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Gene Transfer into Rat Brain Using Adenoviral Vectors

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Abstract

Viral vector-mediated gene delivery is an attractive procedure for introducing genes into the brain, both for purposes of basic neuroscience research and to develop gene therapy for neurological diseases. Replication-defective adenoviruses possess many features which make them ideal vectors for this purpose—efficiently transducing terminally differentiated cells such as neurons and glial cells, resulting in high levels of transgene expression *in vivo*. Also, in the absence of anti-adenovirus immunity, these vectors can sustain very long-term transgene expression within the brain parenchyma. This unit provides protocols for the stereotactic injection of adenoviral vectors into the brain, followed by protocols to detect transgene expression or infiltrates of immune cells by immunocytochemistry or immunofluorescence. ELISPOT and neutralizing antibody assay methodologies are provided to quantitate the levels of cellular and humoral immune responses against adenoviruses. Quantitation of adenoviral vector genomes within the rat brain using qPCR is also described. *Curr. Protoc. Neurosci.* 50:4.24.1–4.24.49. © 2010 by John Wiley & Sons, Inc.

Keywords

viral vectors; gene therapy; immune response; T cells; B cells; brain inflammation; macrophages; gene transfer; immunocytochemistry; qPCR; ELISPOT assay; adenovirus

INTRODUCTION

Viral vector-mediated gene delivery is an attractive procedure for introducing genes into the brain, both for purposes of neurobiological research and for gene therapy of neurological diseases. Replication-defective adenoviruses possess many features that make them ideal vectors for this purpose. Adenoviruses are easily purified to the high titers required for *in vivo* administration, and they are efficient in transducing terminally differentiated cells such as neurons and glial cells, resulting in high levels of transgene expression. At doses of 1×10^8 IU or less, highly purified, LPS-free adenoviral vectors delivered to the brain parenchyma cause minimal inflammation or toxicity, and vector-mediated transgene expression is stable for up to 12 months and spatially restricted to the region of vector administration or areas that project to the injection site (Thomas et al., 2001a; Barcia et al., 2006a).

This unit focuses on methods that have been tailored to the injection of viral vectors into the brain, and the subsequent detection of transgene expression and local inflammation. Basic Protocol 1 here complements Basic Protocol 1 in *UNIT 3.10*, and describes a procedure for the direct injection of adenovirus into the CNS using stereotactic guidance, while Support Protocol 1 in this unit is similar to Basic Protocol 2 in *UNIT 1.1*, and describes preparation of brain sections for immunohistochemical analysis. However, adenovirus-specific modifications are emphasized for these two procedures. Several methods for evaluating gene transfer to the brain

following in vivo administration of the adenoviral vectors are also presented in this unit. A method for detecting vector-mediated transgene expression by horseradish peroxidase (HRP)–based immunohisto-chemical staining is described (see Basic Protocol 2). Fluorescence-based immunohisto-chemical staining to detect more than one marker antigen within a single brain section is described in Alternate Protocol 1.

Adenoviruses are immunogenic and have been used to vaccinate against infection in humans. Adenovirus-derived vectors are also immunogenic and can elicit inflammatory and cytotoxic effects in the brain that are vector dose dependent (Thomas et al., 2001a; Barcia et al., 2006a; Zirger et al., 2006). In addition to their potentially harmful side effects, inflammatory, immune, and cytopathogenic responses also have a profound impact upon the efficacy of gene transfer to the brain (Thomas et al. 2001a,b; Barcia et al., 2006a,b; King et al., 2008). It is therefore necessary to evaluate vector-mediated inflammatory responses and toxicity concomitantly with the evaluation of vector-mediated gene transfer. HRP-based immunohistochemistry (see Basic Protocol 2) or fluorescence-based immunohistochemistry (see Alternate Protocol 1) can be used with the appropriate antibodies to visualize the acute and/or chronic infiltration of immune and inflammatory cells, the activation of brain microglia and astrocytes, the loss of glial cell or neuronal markers (indicating cell loss through acute or chronic cytotoxicity), and/or the integrity of brain myelin after in vivo administration of adenovirus vectors to the brain. More global visualizations of brain structure and infiltrating cells can be obtained by staining cell nuclei with cresyl violet, which is described for Vibratome brain sections (see Basic Protocol 3), together with an alternative method for staining brain myelin with Luxol fast blue. Staining semi-thin plastic-embedded sections with toluidine blue (see Basic Protocol 4) allows even better resolution of the cellular structure of the brain. The preparation of APES-coated slides required for these applications is described in Support Protocol 2.

If vector-mediated transgene expression is observed to decrease or disappear over time, it is usually a result of inflammatory and/or immune responses against the vector or transgene. In such cases, it may be desirable to determine whether the vector genome has been eliminated from the brain (Barcia et al., 2006b; Puntel et al., 2006). Quantitative PCR–based detection of vector genomes from rat brains is described for this purpose (see Basic Protocol 5).

Adenovirus vectors injected into the brain parenchyma (particularly vectors encoding proinflammatory molecules) can cause disruption of the blood-brain barrier (BBB), facilitating the infiltration of inflammatory cells from the circulation. A method for determining the extent of breakage of the blood-brain barrier following administration of adenovirus vectors to the brain is described (see Basic Protocol 6).

Adenovirus vectors administered to the brain parenchyma are able to persist for long periods of time (at least up to 12 months, compared with 2 to 3 weeks after delivery of identical vectors to peripheral tissues such as the liver). The persistence of vector in the brains of naive animals compared to peripheral tissues is thought to be due to the failure to elicit an effective anti-adenoviral T cell response through intraparenchymal vector injection. However, an effective immune response capable of eliminating vector or transgene expression from the brain can be elicited by priming the immune response through injection of adenoviral vectors into the skin (see Basic Protocol 7). The induction of an anti-adenovirus humoral immune response can be assessed using an adenovirus-specific neutralizing antibody assay (Basic Protocol 8) and the induction of an anti-adenovirus cellular immune response can be assessed using an adenovirus specific ELISPOT assay (Basic Protocol 9).

Lastly, since collaborative efforts between different laboratories often involve the transport of valuable vector stocks from one lab to another, a protocol is provided for the optimal transport of viral vector without loss of vector titer (see Support Protocol 3).

The majority of current gene-therapy protocols utilizing adenoviral vectors have involved “first-generation” vectors. These are recombinant vectors which are rendered nonreplicative by deletion of the E1 region from the viral genome. Detailed protocols for the generation and purification of first-generation adenoviral vectors are presented in *UNIT 4.23*. Before any vector preparation is used in vivo, it must be accurately titered and subjected to the stringent quality control tests described in *UNIT 4.23*.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

IN VIVO ADENOVIRUS-MEDIATED GENE TRANSFER INTO THE CNS OF ADULT RATS

UNIT 3.10 describes administration of inoculum to a precise location within the adult mouse CNS using stereotactic guidance. In the protocol below, the technique is adapted to viral vector injection in the adult rat brain using coordinates from Paxinos and Watson (1998). The stereotactic frame, the overall setup, and the sequence of events in this protocol are modified from the procedure described in *UNIT 3.10*. Briefly, the rat is manually restrained, followed by intraperitoneal injection of anesthetics [ketamine (75 mg/kg), and dexmedetomidine (0.25 mg/kg)]. Following anesthesia and prior to surgery, the analgesic Carprofen (5 mg/kg) is administered subcutaneously.

Materials

Adenoviral vector (*UNIT 4.23*)

Dulbecco's PBS, pH 7.4, without Ca or Mg (Cellgro, cat. no. 21–031 CV)

Adult rat (250 g body weight; rats 200 to 350 g may be fitted into the frame; however the stereotactic coordinates may not be accurate with larger or smaller animals, since standard atlases illustrate the brain of 250-g rats)

Anesthetics and analgesics (also see *APPENDIX 4B*)

Ketamine

Dexmedetomidine (Orion Corp., <http://www.orion.fi/>)

Carprofen (Hospira, <http://www.hospira.com/>)

Xylazine

Atipamezole (Antisedan; Orion Corp., <http://www.orion.fi/>)

Buprenorphine (Buprenex)

Betadine (povidone-iodine)

Ophthalmic ointment (Puralube)

Lactated Ringer's solution

Rat chow

Oxygenated Tyrode's solution with heparin (see recipe)

4% (w/v) paraformaldehyde in PBS

Stereotactic frame with rat adapter and blunt ear bars (Stoelting)

Stereomicroscope (e.g., Zeiss Stemi 1000 zoom) equipped with 16× eyepieces and 0.4× auxiliary objective lens, and mounted on hinged coupling arm on a heavy foot stand (or equivalent)

Electric drill with 1.75-mm drill bit (Stoelting)

Heat pad

Fiber-optic illuminator with twin goose-neck pipes (Leica)

Surgical equipment, sterile:

Surgical shavers (Stoelting)

Scalpel and blades

Skin retractors

Cotton swabs

Curved and straight forceps

Holding scissors

Sharp scissors

Sterile gauze

10-μl, 26-G Hamilton syringe with needle (model 701RN, Fisher)

3–0 nylon sutures

Petri dish, plastic

Additional reagents and equipment for rodent anesthesia (*APPENDIX 4B*) and perfusion (Support Protocol 1)

Prepare animal and adenovirus suspension

1. Position the stereotactic frame relative to the surgical microscope and light source such that the microscope is focused on the ear bars of the frame (Fig. 4.24.1). Lay out the drill, heat pad, and sterilized surgical tools next to the frame (Fig. 4.24.2).

Before commencing surgery, it is important to ensure that the position of the microscope relative to the stereotactic unit be such that the experimenter can operate comfortably. Only minimal adjustments should be necessary once the animal is fitted into the frame.

2. Dilute the adenovirus preparation with sterile PBS (pH 7.4) such that the required number of infectious units can be administered in the appropriate volume (see Critical Parameters). Store on ice in a microcentrifuge tube until required.

Careful consideration should be given to the dose of vector administered. As discussed in the Commentary (see Critical Parameters), high doses of vector (i.e., above 10^8 infectious units) are generally associated with transient transgene expression and severe inflammation, while low doses (below 10^6 infectious units) result in minimal transduction (depending on promoter strength). Generally, administration of 10^7 infectious units of vector is optimal (when using vectors expressing transgenes from the major immediate early human CMV promoter) in terms of providing relatively stable transgene

expression with minimal inflammation. Both the total amount of infectious units and the total amount of physical viral particles (see UNIT 4.23) need to be considered when assessing viral vector toxicity. Use of stronger promoters allows doses to be reduced (see Gerdes et al, 2000).

The vector can be kept for several hours on ice. It is not advisable to refreeze the diluted vector, as this can result in a significant drop in infectious unit titer.

4. Anesthetize rat by i.p. injection of ketamine (75 mg/kg) and dex-medetomidine (0.25 mg/kg). After anesthesia but before surgery, administer 5 mg/kg Carprofen subcutaneously as an analgesic (also see APPENDIX 4B). When animal is fully anesthetized, remove the hair overlaying the skull using surgical clippers. Place the animal in the stereotactic apparatus and partially immobilize the skull. Slide the ear bars into the respective ear canals and tighten in place so as to eliminate any medio-lateral head movement while maintaining rotation in the dorso-ventral axis (refer to Fig. 4.24.3). Place the heat pad underneath the animal to prevent hypothermia during surgical anesthesia.

Stereotactic surgery is generally performed on adult rats of 250 g body weight. Difficulties may be experienced in fitting the nose of larger animals into the space available, in which case the stereotactic coordinates will not be accurate, since standard stereotactic atlases illustrate the brain of 250 g rats. If using mice, see UNIT 3.10.

Care should be taken that the positioning of the animal does not cause any respiratory distress, which is monitored by maintenance of regular respiration. As with all surgical procedures, the breathing rate of the animal should be carefully monitored throughout the operation.

6. When the animal is positioned firmly and correctly within the frame, disinfect the incision site with Betadine. Place a drop of ophthalmic ointment into each of the eyes.

Perform stereotactic injection of vector

7. Ensure that the animal is fully anesthetized by checking the lack of responses to footpad and tail pinching. Using a scalpel, make a 2-cm midline incision into the skin, from above the eyes to the level of the ears.
8. Use skin retractors to hold back the skin on either side of the incision.
Bregma (the junction of the sagittal and transverse sutures) should be clearly visible within the region of exposed skull (see Fig. 4.24.4).
9. Remove the connective tissue covering the top of the skull by cleaning the cranium with a cotton swab.
10. Load the 10- μ l Hamilton syringe with the vector suspension and expel a small amount of the vector suspension onto a cotton swab to verify that the needle is not blocked. Clamp the needle into position on the frame.

It is important to ensure that the needle and syringe are clean and in good condition prior to beginning surgery.

The volume of vector suspension injected into the CNS will depend on the anatomical site. No more than 2 μ l of vector suspension should be injected into the brain parenchyma; however, up to 30 μ l of vector can be administered to the ventricles.

11. Direct the light beams from the fiber-optic light pipes onto the exposed skull and focus the microscope onto bregma.

It is only necessary to use the fiber-optic light source when using the microscope. At all other times the light beams should be switched off or directed away from the animal, as they generate considerable heat.

12. Position the syringe over the skull and tighten it into place. While viewing the brain through the microscope, position the needle using the three slide rules so that the needle tip is directly over bregma.
13. Read the coordinates of bregma from the frame. Calculate the new coordinates of the site of injection by adding or subtracting the appropriate lateral and anterior/posterior values from bregma. Move the needle to these new coordinates.

The coordinates of the injection site relative to bregma are found by reference to an atlas of the rat brain (e.g., Paxinos and Watson, 1998).

14. Lower the needle at the new coordinates, so that it is just touching the surface of the skull (be careful not to damage the needle point), and mark this position with a small dot using a very fine marker pen (Fig. 4.24.4). Raise the needle to allow drilling.
15. Viewing the surface of the skull through the surgical microscope, drill a small hole approximately 2 mm in diameter most of the way through the skull at the position marked by the small dot.

Drilling into bone generates considerable heat. It is therefore advisable to drill in short bursts, intermittently bathing the hole with cold sterile saline solution, which can then be removed with a cotton swab before recommencing drilling. The hole may bleed during drilling if blood vessels coursing through the bone are ruptured. This should cause only very limited bleeding, which can be staunches with a cotton swab if necessary. It is sometimes necessary to refocus the microscope on the bottom of the deepening hole during drilling.

16. Stop drilling when the base of the hole becomes translucent. Perforate the remaining thin layer of skull with a sterile needle, and, using a pair of sharp, curved forceps, carefully remove this remaining layer of bone to expose the dura mater.

Clear CSF often leaks into the hole when the skull is perforated. Soak this up with a cotton swab.

17. Using a sterile, bent needle, carefully perforate the dura and remove as much of the membrane as needed to expose the surface of the brain.

It is extremely important to distinguish between the dura (which is whitish, opalescent, and elastic) and the brain itself (which is darker and more yellow in color) to avoid lesioning the brain surface with the needle.

18. Lower the Hamilton needle into the hole until it just touches the surface of the brain and read the vertical coordinate of this position. Calculate the new vertical coordinate of the site of injection.

It may be necessary to adjust the coordinates slightly to avoid rupturing large blood vessels running across the surface of the brain when inserting the needle into brain.

19. Lower the needle into the brain to the site of injection and wait 2 to 3 min before slowly depressing the syringe plunger by 0.5 μ l, over a further minute (Fig. 4.24.5). Wait 1 min for the vector solution to infuse into the brain. Inject a further 0.5 μ l of

vector and wait a further minute. Repeat until the entire 2 μ l of vector has been administered. Wait for 5 min after the final administration.

For large areas of the brain parenchyma, e.g., the caudate putamen, the needle can be raised by 0.1 mm after each 0.5- μ l injection to prevent build-up of pressure that would result from injecting the entire volume of solution in one site only. For other areas within the CNS, such as the substantia nigra (which is comparatively small) or the ventricles, this is either not expedient or not necessary.

A pump—e.g., ultramicropump (UMP; World-Precision Instruments)—which can be attached to the Hamilton syringe for automated injection, makes vector administration less laborious.

Superfine glass needles can be fitted with wax over the Hamilton needle to reduce physical damage to the brain during injection.

20. Remove the needle from the brain very slowly and close the skin incision using 3–0 nylon sutures.

Allow animal to recover

21. Swab the sutured area with sterile saline, remove the animal from the stereotactic apparatus, and allow it to recover after reversal of anesthesia with a single intraperitoneal injection of atipamezole (1 mg/kg) and single subcutaneous injections of buprenorphine for pain relief and warm lactated Ringer's (20 μ l/g body weight, subcutaneously) to prevent dehydration.
22. Keep animals under close observation until fully recovered from the anesthesia, then return them to their main cage. Post-stereotactic surgery, provide the rats with water-soaked chow in a plastic petri dish placed on the cage floor. Remove nylon sutures 10 to 14 days post-operatively.

In most cases the animals recover from the surgery quickly (within 10 to 15 min) and show no behavioral signs of neurological dysfunction.

The surgical procedure is relatively quick, and optimally the animal should be under anesthesia for no longer than 40 min. If several animals are to be operated on in succession, it is often not practicable to sterilize the surgical tools after each operation. In such cases, it is imperative to thoroughly clean the Hamilton needle and other tools between animals, first by soaking in an enzymatic cleaning solution (specially formulated for surgical tools, e.g., Endozyme), then rinsing several times with 70% ethanol, and finally, sterile saline. The drill head can also be cleaned in this manner between operations.

Perform terminal perfusion and collect specimens

At the appropriate timepoint, euthanize the rat via terminal perfusion.

23. To induce deep anesthesia, manually restrain the rat and administer ketamine (80 mg/kg) and xylazine (8 mg/kg) via intraperitoneal injection. Ensure that the animal is fully anesthetized by checking the lack of responses to footpad and tail pinching.
24. Lay the rat with its dorsal side down and fix each of the footpads into place. Make an incision into the abdomen and cut the diaphragm to expose the thoracic cavity.
25. Open the thoracic cavity by completely by cutting the ribs, and lifting the anterior part of the rib cage to expose the heart.

26. Perfuse the animal with oxygenated Tyrode's solution containing heparin using the apparatus and technique described in Support Protocol 1. Prepare brain tissue as described in Support Protocol 1.
27. *If fixed tissue is required for the downstream application (i.e., immunohistochemistry):* After perfusion with oxygenated Tyrodes/heparin as in step 26, perfuse animal with fixative (4% paraformaldehyde in PBS) using the apparatus and technique described in Support Protocol 1.

The brain is then removed from the cranium, as well as other organs, and processed further (see Support Protocol 1 and UNIT 1.1).

EVALUATION OF GENE TRANSFER, INFLAMMATORY RESPONSES, AND VECTOR-MEDIATED TOXICITY FOLLOWING IN VIVO ADMINISTRATION OF RECOMBINANT ADENOVIRUS VECTORS

There are many different methods for evaluating adenovirus-mediated gene transfer in an in vivo paradigm. Immunohistochemical detection of the transgene product within brain sections is a widely used procedure, *UNIT 1.2* describes a method in which serial brain sections are processed using "free-floating" horseradish peroxidase-based immunohistochemistry to detect the transgene product. In the protocol below, a modification of the ABC method described in *UNIT 1.2* is presented, in which nickel chloride is added to the substrate solution to generate a blue/black precipitate (which is easier to photograph than the reddish/brown precipitate obtained with DAB alone). Further, an additional initial blocking step is incorporated whereby the sections are incubated with H_2O_2 to inactivate endogenous peroxidase activity. Triton X-100 is also included throughout the incubations to improve the staining of various epitopes. Also, glucose oxidase is incorporated into the staining solution to generate H_2O_2 from the oxidation of glucose, thus allowing staining to proceed more slowly (see Fig. 4.24.6).

Adenovirus vectors are inflammatory and immunogenic, and can be cytotoxic at high multiplicities of infection. It is therefore expedient (particularly when adenovirus vectors are used for neurological gene therapy applications) to evaluate the inflammatory, immune, and cytotoxic responses to vector delivery, simultaneously with any evaluation of vector-mediated gene transfer. The HRP-based immunohistochemistry protocol described here can be used with appropriate antibodies to visualize the acute and/or chronic infiltration of immune and inflammatory cells, the activation of brain microglia and astrocytes, the loss of glial cell or neuronal markers (indicating cell loss through acute or chronic cytotoxicity), and/or the integrity of brain myelination after in vivo administration of adenovirus vectors to the brain (see Fig. 4.24.12 and Fig. 4.24.13 for some examples). Table 4.24.1 shows some of the antibodies that have been used to investigate host responses to adenoviral vector administration in the rat striatum. All antibodies listed work well at the given dilutions, using Vibratome sections from brains that have been post-fixed in 4% paraformaldehyde solution for 48 hr.

For simultaneous analysis of the expression of two different epitopes (e.g., a cell type-specific marker and a transgene marker), fluorescence-based immunohistochemistry is often more appropriate (see Alternate Protocol 1). Microscopic analysis of fluorescently labeled cells requires tissue sections (optimally ~40 to 60 μm), generated by Vibratome sectioning, *UNIT 1.1* describes cryostat sectioning of frozen brain tissue, which can be used to generate sections as thin as 5 μm . The choice of fixed versus frozen brain tissue sections will depend on the characteristics of the primary antibodies used.

Materials

Brain sections (see Support Protocol 1)

TBS/Triton: Tris-buffered saline (TBS; *APPENDIX 2A*) containing 0.5% (v/v) Triton X-100

Citrate buffer (*optional*; 10 mM citric acid in H₂O, adjusted to pH 6 with 0.1 N NaOH), prewarmed to 60°C

0.3% (v/v) H₂O₂ in PBS

TBS/Triton (see above) containing 10% horse serum (TBS/Triton/10% HS)

Primary antibody recognizing epitope of interest (e.g., transgene or immune cell marker)

TBS/Triton/1% HS (see above) with 0.1% (w/v) sodium azide

Biotinylated secondary antibody (biotinylated antibody against immunoglobulin of species in which primary antibody was raised)

Vectastain ABC Elite kit (Vector Laboratories)

Dulbecco's PBS, pH 7.4, without Ca or Mg (Cellgro, cat. no. 21-031 CV)

0.1 M sodium acetate, pH 6.0

DAB staining solution (see recipe)

Soft-bristled paintbrush

6-well tissue culture plates

Platform shaker

Gelatin-coated glass slides (*UNIT 1.1*)

Additional reagents and equipment for dehydrating and coverslipping sections (*UNIT 1.2*)

Prepare tissue sections for staining

1. Using a soft-bristled paintbrush, transfer the brain sections into 6-well plates containing approximately 5 ml of TBS/Triton. Wash the sections by shaking the 6-well plates at room temperature on a platform shaker for 5 min.
2. Remove the TBS/Triton using a plastic transfer pipet and discard.

Be very careful not to damage the fragile sections with the pipet, or accidentally suck up the sections along with the buffer. The Triton X-100 will permeabilize the tissue.

- 2a. *Optional:* For antigen retrieval, fill each well with citrate buffer prewarmed to 60°C. Allow to cool to room temperature (~20 min). Remove the citrate buffer before proceeding to step 3.
3. Add 2 ml of 0.3% H₂O₂ to each well and incubate with shaking for 20 min at room temperature to inactivate endogenous peroxidase.

If the sections have been stored in PBS without azide, the sections will appear to fizz as the peroxidase metabolizes the H₂O₂ to oxygen and water. Azide inhibits peroxidase activity, and sections stored in the presence of azide should be rinsed with PBS before the H₂O₂ treatment.

4. Wash the sections three times, each time by incubating with 2 ml of TBS/Triton with shaking for 5 min.
5. Block nonspecific antibody-binding sites and Fc receptors by incubating the sections with 1 ml of TBS/Triton/10% HS for 1 hr with shaking.

Ensure that all of the sections are immersed in the blocking solution and are not stuck to the sides of the wells.

Often, it is recommended that nonspecific binding be blocked using serum from the species in which the secondary antibody was generated, e.g., if the secondary antibody is rabbit anti-mouse, then the sections may be blocked with rabbit serum instead of horse serum. The authors have found, however, that horse serum provides the best blocking for a wide range of antibody combinations, irrespective of the origin of the secondary antibody.

6. Wash the sections once for 5 min in TBS/Triton/1% HS, with shaking.

Incubate with primary and secondary antibodies

7. Incubate the sections with the primary antibody, diluted to the required extent in TBS/Triton/1% HS/0.1% sodium azide, for 48 hr with shaking.

This incubation should be performed at room temperature with plates sealed in Parafilm.

Since antibodies are expensive, it is desirable to perform this incubation in as small a volume as possible. A volume of 1.0 to 1.5 ml is generally sufficient to cover all the sections.

Before using an antibody for the first time, it is advisable to determine the optimal dilution factor by performing a small titration experiment incorporating three or four different dilutions. The data sheets provided with commercially available antibodies will often recommend a dilution factor, and this can be used as a starting point. Primary antibodies can be reused if stored at 4°C with 0.01% sodium azide added to the storage buffer.

8. Wash the sections five times in TBS/Triton using the technique described in step 4.
9. Incubate with the biotinylated secondary antibody, diluted appropriately in TBS/Triton/1% HS (no azide), overnight.

Develop color

10. Toward the end of the incubation period, prepare the avidin/biotinylated HRP complex (Solution AB) using the Vectastain ABC Elite kit by adding 1 drop of Solution A and 1 drop of Solution B to 10 ml PBS.

Larger quantities of Solution AB can be prepared in a 50-ml Falcon tube, if many wells are being processed simultaneously.

11. Wash sections five times in TBS/Triton using the technique described in step 4.
12. Incubate the sections for 3 hr in 1 ml of Solution AB, with shaking.
13. Wash three times, each time with 2 ml of PBS using the technique described in step 4.
14. Wash twice, each time with 2 ml of 0.1 M sodium acetate (pH 6.0) using the technique described in step 4.

15. Stain the sections by incubating in 1 ml of DAB staining solution with gentle shaking for 1 to 7 min. Add 5 ml of PBS containing 0.1% sodium azide to stop the development of the stain at the appropriate time.

Carefully monitor the development of the stain; the sections will become faintly purple/black throughout. Specific staining of cells will sometimes be visible only under the microscope after mounting, dehydration, and coverslipping. To avoid overdeveloping, stain sections from only one or two wells at a time (the other sections can be left in sodium acetate until required), but ensure that all sections stained at different times are developed for the same length of time.

Freshly prepare the required volume of DAB solution for all the wells immediately before use and discard unused solution (DAB is photosensitive).

CAUTION: *The staining solution is toxic and the DAB (a mutagenic substance) should be precipitated and inactivated with bleach before discarding.*

16. Wash the stained sections twice with 2 ml sodium acetate (pH 6.0) and twice with 3 ml of PBS using the technique described in step 4.

If the PBS is replaced with PBS containing 0.1% sodium azide, the sections can be stored in the 6-well plates at 4°C for several days before mounting on glass slides.

Mount sections on slides

17. Using a soft-bristled paintbrush, transfer the sections from the well into a clear container (e.g., 15-cm tissue culture dish) filled with PBS. Moisten gelatin-coated slide with PBS. Partially submerge the slide in the PBS and float the sections onto the slide using the paintbrush. Arrange the sections on the slide in anatomical order if possible (approximately six rat brain sections will fit on each slide).

Filter the PBS before use to eliminate fibers and any debris that may stick to the sections.

18. Allow the sections to air dry for several hours so that they become fixed onto the gelatin-coated slides.

The slides may be left to air dry overnight; however it is important to leave them in a dust-free environment to prevent debris and fibers from sticking to the sections.

19. Dehydrate the sections and coverslip as described in UNIT 1.2.

Figure 4.24.7 shows detection of adenoviral vector-mediated expression of the transgene β -galactosidase by this method. It is also possible to visualize the expression of β -galactosidase using a simple and fast chromogenic staining procedure in which the brain sections are incubated in a solution of Xgal for a minimum of 3 hr in the dark (see UNIT 4.23). However, this stain is more nonspecific (Rosenberg et al., 1992) and is less sensitive than β -galactosidase immunohistochemistry.

FLUORESCENCE-BASED IMMUNOHISTOCHEMICAL STAINING OF BRAIN SECTIONS

UNIT 1.2, as well as Basic Protocol 2 in this unit, describe the immunohistochemical localization of a single marker antigen within tissue sections. Sometimes it may be desirable to detect two different marker antigens simultaneously within a single brain section (e.g., to

determine which subpopulation of brain cells has been transduced by codetecting a cell type-specific marker with the transgene marker). The following protocol describes double-labeling of tissue sections using two different fluorescently labeled antibodies. To accurately determine the subpopulation of cells which is double-labeled, confocal microscopy will greatly improve the resolution and quality of data. This protocol describes free-floating immunofluorescent labeling of Vibratome sections; however the method can be adapted for use on frozen sections.

Additional Materials (also see Basic Protocol 2)

Blocking serum (from the species in which the secondary antibodies were generated)

Two primary antibodies recognizing epitopes of interest, generated in different species

Appropriate secondary antibodies labeled with two different fluorescent markers (e.g., Alexa 488 and Alexa 594)

Prolong-Gold Antifade Reagent (Invitrogen, cat. no. P36930)

Fluorescence microscope

1. Using a soft-bristled paintbrush, transfer the brain sections into 6-well plates containing ~5 ml of TBS/Triton. Wash the sections by shaking the 6-well plates at room temperature on a platform shaker for 5 min. Remove the TBS/Triton.
- 1a. *Optional:* For antigen retrieval, fill each well with citrate buffer prewarmed to 60° C. Allow to cool to room temperature (~20 min). Remove the citrate buffer before proceeding to step 2.
2. Block nonspecific antibody binding sites by incubating the sections for 1 hr in TBS/Triton containing 10% (v/v) blocking serum.

The blocking serum should be from the species in which the first secondary antibody to be used was generated, e.g., if the secondary antibody is rabbit anti-mouse, use rabbit serum to block. The authors have found, however, that horse serum provides the best blocking for a wide range of antibody combinations, irrespective of the origin of the secondary antibody.

3. Wash the sections once for 5 min with 1 ml TBS/Triton containing 1% blocking serum, with shaking. Remove the TBS/Triton.
4. Incubate the sections with the primary antibody, diluted in the required amount of TBS/Triton/1%HS/0.1% sodium azide for 48 hr with shaking.

It is possible to use several primary antibodies simultaneously as long as the primary antibodies were raised in different species.

5. Wash the sections five times, each time with 1 ml TBS/Triton for 5 min with shaking.
6. Incubate the sections overnight with the appropriate fluorescent secondary antibody diluted in TBS/Triton/1% blocking serum.

Where possible, this step and all subsequent steps should be performed away from strong light to avoid bleaching the fluorescent label. The 6-well plates can be covered with foil or other lightproof material while shaking.

7. Wash the sections five times, each time with 1 ml TBS/Triton for 5 min with shaking.
8. Repeat steps 2 to 7 for the second set of antibodies.
9. Mount the sections on glass slides (see Basic Protocol 1, step 17) and, as soon as they have dried enough that the sections are no longer at risk of moving on the slide, coverslip them using Prolong-Gold Antifade Reagent.

10. Visualize labeled cells using a fluorescence microscope.

Double-labeled cells can be visualized by overlaying the two patterns of fluorescence for the same field.

SIMULTANEOUS EVALUATION OF VECTOR-INDUCED INFLAMMATION AND DEMYELINATION WITH LUXOL FAST BLUE AND CRESYL VIOLET

A global visualization of infiltrating cells and brain structure can be provided by staining cell nuclei with cresyl violet. The following protocol describes a staining procedure wherein Vibratome brain sections are histologically stained with both cresyl violet and Luxol fast blue for the simultaneous detection of cell nuclei (to visualize areas of inflammation) and myelin (to detect areas of demyelination). If desired, each stain can be used individually for the separate detection of cell nuclei and Nissl substance (cresyl violet) or myelin (Luxol fast blue). Staining of nuclei with cresyl violet is useful for the visualization of the total overall pattern of distribution of inflammatory cells infiltrating the brain after injection of adenovirus, whereas Luxol fast blue staining enables the detection of demyelination, which may occur concomitantly with adenovirus-induced inflammation. Luxol fast blue staining will be reduced in tissues displaying either demyelination or edema. When examining a novel experimental paradigm, it is important to confirm actual demyelination using specific myelin antibodies (see Table 4.24.1). Edema and many aspects of cellular and axonal integrity can also be analyzed by staining semi-thin plastic-embedded sections with toluidine blue (see Basic Protocol 4).

Materials

Free-floating Vibratome-cut brain sections (see Support Protocol 1)

50%, 70%, 80%, and 96% ethanol

Luxol fast blue (LFB) solution (see recipe)

95% methylated spirits

0.05% (w/v) lithium carbonate

0.1% (w/v) cresyl violet in 1% (v/v) acetic acid

APES-coated glass slides (see Support Protocol 2) 60°C oven

Additional reagents and equipment for mounting sections on slides (see Basic Protocol 2, step 17) and dehydrating and coverslipping sections (*UNIT 1.2*)

1. Mount free-floating Vibratome-cut brain sections on APES-coated slides using the technique described in step 17 of Basic Protocol 2 for gelatin-coated slides.

APES-coated slides are used here in preference to gelatin-coated slides, as the sections tend to detach from the latter after incubation in the LFB solution.

2. Allow the sections to air dry and adhere to the slides.
3. Dehydrate the sections by sequentially immersing the slides for 10 min in each of the following dilutions of ethanol: 50%, 70%, 80%, and 96%.
4. Immerse the slides in filtered LFB solution in a glass jar with a lid and incubate the sections overnight (or for 16 hr) in a 60°C oven.
5. Rinse the sections by immersion in 95% methylated spirits for 5 min, followed by distilled water for 5 min.

At this point, the sections will be uniformly stained a deep blue. Differentiation (i.e., destaining) in lithium carbonate and ethanol is required to wash the stain from the nonmyelinated regions.

6. Start the differentiation process by agitating each slide for a few seconds in a jar of 0.05% lithium carbonate.

Fresh 0.05% lithium carbonate should be used after agitation of several slides, as this solution loses its differentiating ability after a few uses.

7. Continue to differentiate by agitation in 70% ethanol.
8. Rinse the slides in distilled water and examine the sections under the microscope.

White matter (myelinated regions) should appear blue, while the rest of the brain should be pale.

9. Repeat the differentiation steps if necessary until the gray and white matter are clearly distinguishable.
10. Counterstain by incubating the sections in 0.1% cresyl violet in 1% acetic acid for 15 min.
11. Wash the sections in distilled water, then dehydrate rapidly through graded alcohols and coverslip as described in steps 28 to 31 of Basic Protocol 1, *UNIT 1.2*.

After staining with cresyl violet, nuclei and Nissl substance should appear purple.

Perform this staining section by section since it is highly variable.

EVALUATION OF VECTOR-MEDIATED TOXICITY BY TOLUIDINE BLUE STAINING

Toluidine blue staining of semi-thin plastic embedded sections (standard preparation for electron microscopy analysis; see *UNIT 1.2*) containing the injection site enables an evaluation of the integrity of the structure of neural cell bodies and processes, and allows a determination of the presence of extracellular edema within the tissue (Dewey et al., 1999).

Materials

1% toluidine blue (see recipe)

Semi-thin plastic embedded 5- μ m brain sections (see *UNIT 1.2*) mounted on gelatin-coated (see *UNIT 1.1*) or APES-coated (see Support Protocol 2) glass slides

Additional reagents and equipment for dehydrating and coverslipping sections (*UNIT 1.2*)

1. Place a drop of 1% toluidine blue onto the brain section and leave for 1 to 2 min at room temperature.
2. Wash off the stain by immersing the slide in distilled water.
3. Dehydrate and coverslip as described in Basic Protocol 2 of *UNIT 1.2*.
4. View using light microscopy.

DETECTION OF VECTOR GENOMES IN RAT BRAINS BY QUANTITATIVE PCR

It is possible to quantitate the levels of vector genomes within the brain or other organs. This protocol describes a quantitative PCR (qPCR)-based method for detecting adenoviral genomes

within the rat brain (Puntel et al., 2006). A primer/probe set is presented with qPCR reaction parameters for quantitation of the vector genomes present in the total DNA extracted from brain tissue. The primer/probe set described below identifies a region of the L3 gene of the human adenovirus serotype 5 genome. It is essential to isolate vector genomes from brains of animals perfused with oxygenated Tyrode's solution in absence of fixative. It is also possible to quantitate vector genomes in other peripheral organs such as liver, lung, and testes.

Due to the high sensitivity of this technique, particular care should be taken when setting up a PCR reaction to avoid cross-contamination between samples; use of aerosol-resistant filter pipet tips will reduce this risk. All tubes, tips, and solutions should be designated for this procedure only and should not be stored in close proximity to any vector stocks. Predesignated PCR-only pipets and racks should be used.

Materials

Brains from rats perfused without fixative (see Support Protocol 1)

DNeasy Blood and Tissue Kit (Qiagen, cat. no. 69506)

100% and 70% ethanol, molecular biology grade

Adenovirus L3 primer/probe set (consists of custom-made oligos synthesized, e.g., by Operon or Eurofins; see Puntel et al., 2006):

1 nmol/μl forward primer: 5'-GAGTTGGCACCCCTATTCGA-3'

1 nmol/μl reverse primer: 5'-ATGCCACATCCGTTGACTTG-3'

100 pmol probe: 6-VIC-5'-CCACCCGTGTGTACCTGGTGGACA-3'-TAMRA

2× QuantiTect Probe PCR Master Mix (Qiagen)

DEPC-treated H₂O (Fluka)

Standard plasmid containing the target sequence (e.g., pSt) for preparing standard curve (also see Puntel et al., 2006)

1-mm Alto rat brain acrylic matrix (CellPoint Scientific)

Digital single-channel and multichannel pipet (Eppendorf) with aerosol-barrier tips and sterile disposable solution basins

Sterile 96-well PCR plates

Optical support base (Applied Biosystems, cat. no. 4308775)

Optical adhesive cover (Applied Biosystems, cat. no. 431171)

Compression pad (Applied Biosystems, cat. no. 431171)

SDS Enterprise Database software (Applied Biosystems)

Real Time Quantitative PCR instrument (Applied Biosystems)

Beckman Allegra 6R centrifuge/GH 3.8A rotor with microplate adaptor

Prepare DNA template

1. Dissect the area of the brain surrounding the injection site using a razor blade and a rat brain matrix (Fig. 4.24.8).
2. Isolate vector genomes using the DNeasy Blood and Tissue Kit (Qiagen) as described in the manufacturer's instructions.

Measuring OD₂₆₀ of the DNA samples (APPENDIX 1K) can be a useful parameter for later expressing the results, mainly when working with DNA extracted from tissue

Perform quantitative PCR

3. Prepare the qPCR reaction mixture:

4 µl 1 nmol/µl forward primer

4 µl 1 nmol/µl reverse primer

10 µl 100 pmol/µl probe

2500 µl 2× PCR master mix

2482 µl DEPC-treated H₂O.

The reaction volume described fills a 96-well plate. Protect from light and store on ice.

4. Transfer 45 µl of qPCR reaction mixture into each well of a sterile 96-well PCR plate using a multichannel pipettor (it is critical to add the mixture directly to the bottom of the well). Tap the plate gently and use a pipet tip to remove any bubbles. Store at 4°C in the dark until use using the optical support base.

It is essential to pipet carefully to avoid bubbles, as these will interfere with the data collection.

5. If needed, make dilutions for each DNA sample (isolated in step 2) using DEPC-treated water, and keep on ice. Run each sample in triplicate

Prepare DNA dilutions depending on the expected number of copies present in the sample. If a high copy number is expected, make dilutions of sample DNA at 10⁻³, 10⁻⁴, and 10⁻⁵. Otherwise use 5 µl of neat DNA.

6. Prepare fresh dilutions for standard curve for each assay. To do so, prepare serial dilutions of a standard plasmid containing the target sequence (e.g., pSt) with concentrations ranging from 10⁷ molecules/5 µl to 10² molecules/5 µl in DEPC-treated water (for standard plasmid details see Puntel et al., 2006). Run each point on the standard curve in triplicate. Keep on ice.

DNA has the tendency to bind to plastic. Do not let the standard curve dilutions sit for extended periods of time. Storage of the dilution tubes on ice may reduce the binding of DNA to the plastic tubes.

7. Add 5 µl of each standard curve point and the vector DNA template to the plate in triplicate. Be sure to pipet carefully to avoid bubbles. In order to avoid bubbles in this step, deliver the volume of template onto the wall of the well, not the bottom.

It is convenient to use an identical plate design for each assay.

8. Protect from light by placing onto an optical support base.
9. Seal the plate with an optical adhesive cover, compression pad (gray side down), and an applicator as described per manufacturer's instructions.
10. Centrifuge for 2 min at 162 × g (1000 rpm in a Beckman Allegra centrifuge/GH 3.8A rotor using microplate rotor adaptor), 4°C.

11. Enter the file name, date, and probe dyes, and run parameters using the SDS Enterprise Database software according to the manufacturer's instructions. Use the following reaction conditions:

1 cycle:	2 min	50°C
1 cycle:	10 min	95°C
40 cycles:	15 sec	95°C
	1 min	60°C.

12. Insert the plate into the Real-Time Quantitative PCR instrument and run the instrument per manufacturer's instructions.

Perform data analysis

13. Check the standard plot (R² should be close to 1, repetition values should overlap almost perfectly).
14. Check the Amplification plot (for 10⁷ standard plasmid copies, the curve will cross the threshold between cycles 15 and 20; for 10² standard plasmid copies the curve will cross the threshold between cycles 35 and 40).
15. Check the SDS software for the display of the threshold value and the average of the quantification for each well of the plate. To obtain the number of genome copies per ml, multiply the average value by 200 (volume factor = 1000 µl/5 µl) and by the dilution factor if any dilution was done with the template.

Note that the range of detection for this technique is, as the standard curve describes, between 10² and 10⁷ genome copies. Thus, values lower than 10² or higher than 10⁷ are beyond of the limit of quantification. There are several solutions to overcome this problem: for samples with values lower than 10², increasing DNA concentration is recommended; for samples with higher values than 10⁷, higher dilutions of the template are recommended; for values around 10², carefully check that the three replicates of the sample show close values; repetition of the experiment is recommended.

ASSESSMENT OF BLOOD-BRAIN BARRIER INTEGRITY IN THE RAT BRAIN

Adenoviruses are immunogenic and will cause a dose-dependent brain inflammation (see Critical Parameters). Adenovirus vectors per se injected into the brain parenchyma, or the use of adenoviruses encoding proinflammatory molecules (Bell et al., 1996), can cause disruption of the blood-brain barrier (BBB) with concomitant infiltration of inflammatory cells from the circulation. While certain cell types (e.g., activated T lymphocytes and antigen-specific B cells) are able to cross an intact BBB, other cell types can only infiltrate the brain once the BBB is broken or opened. Mechanical or vector-induced disruptions to the integrity of the BBB are usually repaired within a few days (although this could depend upon the transgene being expressed by the vector, i.e., if transgenes are pro-inflammatory (M.G. Castro and P.R. Lowenstein, unpub. observ.). Any assessment of vector-induced BBB permeability should therefore be performed within the first few days after surgery.

The following protocol (modified from Hanker et al., 1977) involves injection of the plant enzyme horseradish peroxidase (HRP) into the bloodstream of the rat via the tail vein. Under normal conditions, the intact BBB will exclude circulating HRP from the brain. A permeabilized BBB will, however, allow the HRP to enter the brain within minutes after

administration. The extent of BBB permeabilization can subsequently be visualized by staining Vibratome-cut brain sections according to the Hanker-Yates method, i.e., by incubating the sections with a horseradish peroxidase (HRP) substrate solution, the components of which are metabolized to form a colored reaction product. Typical results using two different doses of first-generation adenovirus vector-expressing β -galactosidase are shown in Figure 4.24.9. Injection of 1 to 5 μ g of lipopolysaccharide (LPS) into the rat striatum provides an excellent positive control for BBB breakage.

Alternatively, clinically used blood pool agents such as gadolinium-derived compounds (e.g., gadoteridol) given as an IV bolus injection and imaged using magnetic resonance angiography have shown benefits over traditionally used extracellular contrast media, and have gained increasing usage in the laboratory setting (Prohance, Bracco Diagnostics). Moreover, intravenous injections of variably sized dextrans conjugated to a number of different fluorochromes such as Texas Red, tetramethylrhodamine, and FITC (available from Invitrogen) have been successfully employed in order to label the intraluminal vascular space in studies requiring the visualization of parenchymal blood vessels through fluorescence microscopy.

Materials

Horseradish peroxidase (HRP; Type II; Sigma)

Normal saline: 0.9% (w/v) NaCl, sterile

Adult rat

Dulbecco's PBS, pH 7.4, without Ca or Mg (Cellgro, cat. no. 21-031 CV)

Hanker-Yates solution A (see recipe)

Hanker-Yates solution B (see recipe)

Towel or other restraining device for rats

0.5- μ l microfine disposable needle-fitted syringe (e.g., 0.3-mm $\times$ 12.7-mm, Becton, Dickinson)

6-well tissue culture dishes

Platform shaker

Gelatin-coated slides (*UNIT 1.1*)

Additional reagents and equipment for perfusion fixation and Vibratome sectioning (see Support Protocol 1 and *UNIT 1.1*) and mounting with DPX mountant (*UNIT 1.2*, Basic Protocol 1, steps 27 to 31)

Administer HRP

1. Dissolve 17.5 mg HRP in 1 ml of sterile normal saline. Keep this solution on ice and out of the light as much as possible.
Try to prepare this immediately before use to avoid excessive exposure to light, which may cause enzymatic denaturation.
2. Restrain the rat (this can be done by wrapping the animal in a towel so that only the tail protrudes) and dilate the tail vein by placing the tail into a beaker of warm water (about 40°C) for ~5 min, or until the veins are visibly dilated.
3. Inject 400 μ l of the freshly prepared HRP solution into dilated tail vein using a 0.5- μ l microfine needle-fitted syringe.

4. Return the animal to the cage and wait for 20 min, then perform perfusion fixation (see Support Protocol 1).

The animal should be perfused within 30 min after injecting the HRP.

5. Section the brain using a Vibratome as described in Support Protocol 1 and store in PBS, pH 7.4, until ready to perform the HRP detection assay.

Do not put sodium azide into the PBS to preserve the brain sections, since this will inhibit the horseradish peroxidase enzyme. This means that the sections cannot be stored for more than a few weeks; ideally, the detection reaction should be performed within a few days.

Detect HRP in the brain

6. Put the brain sections in the wells of a 6-well tissue culture dish containing 4 to 5 ml of Hanker-Yates solution A and incubate on a shaker for 15 min at room temperature.

CAUTION: Remember to wear gloves when handling the Hanker-Yates solutions, which are very toxic.

If a positive control is desired, brain sections from a rat intrastrially injected with 1 to 5 µg of lipopolysaccharide (LPS) can be included.

7. Remove and discard this solution and wash sections twice in PBS, pH 7.4, allowing 3 to 5 min for each wash.
8. Transfer the sections into another 6-well plate containing 4 to 5 ml of freshly prepared Hanker-Yates solution B.
9. Incubate the plate containing the section in the dark (plate can be covered with foil) on a shaker for 15 min to develop the colored reaction product.

If the blood-brain barrier has been damaged, a dark brown reaction product will be formed wherever the HRP has leaked into the brain.

10. Wash sections twice in PBS for a total of 5 min.
11. Mount sections on gelatin-coated slides and allow to dry before coverslipping with DPX mountant as described in *UNIT 1.2*, Basic Protocol 1, steps 27 to 31.

ACTIVATION OF A STRONG ANTI-VIRAL IMMUNE RESPONSE BY INTRADERMAL ADMINISTRATION OF ADENOVIRUS VECTOR

Adenovirus vectors injected into the brains of naive animals are able to sustain transgene expression for at least 1 year (Barcia et al., 2007), compared with those injected into peripheral organs such as the liver, where transgene expression is generally eliminated within 2 to 3 weeks. The relative persistence of vector-mediated expression in the brain is attributed to the failure to elicit an effective anti-adenoviral T cell response following the intraparenchymal vector injection. A strong anti-adenoviral T cell response can be primed through peripheral infection with adenovirus, either prior to or after the intraparenchymal injection. After peripheral exposure to vector, activated anti-adenoviral T cells can cross the blood-brain barrier and facilitate the elimination of adenoviral vector-mediated transgene expression in the brain. Use of animals peripherally primed against adenovirus (rather than naive animals) may provide a more clinically relevant model for neurological gene therapy applications using adenovirus vectors, since most of the human population will have been exposed to, or will be at risk of, a peripheral infection with adenovirus. The following protocol describes a method for activating a strong antiviral immune response by intradermal injection of vector. This protocol has been

used to prime animals 30 to 60 days after, or 2 weeks before, intracranial injection of vector (Thomas et al., 2000; Thomas et al., 2001b; Xiong et al., 2006; Barcia et al., 2006b, 2007, 2008; King et al., 2008). Although peripheral anti-adenoviral immune priming *before* the intracranial injection is a paradigm more likely to represent a clinical scenario, immunizing 30 to 60 days *after* the intracranial injection allows any early acute brain inflammation elicited by the initial intracranial injection to subside before analyzing the effect of a peripherally induced anti-viral immune response upon vector-mediated transgene expression in the brain.

Materials

Adult rat

Normal saline: 0.9% (w/v) NaCl, sterile

Adenoviral vector aliquots in 0.5-ml microcentrifuge tubes on ice: each tube should contain 100 μ l of vector diluted in 0.9% (w/v) NaCl such that each 100 μ l contains 5×10^8 infectious units of vector

Gas anesthetic trolley with the following components:

Isoflurane gas anesthetic

Isoflurane vaporizer (e.g., Fluotec; GE Healthcare)

Medical oxygen cylinder

Medical nitrous oxide cylinder

Induction chamber

Isoflurane scavenger (e.g., Fluovac; Harvard Apparatus)

Desiccator jar (optional)

Surgical shavers (Stoelting)

Gauze surgical swabs

1.0 ml microfine disposable needle-fitted syringe (e.g., 0.3 mm $\times$ 12.7-mm, Becton, Dickinson)

1. Place the animal in the induction chamber and anesthetize with 4% (v/v) isoflurane gas vaporized with oxygen at a flow rate of 1500 ml/min, and nitrous oxide at a flow rate of 750 ml/min.
2. When the animal is fully anesthetized, remove it from the induction chamber and place it on a surgical table with its nose inserted into the outlet tubing of the anesthetic trolley. Adjust the isoflurane level to 1.5% with respect to the the oxygen carrier gas.

Alternatively, animals may also be briefly anesthetized using a desiccator jar with isoflurane. When using this method, it is essential to place the desiccator jar in a chemical fume hood to scavenge any excess vapors.

3. When the animal is fully anesthetized, using the surgical clippers, shave a patch of about 3 cm² of fur from the top of animal's back. Clean the area by swabbing with sterile saline.
4. Withdraw 100 μ l of the vector suspension (containing 1×10^9 infectious units of vector) into the 1.0-ml syringe.
5. Pinch the shaved patch of skin with one hand so that a ridge protrudes between the thumb and forefinger.

6. With the bevel uppermost, insert the needle horizontally into the dermis. Gently push the needle through the ridge of skin until at least 1 cm is inserted.
The needle should be pushed horizontally as near to the top of the ridge as possible to avoid injecting the vector subcutaneously.
7. With the needle still inserted, allow the skin to relax to its natural position. Slowly depress the syringe plunger to inject the vector into the dermis.
The vector solution should form a blister-like “bleb” within the layer of skin. This normally disappears within 10 min.
8. Gently withdraw the needle from the skin before switching off the anesthetic gas and allowing the animal to recover.
The used needle should be soaked in bleach or 1% Virkon before discarding and auto-claving.
9. At the desired time point, proceed to assessment of anti-adenovirus neutralizing antibodies (Basic Protocol 8) or assessment of frequency of anti-adenovirus-specific IFN- γ secreting lymphocytes (Basic Protocol 9).

ASSESSMENT OF ANTI-ADENOVIRUS NEUTRALIZING ANTIBODIES

Systemic administration of adenovirus by intradermal injection elicits both humoral and cell-mediated immune responses specific for adenovirus. Before conducting large experiments with multiple timepoints, it is important to confirm the induction of an anti-adenovirus humoral immune response following immunization. Below is a protocol to assess the levels of anti-adenovirus circulating neutralizing antibodies (Thomas et al., 2000; Barcia et al., 2007; Candolfi et al., 2007; King et al., 2008). This assay should be conducted at 14 days post-immunization during the peak production of neutralizing antibodies.

Materials

Adult immunized rats (Basic Protocol 7)

293 HEK cells (ATCC no. CRL-1573)

293 cell complete medium (see recipe)

Well characterized positive and negative plasma, to be used as assay controls

Ad-hCMV-lacZ (first-generation adenovirus expressing β -galactosidase; prepare by co-transfection of 293 cells with the shuttle vector pAL19-LacZ and pJM17, as described in UNIT 4.23)

Dulbecco's PBS, pH 7.4, without Ca or Mg (Cellgro, cat. no. 21-031 CV)

4% (w/v) paraformaldehyde in PBS

Gas anesthetic trolley with the following components:

Isolurane gas anesthetic

Isoflurane vaporizer (e.g., Fluotec; GE Healthcare)

Medical oxygen cylinder

Medical nitrous oxide cylinder

Induction chamber

Isoflurane scavenger (e.g., Fluovac; Harvard Apparatus)

Desiccator jar (optional)

Heparinized capillary tube

Gauze pad

Vacutainer tubes (BD Biosciences, cat. no. 367981, optional)

Sterile 96-well tissue culture plates

Additional reagents and equipment for anesthesia of rat (see Basic Protocol 7) blood collection (*APPENDIX 4G*) and Xgal staining (*UNIT 4.23*)

Prepare plasma

1. Collect plasma from animal 2 weeks after immunization with adenovirus via retro-orbital blood collection (also see *APPENDIX 4G*).
 - a. Anesthetize the rat using isoflurane administered by either a desiccator jar inside of a fume hood or via a precision vaporizer anesthesia machine as described above in Basic Protocol 7.
 - b. Following anesthesia induction, manually position the rat to collect blood from the vein plexus behind the rat's eyeball (up to 600 μ l total volume).
 - c. Gently insert a heparinized capillary tube below the eye at an $\sim 45^\circ$ angle into the space between the globe and the lower eyelid.
 - d. Gently twist the capillary tube between thumb and forefinger to rupture the vein plexus behind the eyeball and allow blood to the capillary tube.
 - e. At the conclusion of the blood-collection gently press a gauze pad over the closed eyelids for a few seconds until the bleeding has stopped and the animal is returned to its cage.
 - f. Microcentrifuge collected blood for 20 min at 1200 rpm, 4°C , to pellet down red blood cells. Carefully transfer plasma to a clean tube, and store at -70°C until use.

Blood can also be collected from animals during euthanasia under anesthesia, before perfusion. To do so, puncture the right atrium of the heart and collect the blood into a BD Vacutainer tube. Isolate plasma as described in step 1f, and freeze at -70°C until use.

Prepare infection and dilution plates

2. Prepare the Infection Plate by seeding 15,000 293 cells per well in 100 μ l 293 cell complete medium in a sterile 96-well tissue culture plate. Run samples in duplicate. Be sure to seed enough cells so that wells are reserved for Plate and Assay controls. Incubate overnight. Mark the control wells as follows:
 - a. *Plate controls:* Positive (Ad-hCMV-lacZ-infected) and negative (no vector, no plasma) control wells, on each plate.

The Ad-hCMV-lacZ will be added to both the experimental wells and the Plate control wells at step 7 (see below). Note that control plasma samples are run for each plate.
 - b. *Assay Positive control:* a well characterized positive plasma, in a 1:1 dilution.

- c. *Assay Negative control*: a well characterized negative plasma, in a 1:1 dilution.

Incubate overnight at 37°C.

3. The next day, inactivate the complement by heating the plasma at 56°C for 30 min. Perform this step on the experimental plasma prepared in step 1 as well as on the Assay Positive and Negative control plasmas.
4. In a sterile 96-well tissue culture plate (Dilution Plate), perform 2-fold serial dilutions of the plasma in 293 cell complete medium from 1:1 to 1:4096 (12 dilutions). To perform the serial dilutions, use 120 µl plasma: 120 µl medium (yielding volume enough for two wells). Perform the same serial dilutions on the Assay Positive and Negative control plasma.
5. Add 3×10^5 IU of Ad-hCMV-LacZ (diluted in 10 µl of MEM) to each well of the Dilution Plate.
6. Incubate the Dilution Plate for 90 min at 37°C.
7. Remove medium from the cells in each well of the Infection Plate and transfer 50 µl of vector/plasma mixture from the Dilution Plate into the corresponding duplicate wells of the Infection Plate.
8. Incubate 1 hr at 37°C.
9. Add another 50 µl of 293 cell complete medium to each well.
10. Incubate for 20 hr at 37°C.

Perform fixation and staining

11. Aspirate medium and briefly rinse each well with PBS. Aspirate.
12. Add 100 µl of 4% paraformaldehyde. Incubate 5 min at room temperature.
13. Aspirate paraformaldehyde and briefly rinse each well with PBS. Aspirate PBS.
14. Stain with Xgal technique as described in *UNIT 4.23*.
15. Calculate neutralizing antibody titer for each animal as the reciprocal of the highest dilution of plasma at which 50% of Ad-hCMV-LacZ transduction is inhibited.

ASSESSMENT OF THE FREQUENCY OF ANTI-ADENOVIRUS-SPECIFIC IFN- γ -SECRETING LYMPHOCYTES

It is often necessary to evaluate whether individual immunized animals successfully mounted an anti-adenoviral immune response. Below is a protocol to assess the frequency of anti-adenovirus-specific IFN- γ -secreting lymphocytes from the spleens of immunized animals (Barcia et al., 2007; King et al., 2008). This assay will give the most robust signal when performed at 7 days post-immunization; however the assay may be performed up to 2 weeks post-immunization (Xiong et al., 2008). This technique may also be used to assess the frequency of IFN- γ -secreting lymphocytes specific for other antigens such as transgenes, tumor antigens, etc.

Note that alternative methods exist for assessing the frequency of anti-adenovirus-specific IFN- γ -secreting lymphocytes from the spleens of immunized animals by using peptide-MHC tetramers to analyze in vivo precursor frequency and evaluate intracellular cytokine production of antigen-specific T cells.

Materials

35% (v/v) ethanol, filtered through 0.2- μ m filter

Rat IFN- γ ELISpot Development Module (R&D Systems, cat. no. SEL585) including:

Capture Antibody Concentrate

Detection Antibody Concentrate

Dulbecco's PBS, pH 7.4, without Ca or Mg (Cellgro, cat. no. 21-031 CV)

Complete RPMI (see recipe), sterile

Adult immunized rats (Basic Protocol 7)

10 $\times$ ACK solution (see recipe)

Fetal bovine serum (FBS), heat inactivated 30 min at 56°C

Dimethylsulfoxide (DMSO)

Liquid N₂

70% ethanol

Stimulants (will vary by assay; those below are in general use):

Viral antigen: heat-inactivated adenovirus (see recipe)

Cell lysate (see recipe)

Protein and peptide antigens: concanavalin A (ConA; see recipe)

Recombinant rat IFN- γ (R&D Systems, cat. no. 585-IF) as positive control (use at 200 ng IFN- γ per well in 200 μ l complete medium)

Wash buffer: 0.05% Tween 20 in PBS, pH 7.2 to 7.4, filtered through 0.2- μ m filter

Reagent diluent: 1% (w/v) BSA in PBS, pH 7.2 to 7.4, filtered through 0.2- μ m filter

ELISpot Blue Color Module (R&D Systems, cat. no. SEL002) including:

Streptavidin-AP Concentrate

BCIP/NBT Chromogen

96-well filtration plate (Multiscreen HTS, IP; Millipore, cat. no. MSIPS4510)

Surgical instruments, sterile

15- and 50-ml conical polypropylene centrifuge tubes (e.g., Falcon), sterile

12-well tissue culture plate

10-ml syringe

Refrigerated centrifuge

Cryovials

Wash bottle

Dissecting microscope

Automated imager (Axioskop 2 mot plus; Zeiss) and KS ELISPOT software (v. 4.7; Zeiss), or scanner with 6000 to 1200 dpi resolution

Eli-Puncher Kit (ZellNet Consulting; <http://zellnet.com/>)

ImageJ software (<http://rsbweb.nih.gov/ij/applet/>)

Additional reagents and equipment for basic cell culture techniques including counting viable cells by trypan blue exclusion (*APPENDIX 3B*)

Day 0

Perform procedures under sterile conditions in a tissue culture hood.

1. Pipet 15 μ l of 35% ethanol into the center of each well of a 96-well filtration plate and immediately add 200 μ l of PBS.
2. Flick out into large beaker.
3. Wash wells two more times, each time with 200 μ l PBS as described in steps 1 and 2.
4. While the third wash of PBS is in the wells, dilute Capture Antibody Concentrate from the frozen aliquot supplied in the IFN- γ ELISpot Development Module 1:60 in PBS. Prepare sufficient reagent to add 60 μ l per well.
5. Flick out PBS and add 60 μ l of diluted capture antibody to each well. Attempt to avoid bubbles.
6. Seal plate edges with Parafilm and store at 4°C until use.

Optimally, plate should be left at 4°C overnight; at minimum it should be left at 4°C for 3 hr.

Day 1

Perform procedures under sterile conditions in a tissue culture hood.

7. Discard capture antibody and wash wells three times, each time with 200 μ l sterile PBS as described above. Add 200 μ l complete RPMI to each well and incubate for 2 hr at room temperature to block the membrane.

It is often convenient to prepare splenocyte sample, during the blocking process.

Prepare splenocytes

8. Harvest spleen after establishing deep anesthesia (Basic Protocol 1, step 23), but before perfusion. Be sure to harvest spleen without contaminating hairs, and transfer it to 50-ml tube containing 5 ml of sterile complete RPMI.
9. Store spleen on ice while processing remaining animals.
10. To prepare 1 $\times$ ACK solution, dilute 1 ml 10 $\times$ ACK solution with 9 ml autoclaved water and keep on ice.
11. For each spleen, add 3 ml of complete RPMI to a well of a 12-well plate.
12. Pour off RPMI from the spleen in the 50-ml tube.
13. Add ~5 ml of complete RPMI to wash the spleen.
14. Pour off RPMI and transfer the spleen to a well containing complete RPMI.
15. Crush spleen with the flat side of the plunger from a sterile 10-ml syringe.

Tissue should be crushed until no large pieces remain and the suspension is opaque with released cells.

16. Discard the fragments of spleen.
17. Use a 1-ml pipet to transfer the supernatant into 15-ml tube.
18. Centrifuge 5 min at $200 \times g$, 4°C . Pour off supernatant.
The pellet should appear red in color.
19. Resuspend pellet with 3 ml of $1\times$ ACK solution (see step 10) and incubate on ice for 3 min.
20. Add 7 ml of complete RPMI and centrifuge 5 min at $200 \times g$, 4°C .
The pellet should now appear creamy cinnamon-brown in color. If still red, redo the ACK procedure.
21. Pour off supernatant. Resuspend the pellet with 5 ml of complete RPMI.
22. Centrifuge 5 min at $200 \times g$, 4°C . Pour off supernatant.
23. Resuspend the pellet with 1 ml of complete RPMI if the splenocytes are to be used immediately, or in neat FBS (heat-inactivated 30 min at 56°C) if they are to be frozen between harvesting and further processing.

To freeze splenocytes:

- a. Pellet from step 22 should be resuspended at 20 million cells/ml in FBS.
- b. Transfer 0.5 ml of splenocyte suspension to a cryovial.
- c. Add 0.5 ml of 80% FBS, 20% DMSO to each vial, close firmly, mix briefly and immediately transfer to middle (not outside rows) of freezer box at room temperature. Immediately transfer the box to a -80°C freezer.
- d. After 24 hr, transfer the vials to liquid nitrogen.

To thaw splenocytes:

- a. Remove the vials from liquid nitrogen and keep them buried in dry ice.
 - b. Thaw one vial rapidly in a 37°C water bath, and immediately transfer to tissue culture hood. Wipe vial with 70% ethanol and add the contents to 12 ml of complete RPMI in a 15-ml centrifuge tube. It is critical to remove the vial from the water bath as soon as the splenocyte suspension has completely thawed.
 - c. Repeat with a second vial.
 - d. Spin both tubes 5 min at $200 \times g$, room temperature, and resuspend each pellet in 1 ml of complete RPMI.
 - e. Transfer cell suspensions to wells of a 24-well plate and place in a 37°C , 5% CO_2 incubator.
 - f. Repeat with consecutive pairs of vials until all thawed and resuspended. Incubate overnight in a 24-well plate in the 37°C , 5% CO_2 incubator. Proceed to step 24.
24. Count live splenocytes by trypan blue exclusion assay (APPENDIX 3B).
Dead cells will appear "blue" and should not be counted.

25. Discard blocking solution from the pre-coated 96-well plate.
26. Wash wells three times with sterile PBS. After the final wash, remove any remaining liquid by inverting the plate and blotting it against a clean paper towel.
27. Adjust cell density to 1×10^6 cells per 170 μ l complete RPMI medium. Add 170 μ l of the cell suspension to each well.
28. Add stimulants according to design layout for experiment.

Details of stimulants will vary by assay, but those listed below are in general use.

- a. *Viral antigens:* 10^9 IU equivalent of heat-inactivated adenoviral vector per ml.
- b. *Cell lysates:* 100 μ g protein equivalent per ml, e.g., 20 μ l per well of lysate at 1 mg protein/ml.
- c. *Peptide and protein antigens.*
- d. *Concanavalin A (Con A).*

Each data point (i.e., cells from given source treated with given stimulant) should be assayed in triplicate, i.e., three identical wells should be planned. Inevitably, some wells yield anomalous results, making that well uninterpretable unless there are two other wells to compare it with. Viability of each cell source, (e.g., each spleen) should be tested with two wells stimulated with Con A. For each stimulant, test three wells with the stimulant and three wells without the stimulant. In addition, each plate should have three wells with medium without cells, and at least one well with recombinant rat IFN- γ .

29. Add complete RPMI to make the final volume 200 μ l in each well.
30. Incubate at 37°C in a humidified 5% CO₂ incubator for 24 to 48 hr.

Day 3

From this point on, procedures can be performed under nonsterile conditions.

31. Discard contents of wells and wash four times with wash buffer. After the final wash, remove any remaining liquid by inverting the plate and blotting it against a clean paper towel.
32. Dilute Detection Antibody Concentrate (supplied in the IFN- γ ELISpot Development Module) 1:60 in reagent diluent.
33. Add 60 μ l of diluted Detection Antibody to each well.
34. Seal plate edges with Parafilm and incubate overnight at 4°C.

Day 4

35. Dilute Streptavidin-AP Concentrate (supplied in the ELISpot Blue Color Module) 1:60 in reagent diluent.
36. Discard Detection Antibody from wells and wash four times with wash buffer. After the final wash, remove any remaining liquid by inverting the plate and blotting it against a clean paper towel.
37. Add 60 μ l of diluted Streptavidin-AP to each well.
38. Incubate for 2 hr at room temperature.

39. Aliquot enough BCIP/NBT Chromogen (supplied in the ELISpot Blue Color Module) for 100 μ l/well with a pipet, and put it in a 15-ml tube.
40. Use wash bottle to wash plate with wash buffer three times, then wash with sterile water. Remove any remaining liquid by inverting the plate and blotting it against a clean paper towel.
41. Add 100 μ l of BCIP/NBT to each well.
42. Incubate in the dark for ~30 min at room temperature. Check visually every 5 min for spots in positive controls.

It is critical not to over-incubate.

43. Discard chromogen.
44. Rinse plate with running distilled water.
45. Invert the plate and tap to remove excess water against a paper towel.
46. Remove the flexible plastic underdrain from the bottom of microplate and wipe the bottom of plate thoroughly with paper towels. Dry thoroughly.

Be careful not to damage the membranes.

47. Count spots using a dissecting microscope (in the case of lower number than 100 spots) or an automated ELISPOT Reader (SpotScan, spotreader). Alternatively, punch out the ELISpot membranes using an Eli-Puncher kit, scan them with a high-resolution scanner, and count spots on the image manually or using ImageJ (NIH Image software, freeware).

PERFUSION, FIXATION, AND SECTIONING OF BRAIN TISSUE

UNIT 1.1 describes a method for perfusion fixation of animals with saline and fixative. In the protocol below, the animal is instead perfused with oxygenated Tyrode's solution containing heparin prior to perfusing the fixative. Use of oxygenated Tyrode's solution maintains the supply of oxygen to the brain and tissues and avoids clotting during blood clearance, thus preventing cellular death prior to perfusion of the fixative. Additionally, oxygen improves the chemical fixation reaction. If a peristaltic pump is not available, it can be replaced with the apparatus shown in Figure 4.24.10, which uses gravity to facilitate the flow of Tyrode's and fixative. Also, the descending aorta is clamped just below the liver prior to starting the perfusion of fixative, to improve the flow of fixative to the brain. Once the perfused-fixed brains have been removed from the animal as described in *UNIT 1.1*, a period of post-fixing is required before the brains can be sectioned using a Vibratome, or stored in PBS/0.01% azide at 4°C. Post-fixing for 5 hr is optimal for subsequent immunohistochemical analysis (see Basic Protocol 2 and Troubleshooting). Under-fixing the brains can make subsequent sectioning very difficult, whereas over-fixing can lead to the masking or destruction of epitopes to be detected by immunohistochemical analysis, or of nucleic acids for molecular analysis. Since paraformaldehyde leaches out from the fixed brains into the PBS-azide, it is advisable to change the storage solution at regular intervals if the brains are to be kept at 4°C for long periods before processing. The duration of post-fixing required will depend upon the quality of initial fixation. If the brains are to be frozen for cryostat sectioning, then infiltration with sucrose is required, as described in *UNIT 1.1*. Sucrose infiltration is not required for Vibratome sectioning. This protocol describes a procedure for collecting and storing Vibratome-cut brain sections for subsequent immunohistochemical analysis. Cryostat sectioning is described in *UNIT 1.1*.

Materials

Cylinder of pre-mixed 95% oxygen, 5% carbon dioxide with regulator and tubing

Tyrode's solution with heparin (see recipe)

Fixative: 4% (w/v) paraformaldehyde in PBS

Experimental rat (e.g., following step 22 of Basic Protocol 1)

Ketamine/xylazine anesthetics (*APPENDIX 4B*)

Optional (fixative if electron microscopy is downstream application): 4% (w/v) paraformaldehyde/0.1% (w/v) glutaraldehyde in 0.1 M cacodylate buffer

Dulbecco's PBS, pH 7.4, without Ca or Mg (Cellgro, cat. no. 21-031 CV)

Sodium azide (optional)

Superglue

Peristaltic perfusion pump (e.g., Masterflex, Cole-Parmer) or gravity-fed device (Fig. 4.24.10)

Tubing

Two-way valve

Dissecting board (e.g., slab of styrofoam) in tray

Large and small scissors

Forceps

Aluminum-hub blunt perfusion needle (e.g., Monoject 81-202314, Cardinal Health, <http://www.cardinal.com/>)

Dissecting pins

Hemostat

Bone rongeurs

Small spatula

Sodium azide (optional)

6-well tissue culture plate

Cutting platform

Vibratome (e.g., model VT1000S, Leica)

Soft-bristled paintbrush

Perfuse and fix rat

1. Set up the perfusion equipment in a fume hood (if using aldehyde fixative) so that the oxygen/carbon dioxide cylinder, the bottles of Tyrode's solution and fixative, the perfusion pump with its tubing, the tray in which the rat is to be contained during perfusion, and the scissors and forceps are all within reach.

It is also convenient to prepare labeled 50-ml tubes containing 10 to 20 ml of fixative into which the perfused brains can be collected for transfer to the refrigerator.

2. Connect the tubing to the outlet of the perfusion pump and open the valve on the regulator so that gas escapes from the tube slowly. Immerse the end of the tube in the Tyrode's solution and adjust flow of O₂/CO₂ gas until a gentle stream of bubbles

is obtained. Allow the solution to equilibrate with the gas for 10 to 20 min, then shut off the gas and remove the tube.

3. Put one of the input tubes of the perfusion pump into the Tyrode's solution bottle and the other into the fixative and adjust the rate of the pump to deliver a slow, steady flow of Tyrode's via the output tubing to the blunt-ended perfusion needle.

Solution should flow from the needle at about 1 drop/sec. Ensure that there are no bubbles anywhere in the tubing, and that the tubing between the fixative bottle and the two-way valve is also full of solution. Once adjustments are satisfactory, turn off the pump.

4. To irreversibly overdose with anesthesia, manually restrain the rat and administer ketamine (80 mg/kg) and xylazine (8 mg/kg) via intraperitoneal injection. Ensure that the animal is fully anesthetized by checking the lack of responses to footpad and tail pinching.
5. Lay the rat with its dorsal side down on the dissection board in the tray and fix each of the footpads to the board with a dissecting pin.
6. Make an incision into the abdomen, cut the diaphragm to expose the thoracic cavity, and then expose the heart by completely cutting the ribs and lifting the anterior part of the rib cage.
7. Holding the heart gently with forceps, turn on the perfusion pump and carefully insert the perfusion needle into the left ventricle. As soon as the needle is inserted, snip the right auricle with the small scissors; blood should flow freely from the cut.
8. Continue perfusing the animal with oxygenated Tyrode's solution until the organs are exsanguinated and the fluid flowing from the cut auricle is no longer bloody, and then clamp the aorta just below the liver with the hemostat and switch the two-way valve to the fixative.
9. *If fixed tissue is required for the downstream application (i.e., immunohistochemistry):* Perfuse with fixative (i.e., 4% paraformaldehyde for light microscopy or 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M cacodylate buffer for electron microscopy). Continue perfusion until muscular contractions of the forelimbs appear and subside, then turn off the pump, remove the needle, and proceed to removing the brain.
10. Cut off the head, cut the skin off the skull by cutting forward sagittally with scissors, and then remove the skull piece by piece with the rongeurs, starting at the foramen magnum and working forward through the interparietal, the parietal, and the frontal bones. When the brain is largely exposed, cut through the olfactory bulbs, ease the brain out with a small spatula, and drop it into a 50-ml tube containing fixative.
11. Store tissue at 4°C until fixed.
This may take anywhere from a few hours to a few days.
12. Once fixed, replace the fixative with PBS. If the brains are to be stored for more than a few days, add sodium azide to the PBS at 1 g/liter.

Section tissue

13. Add ~4 ml of PBS to each well of a labeled 6-well tissue culture dish.

This will be the storage container for the brain sections.

14. Take a fixed brain from step 11 and mount the part containing the injection site on a cutting platform using Superglue. For example, if the injection site is in the forebrain, cut the brain coronally through the back of the occipital cortex to remove the cerebellum and the hindbrain, and then glue the cut surface of the forebrain to the platform.
15. Put the platform and brain in the Vibratome and section the tissue at 20- to 70- μ m intervals according to the manufacturer's instructions. Use a soft-bristled paintbrush to transfer the brain sections from the cutting chamber to wells of the 6-well plate.

Always immerse the brains in PBS (not water) during sectioning and use a fresh blade to section each brain.

16. Place serial sections in consecutive wells of the 6-well plate (i.e., put the first section in well 1, the second in well 2, the seventh in well 1, the eighth in well 2, etc.), so that each well contains a similar representation of sections throughout the injection site/area of interest.

Six wells will facilitate analysis of the brain using six different immunohistochemical markers, allowing a reconstruction of patterns of transgene expression and inflammatory and immune cell infiltrates throughout the brain. If required, the brain can be sectioned more thinly and collected into a greater number of wells using a 12-well tissue culture plate.

17. Store the sections at 4°C.

If the sections are to be stored for more than 1 week, it is essential to add sodium azide (to a final concentration of 0.02%) to each of the wells to prevent microbial growth. Sections stored at 4°C in PBS with azide can be successfully immunolabeled months or even years later, but sodium azide degrades spontaneously and needs to be replenished at regular intervals (e.g., every couple of months). It is also highly toxic and can cause death if ingested or injected.

PREPARATION OF GELATIN OR APES-COATED SLIDES

Gelatin-coated slides (see *UNIT 1.1*) can be used for most of the immunohistochemical protocols described in the current unit. For applications where slide-mounted sections are stained (e.g., Luxol fast blue staining), APES-coated slides are more appropriate since gelatin is water-soluble and the brain sections can become detached when the slides are incubated in solution for long periods of time.

Materials

Gelatin (Type B G7–500; Fisher Scientific, cat. no. CAS9000-70-8)
 95% ethanol
 Acid alcohol: 1% (w/v) hydrochloric acid in 70% methylated spirits
 3% (v/v) 3-aminopropyl-trioxysilane (APES; Sigma) in acetone
 Acetone
 Glass microscope slides
 Slide rack
 37° or 50°C drying oven
 Glass microscope slides

37°C or 50°C drying oven

To prepare gelatin-coated slides

- 1a. Prepare 1 % (w/v) gelatin solution in distilled water by heating until gelatin dissolves.
- 2a. Submerge microscope slides in gelatin solution for 5 to 10 min.
- 3a. Dry slides overnight at 37°C in a drying oven.

The coated slides can be stored for several months.

To prepare APES-coated slides

- 1b. Place glass microscope slides in a slide rack and degrease them by immersion in 95% ethanol for at least 2 min.
- 2b. Rinse the slides three times in tap water, then once in deionized distilled water, and once in acid alcohol.
- 3b. Leave the slides to air dry.
- 4b. Immerse the rack of slides in freshly prepared 3% APES in acetone for 2 min.
- 5b. Rinse the slides by immersion in acetone for 2 min.
- 6b. Wash the slides in distilled water and dry in the oven at 37°C or 50°C overnight.

The coated slides can be stored for several months at room temperature.

TRANSPORT OF RECOMBINANT ADENOVIRAL VECTORS ON DRY ICE

Clinical trials and collaborations between research groups using recombinant adenoviral vectors require the use of dry-ice transport between laboratories in order to keep the vector stocks frozen. Transport on dry ice is essential when the vector is to be used immediately (without retitration), as the titer used for the experiments will be the one determined by the laboratory that manufactured and shipped the vector. It is recommended that the vector be retitrated after shipment (*UNIT 4.23*), as titration protocols (and therefore results) vary from laboratory to laboratory. This is especially important when comparing the effects of two or more viruses against each other.

It has recently been observed that significant loss of viral titer occurs during dry ice transport (Nyberg-Hoffman and Aguilar-Cordova, 1999). Thawing of the dry ice over 24 hr results in the increased production of CO₂ gas in the transport container, which can penetrate the tubes containing the viruses, thereby decreasing the pH of the vector solution. The viral capsid is unstable at acid pH, and virus aliquots in small microcentrifuge tubes placed directly into dry ice for 48 hr can lose up to 6 log units of infectious titer. A common method of vector transport currently utilized is to place the microcentrifuge tubes containing the vector aliquots into 50-ml polypropylene centrifuge tubes before placing in dry ice. However, this precaution is still not sufficient for maintaining titer; a 2-log decrease in titer was observed when vector was stored in this manner in dry ice for 48 hr. A combination of sealing the aliquot in Parafilm, placing this into a 50-ml centrifuge tube, then placing the tube into two polythene bags, maintains the adenoviral vector titer after storage in dry ice for 48 hr. Alternative methods are described in the original manuscript by Nyberg-Hoffman and Aguilar-Cordova (1999).

Materials

Adenoviral victor aliquotted into labeled 0.5-ml Treff Lab tubes or other suitable tubes

Dry ice

50-ml polypropylene centrifuge tubes (Greiner)

Minigrip resealable polythene bags (Fisher)

Parafilm, cut into squares 3 cm × 3 cm

Polystyrene box

Cardboard box (into which the polystyrene box snugly fits)

Brown tape

NOTE: Each step performed below is done individually at short intervals in the -80°C freezer to keep the aliquots frozen. Freeze/thawing of the aliquot may affect the viral titer.

Prepare aliquots for dry ice shipment

1. Label the 50-ml polypropylene centrifuge tubes and Minigrip resealable polythene bags with the name and titer of the vector and the number of aliquots to be shipped.
2. Place the tubes and bags in the -80°C freezer to cool.
3. Quickly wrap each vector aliquot completely with a 3 × 3-cm Parafilm square.
4. Place the aliquots in 50-ml centrifuge tubes and firmly screw the caps on.
5. Place the centrifuge tube into two nested polythene bags. Remove excess air and seal completely.

Keep the packaged viruses at -80°C until the dry ice container is ready.

It is recommended that two nested polythene bags be used, as a single bag could crack or the seal could be broken when exposed to dry ice. Nyberg-Hoffman and Aguilar-Cordova (1999) report the use of Kapak bags to maintain vector viability.

Prepare box for shipment

6. Obtain enough dry ice pellets to completely fill the polystyrene box used for the shipment.

Polystyrene boxes holding 3 kg of dry ice are sufficient for shipments lasting 48 hr. For longer shipments (i.e., U.S.A. to Europe, or between countries with strict customs regulations), use a larger box holding 5 to 7 kg of dry ice so that the dry ice will last at least 72 hr.

7. Fill the polystyrene box with 3 cm of dry ice. Take this box and the rest of the dry ice to the -80°C freezer.
8. Place the vector aliquots (prepared as above) on top of the 3-cm layer of dry ice.
9. Immediately pour the rest of the dry ice on top, filling the box completely. Shake the box to settle the pellets and top up the box if necessary.

It is essential to completely fill the box. The dry ice will sublime more quickly if there is a gap.

10. Place the lid on the polystyrene box and seal with tape.

Place the box in the cardboard box and seal ready for shipment.

REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

293 cell complete medium

MEM (Cellgro, cat. no. 15-010-cv)
 10% fetal bovine serum (Omega Scientific, cat. no. FB-O1;
<http://www.omegascientific.com/>)
 50 µg/ml Pen-Strep (Cellgro, cat. no. 30-001-CI)
 1× nonessential amino acids (Cellgro, cat. no. 25-025-CI)
 292 µg/ml L-glutamine (Cellgro, cat. no. 25-005-CI)

ACK solution

0.1 mM disodium EDTA (Sigma, cat. no. E-5134)
 0.01 M KHCO₃
 0.15 M NH₄Cl (Sigma, cat. no. A-0171)
 Adjust to pH 7.4 using 1 N HCl
 Filter sterilize through a 0.2-µm filter
 Store up to 1 month at room temperature

Adenovirus, heat-inactivated

Dilute vector to a concentration of 2×10^{10} IU/ml in complete RPMI (see recipe) in a 1.5-ml microcentrifuge tube. The required final concentration in the wells is 1×10^9 IU/ml. Incubate in a water bath at 70°C for 20 min. Place on ice.

Determination of adenoviral titer is described in UNIT 4.23.

The heat-inactivated adenovirus is used as a stimulant for assessing the frequency of adenovirus-specific IFN-γ-secreting lymphocytes (see Basic Protocol 9).

Cell lysate, 100 µg/ml

Harvest 5×10^6 cells of interest (e.g., tumor cell lines or cells transfected with antigen of interest, i.e., the antigen with which the animals donating the lymphocytes have been immunized) inside a tissue culture hood by trypsinization of the monolayer (**APPENDIX 3B**) and resuspend in Dulbecco's PBS, without calcium or magnesium (Cellgro, cat. no. 21-031 CV) in a 15-ml tube. Centrifuge 2 min at $234 \times g$. Resuspend cell pellet in cryovial with 1 ml PBS. Freeze resuspended cells in liquid nitrogen and thaw in water bath (37°C); repeat this freeze-thaw procedure three times. Perform BCA protein assay (kit available from various manufacturers) to estimate protein concentration of cell lysates as described by manufacturer.

The cell lysate containing the antigen with which the animals donating the lymphocytes have been immunized is used as a stimulant for assessing the frequency of adenovirus-specific IFN-γ-secreting lymphocytes (see Basic Protocol 9). It is an alternative method for preparation of stimulants to assess the frequency of IFN-γ-secreting lymphocytes specific for tumor associated or other neo-antigens.

Concanavalin A

Reconstitute lyophilized powder (Sigma, cat. no. C 2010) in 10 mM HEPES-buffered saline, pH 8.5, containing 0.1 mM Ca^{2+} , to prepare a 20 mg/ml stock solution. Store in aliquots at -20°C . Aliquots are thawed and diluted at 1 μl stock solution to 600 μl complete RPMI (see recipe) immediately before use, and added at 10 μl per well if the wells are 200 μl total. The final concentration of concanavalin A is 1.7 $\mu\text{g}/\text{ml}$.

Concanavalin A is used as a stimulant for assessing the frequency of adenovirus-specific IFN- γ -secreting lymphocytes (see Basic Protocol 9).

DAB staining solution

DAB solution 1:

7.25 g nickel ammonium sulfate

600 mg glucose

120 mg ammonium chloride

150 ml 0.2 M sodium acetate, pH 6.0

Store up to several months in the dark at 4°C

DAB solution 2:

15 mg diaminobenzidine (DAB; Sigma)

1 mg glucose oxidase (Sigma)

15 ml distilled H_2O

Prepare fresh, directly before use

Mix equal volumes of DAB solutions 1 and 2 immediately before use.

Digestion buffer

10 mM Tris-Cl, pH 8.0 (APPENDIX 2A)

10 mM NaCl

25 mM EDTA

1% (w/v) SDS

4 mg/ml proteinase K

Prepare fresh before use

Hanker-Yates solution A

25 ml 0.1 M sodium cacodylate, pH 5.1, containing:

150 mg cobalt chloride

100 mg nickel ammonium sulfate

12.5 mg *p*-phenylenediamine

25 mg catechol

Stored at room temperature up to 1 month (or longer)

CAUTION: *Solution is toxic!*

Hanker-Yates solution B

25 ml 0.1 M sodium cacodylate, pH 5.1, containing

100 mg nickel ammonium sulfate

12.5 mg *p*-phenylenediamine

25 mg catechol

50 µl 30% hydrogen peroxide

Prepare immediately before use

CAUTION: *Solution is toxic!*

Luxol fast blue (LFB) solution

Prepare in 95% methylated spirits:

0.1% (w/v) Luxol fast blue (Sigma)

10% acetic acid

Store up to 6 months at room temperature and filter before each use

RPMI medium, complete

1× RPMI 1640 (Cellgro, cat. no. 10-040-CV)

10% fetal bovine serum (FBS)

50 U/ml penicillin

50 µg/ml streptomycin

50 µM 2-mercaptoethanol (2-ME)

Toluidine blue, 1%

1 g toluidine blue

1 g disodium tetraborate

100 ml distilled water

Dissolve the disodium tetraborate in the water. Add the toluidine blue and dissolve by sonication. Filter before use. Store up to 6 months at room temperature.

Tyrode's solution with heparin, oxygenated

For 1 liter:

8 g NaCl (132 mM)

1 ml 26.5% CaCl₂·2H₂O (1.8 mM final)

1 ml 5% (w/v) NaH₂PO₄·2H₂O (0.32 mM final)

1 g glucose (5.56 mM final)

1 g NaHCO₃ (11.6 mM final)

0.2 g KCl (2.68 mM final)

Prepare fresh immediately before use

Just prior to use, add 100 μ l of 1000 U/ml heparin solution to each liter of Tyrode's and gas with 95% O₂/5% CO₂ for 30 min.

COMMENTARY

Background Information

Adenovirus-mediated gene transfer to the periphery—First-generation adenoviruses have been used to successfully deliver genes into a wide variety of non-CNS tissues and organs in animal models of human disease and in several human phase I clinical trials (Hedman et al., 2009; Li et al., 2009; Opyrchal et al., 2009; Xing et al., 2009). These studies have shown that although gene transfer is efficient, expression of the transgene is consistently transient (rarely sustained beyond 3 weeks; Chen et al., 2000; Jeong et al., 2008; *UNIT 4.23*) and administration of vector is associated with the development of local inflammation. The vector-induced immune response outside of the brain is biphasic and consists of an initial innate response which rapidly eliminates many transduced cells, followed by adaptive cellular and humoral mechanisms. The precise delineation of the components of the anti-vector immune response has been confounded by differences in host backgrounds, routes of vector administration, and, particularly, by strong immune responses against certain transgenes. It is widely accepted, however, that a cytotoxic T lymphocyte response directed against transduced cells presenting vector antigens in conjunction with surface MHC class I molecules is a principal cause of the decline in transgene expression observed after adenovirus-mediated gene transfer to peripheral tissues (Yang et al., 1994, 1995). The use of high-capacity vectors can reduce the acute inflammation and provide extended transgene expression (Morral et al., 1998; Kreppel et al., 2002; Brunetti-Pierri et al., 2009). High-capacity adenoviral vectors are thought to be less inflammatory because of their total absence of any original genomic adenoviral sequences. In addition, once the high-capacity adenoviral vectors have un-coated and delivered the vector genome to the cell's nucleus, the vector genome essentially becomes invisible to the host's immune system, as the vector genome will not express any viral protein. Further, high-capacity adenoviral vectors can be engineered to encode molecules that inhibit inflammatory and immune responses, and their viral capsids can be made less evident to the innate immune system by chemical modification of the adenovirus capsids with synthetic polymers.

Adenovirus-mediated gene transfer to the brain—In contrast to the transient transgene expression observed in the periphery, adenovirus vectors delivered to the CNS of naive animals can mediate prolonged transgene expression. The first demonstrations of adenoviral transduction of the brain parenchyma showed substantial vector-mediated transgene expression for at least 2 months (Akli et al., 1993; Davidson et al., 1993; Le Gal La Salle et al., 1993). More recent studies from our lab and others have now shown that transgene expression from Ad vectors carefully delivered to the brain parenchyma can last between 6 and 18 months (Byrnes et al., 1995; Kajiwara et al., 1997; Ideguchi et al., 2000; Thomas et al., 2000; Zou et al., 2000; Zermansky et al., 2001; Amalfitano and Parks, 2002; Glover et al., 2003). Thus, with present highly purified and LPS-free vector preparations, adenoviral-mediated gene delivery is long term. This difference can be ascribed to the distinct immunological status of the CNS as compared with other organs. The brain parenchyma is commonly described as having an immune-privileged status; allografts can survive for long periods of time without immunosuppression, and lipopolysaccharide or pro-inflammatory cytokines injected into the parenchyma induce only minimal inflammation compared to other organs (Montero-Menei et al., 1994). The CNS is not, however, devoid of immune reactions; rather it utilizes a complex interplay between different mechanisms to raise the threshold for and to regulate the progression of immune responses. Communication between the CNS and the immune system is retarded by the lack of a conventional lymphatic drainage system or resident professional antigen-presenting dendritic cells within the brain parenchyma, and by

the physical obstruction to leukocyte surveillance presented by the blood-brain barrier (which in rodents is formed by a tight endothelial barrier that strictly regulates the entry of substances and cells into the brain). Activated T cells and monocytes are, however, able to cross the intact blood-brain barrier, and there is evidence to suggest that, despite the lack of conventional lymphatics, proteins can drain from the brain into deep cervical lymph nodes (Cserr and Knopf, 1997; Weller et al., 2009). Thus, these mechanisms alone do not constitute the immune privilege. Recent evidence suggests that the CNS possesses its own innate immune system composed of microglia and astrocytes, which can actively suppress or shape the immune response by direct glia:immune cell interactions or by producing specific cytokine and chemokine profiles (Matyszak, 1998; Carson and Sutcliffe, 1999; Lowenstein, 2002; Mi et al., 2004; Bechmann et al., 2007; Galea et al., 2007; Popovich and Longbrake, 2008).

Immunogenicity of adenovirus vectors in the brain—Adenovirus vectors injected into the parenchyma stimulate an inflammatory response; local microglia and astrocytes are activated strongly, and inflammatory and immune cells infiltrate across the blood-brain barrier to the site of infection (Byrnes et al., 1995, 1996a; Dewey et al., 1999; Zirger et al., 2006). As previously mentioned, lysis of adenovirus-infected cells by CTLs is a principal cause of the transient transgene expression seen after vector delivery to the periphery. Depletion of CD4⁺ or CD8⁺ T cells before adenovirus administration to the brain reduces the level of inflammation seen from 6 days after vector injection, but such depletion has no effect on the level or longevity of transgene expression (Byrnes et al., 1996b). Thus, even if CD4⁺ or CD8⁺ cells mediate, in part, the inflammation, persistent adenovirus-mediated transgene expression in the brain could be a result of an ineffective T cell response which fails to clear transduced cells (Wood et al., 1996).

Although low doses of adenovirus vectors injected into the brain parenchyma do not seem able to elicit an effective systemic T cell response, a strong T cell response can be stimulated by subsequent peripheral exposure to adenovirus. In such cases, transgene expression in the brain declines rapidly and is accompanied by severe inflammation, characterized by T cell and dendritic cell infiltration, intense microglial activation, and demyelination (Byrnes et al., 1996a; Thomas et al., 2000). If animals are peripherally sensitized to adenovirus prior to injection of vector into the brain, transgene expression is transient and is again accompanied by severe inflammation (Ohmoto et al., 1999; Barcia et al., 2006a,b, 2007, 2008).

In this unit, vector toxicity is based on titers expressed as infectious units. Vector toxicity will also depend on the total number of physical viral particles administered. This is measured by optical absorbance (see *UNIT 4.23*). In the authors' laboratory, vector stocks routinely contain 20 physical viral particles per infectious unit. This ratio must be determined for each individual viral stock prepared, in order to accurately gauge vector quality and related toxicity.

In contrast to the brain parenchyma, efficient immune priming occurs after vector infection of the CSF. Thus, the ventricles and the brain parenchyma represent immunologically distinct compartments, and the immune responses to intracerebral vector infection depend on the anatomical site to which the vector is delivered (Stevenson et al., 1997; Larocque et al., submitted). Adenoviral vectors injected into the lateral ventricle of adult rats elicit a rapid febrile response, which is not seen after vector delivery to the striatum. The fever response to adenoviruses administered to the CSF occurs concurrently with an increase in the concentrations of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6, although it has been shown that IL-1 is the key mediator of adenovirus-induced fever (Cartmell et al., 1999). Thus, it is important to carefully monitor whether vectors injected into the brain parenchyma reach the ventricles.

Prospects for adenovirus-mediated gene transfer to the brain—There have been many advances over the last 10 years in the construction and use of adenoviral vectors for gene transfer into the brain, and their use for gene therapy of brain diseases, described in detail elsewhere throughout this chapter. Improvements in adenoviral vectors that facilitate their use for gene transfer to the brain include more stringent quality controls for vector preparations before their inoculation into animals, as described herein. It is important to note that in our laboratory, unless they are deemed virtually free of LPS and RCA, vectors are discarded and never used for gene-transfer experiments. Using such high-quality viral vector preparations, and keeping the vector dose below the threshold of 1×10^8 IU/injection site, we and others have achieved reliable transgene expression for up to 12 months in naive animals (Barcia et al., 2007). In addition, the use of high-capacity adenoviral vectors allows long-term expression in the brain, even in the presence of preexisting immune responses against adenovirus (Barcia et al., 2007).

Adenoviral vectors have now also been harnessed for the treatment of human brain diseases, including brain tumors such as glioblastoma multiforme grade IV, the most aggressive of brain tumors. Various approaches have been taken into the clinic since 2000. For example, Ylä-Herttuala and co-workers have pioneered the use of adenoviral vectors expressing HSV-1 derived thymidine kinase for the treatment of glioblastoma multiforme grade IV (Määttä et al., 2009), including the recent completion of an encouraging Phase III multicentric clinical trial. Further vectors being tested in the context of ongoing and future clinical trials for glioblastoma multiforme grade IV are oncolytic adenoviruses (Jiang et al., 2006; Nandi and Lesniak, 2009), and the combination of adenoviruses expressing TK and Flt3L (Candolfi et al., 2009).

Critical Parameters

Obtaining long-term adenoviral vector-mediated transgene expression with minimal toxicity—The applications of adenovirus-mediated gene transfer into the brain are diverse. For many applications, long-term expression of the transgene of interest with minimal vector-associated toxicity (immune-mediated or direct vector-mediated) is desirable. The following parameters, in addition to those discussed above, can affect the longevity, stability, and/or toxicity of adenovirus vector-mediated transgene expression:

- the dose of vector administered;
- the transgene expressed by the vector;
- the promoter driving expression of the transgene;
- the nature of the vector backbone (i.e., first-generation versus high-capacity vectors);
- the immune status of the animal prior to vector delivery, and the environment (i.e., “clean” versus “specific-pathogen-free”) in which the animals are kept;
- the volume in which the vector is delivered;
- the purity of the adenovirus preparation.

Each of these criteria is discussed below.

Dose administered—The balance between achieving high-level, widespread and sustained transgene expression is precarious with most vector backbone–promoter–transgene combinations that are easily available at the present time. Injection of 10^8 to 10^9 infectious units of adenovirus vector into the adult rat caudate generally results in extensive transgene expression throughout the ipsilateral striatum; however this is transient, with little expression remaining by 30 days post-vector-administration (see Figure 4.24.7). The eventual loss of transgene expression from high doses of vector is likely to be a consequence of direct vector-

mediated acute cytotoxicity caused by infection of neuronal cells at high multiplicities of infection, and also the elicitation of severe chronic inflammation (Thomas et al., 2000). Administration of 10^6 to 10^7 infectious units generally results in more spatially restricted, but prolonged, transgene expression (Figure 4.24.7), with accompanying inflammation that is transient and does not decrease transgene expression. Administration of fewer than 10^6 infectious units of vector results in very little or no transduction (unless a very strong promoter is used; see discussion below and Fig. 4.24.11). All vector preparations should therefore be accurately titrated for infectious units before *in vivo* use, as described in *UNIT 4.23*. It is also necessary to determine the concentration of physical particles and the ratio of particles to infectious units for each batch of vector (also described in *UNIT 4.23*). Since vector titers may vary across different laboratories, each individual lab should determine the dose of vector resulting in optimal gene transfer, based on their own determination of titer. As discussed below, the dose for optimal long-term transgene expression will depend upon the constituents of the vector genome (i.e., vector backbone, promoter and transgene). Generally, the dose should be sufficient to obtain maximal spread of vector-mediated transgene expression in the complete absence of acute direct vector-mediated cytotoxicity. Cytotoxic lesions can be identified by the acute absence of GFAP (glial fibrillary acidic protein: astrocytes) or NeuN (neuronal nuclei) immunoreactivity within the region corresponding to the area of viral spread (see Fig. 4.24.12), or through the appearance of reactive astrogliosis. The severity of the lesion increases with increasing viral dose (Thomas et al., 2000; Zirger et al., 2006). Note that damage caused by inserting the needle will also result in GFAP and NeuN loss, restricted to the needle track.

Vector backbone, immune status of the animal, and environmental conditions

—As discussed above, long-term transgene expression is possible from first-generation adenovirus vectors only in the brains of naive animals. Peripheral immunization with adenovirus activates a strong anti-adenoviral immune response that is able to eliminate vector-mediated expression from the brain. Since most humans will have been subjected to a peripheral infection with adenovirus, use of peripherally primed animals (see Basic Protocol 7), rather than naive animals, may provide a more clinically relevant model for neurological gene therapy applications using adenovirus vectors.

In contrast to first-generation vectors, new-generation, high-capacity adenovirus vectors injected into the rat brain parenchyma are able to sustain prolonged transgene expression even in the presence of an anti-adenoviral T cell response elicited through intradermal injection of adenovirus (Thomas et al., 2000; Barcia et al., 2006a). High-capacity adenoviral vectors are deleted of all viral genes, retaining only the minimal elements of the viral genome required for replication *in vitro* and packaging (Morsy and Caskey, 1999). Thus, at least at moderate doses of vector (10^7 pfu), the vector backbone has profound consequences upon the stability of adenoviral vector-mediated transgene expression in the brain.

Ohmoto et al. (1999) have shown that a “clean” environment reduces vector-induced inflammation and improves longevity of transgene expression.

Specific and nonspecific side-effects of transgenes—Transgenes encoding known immunomodulatory molecules will have an effect upon the nature of the immune and inflammatory responses to the vector (Bell et al., 1996); however, other transgene products can also contribute to vector toxicity. It is therefore important to characterize the “side effects” of each different vector used *in vivo*. The importance of thoroughly investigating long-term as well as short-term toxicity is illustrated by Dewey et al. (1999) and Cowsill et al. (2000), who showed that coadministration of adenovirus vectors expressing HSV-1 thymidine kinase and ganciclovir to the brains of rats for glioma gene therapy caused unexpected ongoing chronic inflammation and brain cytotoxicity in long-term survivors.

Choice of promoter—The choice of promoter driving the expression of a transgene of interest will greatly affect the levels of transgene expression achievable from any given dose of adenovirus vector. Some applications of adenovirus-mediated gene transfer to the brain may require the restriction of transgene expression to within a certain subpopulation of neural cells. This can be achieved by “transcriptional targeting” through the use of cell-type-specific promoters. Cell-type-specific promoters are generally much weaker than the strong “promiscuous” promoters derived from viruses—e.g., cytomegalovirus (CMV) or Rous Sarcoma virus (RSV)—which are able to drive high levels of transgene expression within many cell types (Smith-Arica et al., 2000). Different virus-derived “strong” promoters also display markedly different efficiencies. Figure 4.24.11 shows the levels of β -galactosidase expression achievable from two doses of adenovirus vector expressing the transgene under the control of the major intermediate early *human* CMV promoter (Ad-hCMV-lacZ; Shering et al., 1997). When the same transgene is expressed from a different vector containing the much stronger major intermediate early *murine* CMV promoter (Ad-mCMV-lacZ), substantial amounts of β -galactosidase expression can be detected even at the very low vector dose of 1×10^4 infectious units. No transgene expression is detectable from the same dose of Ad-hCMV-lacZ—i.e., it appears that only cells infected with this vector at a high multiplicity will produce detectable levels of transgene product (Gerdes et al., 2000). Hence, the use of very strong promoters can facilitate substantial and widespread expression of transgene at vector doses that elicit negligible inflammatory and toxic side effects. The choice of promoter is crucial to the efficiency of viral vector-mediated gene transfer to the brain.

Volume administered—As previously discussed, the brain parenchyma and ventricles constitute immunologically distinct compartments, and immune priming can take place after vector infection of the CSF (but not the parenchyma). Vector administered to the striatum in large volumes may leak into the CSF; therefore, the vector preparation should be of sufficiently high titer that the required dose can be delivered in a volume not exceeding 2 to 4 μ l (keeping the volume closer to 2 μ l if vector titer allows). Vector administered to rat ventricles can be delivered in larger volumes, but should still be kept as close to 2 to 4 μ l as possible.

Vector purity—Vector purity should be assessed by calculating the ratio of absorbance at 260 nm to that at 280 nm (pure adenovirus type 5 has a 260:280 ratio of 1:3; a protocol for this determination is given in *UNIT 4.23*). In addition to determining vector purity by optical absorbance, it is imperative to ensure that the preparation is free of contamination from both replication competent adenovirus (RCA) and lipopolysaccharide (LPS or endotoxin). Emergence of RCA during propagation of first-generation adenoviral vectors on 293 cells is common, and injection of RCA-contaminated vector into the brain can cause severe inflammation and tissue damage, as illustrated in Figure 4.24.13. Alternative cell lines to the standard 293 cell line have recently been developed to reduce the probability of generating RCA during vector propagation (Fallaux et al., 1996). LPS is a component of the cell wall of Gram-negative bacteria and is an extremely potent stimulator of the mammalian immune system. Many studies have used injection of LPS into the brain parenchyma to investigate inflammatory responses within the CNS. Unfortunately, LPS is another frequent contaminant of adenoviral vector preparations, and it has been shown that its toxicity to primary cultures of human cells is increased in the presence of adenovirus (Cotten et al., 1994). The level at which contaminating LPS is toxic when administered in vivo with adenovirus has not been determined, but the authors do not use vector stocks containing more than 2 endotoxin units per ml for in vivo experiments.

Other factors affecting long-term transgene expression—In a nonsensitized animal, adenovirus vectors can mediate prolonged transgene expression, but levels of expression decline over time. The cause of this decline is not clear, but a putative mechanism may be the

gradual inactivation of the viral promoters—i.e., cytomegalovirus (CMV) or Rous Sarcoma virus (RSV) elements—that are generally used to drive transgene expression in adenovirus vectors. The inactivation of viral promoters used in genetically modified skin fibroblasts transplanted in vivo has been documented (Palmer et al., 1991). In further support of the hypothesis of promoter inactivation, adenoviral vector genomes have been detected in transduced muscle tissue long after transgene expression has disappeared (Chen et al., 1999). Use of CNS-specific housekeeping promoters may ameliorate shut-down of transgene expression, and, indeed, use of such promoters confers the additional advantage of targeting expression to a specific subpopulation of cells within the CNS. Such promoters include the glial fibrillary-acidic protein (GFAP) promoter specific for astrocytes, the neuron-specific enolase (NSE) promoter specific for neurons, and the myelin basic protein (MBP) promoter for targeting oligodendrocytes (Morelli et al., 1999; Smith-Arica et al., 2000). Importantly, most cell-type specific promoters used to date are weaker than most virus-derived promoters (Smith-Arica et al., 2000). Other factors that may explain the diminution of transgene expression over time include cell turnover and direct toxicity of viral or transgene products to infected cells.

Other considerations

Surgical technique: Poor surgical technique can generate lesions within the brain that will jeopardize the experiment and will certainly affect the quality of the data. It is therefore essential to practice the procedure on dead animals to gain competence before starting an experiment. The Hamilton needle should be examined under the microscope before using, as bent needles can also cause lesions within the brain. For very precise injections, it may be desirable to grind the tip of the needle to remove the bevel, or to use superfine glass needles, fixed with wax to the tip of the Hamilton needle.

When setting up a stereotactic apparatus, it is advisable to perform preliminary experiments injecting adenovirus expressing β -galactosidase, to ensure that the correct area of the CNS is targeted using a particular set of coordinates. The animal can be sacrificed 2 to 5 days after surgery and brain sections analyzed by Xgal histochemistry.

Retrograde transport of adenovirus vectors: Adenovirus vectors injected into the brain parenchyma will infect neurons, astrocytes, and microglial cells surrounding the injection site. In addition, vector taken up by neuronal axons in the injection site will be transported in a retrograde manner to neuronal cell bodies in distant sites. This phenomenon can be exploited to specifically transduce neuronal populations in areas of the brain which are difficult to target by direct injection. Such delivery confers the additional advantage of avoiding undesirable side effects associated with tissue damage at the site of interest. Retrograde transport of vector is particularly suited to studies of neurodegeneration in rat models of Parkinson's disease (see *UNIT 9.4*); adenovirus vectors encoding neurotrophic factors can be injected into the striatum, from where they will be transported along dopaminergic neuron axons to the substantia nigra. The efficiency of this procedure should be tested with each new vector, and in each neuronal system.

Troubleshooting

Sectioning—It should be possible to generate good-quality intact Vibratome sections: 40 to 60 μ m from properly fixed material using a high-quality Vibratome. Underfixed tissue will disintegrate during sectioning. Refixing the brains for 2 to 3 hr in 4% paraformaldehyde will generally ameliorate the problem; however, it is important not to overfix the material as this will compromise the quality of the subsequent samples for immunohistochemistry and PCR. Lesions within the brain also tend to disintegrate during sectioning; in these cases it may be necessary to cut thicker sections in order to preserve the integrity of the lesioned area.

Immunohistochemistry—Problems encountered during immunohistochemical tissue staining generally fall into two categories: absence of staining or high levels of nonspecific background staining. Each primary antibody used should be titrated to determine the dilution required for optimal staining, using tissue sections that are known to be positive for the epitope of interest (these sections may be derived from other organs such as spleen or liver). If a range of optimal dilutions is not recommended by the supplier, then try a range of dilutions from 1:100 to 1:2000 as a starting point. When performing immunohistochemistry on brain sections, it is expedient to include a positive control for each marker under investigation to ensure that absence of staining within the brain tissue is not the result of omission of one of the reagents. Fixation or tissue processing (e.g., paraffin embedding) can mask some antigenic processes, particularly those on the cell surface. Incubation of sections in 0.01 M sodium citrate (pH 7.6) for one minute at 60°C (heated in a microwave) can retrieve some epitopes without severe tissue damage.

If the optimal dilution of each antibody has been determined in a preliminary experiment, the levels of background staining should be very low. High background staining may result from insufficient washing, or, more likely, from overdeveloping with the DAB reagent. Since the color reaction proceeds rapidly, it is advisable to stain each well of brain sections individually (if multiple samples are being processed simultaneously) and to carefully monitor color development by eye (rather than with a timer) to avoid overstaining.

Anticipated Results

The results of adenovirus vector-mediated gene transfer into the brain will depend upon the experimental design and the factors discussed in Critical Parameters. Several figures illustrate examples of results obtained with different doses of vector (Fig. 4.24.7, Fig. 4.24.9, Fig. 4.24.11, and Fig. 4.24.12), different promoters (Fig. 4.24.11), and vector stocks of variable purity (Fig. 4.24.13).

Time Considerations

An in vivo experiment involving stereotactic delivery of adenovirus to the brain is a major undertaking which requires considerable preparation and labor input. The duration of the experiment and the time taken to generate data will depend upon the questions being posed and the number of animals required to answer the question in a statistically significant manner. Generation and titration of the viral vectors requires several weeks, and all the quality-control tests (e.g., LPS/RCA assays) must be completed for each batch of vector before in vivo administration. Stereotactic delivery of the adenoviral vector is in itself a labor-intensive process. With practice, the surgical procedure should take no more than 40 min per animal, and hence several animals can be operated upon in 1 day. The time required to process the data is again dependent upon the number of animals involved in the experiment and the type of analysis undertaken.

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Figure 4.24.1.
Optimal position of stereotactic frame, fiber-optic light source, and surgical microscope.

Figure 4.24.2.

Drill and sterilized surgical tools required for stereotactic injection of adenoviral vectors into the rat brain. From left to right: drill, scissors, forceps with suture, syringe with bent needle, retractor, scalpel, forceps.

Figure 4.24.3.

Correct placement of anesthetized rat in the stereotactic apparatus. **(A)** The ear bars are gently inserted into each ear canal so the head of the animal is firmly in place and does not wobble. **(B)** It is critical to ensure the animal's tongue is extended outside of the mouth to prevent choking during surgery.

Figure 4.24.4.

The syringe is positioned over the future injection site and lowered to the skull. A marker pen is used to mark the position of the needle on the skull. The skin overlying the skull is held back with a retractor.

Figure 4.24.5.

The Hamilton syringe is slowly depressed to inject adenoviral vector into the brain. (A) 2 to 3 minutes after the needle is inserted into the brain, the syringe plunger is slowly depressed to inject 0.5 μ l over the course of 1 min. (B) Precise finger position is used to control the speed of the injection.

Figure 4.24.6.

Staining reaction for immunohistochemistry, using glucose oxidase (GOD), horseradish peroxidase (HRP), and 3'3'-diaminobenzidine (DAB).

Figure 4.24.7.

Transgene expression at 3 and 30 days post-infection with different doses of vector and different vector backbones. Panels (A–D) show the immunohistochemical detection of the transgene product β -galactosidase (β -Gal) after administration of either 1×10^7 infectious units (panels A and B) or 1×10^9 infectious units (panels C and D) of a first-generation adenovirus vector expressing β -Gal from the hCMV promoter. Transgene expression remains stable over the 30-day period after injection of 10^7 infectious units, but is almost eliminated after injection of 10^9 infectious units. Panels E and F show transgene expression from 1×10^7 infectious units of a high-capacity adenovirus vector, expressing β -galactosidase from the hCMV promoter.

Levels of expression from the high-capacity vector are similar to those from the equivalent dose of first-generation vector (from Thomas et al., 2000).

Figure 4.24.8.

The area of the brain surrounding the injection site is dissected. (A) The brain is placed in the rat brain matrix and a razor blade is used to make a dorso-ventral cut separating the forebrain from the midbrain. (B) A second razor blade is used to make a dorso-ventral cut to remove the olfactory bulb. (C) A third razor blade is used to separate the right and left forebrain hemispheres.

Figure 4.24.9.

Detection of blood-brain barrier permeability 2 days after administration of (A) saline, (B) 1×10^7 infectious units of adenovirus vector, or (C) 1×10^9 infectious units of adenovirus vector. Animals were injected with HRP via the tail vein 20 min before perfusion-fixation, and HRP within the brain was detected according to the Hanker-Yates method (E. Abordo-Adesida, M. Castro, and PR. Lowenstein, unpub. observ.).

Figure 4.24.10.
Apparatus for perfusion-fixation by gravity.

Figure 4.24.11.

Transgene expression and inflammation in brains injected with 1×10^4 infectious units (panels **A**, **B**, **E**, and **F**) or 1×10^7 infectious units (panels **C**, **D**, **G**, and **H**) of a first-generation adenovirus vector expressing β -galactosidase from the major intermediate early human CMV promoter (hCMV; panels **B**, **F**, **D**, and **H**) or the major intermediate early murine CMV promoter (mCMV; panels **A**, **E**, **C** and **G**). Expression from the mCMV promoter is approximately 3 log units higher than from the hCMV promoter, and thus substantial levels of transgene expression can be achieved from low doses of vector which elicit minimal levels of inflammation—evidenced here by ED1 staining of activated microglial cells and macrophages (from Gerdes et al., 2000).

Figure 4.24.12.

Immunohistochemical detection of GFAP (A,C; activated astrocytes) or NeuN (B,D; neuronal nuclei) 3 days after injection of 1×10^7 or 1×10^9 infectious units of a first-generation adenovirus vector expressing β -galactosidase. Low-power images are shown on the left of each panel. The small box in the low-power images delineates the region which is shown at higher power on the right of each panel. Absence of GFAP or NeuN immunoreactivity within the injection site indicates astrocyte/neuronal death due to direct vector-mediated cytotoxicity or damage caused by the needle injection. Note the lesion in brains injected with 1×10^9 infectious units of vector extends throughout the region corresponding with the area of vector spread (Thomas et al., 2000).

Figure 4.24.13.

Effect of injecting an RCA-contaminated stock of adenovirus vector into the brain. Panels **A–D** show the immunohistochemical detection of activated astrocytes (GFAP; panels **A** and **B**) and activated microglial cells and macrophages (ED1; panels **C** and **D**), 14 days after injection of 1×10^9 infectious units of adenovirus vector, either RCA-free (panels **B** and **D**), or heavily contaminated with RCA (panels **A** and **C**). Although the high dose of RCA-free vector has caused considerable inflammation (i.e., astrocyte activation and macrophage infiltration), injection of the same dose of RCA-contaminated vector has caused massive inflammation and tissue necrosis (E. Abordo-Adesida, M. Castro, and PR. Lowenstein, unpub. observ.).

Table 4.24.1

Antibodies Used to Detect Inflammation, Immune Responses, and/or Adenovirus Vector-Mediated Cytotoxicity in the Rat Brain

Antibody	Specificity within the brain	Dilution	Source
<i>Inflammatory and immune responses:</i>			
ED1	Infiltrating monocytes and activated macrophages/microglia	1:1000	Serotec
CD43	Infiltrating lymphocytes	1:500	Serotec
CD4	Helper T cells (perivascular microglial cells are also recognized)	1:200	Serotec
CD8	Cytotoxic T lymphocytes and natural killer cells.	1:500	Serotec
CD8 β	Cytotoxic T lymphocytes	1:2000	Pharmingen
CD161	Natural killer cells	1:2000	Serotec
CD45RA	B cells	1:2000	Pharmingen
OX-6	MHC class II	1:200	Serotec
OX-18	MHC class I	1:200	Serotec
OX-62	Dendritic cells and $\gamma\delta$ T cells	1:20	Serotec
ICAMI	Perivascular microglia/activated endothelial cells	1:100	Serotec
<i>Virus-mediated acute cytotoxicity:</i>			
GFAP	Glial fibrillary acidic protein; activated astrocytes	1:200	Roche
NeuN	Neuronal nuclei	1:50	Autogen Bioclear
<i>Myelin integrity:</i>			
MBP	Myelin basic protein	1:2000	Dako