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Tai, DS
Hu, C
Lee, CCI
et al.

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Development of Operational Immunologic Tolerance with Neonatal Gene Transfer in Nonhuman Primates: Preliminary Studies

Denise S. Tai, MD[1], **Chuhong Hu, MD**[1], **C. Chang I. Lee, PhD**[2], **Michele Martinez, MS**[2], **Gloria Cantero, PhD**[1], **Elizabeth H. Kim, BA**[1], **Alice F. Tarantal, PhD**[2*], and **Gerald S. Lipshutz, MD MS**[1,*]

[1]Department of Surgery, David Geffen School of Medicine, University of California, Los Angeles, CA

[2]California National Primate Research Center and Departments of Pediatrics and Cell Biology and Human Anatomy, University of California, Davis, CA

Abstract

Achieving persistent expression is a prerequisite for effective genetic therapies for inherited disorders. These proof-of-concept studies focused on AAV administration to newborn monkeys. Serotype rh10 AAV expressing ovalbumin and green fluorescent protein (GFP) was administered intravenously at birth and compared to vehicle controls. At 4 months postnatal age a second injection was administered intramuscularly, followed by vaccination at 1 year of age with ovalbumin and GFP. Ovalbumin was highest 2 weeks post-administration in the treated monkey, which declined but remained detectable thereafter; controls demonstrated no expression. Long-term AAV genome copies were present in myocytes. At 4 weeks, neutralizing antibodies to rh10 were present in the experimental animal only. With AAV9 administration at 4 months, controls showed transient ovalbumin expression that disappeared with development of strong anti-ovalbumin and anti-GFP antibodies. In contrast, increased and maintained ovalbumin expression was noted in the monkey administered AAV at birth, without antibody development. After vaccination, the experimental monkey maintained levels of ovalbumin without antibodies, whereas controls demonstrated high levels of antibodies. These preliminary studies suggest that newborn AAV administration expressing secreted and intracellular xenogenic proteins may result in persistent expression in muscle, and subsequent vector administration can result in augmented expression without humoral immune responses.

Keywords

adeno-associated virus; gene therapy; neonate; nonhuman primate; tolerance; Ovalbumin

Please address correspondence to: G.S.L. (glipshutz@mednet.ucla.edu), Gerald S. Lipshutz MD, 77-120 CHS, Department of Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095-7054, Telephone: 310-267-9592, Fax: 310-267-359.

*Co-Senior authors

Conflicts of Interest

The authors have nothing to disclose.

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Introduction

Significant advances have been made toward the successful treatment of inherited diseases by gene transfer[1–10]. However, in some individuals with inherited disorders, the normal protein may be recognized as non-self or as a 'neoantigen', and the introduction or expression of that protein may result in the induction of a neutralizing immune response[11–13]. Thus, the success of gene therapy is dependent on the development of a state of tolerance or immunological anergy to secreted protein products. Achieving this could have an impact on the treatment of human disorders of secretory proteins, such as hemophilia and Pompe disease, where inhibitory antibodies can develop and complicate recombinant protein-based therapies[14].

Viral vector gene delivery before the development of immune competence may have important advantages for induction of tolerance to therapeutic gene products (and not to viral capsid antigens). Prior studies in mice have shown that the immune system does not respond to transgene-encoded or viral capsid proteins when adeno-associated virus (AAV) is administered *in utero* or in the early neonatal period[15–18]. If a state of immunological tolerance and/or anergy to the transgene-encoded protein could be established during fetal or neonatal life, re-administration of a viral vector postnatally and with an alternative serotype[19] would allow for augmentation of expression, with the goal of achieving therapeutic protein levels. In addition, if gene therapy alone failed to achieve therapeutic levels of protein, the development of tolerance would permit the exogenous administration of protein (e.g., factor VIII, factor IX, or lysosomal enzymes) postnatally without the concern for the development of inhibitory antibodies. Because many inherited disorders can be identified during the fetal period, fetal or newborn gene transfer could provide the ideal time for treatment and may prevent the pathology associated with disease[20–21].

Studies suggesting the potential for immunologic tolerance to develop to antigens presented in the neonatal period have been demonstrated primarily in murine models[16–18, 22] with few having been conducted in nonhuman primates at a clinically relevant time where intervention could be performed in humans. Humans are born with a considerably more mature immune system than rodents and are capable of generating effective T and B cell responses with populated peripheral lymphoid tissues, suggesting the newborn mouse is immunologically equivalent to a second trimester human fetus. Murine findings (e.g., reduced expression of CD40 ligand and diversity of T cell receptors, enhanced Th2 and diminished Th1 responses, and lack of peripheral effector T cells prior to postnatal day 2 among others), while interesting, are not necessarily predictive of the human immune response[23–25]. The significant differences in the state of immunologic development at birth between rodent and primate immune systems have led to findings that are difficult to translate to the human clinical setting, as tolerance is easier to achieve in mice[26–28]. In addition, strain-specific differences in murine models have also affected results of gene transfer experiments and, at times, have underestimated human immune responses. Thus, studies with the potential for translation to human therapy must be tested in preclinical nonhuman primates where immunologic ontogeny is closer to humans[29]. The results described herein represent a pilot study of such an intervention.

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Results and Discussion

At birth one newborn rhesus macaque was intravenously (IV) administered 8×10^{12} genome copies (gc)/kg of AAV serotype rh10 expressing ovalbumin (OVA) and GFP (OVA-2A-GFP) while two newborns received saline intravenously (IV) and served as vehicle controls (Figure 1A and B). All newborns had anti-AAV titers ≤ 1:5 at birth to all tested AAV serotypes (Table 1). Sampling blood at two weeks of age in both groups of animals, the monkey that received AAV rh10 OVA-2A-GFP at birth demonstrated elevated neutralizing antibody (NAb) titers to AAV8 (320) and AAV rh10 (2560). In contrast, neonatal injection in mice often does not result in an immune response to viral vectors;[16, 18] this difference is likely due to the state of immune maturity at birth. The development of a NAb response here to serotypes 8 and rh10 prompted the selection of AAV9 as the serotype of choice for the second postnatal injection at 4 months (Table 1). The injection was performed intramuscularly (IM) because of low-level anti-AAV9 (1:10) NAb in the experimental animal. At one year all monkeys received 50 μg IM challenges of purified OVA and recombinant GFP proteins with adjuvant to evaluate if previously established tolerance to transgene-encoded proteins could be broken. Serial blood samples were collected, and at approximately 1 year 4 months postnatal age tissue harvests were performed.

All complete blood counts (CBCs) and clinical chemistry panels were within the normative range for all animals in this age group (data not shown). Growth trajectories and development were also within normal limits when compared to concurrent and historical controls (Figure 2); animals increased ~5.8 times in weight from 0.48 ± 0.03 kg at birth to 2.8 ± 0.22 kg at approximately 1 year of age. By comparison, the average human newborn weighs 3.4 kg[30–31] at birth (95% in the range 2.5–4.6 kg), generally doubles its weight by 5 months, and triples by 1 year[32].

Growth trajectory is an important consideration for neonatal gene therapy (Figure 2). Previous murine studies have demonstrated that neonatal IV administration of AAV results in high early expression in the liver and lower expression in myocytes[15]. With hepatic proliferation, substantial loss of expression occurs in the rapidly developing neonatal mouse liver[15, 18, 33]. Prior studies by Wilson et al. with AAV8-mediated transduction in one-week-old rhesus monkeys were found to be safe and to produce high hepatic GFP transduction using the liver-specific promoter thyroxine binding globulin[35]. However, in those studies, significant dilution of vector genomes and thus loss of transgene expression was noted in the five weeks following vector administration. This passive loss and active degradation of non-integrated AAV DNA in rapidly dividing tissues such as the liver is an important challenge to the preservation of antigen expression and the potential need for successive vector therapy. As the average weight of the human liver at birth is 78 grams, which doubles by 4 months, and increases to 288 grams by one year (an increase of 3.7 times[34]), a similar fold increase in size would be expected in the nonhuman primate and likely explains the findings of substantial loss of hepatocyte expression in those studies.

In our investigation, the monkey that received rh10 OVA-2A-GFP at birth showed a peak in OVA expression two weeks after vector administration, which remained stable for approximately three months (Figure 3); in contrast to the studies by Wilson et al., ours

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longitudinally measured secreted OVA, allowing for greater sensitivity of detection. By four months of age, OVA levels were noted to decline (which likely was related to the growth trajectory in this age group and loss from hepatocytes) but remained detectable. As the chicken ß-actin promoter/CMV enhancer was utilized in the studies described herein in order to provide ubiquitous expression, complete loss of expression (by detection of circulating plasma ovalbumin) was not expected or found, likely because myocytes, which are postmitotic at birth, produced the majority of expression. It is this persistence of protein expression that we propose is key to the maintenance of immune unresponsiveness[36–37].

The humoral immune system was not responsible for the decline in OVA expression seen early in the rhesus monkey administered AAV at birth. Humoral immune responses against OVA and GFP were examined after neonatal injection at 4 months of age (Figure 4). The monkey injected at birth (shown as #3 in Figure 4) did not demonstrate antibody development to OVA or GFP after the initial injection. Upon administration of a second postnatal dose of AAV (serotype 9 OVA-2A-GFP), OVA expression increased and remained elevated (Figure 3, green line). There was no substantial decline in OVA expression after OVA and GFP vaccinations at 12 months postnatal age. Similarly this monkey (#3 in Figure 4) did not demonstrate high-level antibody development to OVA or GFP after vaccination with OVA and GFP in adjuvant.

Conversely, the vehicle-injected control animals showed no evidence of serum OVA expression when examined to 4 months postnatal age (red and blue lines, Figure 3). When AAV9 was administered IM at 4 months, the sample time point immediately following injection showed that both monkeys had low levels of OVA expression and subsequently disappeared and remained undetectable. Humoral immune responses against OVA and GFP were evaluated by ELISA (Figure 4). While the two control monkeys (#1, #2) showed no evidence of high titer antibodies to OVA and GFP before IM injection, they concomitantly developed high antibody titers after postnatal AAV9 injection at 4 months postnatal age, consistent with a primary humoral immune response which persisted. The pattern of the humoral immune response to GFP was similar to that of OVA. As expected, protein vaccination at 1 year of age did not change the established humoral immunity in these two animals.

At tissue harvest no gross abnormalities were found. The rectus femoris muscle (the site of the AAV9 IM injection) of the treated animal clearly expressed GFP by both gross examination and whole tissue fluorescence (Figure 5A) (muscle not injected from the same experimental animal is shown in Figure 5B) and by immunohistochemistry (Figure 5C); high AAV gc were found at this site (75.6 genome copies/diploid nucleus).

In general, the IV injection in the experimental animal produced higher vector genome copies in many tissues when compared to the controls (Figure 6). With the IM injection at 4 months, it appears that some vector may have spread from the injected muscle to the systemic circulation, resulting in low-level transduction of tissues including the central nervous system (CNS), cardiothoracic organs, kidneys and adrenals, gastrointestinal tract, and musculoskeletal system. The differences in viral copy numbers in tissues of the experimental animal demonstrate higher (when compared to the controls, but still low-level)

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transduction in the CNS, suggesting that the brain and spinal cord may be more accessible with a systemic injection at birth. The cardiothoracic system (lungs, heart, and aorta) of the experimental animal was transduced at higher levels than other organs and tissues. The kidneys and adrenal glands were also transduced in the experimental animals, however less than the cardiothoracic organs. The gastrointestinal tract was generally poorly transduced by AAV in both groups with low copy numbers in the stomach, pancreas, small intestine, colon, and omentum. The exceptions were the esophagus and peritoneum in the experimental animal where expression was higher, albeit still low compared to the aorta and ventricles.

We and others when studying mice[15], and studies performed by Wilson et al.[35] in infant rhesus monkeys, have demonstrated the loss of AAV genome copies in the growing liver. In these studies, we found few transgene-expressing hepatocytes in the liver (Figure 5D); they were widely located with no clusters of expressing cells. Transgene-expressing cells were found in all lobes of the liver: quadrate, caudate, left, and right. In the studies on AAV copy numbers in the liver, there was no substantial difference between the experimental and controls suggesting that the AAV copy number detected at 16 months was due to the 4 month injection and that the copies resulting from the neonatal injection in the experimental animal were lost with hepatocyte division, consistent with the Wilson et al. infant rhesus monkey study[35]. Finally, with the exception of the muscle injection site, low-level transduction of muscle at widespread locations occurred and, importantly, persisted with the injection at birth. We believe that it is this persistence of transgene-encoded protein expression that is important in part to establish and maintain operational tolerance. Thus, the expression in the cardiothoracic organs and skeletal myocytes may be involved as the primary sites of maintenance of expression and source of production of antigen to the immune system.

Taken together, these preliminary findings suggest that establishment of operational tolerance is possible in nonhuman primates by the administration at birth of AAV expressing xenogenic proteins. This results in transgene protein expression without the development of a humoral immune response with either subsequent augmentation of expression with AAV serotype switched readministration, or with protein vaccination with adjuvant.

Advancements in prenatal diagnosis continue to influence the decision of when treatment should or could be initiated for inherited genetic diseases. In humans, techniques for detecting fetal genetic material in the maternal circulation raise the possibility of using maternal blood extensively to diagnose fetal disease as “cell-free” nucleic acids can be isolated from maternal blood as early as the fifth postmenstrual week[38]. Such advancing technology offers a method to alter the timing of disease diagnosis and potential intervention prior to or at birth. For monogenic disorders where there is a deficiency of a protein, it is now clear that the primary obstacle to overcome lies in controlling immune responses to vector and/or to transgene-encoded proteins.

Correction of genetic diseases during this early developmental window offers several potential advantages over postnatal gene transfer. First, it may allow for the correction or prevention of genetic diseases before the onset of pathology. Early gene transfer and long-term expression of therapeutic proteins during development and at birth may limit or

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abrogate the pathologic consequences of genetic mutations and may provide the best opportunity for treatment. Second, transduction efficiency may be enhanced in the fetal and early neonatal period. For instance, proliferative stem cells may be more effectively transduced than more quiescent adult stem cells, and organs inaccessible later in life may also be more readily transduced *in utero* and in the early postnatal period. And third, immune immaturity may be able to overcome the barrier of immune responses that complicates gene transfer postnatally and in adults[2, 39].

While these studies are preliminary and very limited in number, they do suggest that operational tolerance to transgene-encoded proteins can be induced in a nonhuman primate model with an immune system similarly developed at birth to that of the human neonate. Demonstrating reproducibility, and understanding the mechanism, of these findings in larger numbers of animals will be an important next step. If successful, such findings could have an impact on the treatment of human disorders of secretory or intracellular proteins and neuromuscular disorders where inhibitory antibodies can develop and complicate recombinant protein-based therapies. Conceivable applications where tolerance induction is important would include treatment in neonatal hemophiliac boys to prevent inhibitor development if infusion of recombinant factor VIII is needed later in life (if AAV-mediated factor VIII production is insufficient) and in cross-reacting immunologic material negative Pompe patients where large doses of acid alpha-glucosidase would be administered.

Materials and Methods

AAV vector

A bicistronic, serotype rh10 rAAV containing the cDNAs OVA and GFP under the control of the chicken β-actin promoter/CMV enhancer (CBA) was developed (Figure 1). OVA and GFP were selected as xenogenic transgene proteins in order to evaluate humoral and cell mediated immune responses. Construction of the transgene plasmid p1044 OVA-2A-GFP was performed by overlapping PCR to combine the OVA and GFP cDNAs linked by a 2A sequence from foot-and-mouth disease virus (see Supplementary Figure). Three plasmids containing the OVA, GFP, and 2A cDNAs were individually PCR amplified under the following conditions. The plasmid BS-OVA containing the cDNA for secreted OVA (kindly provided by R. Herzog, University of Florida, Gainesville, FL) was amplified for OVA using a forward OVA primer (5′-CGGGATCCATGGGCTCCATCGGTGCAGCA-3′) and reverse primer, 2A-OVA (5′-GACCCTCTTTTGGCTCTAGGGGAAACACATCTGCCAAAG-3′). The plasmid containing the linker 2A DNA (kindly provided by R. Koya, UCLA, Los Angeles, CA) was amplified for 2A using OVA-2A forward primer (5′-TGGCAGATGTGTTTCCCCTAGAGCCAAAAGAGGGTCCGGC-3′) and GFP-2A reverse primer (5′-TCCTCGCCCTTGCTCACCATAGGACCGGGGTTCTCCTCCA-3′). The GFP cDNA was amplified from a plasmid provided by R. Koya using a 2A-GFP forward primer (5′-TGGAGGAGAACCCCGGTCCTATGGTGAGCAAGGGCGAGGA-3′) and GFP reverse primer (5′-CCGGAATTCTTACTTGTACAGCTCGTCCATGCC-3′). The PCR conditions were as follows: initialization at 95°C for 2 minutes followed by 35 cycles of denaturation, annealing and elongation at 95°C × 20 seconds, 60°C × 20 seconds and 72°C × 30 seconds, respectively. Final elongation was performed at 72°C × 3 minutes. The 2A and

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GFP purified DNA products were combined using overlapping PCR, deriving a 2A-GFP segment that was then combined and amplified with the OVA DNA to derive a final product of OVA-2A-GFP. The p1044 plasmid backbone (provided by Julie Johnston, University of Pennsylvania Preclinical Vector Core) was digested with Mlu1 and the OVA-2A-GFP was inserted and ligated by blunt-blunt cloning procedures to create the plasmid 1044 OVA-2A-GFP. Confirmation was performed by sequencing. Vector aliquots were provided by the University of Pennsylvania Preclinical Vector Core[40].

Animals

All animal procedures were approved prior to implementation by the Institutional Animal Care and Use Committee at the University of California, Davis, and were consistent with the requirements of the Animal Welfare Act. Activities related to animal care including diet and housing were performed as per standard operating procedures at the California National Primate Research Center. Normally cycling, adult female rhesus monkeys (*Macaca mulatta*) with a history of prior pregnancy were bred and identified as pregnant according to established methods[41]. All were screened for AAV antibodies and those that were seronegative were selected for the study[35]. Fetal growth and development was monitored sonographically using established protocols and newborns were delivered by cesarean section at term[41,42]. Umbilical cord blood (~15 ml) was collected for CBCs, clinical chemistry panels, serum, and plasma. Infants were raised in the nursery for postnatal studies (see below). Body weights and food intake was recorded daily for the first month of age, then weights were assessed regularly for the duration of the study. Blood samples were collected from a peripheral vessel before and after each injection, and monthly throughout the study period until tissue harvest (Figure 2).

Postnatal Transfers and Tissue Harvests

At birth one newborn was administered 8×10^{12} gc/kg IV of rh10 OVA-2A-GFP viral vector while two newborns received IV saline and served as vehicle controls (Figure 2); administration was performed by right upper extremity peripheral venous injection. All three animals were male. At four months of age all animals received an IM injection of AAV9 OVA-2A-GFP, and at 1 year they received 50 μg IM challenges of purified OVA (Thermo Scientific, Rockford, IL) and recombinant GFP (Vector Laboratories, Burlingame, CA), each prepared 1:1 with alum adjuvant (Thermo Scientific) and prepared and injected under aseptic conditions. Injections were performed in the rectus femoris muscle as multiple ~0.2 μl injections. At approximately 1 year, 4 months postnatal age animals were euthanized by an overdose of pentobarbital and tissue harvests performed with collection of all bilateral organs and the brain, trachea, esophagus, aorta, pericardium, thymus, heart, lung (all lobes), spleen, liver (all lobes), lymph nodes (axillary, inguinal, mesenteric), omentum, peritoneum, pancreas, adrenals, kidneys, reproductive tract, gastrointestinal tract, diaphragm, skin, and muscles (intercostal, thoracic, abdominal, upper and lower limbs). All samples were collected in triplicate and sections of each tissue were placed in phosphate-buffered formalin for histopathology and cryopreserved in OCT.

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Genome Copy Number Determination

Genomic DNA was isolated from tissues using the Gentra Puregene Tissue kit (Qiagen, Valencia, CA) as recommended by the manufacturer. The primers for GFP were as follows: forward 5′- GCAGTGCTTCAGCCGCTAC-3′, reverse 5′-AAGAAGATGGTGCGCTCCTG -3′, and probe 5′ -FAM-CCGACCACATGAAGCAGCACGACTT-TAMSp-3′. The primer sequences for the housekeeping gene ε-globin were as follows: forward 5′-TGGCAAGGAGTTCACCCCT-3′, reverse 5′- AATGGCGACAGCAGACACC-3′, and probe 5′-FAM-TGCAGGCTGCCTGGCAGAAGC-TAMSp-3′. Real-Time quantitative PCR analysis was carried out in 96-well optical plates using the 7900 ABI Sequence Detection System (Applied Biosystems, Foster City, CA) and the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. PCR reactions were run in duplicate in separate wells and contained 1× TaqMan universal master mix with 400 nM of forward and reverse primers and 100 nm probe in a 25-μL reaction volume. The PCR protocol consisted of one cycle of 2 min at 50°C, 15 min at 95°C, followed by 40 cycles at 15 s at 95°C, and 60 s at 60°C.

Immunohistochemistry

Immunohistochemistry for GFP was performed. The four lobes of the liver were examined, four slides each, at four different levels. Briefly, 5 μm paraffin embedded sections were successively deparaffinized with two times xylene washes for 3-minutes, one time 1:1 xylene:100% ethanol for 3 min, two times 100% ethanol for 3 min and then rehydrated with 95% ethanol, 70% ethanol, and 50% ethanol for 3 minutes each. Rehydrated sections were gently rinsed with PBS. Antigen unmasking was performed immersing the rehydrated samples in a Coplin jar filled with 50 mL of 1X Heat Mediated Antigen Retrieval Solution pH 6.0 (Abcam, Cambridge, MA) inside of a vegetable steamer for 20 min at 95–100°C. The staining commenced with 1 h room temperature blocking with 4% goat serum (Gemini Bio-Products, West Sacramento, CA) in PBS. Subsequently 1:200 anti-GFP (FITC) antibody (Fisher Scientific, Catalog number PA146326) was added to 4% goat serum in PBS and incubated overnight at 4°C. The next day, cells were washed three times with PBS for 5 minutes. Slides were mounted with mounting media with DAPI (Invitrogen) and visualized for fluorescence using an Olympus IX71 microscope. Images were captured with cellSens software (Olympus, Center Valley, PA).

AAV NAb Assay

Serum samples obtained at birth (prior to AAV gene transfer) and at 4 weeks postnatal age were singly analyzed for NAb to AAV8, AAV9, and AAV rh10 using an *in vitro* transduction inhibition assay performed on Huh7 cells (University of Pennsylvania) as previously described (without routine *Mycoplasma* screening)[43].

ELISA

ELISAs were performed to determine serum OVA expression levels (ng/mL) at serial time points in serum samples collected. Ninety-six well immunoplates (NUNC, Rochester, NY) were coated with rabbit anti-OVA IgG (Cortex Biochem, Madison, WI, Catalog number

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CR2042RP) diluted 1:5000 in coating buffer and incubated overnight at 4°C. After blocking with 5% fetal calf serum, 100 μl serum samples were added in triplicate and a secondary antibody (rabbit anti-OVA IgG horseradish peroxidase conjugated; Rockland Immunochemicals, Gilbertsville, PA, Catalog number 200-4333) was added at 1:20,000 dilution. After washing, OPD substrate was added and after 6 minutes plates were read at 492 nm. A standard curve was generated using OVA serially diluted from 4 ng/ml. Sample testing was performed in duplicate and results were averaged.

Immune response to OVA and GFP were also assessing using ELISA. Ninety-six well immunoplates (NUNC, Rochester, NY) were coated with OVA (10 μg/mL diluted in coating buffer) or recombinant GFP (6.25 μg/mL diluted in coating buffer) as the capture antigen and incubated overnight at 4°C. Two-fold serial dilutions of serum were prepared beginning from 1:5 to 1:640 and added in duplicate after blocking with 5% fetal calf serum. Goat anti-monkey IgG horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, Catalog number SC2458) at 1:1000 dilution was incubated as the secondary antibody and OPD substrate was used for color development. Results were reported as the optical density (OD) after absorbance reading at 492 nm. Sample testing was performed in duplicate and results were averaged.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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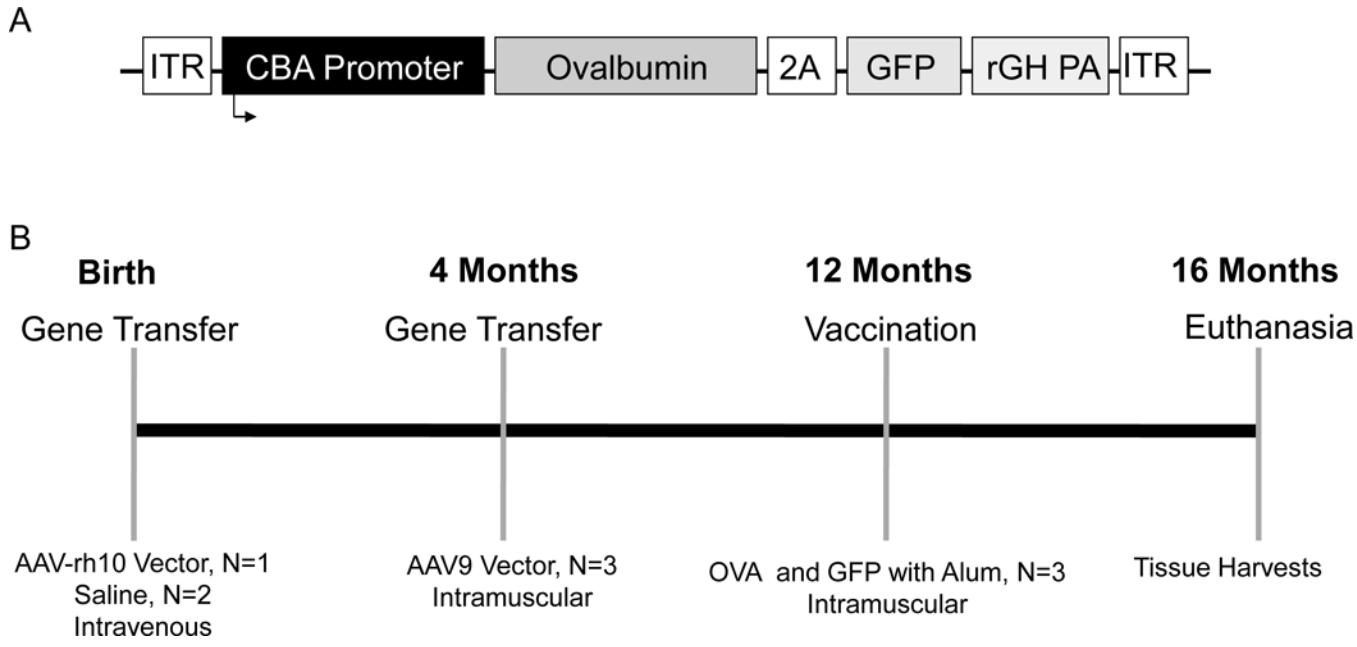

Figure 1.

A) Bicistronic recombinant Adeno-Associated Virus (rAAV). A bicistronic, serotype rh10 rAAV containing the cDNAs for ovalbumin (OVA) and green fluorescent protein (GFP) under the control of the chicken β-actin promoter/CMV enhancer (CBA) was constructed. The plasmid construct includes a linking 2A sequence from foot-and-mouth disease virus and rabbit growth hormone polyadenylation signal (rGHpA). OVA and GFP were selected as xenogenic transgene proteins for the purpose of evaluating humoral immune responses. B) **Overall Study Design**. Rhesus monkeys were delivered by cesarean section at term and nursery reared. At birth, one newborn monkey was intravenously (IV) administered 8×10^{12} genome copies (gc)/kg of AAV-rh10 OVA-2A-GFP viral vector while two monkeys received IV saline and served as vehicle controls. At 4 months of age all monkeys received an intramuscular (IM) injection of 8×10^{12} genome copies gc/kg of AAV9 OVA-2A-GFP. At 1 year of age all monkeys received purified OVA and GFP individually (50 μg IM each) prepared 1:1 with alum adjuvant. At approximately 1 year 4 months postnatal age tissue harvests were performed for a complete analysis.

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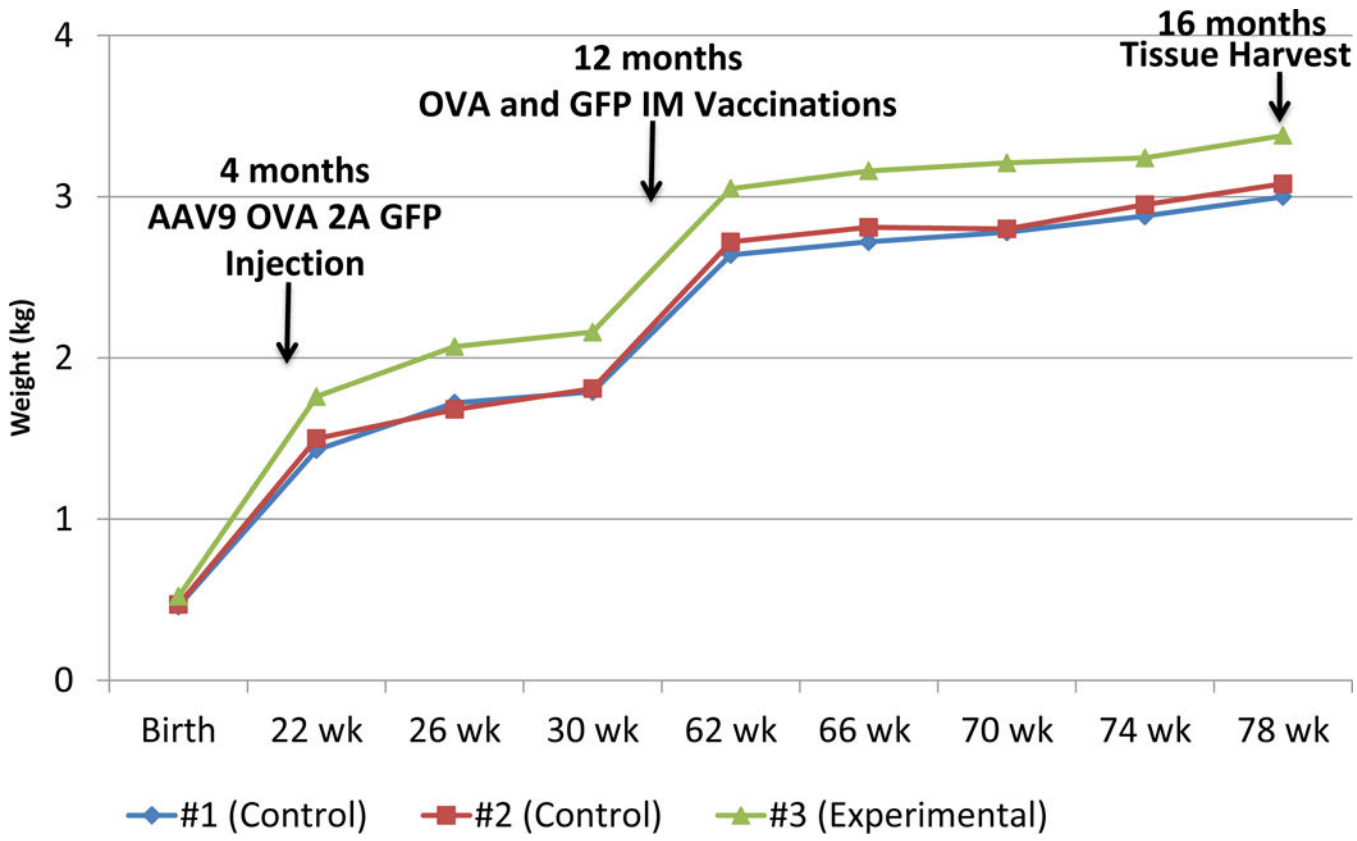

Figure 2. Body Weight

All animals were within the expected weight range during the study and did not differ significantly in weight or weight gain when compared to concurrent or historical controls.

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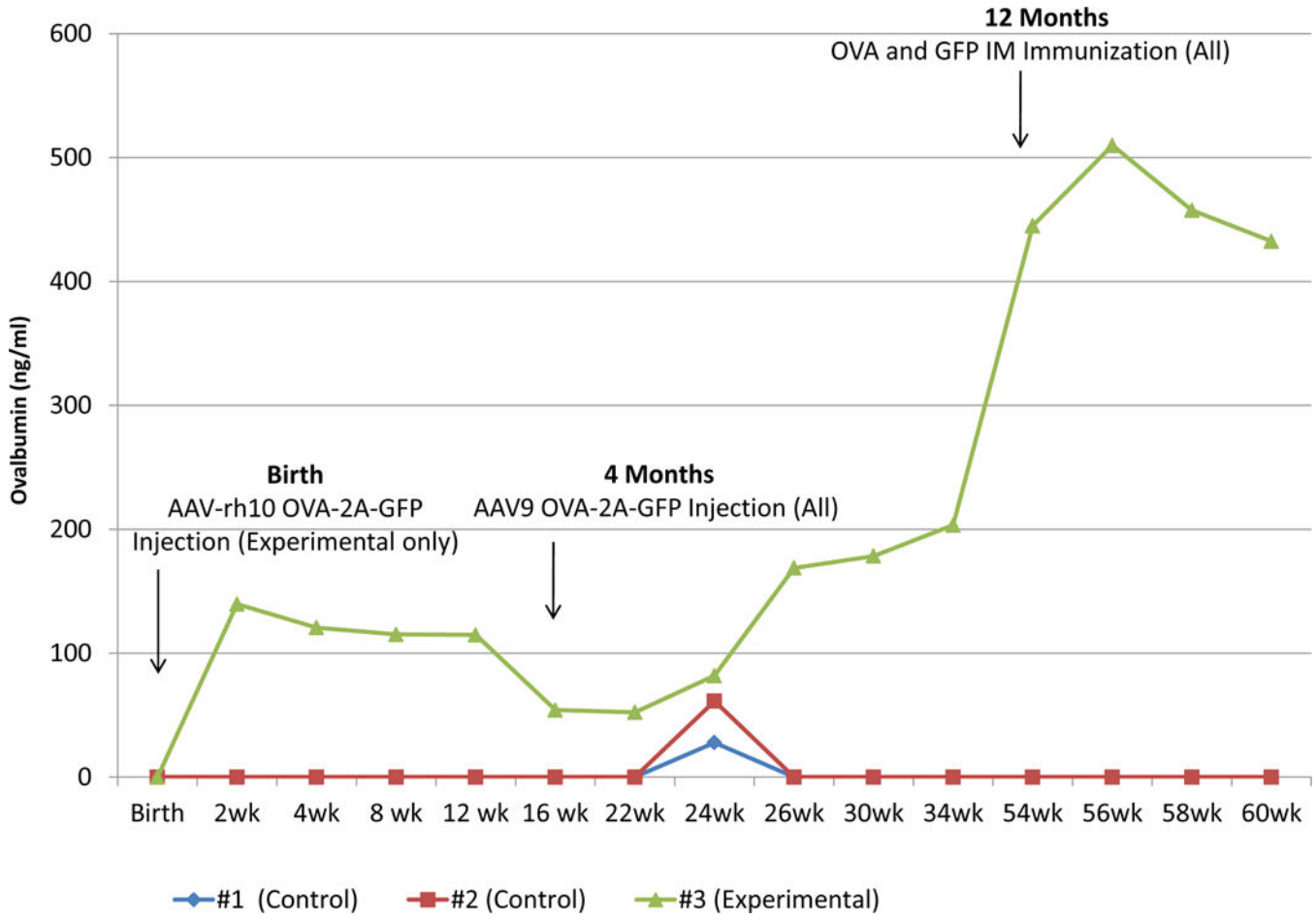

Figure 3. Serum OVA Expression

Serial serum samples were collected from birth until tissue harvest. ELISAs were performed to determine OVA expression (ng/mL) at each time point. In the monkey that received AAV-rh10 OVA-2A-GFP at birth (green line), OVA expression peaked at two weeks after neonatal vector administration and remained stable for approximately three months. By four months of age, OVA levels began to decline but remained detectable. Upon administration of AAV9 OVA-2A-GFP, OVA expression increased and remained elevated. There was no decline in OVA expression after OVA and GFP vaccinations at 12 months postnatal age. Both control monkeys (red and blue lines) had no detectable serum OVA expression until four months, when the AAV9 vector was administered IM. Following AAV9 injection, both monkeys showed low levels of OVA expression that subsequently declined and remained undetectable until tissue harvest.

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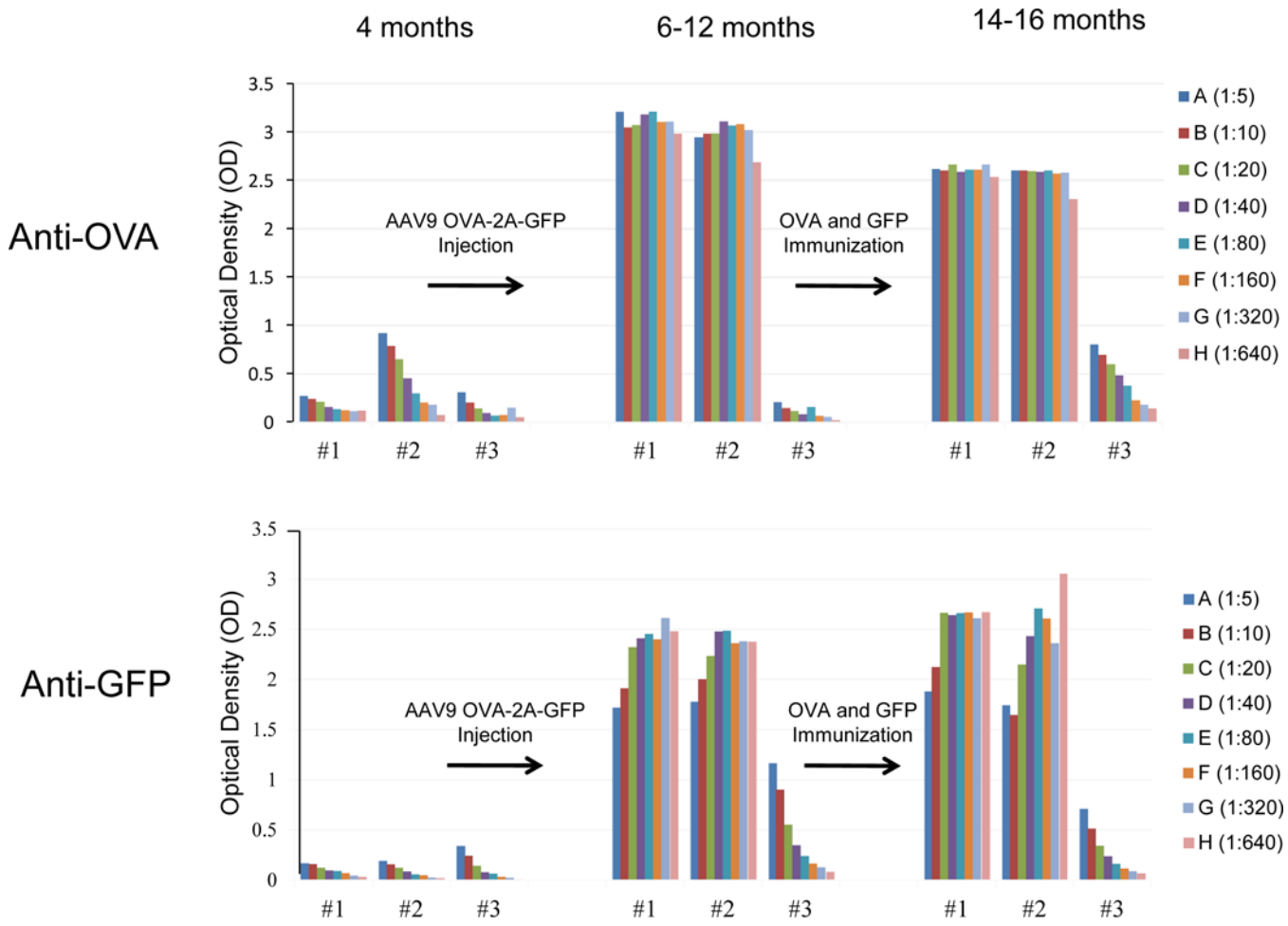

Figure 4. Humoral Immune Responses to Xenogenic Proteins

Humoral immune responses against OVA and GFP were assessed after neonatal AAV injections and OVA and GFP vaccinations by ELISA. Two-fold serial dilutions of serum were prepared beginning from 1:5 to 1:640. Results are reported as the optical density (OD) after absorbance reading at 492 nm. (#1, #2: nonhuman primates administered saline at birth and AAV at 4 months of age; #3: nonhuman primate administered AAV at birth and at 4 months of age.)

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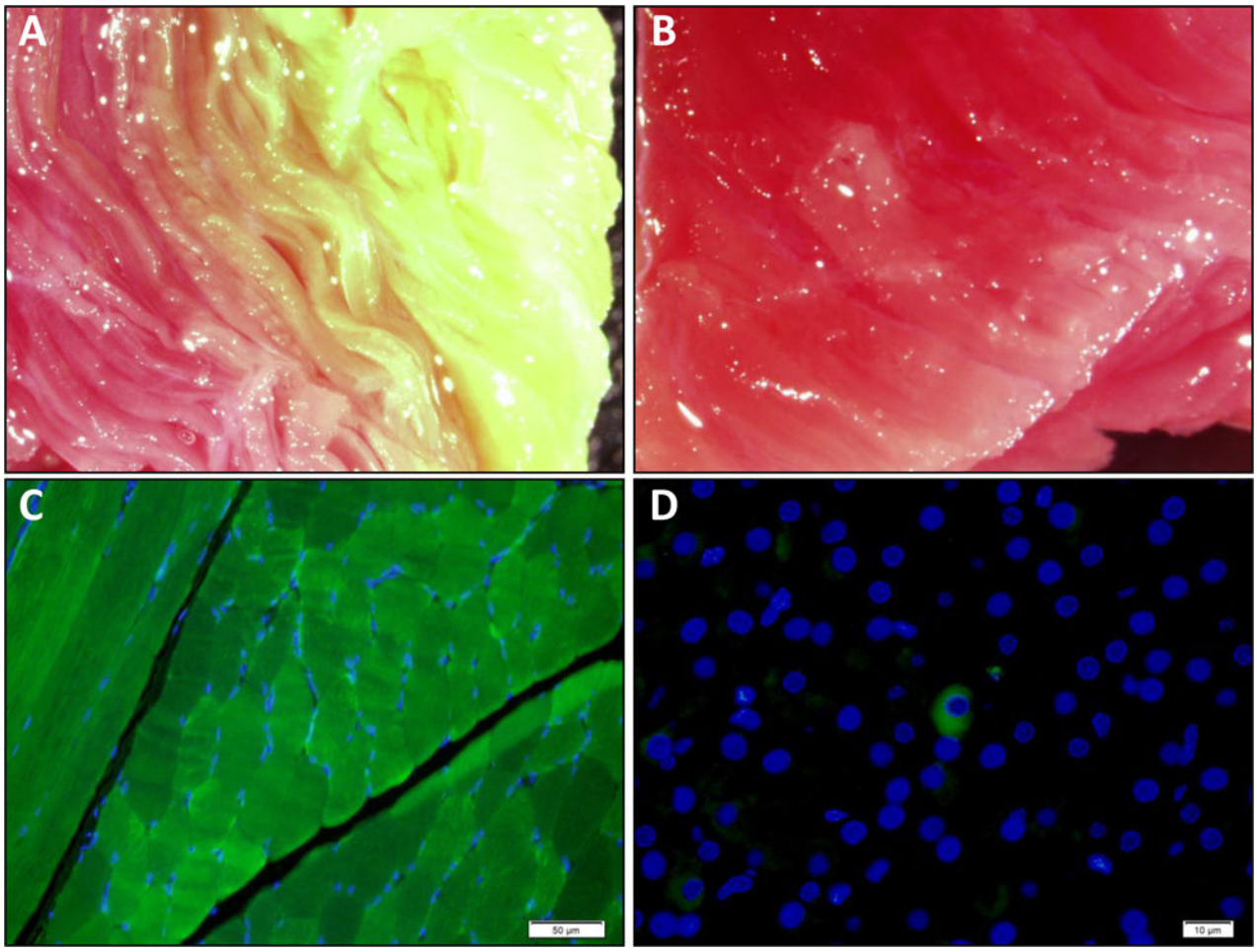

Figure 5. GFP Expression at Tissue Harvest

A. At tissue harvest the rectus femoris muscle strongly expressed GFP grossly. **B**. Adjacent skeletal muscle that was not intramuscularly injected was negative. **C.** Microscopic examination of muscle of the experimental animal demonstrated GFP expression resulting from IM injection. **D**. The liver demonstrates rare scattered transgene-encoded protein positive cells.

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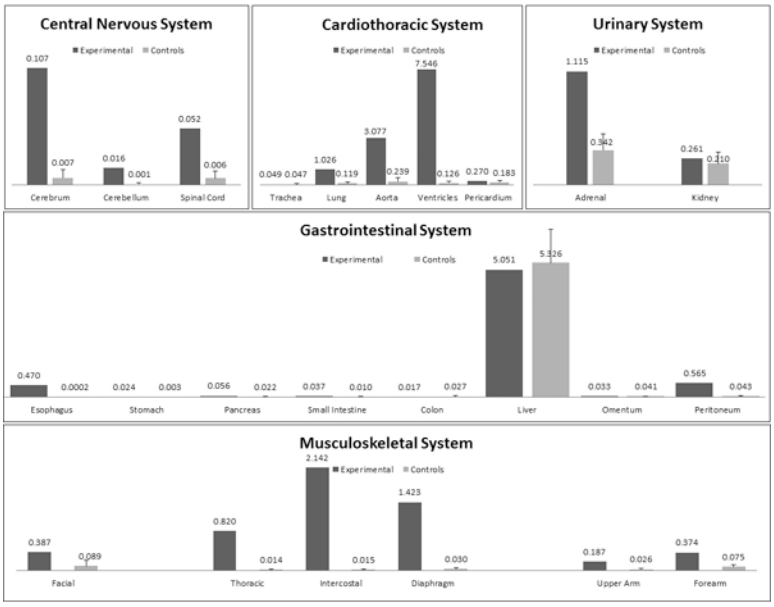

Figure 6. Vector Copy Number in Tissues

At tissue harvest, specimens from the controls and experimental animals were analyzed for sites of AAV transduction following juvenile AAV injection only or injections at birth and as a juvenile, respectively. RT-PCR confirmed that widespread and low-level transduction in the musculoskeletal system and higher expression in the cardiothoracic organs were present in the experimental animal compared to the controls, suggesting these as the sites of persistent transduction after primary injection. (Data is presented as genome copies per diploid nucleus as mean + SD where indicated.)

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Table 1

Prior to selecting the AAV serotype for the second postnatal injection, a neutralizing antibody (NAb) assay was performed to evaluate the serostatus of all rhesus monkeys to selected serotypes. Serum samples obtained from umbilical cord blood at birth and at 4 weeks postnatal age were analyzed for NAb using an *in vitro* transduction inhibition assay. NAb titers were reported as the reciprocal value of the highest serum dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells, with a limit of detection at 1/5 dilution.

NAb 50 in Huh 7 Cells						
	AAV 8		**AAV 9**		**AAV rh10**	
	Birth	**Week 4**	**Birth**	**Week 4**	**Birth**	**Week 4**
1[a]	<5	5	<5	<5	5	10
2[a]	<5	<5	<5	<5	<5	<5
3[b]	<5	**320**	<5	10	<5	**2560**

[a]Rhesus monkey received saline at birth

[b]Rhesus monkey received AAV-rh10 OVA-2A-GFP at birth