, and tested immediately or after 4 hr without exposure to the device, or after exposure to the device by withdrawal into the injection device that was kept on ice for 4 hr.

Toxicology Study Design

The study was performed at Covance (Madison, WI), the drug development business of Laboratory Corporation of America Holdings (LabCorp, Burlington, NC), and was conducted in compliance with Good Laboratory Practice for Nonclinical Laboratory Studies (GLP) requirements. Cynomolgus macaques in three groups ($n=2$ males and 2 females per group; 2 years of age; and weighing between 2.3 and 3.1 kg) each received a subretinal injection of 300 μl containing either vehicle or rAAV2tYF-PR1.7-hCNGB3 at a concentration of 4×10^{11} VG/ml (1.2×10^{11} VG/eye) or 4×10^{12} VG/ml (1.2×10^{12} VG/eye) in the right eye (Table 1). Using previously described methods,²⁶ each injection was administered as two blebs of 150 μl each. On the basis of allometric scaling, these doses bracket the

Table 1. Study design

Group	Number		Dose level		
	Male	Female	Vector concentration (VG/ml)	Injection volume (μl)	Total dose (VG)
1	2	2	0 (control)	300	0
2	2	2	4.0×10^{11}	300	1.2×10^{11}
3	2	2	4.0×10^{12}	300	1.2×10^{12}

Note: Animals were administered test article or vehicle control in the right eye via subretinal injection on study day 1 at a volume of 300 μl /eye (two 150- μl injections). The left eye was not treated.

VG, vector genomes.

doses planned for a phase 1/2 clinical trial (450 μl at concentrations from 2×10^{11} to 2×10^{12} VG/ml). The left eyes were untreated. Residual dosing formulations were frozen for later testing by qPCR to confirm the concentration of vector administered.

Animals were scheduled to be euthanized 91 days after vector administration (study day 92), and samples were collected for evaluation of safety and biodistribution.

Animals were observed twice daily for mortality, clinical abnormalities, and signs of pain or distress. Detailed observations were made at least once during the predose phase, before dosing on study day 1, weekly thereafter, and on the day they were euthanized. Body weights were obtained during the predose phase, on the day of dosing, weekly thereafter, and on the day they were euthanized. An ophthalmic examination (slit-lamp biomicroscopy, indirect ophthalmoscopy, and measurement of intraocular pressure [IOP]) was conducted during the predose phase; on study days 3, 8, and 15; and during study weeks 4, 9, and 13 for all surviving animals. Aqueous cells and flare, and vitreous cells, were scored as previously described.²⁷ Aqueous and vitreous cell scores were assigned using an estimate of cells per single 0.2-mm field of the focused slit-lamp beam as 0 (no cells), trace (1–5 cells), 1+ (5–25 cells), 2+ (25–50 cells), 3+ (50–100 cells), or 4+ (>100 cells). Aqueous flare was scored, on the basis of the presence of protein in the anterior chamber, as 0 (no visible protein), trace (visible only to an experienced observer using a small, bright focal light source and magnification), 1+ (mild), 2+ (moderate), 3+ (moderate but more than 2+), or 4+ (severe). Vitreous haze was scored according to the Standardization of Uveitis Nomenclature (SUN) method.²⁸

Scotopic and photopic electroretinogram (ERG) responses and visual evoked potential (VEP) measurements for each eye were obtained during the predose phase and during study week 13. Blood for hematology, coagulation, and clinical chemistry

analysis was obtained twice during the predose phase and on study days 12 and 89.

Serum samples for measurement of antibodies to AAV (neutralizing antibody assay) and hCNGB3 (ELISA), and for measurement of cell-mediated immune responses to hCNGB3 and AAV capsid peptides by enzyme-linked immunospot (ELISPOT) analysis of peripheral blood mononuclear cells (PBMCs), were obtained during the predose phase and on the day they were euthanized.

At necropsy, a complete external and internal examination was performed, including body orifices and cranial, thoracic, and abdominal organs and tissues. Tissues designated for qPCR analyses, other than brain, were collected immediately after death, using ultraclean techniques, and samples for qPCR were obtained before fixation. Brain, heart, lungs, liver, gall bladder, kidneys (pair), spleen, thymus, adrenals (pair), uterus, ovaries (pair), and testes (pair) were weighed. Brains were removed as soon as feasible after exsanguination, weighed, sectioned sagittally (along the fissure), and collected into 10% neutral buffered formalin. Eyes and optic nerves were collected in modified Davidson's fixative and stored in 10% neutral-buffered formalin. Other tissues were preserved in 10% neutral-buffered formalin.

The eyes were processed for histology and biodistribution as follows. Eyes were enucleated, and the anterior segment was removed. The lens was removed and frozen for qPCR analysis. The anterior segment was divided in half for qPCR and histopathology evaluation. A section of the right eye cup, including the fovea, optic disc, and inferior bleb (from the injected eye), was collected for histopathology. For the remaining calotte, a 6-mm-diameter punch was collected to include the superior bleb from the right eye and to include the macula from the left eye and frozen for qPCR analysis. Samples of vitreous, optic nerve (proximal to globe), and lens were also collected and frozen for qPCR analysis. The remaining intraorbital optic nerve was designated for histopathology. The remaining eyecup (including the remaining vitreous) was fixed and submitted for histopathology.

The following regions of the fixed brain tissue were dissected for qPCR analysis: optic chiasm, optic tract (left and right), lateral geniculate nucleus (LGN), left and right), occipital cortex (left and right), and cerebellum. Samples for qPCR were transferred into 70% ethanol.

Sections of the following tissues were stained with hematoxylin and eosin and examined histologically: adrenal glands, brain, optic chiasm, optic tract (left and right), LGN (left and right), occipital

cortex (left and right), cecum, colon, diaphragm, duodenum, epididymides, esophagus, femur with bone marrow (articular surface of the distal end), gall bladder (drained), heart, ileum, jejunum, kidneys, lesions, liver, lung with large bronchi, lymph nodes (mesenteric), lymph nodes (deep cervical), lymph nodes (preauricular), lymph nodes (inguinal), lymph nodes (mandibular), mammary gland (females), ovaries, pancreas, pituitary gland, sciatic nerve, prostate, skin/subcutis, spleen, stomach, skeletal muscle (gastrocnemius), testes, thymus, thyroid with parathyroid, urinary bladder, and uterus.

Detection of Antibodies to AAV and CNGB3

Antibodies to AAV2tYF capsid were measured using a neutralization assay as previously described.²⁹ Antibodies to CNGB3 were determined by an ELISA based on enhanced chemiluminescence (ECL) detection technology (Meso Scale Discovery [MSD], Gaithersburg, MD). Briefly, diluted monkey test serum, rabbit anti-hCNGB3 antibody (21st Century Biochemicals, Marlborough, MA) as a positive control, or pooled normal monkey serum as a negative control was added to a mixture that contained both biotin-labeled hCNGB3 generated from *Pichia pastoris* (Protein Potential, Rockville, MD) and SULFO-TAG-labeled hCNGB3 and allowed to complex with anti-hCNGB3 antibodies. The labeled antigen-antibody complexes were transferred to a streptavidin-coated plate (MSD), where they were captured. The plate was washed to remove unbound complexes, Read Buffer (MSD) was added to each well of the plate, and the plate was read on a SECTOR Imager 6000 (MSD). Samples with a reading above the cut point were subjected to a confirmatory assay for specificity by adding hCNGB3 antigen to the test samples and incubated before being added to a mixture of biotin-labeled hCNGB3 and SULFO-TAG-labeled hCNGB3. Samples confirmed positive were then titrated to estimate the antibody level.

Quantitation of Vector in Blood and Tissues

DNA was extracted with kits (for frozen tissue) (AutoGen, Holliston, MA) or using a LabCorp proprietary method (for formalin-fixed brain). DNA concentration was determined with Molecular Probes PicoGreen (Thermo Fisher Scientific, Waltham, MA). A 0.2- μ g sample was subjected to TaqMan qPCR (Roche Molecular Diagnostics, Pleasanton, CA) analysis with an Applied Biosystems 7900HT real-time PCR system (Thermo Fisher Scientific), using primers and probe that target the simian virus 40 (SV40) polyadenylation sequence in the vector or

using primers and probe that target human and nonhuman primate glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cycle threshold (C_t) value was determined and compared with a standard curve based on serial dilutions of a plasmid containing known amounts of SV40 or GAPDH sequences. During assay validation the limit of detection (LOD) was determined to be 1 copy per reaction and the lower limit of quantification (LLOQ) was determined to be 10 copies per reaction. When the assay was performed on samples from this study, all no-template control samples had C_t values >38 and each standard curve had a coefficient of variation $<15\%$, R^2 values >0.98 , and a linear range of 10^1 to 10^7 copies per reaction. For samples with low DNA concentrations (lens and vitreous), additional samples were extracted and combined in an attempt to achieve sufficient DNA mass to allow $0.2 \mu\text{g}/\text{reaction}$. For samples with $<0.2 \mu\text{g}/\text{reaction}$ after multiple extractions, the DNA mass per sample was recorded. Samples with DNA mass $<0.03 \mu\text{g}/\text{reaction}$ were spiked with 1×10^3 copies of the GAPDH plasmid per reaction to confirm the absence of inhibitory factors in the sample. GAPDH was amplified from all such samples.

Statistical Analyses

Because of the small number of animals in the study, statistical data analyses were limited to the calculation of means and standard deviation. No hypothesis testing, such as regression or group comparisons, was performed.

RESULTS

Characterization of rAAV2tYF-PR1.7-hCNGB3

The drug product had a vector concentration of 4.2×10^{12} vector genomes (VG)/ml; purity, $>90\%$; endotoxin, less than the LLOQ (lower limit of quantification); and was negative for bacteria and mycoplasma. Concentrations of process residuals in the drug substance used to generate this drug product were 124 ng/ml for herpes simplex virus (HSV) DNA, 3.5 ng/ml for baby hamster kidney (BHK) DNA, 17.2 ng/ml for HSV protein, 70.8 ng/ml for bovine serum albumin, and less than the LLOQ for BHK protein, Benzonase, AVB ligand, and infections HSV.

Results of device compatibility testing demonstrated no significant change in vector concentration after exposure to the administration device for 4 hr.

Toxicology Study Results

The subretinal injection procedure was uneventful except for one animal in the vehicle con-

trol group, which had an intraoperative posterior lens capsule tear. Results of dosing analysis confirmed that the measured concentrations of study agent in the residual dosing formulations were consistent with the concentrations specified in the protocol.

Administration of rAAV2tYF-PR1.7-hCNGB3 had no effect on qualitative food consumption, body weight, or body weight change. There were no intergroup differences in hematology, coagulation, or clinical chemistry parameters, intraocular pressure (IOP), or visual evoked potential (VEP). Scotopic and photopic electroretinogram (ERG) parameters were generally within expected ranges for a given flash condition, except that mild changes were possibly noted in the ERG of the dosed eye for females given the higher dose, although it was difficult to confirm whether this was procedure-related or simply natural variability in this study with a small number of animals per group. ERG changes of this small magnitude were considered unlikely to indicate compromised retinal function.

Ophthalmic Findings

One female given the lower dose of 1.2×10^{11} VG/eye was euthanized at an unscheduled interval on study day 5 because of severe ocular inflammation, based on the recommendation from the veterinary staff. This animal had signs of ocular pain, including eye rubbing and squinting, opaque right eye, and red conjunctiva. Minor clinical pathology findings included mildly increased absolute neutrophil count, mildly decreased albumin and increased globulin concentrations, and mildly increased aspartate aminotransferase activity. Cytology of direct smears of a vitreous aspirate collected at necropsy demonstrated a thin proteinaceous background, with modest numbers of nucleated cells, including an admixture of neutrophils (approximately 85%) and macrophages (approximately 15%). Neutrophils were nondegenerate, and macrophages were occasionally noted with phagocytized pale basophilic globular material (possible test article) within the cytoplasm. An aerobic bacterial culture had no growth after 5 days of incubation.

Macroscopically, the right eye was discolored and had a microscopic correlate of moderate neutrophilic infiltrate. The finding was characterized by the presence of neutrophils in the sclera, iris, ciliary body, vitreous body, optic disc, and multiple layers of retina. The neutrophilic infiltrate in the right eye was considered related to the clinical condition of this animal. The exact cause of the inflammatory response in this animal was unclear. The absence of bacteria on cytology and culture

Figure 1. Ocular inflammation findings. Intensity of parameters in individual animals was scored in a standardized fashion as 0, trace (0.5), 1+, 2+, 3+, or 4+ as described in Research Design and Methods. Each color and symbol represents an individual animal, with four animals per dosing group. One animal in group 2 was euthanized on day 5, based on a clinical diagnosis of endophthalmitis. One animal in group 1 developed a cataract after an intraoperative posterior lens capsule tear; vitreous cells and haze could not be scored after study day 3 in this animal.

results suggested a test article-related effect, although an occult bacterial endophthalmitis could not be conclusively eliminated on this basis alone because septic endophthalmitis does not always demonstrate bacteria on cytology or aerobic culture because of the small sample amounts analyzed and the low number of organisms required to generate fulminant endophthalmitis.³⁰ Inflammation of this severity was not present in any other animals in the lower dose group or any animals in the higher dose group, and the findings noted in this animal may not have been related to the test article.

All animals had a normal ophthalmic examination before vector administration and no abnormal findings were noted in the untreated eyes throughout the study. Subretinal injection sites had pig-

ment mottling at the injection site and the retina appeared flat at all intervals, except on study day 3 in one animal in the higher dose group, in which the injection site appeared mildly elevated.

On the basis of the standard of care for nonhuman primates after ophthalmic surgery at Covance (Madison, WI), topical therapy with atropine and tobramycin/dexamethasone was administered on study days 1–5 and 8–10 in the treated eyes of all animals. Three females (one in each group) also received additional topical atropine and tobramycin/dexamethasone for 21–54 days. Two animals (one in the lower dose group and one in the higher dose group) received an intramuscular injection of analgesic (buprenorphine) on study day 5 and one animal in the higher dose group received intramuscular

injections of a nonsteroidal antiinflammatory drug (flunixin meglumine on study days 5–7 and meloxicam on study days 8–17).

Serial ophthalmic examinations demonstrated a dose-related anterior and posterior segment inflammatory response that was greater than that observed in eyes given vehicle control article. Most of the manifestations of inflammation improved over time except that vitreous cells persisted in vector-treated eyes until the end of the study (Fig. 1).

Subretinal injection of vehicle control article was associated with a transient mild (trace) to moderately severe (3+) anterior segment inflammatory response that resolved by study day 15 except for one animal, which had an intraoperative dosing complication that resulted in a posterior lens capsule tear, and eventually a complete cataract and presumed lens-induced uveitis at study week 4. Posterior segment inflammation was mild and transient. Vitreous haze was sporadic, mild (0.5), and transient, resolving by study day 8 in all eyes (see Fig. 1).

In the lower dose group, one animal described previously was euthanized on study day 5, based on a clinical diagnosis of endophthalmitis. In the other three animals in this group, subretinal injection of rAAV2tYF-PR1.7-hCNGB3 at 1.2×10^{11} VG/eye was associated with an anterior segment inflammatory response, which resolved between study weeks 4 and 9, and a posterior segment inflammatory response, which persisted through study week 13 (Fig. 1). In addition to varying amounts of white vitreous cells and vitreous haze, white peri-

vascular sheathing around retinal blood vessels and white to gray-white subretinal infiltrates within and outside of the injection site were sometimes noted.

In the higher dose group, subretinal injection of rAAV2tYF-PR1.7-hCNGB3 at 1.2×10^{12} VG/eye was associated with an anterior and posterior segment inflammatory response, which persisted through study week 13. Notable posterior segment inflammatory responses included white vitreous cells, vitreous haze, white perivascular sheathing around retinal blood vessels, and white to gray-white subretinal infiltrates within and outside of the injection site. In one instance the inflammation was severe enough to warrant antiinflammatory therapy. Vitreous haze was of greater severity and more persistent than in the other two groups.

Histopathological Findings

There were no test article-related gross necropsy observations. Histopathology demonstrated mononuclear cell infiltrates in the vitreous body/optic disc, of minimal intensity, in the vector-injected eye of all animals at both dose levels (Fig. 2). All other tissues collected for histopathological examination showed no abnormalities.

Antibody and T Cell Responses to AAV and CNGB3

Titers of serum neutralizing antibodies to AAV2tYF are shown in Table 2. An increase in antibody titer was observed in all vector-injected animals by study day 92, and titers were generally

Figure 2. Histological findings. *Top left:* A normal optic disc and retina in an eye injected with the vehicle control article. *Top right:* A normal retina from an uninjected eye. *Inset* shows the absence of mononuclear infiltrates around the blood vessel. The *lower left* panel shows mononuclear infiltrates around the blood vessel in the optic disc of an animal given 4.0×10^{11} vg/mL, and the inset shows a higher magnification of the blood vessel. The *lower right* panel shows mononuclear infiltrates around a retinal blood vessel and in the vitreous body in an animal given 4.0×10^{12} vg/mL, and the inset shows a higher magnification of the optic nerve. The low magnification was 10 $\times$ and high magnification (insets) was 40 $\times$.

Table 2. Serum anti-AAV2tYF antibody titers in cynomolgus macaques

Group	Animal ^a	Sex	Predose	Day 5	Day 92
1	I05376	M	<5		<5
	I05377	M	<5		<5
	I05382	F	<5		<5
	I05383	F	<5		<5
2	I05378	M	10		160
	I05379	M	<5		80
	I05384	F	40	20	
	I05385	F	<5		20
3	I05380	M	10		320
	I05381	M	<5		160
	I05386	F	40		320
	I05387	F	<5		160

^aAnimal I05384 was euthanized on day 5; all other animals were euthanized on day 92.

F, female; M, male.

higher in the animals that received the higher dose of rAAV2tYF-PR1.7-hCNGB3.

No animal developed antibodies to hCNGB3 and no animal had a positive ELISPOT response to AAV2tYF capsid or hCNGB3 peptides (data not shown).

Biodistribution

For biodistribution analysis, DNA was extracted from tissues, 0.2 μ g of DNA was tested by qPCR, and results are reported as vector DNA copies per microgram of host DNA. Samples of lens and vitreous contained little DNA, and the entire extracted sample was dried and used for qPCR analysis. Results are reported as copies per sample (and also as copies per microgram of DNA if the mass of DNA recovered was measurable).

Levels of vector DNA in ocular tissues were related to proximity to the dose site and time of sacrifice. Vector DNA was detected in the retina from the injected eye of seven of eight vector-injected animals (Table 3) and in the lens and vitreous of the injected eye from all vector-injected animals (Table 4). The animal euthanized on study day 5 had the highest level of vector DNA in the retina, anterior chamber, and vitreous of the injected eye and also had low levels of vector DNA detected in the anterior segment and retina from the uninjected eye. One animal in the higher dose group had vector DNA detected in the anterior segment, optic nerve, lens, and vitreous but not in the retina in the injected eye. The reason the retina was negative is not known.

Low levels of vector DNA were detected in a limited number of nonocular tissues from vector-injected animals, including the spleen and the cervical, mandibular, or parotid lymph nodes (Table 5). Vector DNA was not found in the heart, lungs, liver, kidney, testes, or ovaries from any animal.

A low level of vector DNA (15 copies) was also detected in vitreous humor from the right eye of one animal administered vehicle control article. This was investigated and a source of contamination was not determined.

DISCUSSION

Mutations in *CNGB3* are responsible for approximately half of all cases of achromatopsia. Preclinical proof-of-concept studies in mouse and dog models of *CNGB3* achromatopsia demon-

Table 3. Vector DNA in ocular tissue, visual pathways, and cerebellum

	Group 1				Group 2				Group 3			
	I05376	I05377	I05382	I05383	I05378	I05379	I05384	I05385	I05380	I05381	I05386	I05387
Ocular tissue												
Anterior segment (left)	—	—	—	—	—	—	—	—	—	—	—	—
Retina (left)	—	—	—	—	—	—	66	—	—	—	—	—
Optic nerve (left)	—	—	—	—	—	—	3,318	—	—	—	—	—
Anterior segment (right)	—	—	—	—	—	—	17,108	—	—	792	—	—
Retina (right)	—	—	—	—	90,149	141,994	32,778,995	1,512,418	624,453	—	15,146	831,640
Optic nerve (right)	—	—	—	—	—	—	57	—	—	69	—	58
Optic chiasm	—	—	—	—	—	—	—	—	—	—	—	—
Visual pathways												
Optic tract (left)	—	—	—	—	—	—	—	—	—	—	—	—
LGN (left)	—	—	—	—	—	—	—	—	—	—	—	—
Occipital cortex (left)	—	—	—	—	—	—	—	—	—	—	—	—
Optic tract (right)	—	—	—	—	—	—	—	—	—	—	—	—
LGN (right)	—	—	—	—	—	—	—	—	—	—	—	—
Occipital cortex (right)	—	—	—	—	—	—	—	—	—	—	—	—
Cerebellum	—	—	—	—	—	—	—	—	—	—	—	—

Note: Data are expressed as vector genome copies (VG) per microgram of DNA.

Note: Animal I05384 was euthanized on study day 5; all others were euthanized on study day 92.

Symbol and abbreviation: —, less than the lower limit of quantification (50 VG/ μ g of total DNA); LGN, lateral geniculate nucleus.

Table 4. Vector DNA in lens and vitreous

Group	Animal number	Lens (left)			Lens (right)		
		Copies per sample	Mass per sample (μ g)	Copies per μ g	Copies per sample	Mass per sample (μ g)	Copies per μ g
1	I05376	—	0.00	NA	—	0.00	NA
	I05377	—	0.00	NA	—	0.00	NA
	I05382	—	0.00	NA	—	0.00	NA
	I05383	—	0.00	NA	—	0.00	NA
2	I05378	—	0.00	NA	78	0.00	NA
	I05379	—	0.00	NA	458	0.00	NA
	I05384	—	0.00	NA	60,291	0.00	NA
	I05385	—	0.00	NA	149	0.00	NA
3	I05380	—	0.00	NA	332	0.00	NA
	I05381	—	0.00	NA	3,516	0.00	NA
	I05386	—	0.00	NA	494	0.00	NA
	I05387	—	0.00	NA	76,252	0.00	NA

		Vitreous (left)			Vitreous (right)		
		Copies per sample	Mass per sample (μ g)	Copies per μ g	Copies per sample	Mass per sample (μ g)	Copies per μ g
1	I05376	—	0.00	NA	—	0.00	NA
	I05377	—	0.00	NA	—	0.00	NA
	I05382	—	0.00	NA	—	0.00	NA
	I05383	—	0.00	NA	15	0.00	NA
2	I05378	—	0.00	NA	302	0.00	NA
	I05379	—	0.00	NA	13,637	0.00	NA
	I05384	—	0.00	NA	1,008,270	0.02	62,238,893
	I05385	—	0.00	NA	14,255	0.00	NA
3	I05380	—	0.00	NA	3,713	0.00	NA
	I05381	—	0.00	NA	12,825	0.00	NA
	I05386	—	0.00	NA	—	0.00	NA
	I05387	—	0.00	NA	26,701	0.00	NA

Note: Data are expressed as vector genome copies (VG) per microgram of DNA.

Note: Animal I05384 was euthanized on study day 5; all others were euthanized on study day 92.

Symbol and abbreviations: —, less than the lower limit of quantification (50 VG/ μ g of total DNA); NA, not applicable.

strated the ability of AAV vectors expressing human CNGB3 to rescue cone function.^{13,14} The rAAV2tYF-PR1.7-hCNGB3 vector was designed using a 1.7-kb L-opsin promoter that directs strong transgene expression in all three types of primate

cone photoreceptors, a cDNA that was optimized on the basis of human codon usage, and an AAV2tYF capsid that is efficient at transducing primate cone photoreceptors after subretinal injection. Because primate retinas have a thick

Table 5. Vector DNA in nonocular tissues

	Group 1				Group 2				Group 3			
	I05376	I05377	I05382	I05383	I05378	I05379	I05384	I05385	I05380	I05381	I05386	I05387
Nonocular tissue												
Heart	—	—	—	—	—	—	—	—	—	—	—	—
Lung	—	—	—	—	—	—	—	—	—	—	—	—
Liver	—	—	—	—	—	—	—	—	—	—	—	—
Spleen	—	—	—	—	81	49	14	46	189	254	45	57
Kidney	—	—	—	—	—	—	—	—	—	—	—	—
Testes	—	—	—	—	—	—	—	—	—	—	—	—
Ovaries	—	—	—	—	—	—	—	—	—	—	—	—
Lymph nodes												
Cervical (left)	—	—	—	—	—	—	—	—	—	—	—	—
Cervical (right)	—	—	—	—	13,319	—	58	—	—	—	—	—
Mandibular (left)	—	—	—	—	—	—	—	—	—	—	—	165
Mandibular (right)	—	—	—	—	—	—	—	1,645	—	—	—	1,775
Parotid (left)	—	—	—	—	—	—	—	—	—	—	—	—
Parotid (right)	—	—	—	—	105,180	—	—	—	—	—	310	179,649

Symbol: —, less than the lower limit of quantification (10 VG per sample).

Note: Data are expressed as vector genome copies (VG) per microgram of DNA.

Note: Animal I05384 was euthanized on study day 5; all others were euthanized on study day 92.

inner limiting membrane that restricts the ability of AAV vectors to transduce photoreceptor cells after intravitreal delivery,³¹ subretinal injection was the route of delivery used in preclinical studies and will be the route of delivery in planned clinical trials.

The key toxicology finding in the current study was that subretinal administration of rAAV2tYF-PR1.7-hCNGB3 resulted in a dose-related anterior and posterior segment inflammatory response that improved over time. Aqueous cells, sometimes with aqueous flare, were observed at the study day 3 evaluation in all groups and generally resolved or were at the 1+ level by the study week 4 examination. Vitreous cells were observed at the trace or 1+ level in the vehicle control group and resolved by study week 9 or 13. Vitreous cells and vitreous haze were greater in the vector-treated eyes and were greater at the higher dose than at the lower dose. Persistence of vitreous cells for an extended period of time after resolution of vitreous haze, as seen in this study, can also be seen in patients with uveitis, and haze is considered to be a better indicator of activity in posterior uveitis.³² The ocular inflammation observed in this study is similar to the ocular inflammation seen in nonhuman primates after intravitreal injection of rAAV2tYF-CB-hRS1, an AAV vector expressing retinoschisin (RS1) and packaged in AAV2tYF capsids.³³

Routine postoperative medication in this study consisted of topical atropine, antibiotics, and corticosteroids during the first 10 days after surgery and additional dilating and antiinflammatory medications at later time points, based on the clinical evaluation by the attending veterinarian. Previous studies in which humans received subretinal injections of AAV vector expressing RPE65 or REP1 employed a variety of postoperative dilating, antibiotic, and antiinflammatory regimens, including oral corticosteroids in some studies.^{34,35} Results from the present nonclinical study suggest that oral corticosteroids should be considered for use as part of the postoperative antiinflammatory regimen in clinical trials of rAAV2tYF-PR1.7-hCNGB3.

The inflammatory response likely represents a reaction to the AAV capsid. Serum levels of neutralizing antibodies were induced against AAV2tYF in this study at levels similar to those seen in a previous study in which cynomolgus macaques were administered rAAV2tYF-CB-hRS1 by intravitreal injection. This contrasts with studies in mice, in which subretinal injection of AAV vector induced a lower anti-AAV antibody response than intravitreal injection.³⁶ There were no T cell responses to AAV capsid peptides and no antibody or T cell responses to CNGB3.

Other parameters tested demonstrated no evidence of local or systemic toxicity and no changes in IOP, VEP responses or in hematology, coagulation, or clinical chemistry parameters and no clinically important changes in ERG responses. Results of biodistribution studies demonstrated that the vector did not spread widely or consistently outside the injected eye. High levels of vector DNA were found in vector-injected eyes, but minimal or no vector DNA was found in any other tissue. The highest levels were in the animal euthanized on study day 5, which is consistent with other studies that have shown higher levels of vector DNA in animals euthanized 1 or 4 weeks after vector administration compared with animals euthanized 3 months after vector administration.^{33,37,38}

In other studies, dose-dependent restoration of cone photoreceptor function after subretinal injection of rAAV2tYF-PR1.7-hCNGB3 was demonstrated in CNGB3 knockout mice and CNGB3 mutant dogs. In the mouse model, cone ERG responses developed in 31% of eyes treated at a vector concentration of 1×10^{12} VG/ml and 90% of eyes treated at a vector concentration of 4.2×10^{12} VG/ml.³⁹ In the dog model, cone ERG responses developed in two of three eyes treated at a vector concentration of 2×10^{11} VG/ml and eight of eight eyes treated at a vector concentration of 1×10^{12} VG/ml (A. Komaromy and G. Ye, unpublished observations). However, because of species differences in the efficiency of AAV vectors packaged in capsids of different serotypes to enter cells, and of promoters to drive transgene expression after vector entry into cells, caution must be exercised when using efficacy results from studies in mice to assist in defining a minimally effective dose in humans. In a study in nonhuman primates, a vector expressing green fluorescent protein (GFP) driven by the PR1.7 promoter and packaged in AAV2tYF capsids achieved high-level transgene expression in most primate cone photoreceptors when given by subretinal injection at a dose of 2×10^{11} VG/ml and in all primate cone photoreceptors when given at a dose of 5×10^{11} VG/ml. On the basis of results of the efficacy studies in mice and dogs, the GFP expression study in nonhuman primates, and the safety and biodistribution studies in mice and nonhuman primates, we have selected a concentration of 2×10^{11} VG/ml as the initial dose level of rAAV2tYF-PR1.7-hCNGB3 to be evaluated in a phase 1/2 clinical trial.⁴⁰

The method used to produce and purify rAAV2tYF-PR1.7-hCNGB3 is the same as the method used for rAAV2tYF-CB-hRS1.³³ This method resulted in a product with purity >90% and low or

undetectable levels of process residuals, similar to the low levels of process residuals seen in toxicology and clinical batches of an rAAV1-CB-hAAT product that had a favorable safety profile when administered by intramuscular injection in a clinical trial at doses up to 6×10^{12} VG/kg (4.2×10^{14} total VG in a 70-kg person) in subjects with α_1 -antitrypsin deficiency.^{41,42}

In conclusion, subretinal injection of rAAV2TYF-PR1.7-hCNGB3 at a concentration of 4×10^{11} or 4×10^{12} VG/ml was associated with a dose-related anterior and posterior segment inflammatory response that improved over time except that vitreous cells persisted longer than other manifestations of ocular inflammation. Histopathological examination demonstrated minimal mononuclear infiltrates in all vector-injected eyes. Biodistribution studies demonstrated high levels of vector DNA in the injected eye but minimal or no vector DNA in any other tissue. These results support the use of rAAV2TYF-PR1.7-hCNGB3 in clinical studies in patients with achromatopsia. A phase 1/2 clinical trial evaluating rAAV2TYF-PR1.7-hCNGB3 administered by subretinal injection in patients with achromatopsia is scheduled to begin in 2016.⁴⁰

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AUTHOR DISCLOSURE

G.Y., C.G., P.M.R., D.R.K., and J.D.C. are employees and shareholders of AGTC and have a conflict of interest to the extent that this work potentially increases their financial interests. W.W.H. and the University of Florida have a financial interest in the use of AAV therapies and are shareholders of AGTC. Covance is the drug development business of Laboratory Corporation of America Holdings (LabCorp). Content was developed by a scientist (L.M.S.) who at the time was affiliated with the LabCorp Clinical Trials or Tandem Labs brands, now part of Covance. None of the other authors has a competing financial interest.

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