USE OF ADENOVIRAL VECTOR FOR STUDYING TRANSPORT KINETICS OF GREEN FLUORESCENT PROTEIN AND INFLAMMATION IN THE FACIAL NERVE SYSTEM OF THE RAT

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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Chairman: W.J. Streit, Ph.D.
Major Department: Molecular Genetics and Microbiology

Adenoviral vectors have been used in gene delivery studies throughout the nervous system. This study investigated the use of Ad.CMV-GFPh in the rat facial nerve system. The goals were to determine the optimal viral titer, transgenic GFPh kinetics, and inflammatory responses when the vector was administered at the peripheral injury site of the facial nerve system. My results indicate the optimal titer for in vivo transduction of this system to be 5e9 pfu/ml. Comparison of the brains of the control animals to the Ad.CMV-GFPh treated animals indicated no difference in reactive gliosis for the first 7 days post injury. Furthermore, the reactive gliosis in Ad.CMV-GFPh treated animals did not show selective gliosis for neurons expressing GFPh. At the peripheral injury site Ad.CMV-GFPh treated animals had increased mononuclear cell infiltration compared to the control animals, while both groups exhibited mast cell infiltration.

INTRODUCTION AND REVIEW

Adenoviruses

Since the 1970s there has been extensive research on adenoviruses. As a result many characteristics of these viruses are well defined. Adenoviruses are approximately 36kB, linear, double stranded DNA viruses. These are non-enveloped, encapsulated viruses which insert their genome perisomally with respect to the host cell's DNA. Replication is dependent on three stages of gene expression: immediate early (IE) genes E1, early (E) genes E2-E4, and late (L) genes. The expression of the immediate early and early genes must occur prior to the onset of viral DNA replication while the late genes are expressed after the start of viral DNA expression (Hermens and Verhaagen, 1998).

Among the unique characteristics of adenoviruses are their ability to infect a variety of cells both in vitro and in vivo. Adenoviruses are also capable of infecting dividing and non-dividing cells. Infection by adenoviruses begin with an unidentified cell surface receptor mediated internalization of the virus (Wickham et al., 1993). Upon release from the endosomes into the cytoplasm, the nucleocapsid is transported to the nucleus where the viral DNA inserts itself perisomally (Greber et al., 1993). At onset of viral gene expression, the immediate early genes both help to induce and block apoptosis of the infected cells as well as activate expression of the early genes (Rao et al., 1992). E3 encoded proteins are believed to inhibit the presentation of MHC class I antigens at

the cell surface thereby minimizing CTL-mediated cellular lysis of the infected cells. (Ginsberg et al., 1989; Gooding, 1992).

Adenoviruses as Viral Vectors

Adenoviruses are one of 5 viral vector systems commonly used for gene transfer to non-dividing cells such as those of the central nervous system (CNS) (Baumgartner and Shine, 1998). Other vector systems include adeno-associated viruses, defective herpes simplex viruses, recombinant herpes simplex virus, and lentiviruses (Hermens et al., 1997). Because of the extensive research of adenoviruses both in vitro and in vivo, several simplified systems for creating new types of recombinant adenoviruses have been developed (He et al., 1998). For these studies replication defective recombinant adenovirus proved to be the vector of choice for the following reasons: 1) these viruses are capable of infecting non-dividing cells in vitro and in vivo, 2) been shown to be transported retrogradely in the CNS (Davidson and Bohn, 1997), 3) have a capacity for large transgene expression cassettes up to 10 kB (Smith, 1998), 4) can be concentrated to high titers on the order of 10e12 pfu/ml (Hermens and Verhaagen, 1998), 5) demonstrate significantly long transient expression of their transgenes relative to other viral vectors used in the CNS (Byrnes et al., 1995; Byrnes et al., 1996), 6) are relatively safe to used, classified at biosafety level (BSL) 2, and 7) can be created using a number of simplified systems.

The viral vector used in this study was created using the Psi 5 system developed by Stephen Hardy (Hardy et al., 1997). The transgene used was an enhanced/humanized form of jellyfish derived green fluorescent protein (GFPh), and it was driven by the cytomegalovirus (CMV) promoter. The shuttle vector carrying the transgene cassette

was pTRUF7 developed by the University of Florida's vector core. The pTRUF7 is derived from the pAdlox shuttle vector developed by Stephen Hardy. To make new recombinant adenoviruses, the Psi 5 system uses lox P sites on both the viral backbone (i.e. donor virus) and the shuttle vector and is mediated by the recombinase enzyme provided by the Cre 8 cell line in which the co-transfection occurs. (Hardy et al., 1997). The recombinant adenovirus with the GFPh under control of the CMV is referred to as Ad.CMV-GFPh [Fig. 1-1]. This virus constitutes a replication defective recombinant adenovirus with an E1 deletion/substitution and a deletion in part of the E3 region.

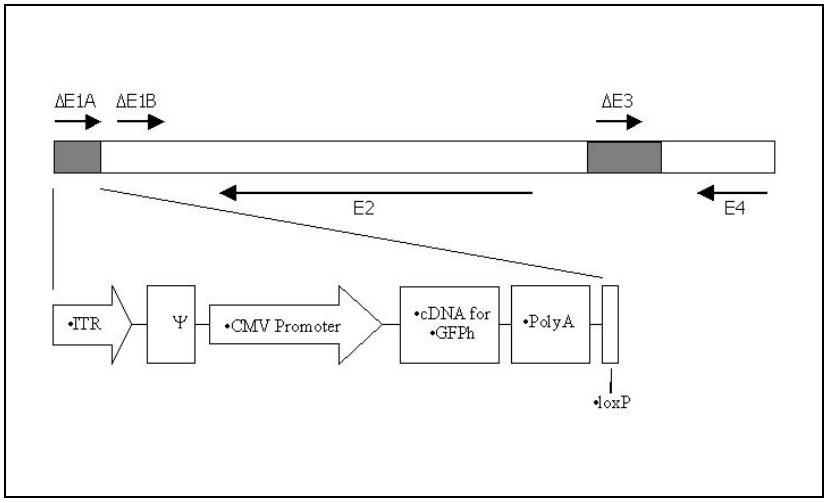

Figure 1-1: Viral vector Ad.CMV-GFPh with inverted terminal repeat (ITR), packaging sequence (Psi), cytomegalovirus (CMV) promoter, GFPh cDNA, polyadenelation signal (poly A), and lox P restriction site

The Facial Nerve and Nucleus Model

The model of the nervous system used for these studies was the rat facial nerve and facial nucleus. This model was selected for several reasons. First of all it consists of

a single population of peripheral motor neurons. When the axons in the periphery of the nerve undergo a lesion or injury they readily regenerate. The inflammatory response to injury alone has been well characterized. And finally the part of the nerve injured is easy to access surgically.

The injury induced to the system was a crush injury to the facial nerve at the injection site. The crush injury to the facial nerve is a simple, consistent, and reproducible procedure. The injury can be verified behaviorally by lack of whisker movement on the injured side. The proximity of the peripheral facial nerve to the facial nucleus may also be a key factor in terms of time between injection, retrograde transport to the nucleus, and expression of the transgene.

The Purpose of this Study

A replication defective recombinant adenovirus under control of the cytomegalovirus (CMV) and carrying the transgene for an enhanced jellyfish green fluorescent protein (GFPh), Ad.CMV-GFPh, was used for three sets of experiments. First, it was used in vitro on mixed glial cultures and neuronal cultures. Next, it was used to determine an optimal titer for delivery in vivo to the facial nerve system. And finally, it was used to determine a time course of GFPh kinetics and inflammation in vivo for the facial nerve system.

MATERIALS AND METHODS

Tissue Cell Cultures

HEK 293 Cultures

A 75 cm^2 flask of Human Embryonic Kidney (HEK) 293 cells at passage 36 and 80-90% confluency was received from the University of Florida vector core. These cells were split, cultured, and stored according to standard aseptic culture techniques. Standard growth medium [90% Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO), 10% Fetal Bovine Serum (FBS) (Gibco Life Technologies, Gaithersburg, MD), antibiotics - 100 units/ml penicillin and 100 цg/ml streptomycin (Mediatcech Cellgro, AK)] was used and the cells were incubated at 37ºC and 5% CO^2 (standard conditions). Two flasks of cells were used to generate a long term stored stock. The cells that were to be stored long term were removed from the monolayer by standard trypsinization and neutralization. After being re-suspended in growth medium, the cells were pelletted, the supernatant was aspirated and replaced with a cryprotectant consisting of 90% DMEM and 10% Dimethyl Sulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA), and triturated 3 times. The new suspension of cells in the cryoprotectant was then aliquoted into 1 ml freezer tubes, placed in a freezer box and frozen at -80°C overnight. The following day the cells were removed from the freezer and stored in a cell bank provided by Gerry Shaw of the University of Florida. The following day two freezer tubes of cells were recovered and the HEK 293 cells cultured to indicate that they were still viable/recoverable after the freezing, storing, and recovery process.

Rat Mixed Glial Cultures

Co-cultures containing astrocytes, oligodendrocytes, and microglia from neonatal rats were graciously provided by Tanya McGraw of the University of Florida. These cultures were generated from 2 day old rat pups based on methods outlined in Guilian and Baker (1996) and maintained in growth medium at standard culture conditions. Approximately every four days the growth medium was aspirated and replaced with fresh growth medium.

Rat Neonatal Neuronal Cultures

Rat neonatal neuronal hippocampal cultures were received from the University of Florida core facility run by Colin Sumners. These cultures were generated from 1day old rat pups after sacrificing the animals and recovering the brain tissue according to the protocol outlined by the instructions accompanying Neurobasal Medium (Gibco Life Technologies, Grand Island, NY). Prior to receiving them, the cells were cultured for 20 days in the Neurobasal Medium in conjunction with B-27 Supplement 50X (Gibco Life Technologies, Grand Island, NY) at 37ºC and 9% CO^2. The cultures were treated with fresh medium every 4 days.

Viral Development and Preparation

First Round of Amplification

A viral lysate sample of the previously described replication defective, recombinant, human serotype 5 adenovirus, Ad.CMV-GFPh, was graciously received from Mary Schleissing of the Barry Byrne's lab at the University of Florida. This sample was amplified using a line of HEK 293 cells. Two 150 mm cell culture plates were seeded with HEK 293 cells at passage 44 in growth medium and incubated for 3 days at standard conditions . At day three the cells reached 75% confluency and were ready for

the first round of amplification of the Ad.CMV-GFPh lysate (1.5 ml). The viral lysate was diluted into 22.5 ml of sterile filtered DMEM to create an infectious medium. The growth medium was aspirated from the two 150 mm plates containing the HEK 293 cells and replaced with 12 ml/plate of infectious medium. The cells with infectious medium were incubated for 2 hours under standard conditions while rocking and rotating every 20 minutes to redistribute the infectious medium. Each dish then had 13 ml of fresh growth medium added to it and was then returned to the incubator. After 5 days the transfected cultures showed signs of full cytopathic effect (cpe). Full cpe was characterized by cells rounding up and lifting off the flask bottom, as well as acidification of the medium. Expression of GFPh was verified by use of an inverted scope with fluorescein filters provided by Gerry Shaw of the University of Florida. The cells were then harvested, pelleted by low speed centrifugation, re-suspended in 0.5 ml of sterile phosphate buffered solution (PBS), and frozen at –80ºC.

Second Round of Amplification

The second round of amplification was conducted on the lysate from the first round of amplification using standard techniques (Tollefson et al., 1999). Forty 150 mm cell culture plates were seeded with HEK 293 cells at passage 47 in 15 ml of growth medium. At 80-90% confluency the HEK 293 cells were ready for transfection. The viral lysate from the previous round of amplification was rapidly thawed in a water bath at 37ºC followed by 2 more rounds of rapid freeze/thaw (-80ºC/37ºC) cycles. An infectious medium solution was prepared from 200 ml of DMEM combined with 1 ml of viral lysate from the first round of amplification. The growth medium was removed from the forty 150 mm plates and replaced with 5 ml/plate of infectious medium, and incubated at standard conditions for 2 hours with rocking and rotating every 20 minutes.

Then each plate had 15 ml of fresh growth medium added to it and then was returned to the incubator. On the 7th day following the transfection, the cells showed signs of full cpe, and GFPh expression was verified. The cells were harvested, pelleted by low speed centrifugation, re-suspended in 8 ml of sterile PBS, and frozen at –80ºC.

Purification, Concentration, and Desalting

The virus from the second round of amplification was then purified and concentrated using standard CsCl banding techniques (Tollefson et al., 1999). The viral lysate was rapidly thawed in a water bath at 37ºC followed by 2 more rounds or rapid freeze/thaw (-80ºC/37ºC) cycles. The cells were then pelleted by low speed centrifugation for 5 minutes. The lysate was added to two 3.5" x 1" Beckman Ultra Clear Centrifuge Tubes (Beckman Coulter, Fullerton, CA). Each tube was underlayed with 9.25 ml of 1.25 g/ml CsCl solution which was subsequently underlayed with 7.4 ml of 1.6 g/ml CsCl solution. The tubes were then overlayed with PBS until it reached the "Beckman" writing on the tube. The tubes were centrifuged for 2 hours at 25,000 RPM and 4ºC in a SW 28 rotor provided by the University of Florida Brain Institute. The lower band containing the virus was then extracted from each tube using an 18 gauge needle and 3 cc syringe. The extracted virus was added to two 1.5" x 0.5" Beckman Ultra Clear Centrifuge Tubes (Beckman Coulter, Fullerton, CA) and then overlayed with 1.35 g/ml CsCl solution until it reached the "Beckman" writing on the tube. The tubes were then covered and inverted several times prior to being centrifuged overnight at 50,000 RPM and 4ºC in a SW 50.1 rotor provided by Maureen Goodenow of the University of Florida. A 3 cc syringe and 18 gauge needle were then used to remove the lower viral containing band from the CsCl gradient.

Finally, the solution containing the virus was desalted by means of a CsCl desalting buffer (137 mM NaCl + 5 mM KCL + 10 mM Tris-HCl at pH 7.4, 1 mM MgCl2 + 10% sucrose) and one Pharmacia PD-10 Sephadex column per 20 plate prep. The columns were washed with 25 ml of the desalting buffer. The virus was then added directly to the columns. Fractions of 8-10 drops were collected. After all of the solution flowed through, 3-4 ml desalting buffer was added and the milky viral containing fractions were collected and aliquoted at 10 µl before freezing at -80ºC. Subsequently the viral construct was verified by restriction analysis.

Titering by Plaque Assay

Eleven 60 mm culture dishes were seeded with HEK 293 cells in growth medium. These cultures were then incubated at standard conditions for 2 days. The cultures were at approximately 60% confluency and showed no signs of clumping or unusually dense areas of cells. These cultures were then used to estimate a viral titer in terms of plaque forming units per milliliter (pfu/ml) (Tollefson et al., 1999).

On the day of transfection 25 ml of a 1.8% agar solution was autoclaved and cooled in a water bath to 57ºC to keep the agar from solidifying. Also, 25 ml of sterile filtered 2 x DMEM and a 1 ml aliquot of FBS was placed in a water bath at 37ºC. Ten, 10 ml serial dilutions of the virus in DMEM (infectious medium) were created starting with 100 µl of purified virus in 9.9 ml of DMEM. Each dilution was 1/10 of the concentration of the previous dilution. Without disturbing the monolayer, the growth medium was aspirated from the cultures. Ten of the cultures each had 1 ml of infectious medium from one of the ten serial dilutions added to it without disturbing the monolayer, and the eleventh plate received 1 ml of DMEM to serve as a control. The dishes were gently rocked to distribute the infectious medium. The eleven 60 mm plates were labeled

indicating the dilution of virus used or the control and were incubated at standard conditions for 1.5 hours while the plates were rocked and rotated every 20 minutes.

Without disturbing the monolayers, the infectious medium and control medium were aspirated from the dishes. Twenty-five ml of the 2 x DMEM was mixed with 1.5 ml of FBS and antibiotics (100 units/ml penicillin and 100 цg/ml streptomycin) and added to the 25 ml of 1.8% agar solution. Carefully, 4 ml of the agar/medium mix was added to each dish by tilting the plate and adding the medium to the side of the dish. The plates were allowed to cool at room temperature undisturbed for 15 minutes so that the agar overlay could solidify. The cultures were then incubated at standard conditions.

One day after transfection the plates were observed for confluency and even growth. No abnormalities were noticed. On the 3rd day following transfection another 3 ml agar overlay was added. Plaques appeared on day 5 and a 3 ml agar overlay containing 0.001% Neutral Red dye (Fisher Scientific, Pittsburgh, PA) was added. On the day following the addition of the overlay containing Neutral Red, the plaques were counted to determine the titer. The plaques were also verified for expression of GFPh.

In Vitro

HEK 293 Transfection

Two 75 cm^2 flasks of HEK 293 cells at passage 39 were cultured to approximately 80-90% confluency. One culture was transfected with 20 цl of stock virus in 4.980 ml of DMEM (i.e. 1:250 or 2e8 pfu/ml) for 1.5 hours at 37ºC and 5% CO^2. The other culture served as a control and received 5 ml of DMEM for 1.5 hours at 37ºC and 5% CO^2. The infectious medium and control medium were then aspirated and replaced with 10 ml of fresh growth medium. After 24 hours, expression of GFPh could be detected in the culture which received infectious medium, where as the control showed

no expression of GFPh. At 48 hours cpe was evident in the transfected culture and not in the control.

Rat Mixed Glial Transfection

Two 75 cm^2 flasks of mixed glial cultures consisting of astrocytes, oligodendrocytes, and microglia were cultured to 80-90% confluency. One culture was transfected with 20 ц l of stock virus in 4.980 ml of DMEM (i.e. 1:250 or 2e8 pfu/ml) for 1.5 hours at 37ºC and 5% CO2. The other culture served as a control and received 5 ml of DMEM for 1.5 hours at 37ºC and 5% CO^2. The infectious medium and control medium were then aspirated and replaced with 10 ml of fresh growth medium. After 24 hours expression of GFPh could be detected in the culture which received infectious medium, where as the control showed no expression of GFPh. At 48 hours minimal cpe was evident in the transfected culture (in terms of acidification and reduction of growth) and not in the control.

Rat Neonatal Neuronal Transfection

Two 35 mm flasks of rat neonatal neurons were cultured to 80-90% confluency. One culture was transfected with 4 ц l stock virus in 1 ml of DMEM (i.e. approximately 1:250 or 2e8 pfu/ml) for 1.5 hours at 37ºC and 9% CO^2. The other culture served as a control and received 1 ml of DMEM for 1.5 hours at 37ºC and 9% CO2. The infectious medium and control medium were then aspirated and replaced with 10 ml of fresh growth medium. After 24 hours expression of GFPh could be detected in the culture which received infectious medium, where as the control showed no expression of GFPh. At 48 hours minimal cpe was evident in the transfected culture (in terms of acidification and reduction of growth) and not in the control.

In Vivo

Dilution Studies

All animals used for the in vivo studies were male adult Sprague-Dawley rats (Harlan, Indianapolis, IN). The virus was administered at four different titers. All surgeries were conducted by W.J. Streit of the University of Florida. The animals were anesthetized and the right facial nerve was surgically exposed. Four animals then received 1 μl injections of virus at 4 different titers via a Hamilton 10 μl syringe as follows: animal D1 received stock virus (titer of 5e10 pfu/ml), D2 received stock virus diluted 1:10 in sterile PBS (titer of 5e9 pfu/ml), D3 received stock virus diluted 1:100 in sterile PBS (titer of 5e8 pfu/ml), and D4 received stock virus diluted 1:1000 in sterile PBS (5e7 pfu/ml). Immediately following the viral injection a pair of 1 mm curved hemostats was used to induce a crush injury in the nerve at the injection site. The hemostats were clamped onto the nerve for 10 seconds. The incision was then closed with 1 or 2 surgical staples. Upon awakening the crush injury was verified behaviorally by the observation that the animals had no movement in the whiskers on their right (i.e. injured) side.

Time Course, GFPh Kinetics, and Inflammation Studies

The set of animals included 4 control animals and 8 experimental animals. All animals were anesthetized and the right facial nerve was surgically exposed. The four control animals each received a 1 μl injection of sterile PBS followed by a crush injury at the injection site as described above. The 8 experimental animals each received a 1 μl injection of stock adenovirus diluted 1:10 in sterile PBS yielding a final titer of approximately 5e9 pfu/ml. The experimental animals also received a crush injury of the facial nerve at the injection site as described above. The incisions were then closed with

1 or 2 surgical staples. Upon awakening the crush injury was verified behaviorally by the observation that none of the animals had movement in the whiskers on their right (i.e. injured) side.

Histology

Dilution Studies

The 4 animals were sacrificed 6 days after receiving their injections of diluted Ad.CMV-GFPh. All animals were sacrificed in the following manner. Each animal was anesthetized with an intraperitoneal (IP) injection of approximately 0.35 ml of sodium pentobarbital diluted 1:2 in PBS. When the animals were sufficiently anesthetized they were perfused intracardially with 200-300 ml of warm PBS + Heparin (heparin diluted in PBS at 1:2500) followed by 200-300 ml of 4% paraformaldehyde (pfa) to fix the tissue. The brains were then extracted and stored in 4% pfa for 2-4 days.

The brains were sectioned, processed, and stained as follows. The cortex was removed and a section of brainstem containing the facial nucleus and nerve was embedded in 3% agarose. The brains were then cut on a virbratome in PBS at a thickness of 50 μm. Thirty-five to 45 sections were processed These sections ran through the facial nucleus and rostral to the point at which the facial nerve could be seen exiting the brain. From each animal 12 to 16 sections were used. The sections were rinsed twice in PBS (5 minutes/rinse) then soaked in PBS overnight at 20ºC. The following day the sections were mounted on Superforst/Plus slides (Fisher Scientific, Pittsburgh, PA) cover slipped with Fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL) as a mounting medium and checked for GFPh expression with a microscope equipped with a fluorescein filter set.

Time Course, GFPh, Kinetics, and Inflammation Studies

The 4 control animals were sacrificed 4, 7, 10, and 14 days after receiving their injections of sterile PBS. The 8 experimental animals were sacrificed 4, 5, 6, 7, 8, 10, 12, and 14 days after receiving their injections of Ad.CMV-GFPh [Table 2-1]. All animals were sacrificed in the manner previously described.

Table 2-1: Summary of kinetics and inflammation studies

Animal ID	Received	Day Sacrificed
C5	PBS	4
E9	Ad.CMV-GFPh	4
E10	Ad.CMV-GFPh	5
E11	Ad.CMV-GFPh	6
C6	PBS	7
E12	Ad.CMV-GFPh	7
E13	Ad.CMV-GFPh	8
C7	PBS	10
E14	Ad.CMV-GFPh	10
E15	Ad.CMV-GFPh	12
C8	PBS	14
E16	Ad.CMV-GFPh	14

The brains from both groups of animals were sectioned, processed and stained in the following manner. The cortex was removed and a section of brain containing the facial nucleus and nerve was embedded in 3% agarose. The brains were then sectioned on a virbratome in PBS at a thickness of 50 цm. Thirty-five to 45 sections were processed for each animal. These sections ran through the facial nucleus and just rostral to the point at which the facial nerve could be seen exiting the brain. From each animal 12 to 16 sections were used for histochemistry to check for gliosis. The sections that were not stained for gliosis were rinsed twice in PBS (5 minutes/rinse) then soaked in

PBS overnight at 20ºC and subsequently mounted the following day on Superforst/Plus slides cover slipped with Fluoromount G as a mounting medium. These sections were then examined under a microscope with a fluorescein filter set for expression of GFPh. The sections for glial staining were rinsed twice in PBS (5 minutes/rinse) then permeated with PBSC + 0.1% TritonX-100 (Fisher Scientific, Pittsburgh, PA) at 20ºC overnight. The sections were then incubated overnight at 20ºC or on a shaker at room temperature for 2 hours in a solution of PBSC + 0.1% TritonX-100 + biotinylated isolectin Gs-I-B4 (10 µg/ml) (Sigma, St. Louis, MD). The tissue was then rinsed in PBS 3 times (5 minutes/rinse). Next, the tissue samples were incubated on a clinical rotator at room temperature for 20 minutes in a solution of PBS + Streptavidin:Texas-Red (5 • g/ml) (Molecular Probes Inc., Eugene, OR). The sections were then rinsed in PBS 3 times (5minutes/rinse). Sections were then mounted on Superfrost/Plus slides and cover slipped with Fluoromount G as a mounting medium. These sections were then viewed under a microscope with the appropriate filters for expression of GFPh and Texas Red. The facial nerves for the 8-14 day control and experimental animals were sectioned, processed, and stained. The nerves were allowed to soak in a cryoprotectant consisting of 30% sucrose in PBS for 2-3 days. The nerves were then mounted in OCT Compound (Sakura Finetek USA, Inc, Torrance, CA) and sectioned longintudally on a cryostat at 20 • m and -19ºC. Each nerve provided 20-34 sections. All of the sections were mounted on subbed slides and allowed to air dry overnight. Half the sections from each animal were then cover slipped with Flouromount G as a mounting medium and checked for signs of GFPh with a microscope and fluorescein filter set. The other half of the sections for each animal were Nissel stained with cresyl violet, dehydrated and cover slipped

using Permount (Fisher Scientific, Pittsburgh, PA) as a mounting medium. The sections were then checked under a microscope for lymphocyte infiltration.

RESULTS

In Vitro

HEK 293 Transfection

The first sign of expression of GFPh in these cells could be detected on the second day after transfection with the use of an inverted microscope and a fluorescein filter set. These cells showed a very high level of expression by day 4 and cytopathic effects such as acidification of the medium and morphological changes (cells rounding up and lifting off of the bottom of the flask) [Fig. 3-1]. By day 5 full cpe could be seen as all of the cells had lifted off the bottom of the flask, and expression of GFPh could be detected in virtually every cell.

Rat Mixed Glial Transfection

Expression of GFPh could be detected in the mixed glial cultures on day 2 after transfection. Within the culture it could be seen that the astrocytes, oligodendrocytes, and microglia all were capable of expressing GFPh and therefor must have been transfected [Fig 3-1]. After 4-5 days it appeared that expression of GFPh and hence the number of cells transfected had peaked. Compared to the controls little cytopathic effect could be seen at this time. The transfected cultures were only slightly more acidic as could be detected by change of growth medium color, and very few of the cells were lifting off the plate.

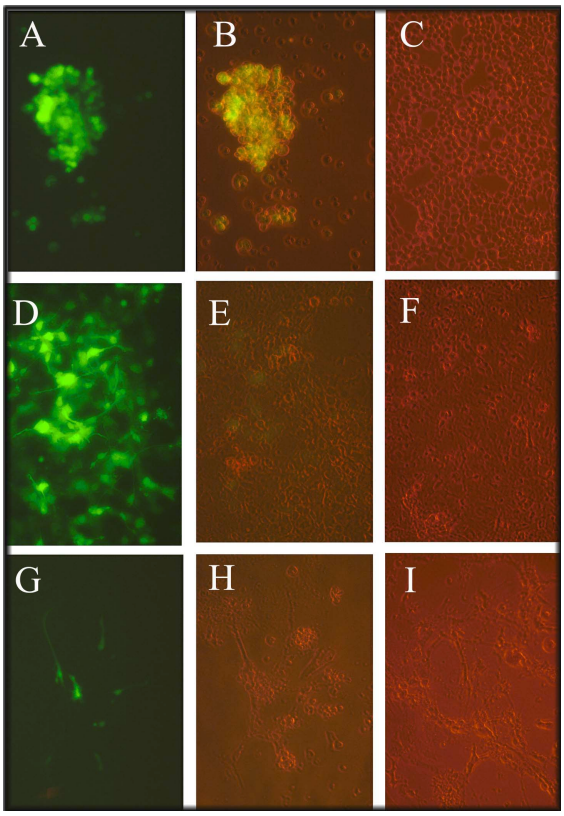

Figure 3-1: In vitro results at 48 hours and 10x. A) Transfected HEK cells with GFP filter, B) transfected HEK cells with light field, C) control HEK with light field, D) transfected mixed glial cells with GFP filter, E) transfected mixed glial cells with light field, F) control mixed glial cells with light field, G) transfected neurons with GFP filter, H) transfected neurons with light field, and I) control neurons with light field.

Rat Neonatal Neuronal Transfection

Two days after transfection of the rat neonatal hippocampal neurons, expression of GFPh could be detected. The cell bodies of the some neurons as well as the processes showed fluorescence [Fig. 3-1]. Again, by day four slight acidification of the growth medium could be seen when compared to the control cultures. The number of cells expressing GFPh seemed to peak around day 4, and the transfection efficiency was noticeably less than that of the mixed glial cultures. Similar to the mixed glial cultures, very few neurons lifted off of the bottom of the plate or showed morphological changes.

In Vivo

Dilutions Studies

Animal D1 which received an injection at 5e10 pfu/ml showed no expression of GFPh in the facial nucleus. Animal D2 at 5e9 pfu/ml showed significant expression while animal D3 at 5e8 pfu/ml showed very little expression of the transgene. Finally, D4 at a dilution of 5e7 pfu/ml showed no expression of GFPh in the facial nucleus [Fig. 3-2].

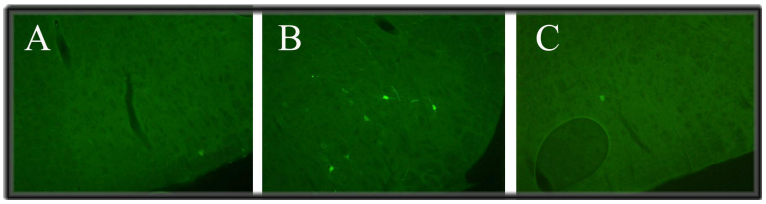

Figure 3-2: Dilution studies 6 days post-injection with Ad.CMV-GFPh at 10X. A) 5e10 pfu/ml, B) 5e9 pfu/ml, and C) 5e8 pfu/ml

Time Course, GFPh Kinetics, and Inflammation Studies

Prior to sacrificing the animals and conducting the histological studies, behavioral observations in regards to whisker were made. In both control and treated animals, right side whisker movement returned around days 12-14 post injury. The histology for the facial nucleus showed expression of GFPh during the entire time course from day 4-14. Moderate expression of GFPh could be seen in the soma and processes of many neurons in the nucleus. The number of cells expressing GFPh that could be seen ranged from 6 to 137 in the 8 experimental animals [Fig. 3-3]. Although not statistically shown with such a small sample of animals, it seemed that expression appeared by day 4, peak expression

occurred around days 6-10, and lasted throughout the 14 days studied. In the later animals the amount of GFPh in the neuronal processes not only appeared to be increasing, but also could be seen migrating towards the periphery of the exiting nerve. This anterograde transport can be clearly seen in the 4-12 day animals [Fig. 3-4]. Also present in the later animals (day 7 and beyond) were some neuronal processes with varicosities resulting in a "beaded" appearance. Until day 7 the microglial histochemistry indicated no apparent difference between the injured control animals and the injured treated animals in terms of reactive gliosis [Fig. 3-5]. The main difference in reactive gliosis appeared in the later animals (at day 12-14) at which time the Ad.CMV-GFPh treated animals showed slightly increased reactive gliosis [Fig. 3-5]. But, when combining green field and red field images, it was clear that the perineuronal reactive gliosis was not specific for neurons that were expressing GFPh [Fig. 3-6].

The second set of these histological studies was focused on GFPh expression and lymphocyte infiltration in the proximity of the injury site. At time points 8-14 days, no expression of GFPh was detected in the peripheral facial nerve of the Ad.CMV-GFPh treated animals. But, comparison of the facial nerves from the control animals with that of the experimental animals after performing a Nissel stain, clearly indicated that there was increased mononuclear cell infiltration in the Ad.CMV-GFPh treated animals. In addition to the mononuclear cell infiltration, perivascular cuffing could be seen in several of the Ad.CMV-GFPh treated animals [Fig. 3-7]. Also nuclear fragmentation, a sign of apoptosis, could be detected based on the Nissel stain of the adenovirus treated animals. The nuclear fragmentation can be seen within the T-cells surrounding the apoptotic

neurons [Fig. 3-8]. Both control and Ad.CMV-GFPh treated animals showed signs of limited mast cell infiltration [Fig. 3-8].

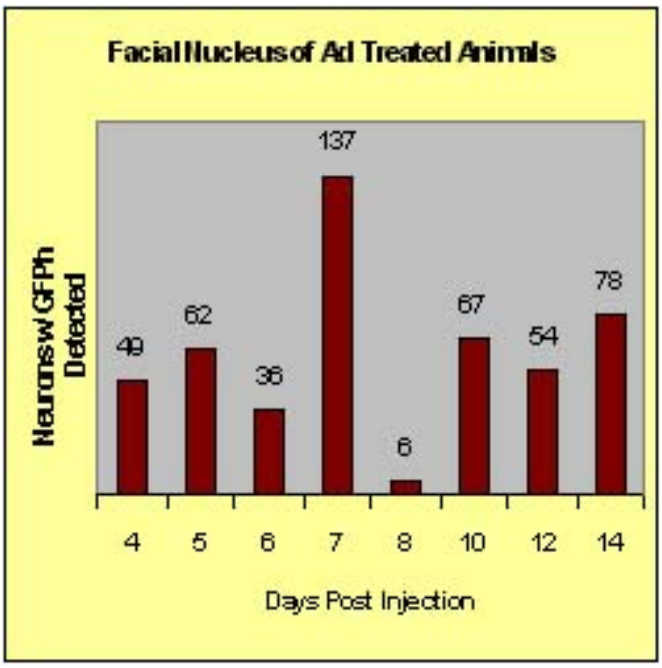

Figure 3-3: Number of neurons expressing GFPh

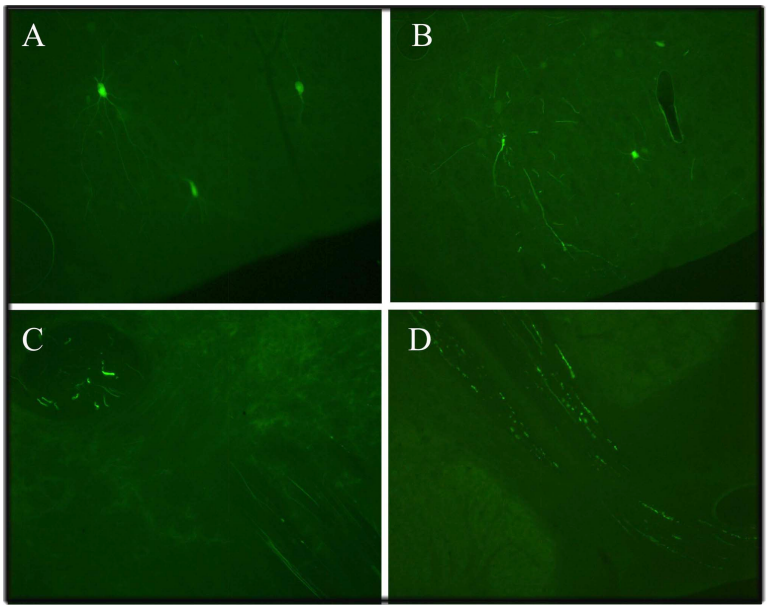

Figure 3-4: GFPh transport kinetics in the facial nerve system post injection at 10X. A) Day 4 in nucleus, B) day 7 in nucleus, C) day 7 in "knee", and D) day 12 at nerve root

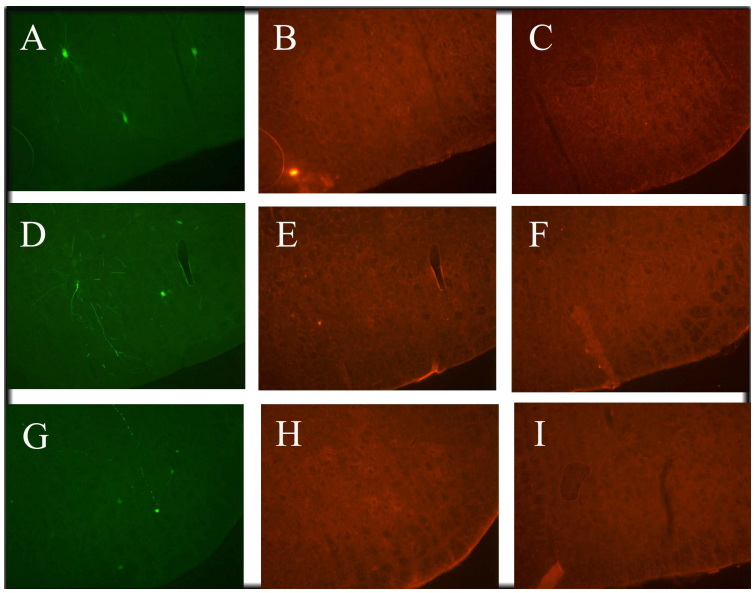

Figure 3-5: Ad.CMV-GFPh and control animals at 10X. A) Day 4 virus, B) 4 day virus, C) 4 day control, D) 7 day virus, E) 7 day virus, F) 7 day control, G) 14 day virus, H) 14 day virus, and I) 14 day control

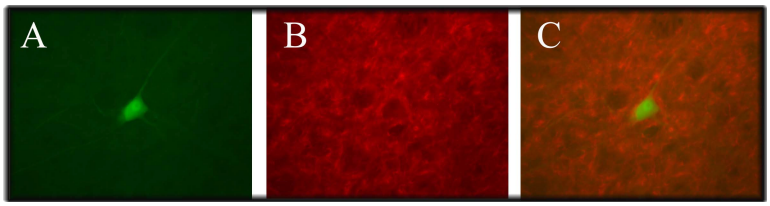

Figure 3-6: Non-selective reactive gliosis 4 days post injection of Ad.CMV-GFPh at 10X. A) With filter for GFP, B) with filter for Texas Red, and C) combined green and red fields.

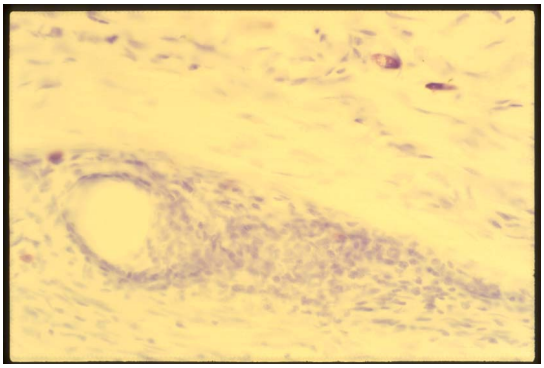

Figure 3-7: Perivascular cuffing in the facial nerve 12 days post injection at 10X

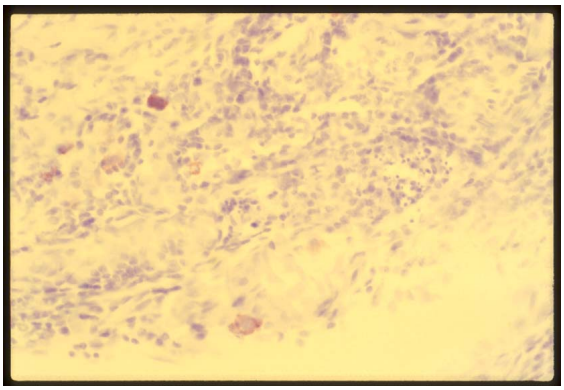

Figure 3-8: Nuclear fragmentation, a sign of apoptosis, and mast cell infiltration in the facial nerve 12 days post injection at 10X

DISCUSSION AND CONCLUSIONS

In Vitro

HEK 293 Transfection

As expected in the HEK 293 cells, there was a high level of GFPh expression because of viral replication. This was due to the E1 transgene expression by the host HEK 293 cells thereby enabling the cascade of the remaining viral proteins to be produced in the transfected cells. The extensive cpe shown by these cells at such an early time point can be attributed to the production of viral proteins which are detrimental to host cells (Hermens and Verhaagen, 1998).

Rat Mixed Glial Transfection

The successful transfection of the mixed glial cultures indicates that several different cell types of the neonatal rat nervous system are suitable targets for this viral vector system expressing the GFPh transgene. These cell types include but may not be limited to astrocytes, oligodendrocytes, and microglia. The fact that the cytopathic effects were minor in these cultures was most likely do to the minimal expression of viral proteins because of the deletions in the E1 regions of the viral genome. Therefore this culture system can serve as a suitable model for gene transfer in non-neuronal cells of the CNS. Some possible applications of such a system include the study of gene delivery by adenovirus under cell type specific promoters in the mixed glial cultures.

Rat Neonatal Neuronal Transfection

The cultures of the neonatal neurons were also capable of being transfected by the Ad.CMV-GFPh as seen by the expression of GFPh in the neurons. These cells seemed to exhibit the least amount of cpe of all the cultures, but also had the lowest transfection rate. This may be related to the unidentified cell surface receptor responsible for mediating the uptake of the virus.

In Vivo

Dilutions Studies

The variability of expression with the titer of the injected adenovirus indicates a concentration dependence for successful transduction in vivo. As reported by others recombinant adenoviruses as vectors become less efficient at extremely high titers (Liu et al., 1997). These studies indicate that the Ad.CMV-GFPh vector should be used at a titer of approximately 5e9 pfu/ml for the facial nerve and nucleus of these types of animals.

Time Course, GFPh Kinetics, and Inflammation Studies

The paralleled recovery of whisker movement amongst both the control and Ad.CMV-GFPh treated animals around day 12 was the first indicator that at this time point pathological effects may not be drastically different. These preliminary observations hinted that behaviorally the recovery of the injured control animal and the injured adenovirus treated animal were similar to some extent.

The histological findings of GFPh expression and transport within the facial nucleus revealed several interesting facts. First of all, this viral vector system was capable of undergoing rapid retrograde axonal transport from the periphery to the nucleus in the brain. Secondly, the viral vector in this model provided expression of the

transgene within 4 days of treatment as opposed to systems such as AAV that can require up to 6 weeks. This indicates that for immediate and short-term treatment studies in rats, this vector system can be useful. Finally, in this system GFPh acted as an anterograde marker. This allows visualization of the neuronal processes leaving the nucleus and heading back towards the periphery. This can be very useful for studying pathways and perhaps marking the original injury site in such models. In short, this system allowed for rapid retrograde transport of the vector, relatively early and robust expression of the transgene, and anterograde labeling of the processes of the motoneurons as they headed back towards the site of the original injury.

The histological findings of inflammation in terms of reactive gliosis within the facial nucleus revealed several facts about the two groups of animals. At the early time points (day 7 and below), the glial response between the two sets of animals was very similar. So much so that while using the microscope with the Texas Red filter set, the two groups of animals were indistinguishable histologically. Also worth noting were the results based on dual labeling of the facial nucleus for GFPh and reactive gliosis. It was clearly seen that perineuronal reactive gliosis was independent of GFPh expression. Some cells that expressed GFPh were surrounded by hypertrophic microglia while some cells expressing GFPh were not surrounded by hypertrophic microglia. This illustrates that inflammatory responses in such a model are not specific for neurons transduced with the virus. The main difference in reactive gliosis appeared in the later time points (days 10-14). At these later times, gliosis began to subside in the control animals, but remained relatively constant in the experimental animals. Rather the Ad.CMV-GFPh treated animal seemed to be in a latent or extended phases of reactive gliosis. Studies carried out

to further time points are required to indicate if remission of gliosis is in fact only delayed in the treated animals when compared to the control animals.

The last set of histological studies looked at GFPh expression in the proximity of the injury site as well as lymphocyte infiltration in the vicinity of the injury site. Both sets of animals showed signs of infiltration by local mast cells in the periphery of the facial nerve. But the adenovirus treated animals showed significant signs of mononuclear cell infiltration and apoptosis where as the control animals showed almost none. Studies conducted out to further time points might indicate the long term pathological effects of this vector on the rat facial nerve system.

Future Research

The next stage of studies would include developing novel, replication defective, recombinant adenoviruses for studying a population of central neurons (neurons in whixh the axons remain within the CNS). First a model system of the CNS would have to be chosen, possibly one of the cortical spinal tracts. Next, an injury and treatment model would have to be developed. A reasonable choice would be a thoracic contusion or hemisection injury followed by treatment with a adenovirus vector with a potentially therapeutic transgene such as a neurotrophic factor or cytokine of interest. Finally, delivery, expression, time course, and immunological responses of the injured animals treated with the novel gene therapy could be compared to that of injured animals treated with saline as a control.

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BIOGRAPHICAL SKETCH

Todd Matthew Alter hails from Coral Springs, Florida. In 1991, he came to Gainesville to attend the University of Florida from which he received his Bachelor of Science degree with honors in engineering sciences. After working at the University of Florida Department of Neuroscience from 1996 to 1998, he was accepted to the same university's Graduate School in the Department of Molecular Genetics and Microbiology. He graduated with a Master of Science degree in medical sciences and has relocated to the Chapel Hill area of North Carolina.