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Cytokine Gene Delivery into the Central Nervous System Using Intrathecally Injected Nonreplicative Viral Vectors

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1. Introduction

The delivery of drugs through the bloodstream in patients affected by central nervous system (CNS)-confined multifocal diseases can be therapeutically ineffective because of the presence of the blood–brain barrier (BBB), which forms an inaccessible wall to the majority of CNS-targeting molecules. The BBB is a specialized endothelial structure formed by the interaction between endothelial cells and astrocytes. It can be distinguished from the normal endothelium for the presence of tight junctions between endothelial cells, which are impermeable to macromolecules and even ions, and for the reduced endocytic activity, which considerably decreases the number of molecules that can cross the BBB in a nonspecific fashion ***(1)***. Only the presence of specific transport mechanisms assures that molecules essential for the brain metabolism (e.g., amino acids and glucose) reach the brain parenchyma.

1.1. CNS Drug Delivery

Chronic inflammatory demyelinating diseases of the CNS, such as multiple sclerosis (MS), might benefit from anti-inflammatory therapies ***(2)***. However, promising treatments such as those based on the systemic administration of anti-inflammatory cytokines did not result in a consistent therapeutic effect in MS patients ***(3)***. The scarce capacity of cytokines to cross the BBB, along with their short half-life and autocrine/paracrine activity, might render necessary

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the delivery of these molecules directly into the CNS. Biological and physical vectors engineered with heterologous genes coding for anti-inflammatory cytokines might represent the appropriate tool to deliver cytokine into the CNS ***(4)***. Results obtained in rodents affected by experimental autoimmune encephalomyelitis (EAE), the animal models of MS, support this working hypothesis ***(5)***.

1.2 The Ependymal Way to Access the CNS

Here, we summarize the technical procedure and the troubleshooting of a novel strategy we recently established to access the CNS using viral vectors engineered with heterologous genes coding for anti-inflammatory cytokines. This approach is based on the injection into the cerebrospinal fluid (CSF) space trough the cisterna magna (i.c.) of nonreplicative viral vectors (**Fig. 1a**). Injected vectors, in turn, infect exclusively neuroectodermal cells lining the CSF space (including the Virchow–Robin spaces) and forming the blood–CSF barrier surrounding both the brain and the spinal cord (i.e., ependymal, choroidal, and leptomeningeal cells) (**Fig. 1b**). The viral genome enters into the nucleus of infected cells and dictates heterologous gene transcription (**Fig. 1c**). The protein coded by the transgene is then translated into the cell cytoplasm and secreted into the CSF (**Fig. 1d**). Secreted proteins diffuse, via the ependymal layer or the pia mater, into the CNS parenchyma, where they are still biologically active and can exert therapeutic activity.

Only vectors fulfilling the following criteria can be used. (1) vectors able to infect nondividing cells because ependymal and leptomeningeal cells cycle at a very slow rate; (2) vectors that can be obtained at very high titers because only very small volumes (up to 10 μL in mice) can be injected; (3) vectors with very low or no immunogenicity because the protocol is designed to interfere with an already ongoing immune reaction; (4) vectors expressing the transgene for the long term because repeated intrathecal injection of the vectors is a not feasible approach in a routine clinical setting.

Several viral vectors fulfill the above-mentioned criteria, among which herpes simplex virus (HSV) type-1-derived vectors, adenoviral vectors (AD), adeno-associated viral vectors (AAV), and lentiviral vectors. However, only HSV-1 and AD vectors, to our knowledge, have been intrathecally delivered, so far.

1.2.1. Herpes Simplex type-1-Derived Vectors

Several features make HSV-1 a likely candidate as a vector for gene transfer: (1) At least one-third of the 152 kb of the HSV-1 genome is made of genes nonessential for replication. These genes can be deleted and substituted by exogenous genes without any detrimental effect for in vitro viral growth. (2) HSV-1 can be easily propagated in several different cell lines (e.g.,

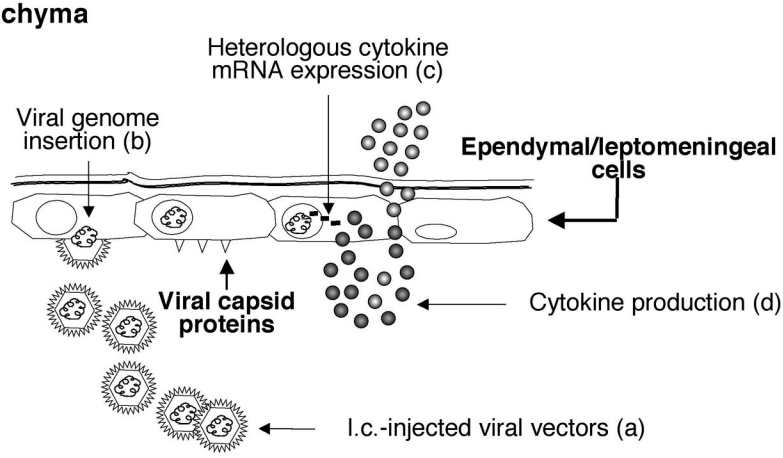

Fig. 1. The ependymal route. Nonreplicative viral vectors engineered to contain a cytokine gene are injected intracisternally in cerebrospinal fluid spaces (a); these vectors insert their genome into the cells lining ventricles and sub-arachnoid spaces (ependymal and leptomeningeal cells) (b) and induce them to transcribe (c) and translate the cytokine gene which is then released (d) into the cerebrospinal fluid. From there the exogenously produced cytokine can travel through the ependymal cell layer into the brain parenchima and exert there its potentially beneficial effect.

complementing cell lines), allowing the generation of high-titer viral stocks. (3) HSV-1 is able to infect several different cell types, regardless of the cell cycle, with high efficiency, making it an ideal candidate vector for several applications (i.e., infection of postmitotic neurons). (4) During the lytic cycle, many HSV-1 genes are expressed with high efficiency. A heterologous gene driven by a viral promoter can, therefore, produce large amounts of protein. (5) HSV-1 is able to persist in a state of latency for the whole life of its host. During latency, the viral genome is circularized and remains as an episome in the cell nucleus. Lytic genes are silent and only latency-specific transcripts are present. The introduction of foreign sequences under the control of latency-specific promoters may allow long-term transcription of transgenes.

We have been working with nonreplicative deletion mutant HSV-1 vectors. These deletions lead to the inability to replicate in normal conditions and lower their cytotoxicity. Immediate–early genes are the main target of the mutations because these genes are both essential for viral replication and responsible for most of the cytopathicity. Immediate–early genes are ICP infected cell

polypeptides ICP0, ICP22, ICP4, ICP27, and ICP47, in order of decreasing toxicity. Among those, only ICP4 and ICP27 are essential for replication, although the lack of some of the others (ICP0, ICP22) produces a marked decrease in viral titers. HSV-1 deletion mutants have been generated lacking three immediate–early genes (ICP4, ICP27, and ICP22) ***(6,7)*** and are propagated on a corresponding complementary cell line producing ICP4 and ICP27 (ICP22 is nonessential for in vitro viral growth). Using these herpetic vectors engineered to express interleukin (IL)-4, interferon (IFN)-γ, and FGF-II, we have obtained encouraging results in both mice and nonhuman primates affected by EAE ***(5,8–13)***.

1.2.2. Adenoviral Vectors

Adenoviruses have a double-stranded DNA genome of about 35–40 kb and lack an envelope. About 50 serotypes have been described, most of which causing, in humans, benign diseases of the respiratory tract. Serotypes 2 and 5 are the most studied for the obtainment of gene therapy vectors. In the adenoviral genome, between two inverted repeats (ITR) functioning as origin of replication, there are complex transcriptional units that can be divided into four early (E) and five late (L) regions. Of those, region E1A, the first expressed after infection, is essential for viral replication. First-generation vectors have been obtained deleting the E1 gene and replacing it with a 8- to 5-kb transgene expression cassette. The E1 gene product was provided in trans by a complementing cell line. Because of the high toxicity and immunogenicity of these vectors, high-capacity (HC), also named helper-dependent (HD) vectors, almost completely devoid of viral sequences, have been developed. HD vectors, which depend on an helper virus for in vitro growth, have only the two ITRs and the packaging signal and are, therefore, able to accomodate up to 30–35 kb of exogenous DNA. Contaminating helper virus is eliminated both by CRE-mediated excision of the packaging signal from its genome and, subsequently, by gradient purification. Main features of these vectors are (1) the ability to infect many different cell types regardless of the cell cycle (including postmytotic cells), (2) reduced toxicity and immunogenicity, and (3) long-term expression of the transgene (up to 6 mo). The genome can be entirely manipulated as a plasmid and grown in bacteria, making its genetic engineering easier. Adenoviral vectors have been used to infect ependymal–leptomeningeal cells, also in nonhuman primates ***(14–17)***.

1.3. Injection of HSV-1 Vectors into the Cisterna Magna to Access the CNS

To access the CSF space of mice, the most common procedure is an intraventricular injection performed using a stereotactic apparatus. This

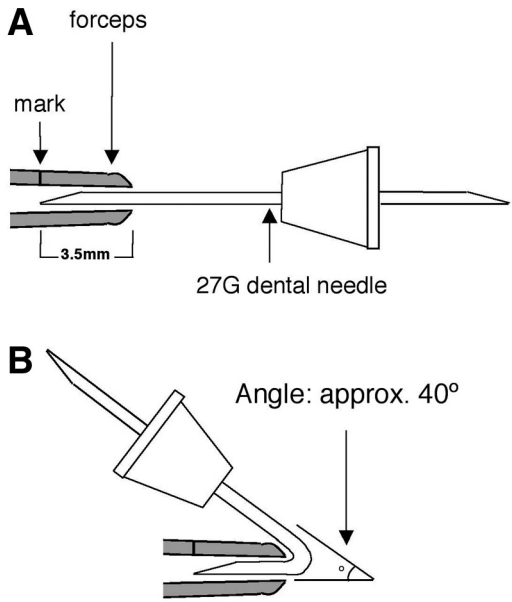

Fig. 2. Needle preparation. Hold the needle tightly with the tip at 3.5 mm from the end of the forceps **(A)**; bend the needle with the forceps at an angle of approx 40°, keeping the cutting edge inside **(B)**.

procedure is, however, time-consuming and limits the number of mice that can be treated in a single experiment. Here, we describe the application of a quick and simple intracisternal (i.c.) injection technique that can be used to deliver cytokine genes within the CNS. We also show a method to sample CSF from mice that represents an essential corollary technique to verify the efficiency of heterologous protein production within the CNS.

2. Materials

2.1. Injection Procedure

1. Flat forceps.
2. Dental needle 27G × 13/16 in. (0.40 × 21 mm) (*see* **Fig. 2**).
3. Bunsen burner.
4. Polyethylene tubing; inner diameter = 0.38 mm (0.015 in.); outer diameter = 1.09 mm (0.043 in.) (Becton Dickinson, cat. no. 427406).
5. 10-µL Hamilton GC syringe.
6. Diethyl ether.
7. Multichannel pipet reservoir.
8. Pipet tip box.

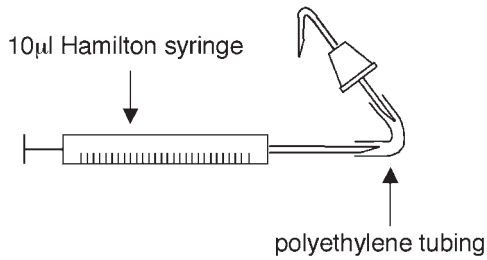

Fig. 3. The injecting device. The injecting device is obtained connecting the bent dental needle to the 10 μL Hamilton syringe through a small (approx 5 mm) piece of polyethylene tubing.

2.2. Cerebrospinal Fluid Sampling

1. 100-μL Pyrex disposable microsampling pipets (Corning).
2. Bunsen burner.
3. Microdissecting scissors.
4. Cloralium hydrate (store at 4°C for up to 2 mo).
5. Disposable scalpel.
6. Cotton swabs.
7. Pipet tip box.
8. Butterfly needle.
9. 10-mL syringe.

3. Method

3.1. Intracisternal Injection of Vectors in Mice

1. Needle preparation. Needles are prepared as described by Ueda et al. ***(18)***. Measure 3.5 mm from the tip of a large forceps and indicate the point with a marker (**Fig. 2A**).
2. Used the marked forceps as a tool to bend a 27-gage dental needle, keeping the cutting edge toward the inside of the loop (**Fig. 2A,B**). The needle should be J shaped, with an angle of approx 40° (**Fig. 2B**).
3. Before recapping, the needle should be briefly flamed.
4. Dental needles have a short needle at the opposite end as well. Connect a 1-cm-long polyethylene tubing (internal diameter 0.38 mm) to the short end. Several needles can be prepared and kept for further use.
5. Connect the dental needle, through the polyethylene tubing, to the needle of a 10-μL Hamilton syringe (**Fig. 3**).
6. Fill the resulting injecting device, aspirating the viral vector containing solution from a reservoir for multichannel pipets.
7. Briefly anesthetize the mouse with ether, lean it on a small box (e.g., a pipet tip box), and bend the head slightly forward (**Fig. 4**). Run the needle along the external surface of the occiput and insert it into the cleft between the occiput and

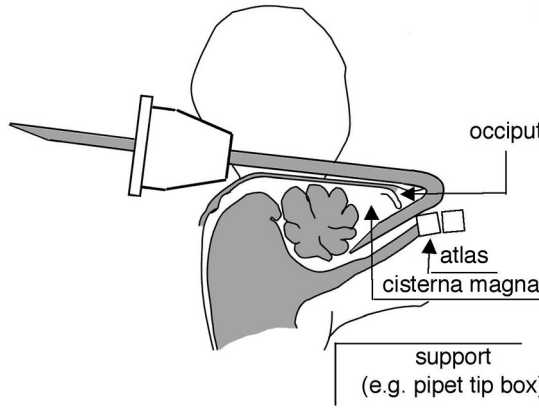

Fig. 4. The anesthetized mouse is put on a support and the head bent forwards. The needle is inserted as shown at the midline in the cleft between atlas and occiput.

the atlas vertebra through the intact skin, muscles, and ligaments in the midline at the back of the neck (**Fig. 4**). The bent part of the needle is kept in close contact with the internal surface of the occiput for the entire length (**Fig. 4**). The operator should learn to recognize the feeling of the needle being "hooked" to the mouse skull.

8. Inject the vector-containing solution in approx 10 s (best done by a second operator) and keep the needle in place a few more seconds before extracting it (*see* **Notes 1 and 2**). The same needle can be used for several mice to be injected with the same vector. Mice recover rapidly from the procedure, showing no evident adverse effects of the injection (*see* **Notes 3–6**).

The entire injection procedure takes less than 1 min. If the procedure has been performed correctly, your vector should have been distributed throughout the ventricular and CSF space (*see* **Note 7**). If a reporter gene-containing vector (e.g., β-galactosidase) is employed, the brain of an injected mouse should appear, upon specific staining, as in **Fig. 5B** (the solid arrow indicates the injection site; dashed arrows indicate viral vector-infected leptomeningeal cells). A sham-injected mouse brain is shown in **Fig. 5A** for comparison.

3.2. Cerebrospinal Fluid Sampling

1. Cerebrospinal fluid can be sampled only at sacrifice, because the procedure permanently lesions neck muscles and the cisterna magna.
2. Glass needle preparation. Keep the center of a 100-µL Pyrex disposable micro-sampling pipet on the flame of a Bunsen burner until it starts to melt. Take the two extremities apart quickly, so that the pipet is divided into two parts with two short terminal cones (*see* **Fig. 6**).

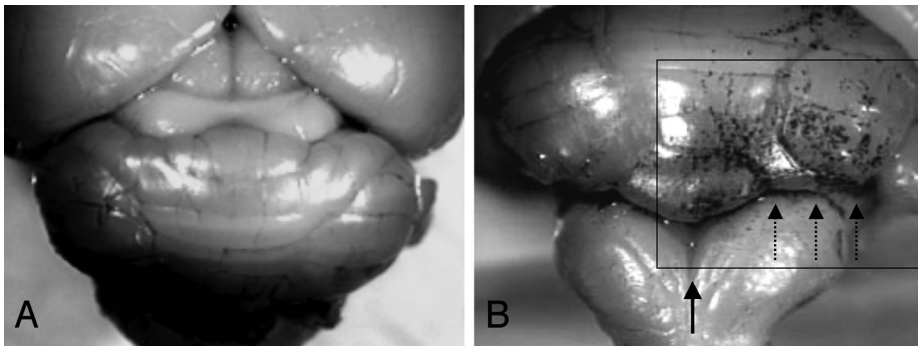

Fig. 5. Dissemination of the d120-IFNγ-lacZ herpetic vector within the CNS of a representative Balb/C mouse after injection of 10^9 plaque forming unit (PFU) of the vector in the mouse' cisterna magna (I. C.). Dark spots in panel **B** indicate the vector-infected cells around the injection site in a representative animal injected I. C. with the vector and sacrificed 72 h later. In panel **A** the same brain area obtained from a sham-injected animal is shown.

3. Using microdissecting scissors, cut the terminal of the cone to obtain a cutting edge as indicated by the dashed lines in **Fig. 6**.
4. Cut off the needle from a butterfly needle and connect the glass needle to its tubing.
5. Anesthetize the mouse with an intraperitoneal injection of cloralium hydrate (400 mg/kg of weight) and check for the absence of corneal and deep pain reflexes.
6. Lean the mouse on a support where you can bend the mouse head forward.
7. With a scalpel, cut the skin of the mouse twice horizontally, above the occiput (above the first vertebras of the column, and once vertically to connect the two previous incisions on the midline).
8. Open the skin, remove the muscles of the neck with a cotton swab and wait for the small hemorrhages to stop (*see* **Note 8**). You should see exposed the dura mater above the cisterna magna as a transparent sierosa (*see* **Fig. 7**).
9. Using the glass needle, punch the dura mater, slightly lateral to the midline (*see* **Note 9**).
10. The CSF enters the pipet by capillarity, usually 5–10 μL.
11. Empty the pipet in an Eppendorf tube by connecting it to a syringe through the tubing of a butterfly needle (after removing the needle) and blowing air into it.
12. Sacrifice the mouse.

4. Notes

1. Applying excessive pressure will cause hydrocephalus.
2. Ten microliters is the highest injectable volume. Injection of more fluid will result in reflux along the needle track.

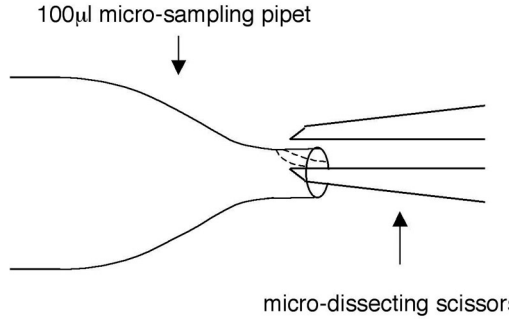

Fig. 6. Preparation of the glasss needle for the collection of the cerebrospinal fluid. The needle is obtained tearing apart a 100 μL micro-sampling pipet on an open flame to obtain a short conical end. A sharp edge is obtained as shown with micro-dissecting scissors.

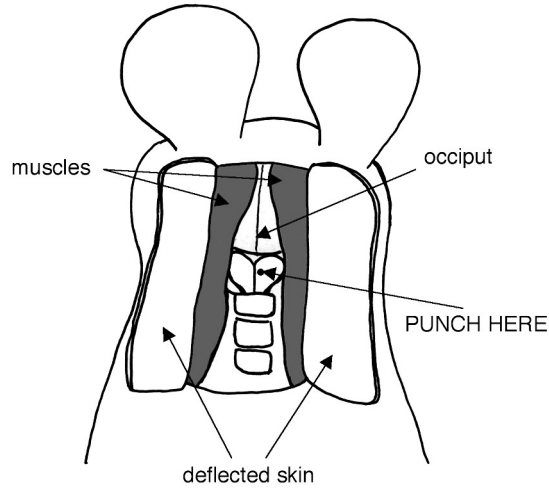

Fig. 7. Cerebrospinal fluid sampling. The anesthetized mouse is put on a support where the mouse head can bent forward. After skin and the muscles of the neck have been removed and the small hemorragies have stopped, use the glass needle to punch the exposed dura mater above the cisterna magna slightly lateral to the midline, as shown.

3. Mice sometimes become ataxic because of cerebellar lesions. The length of 3.5 mm indicated for the bent part of the needle works with most mice strains at different ages. If, however, this becomes a recurrent problem or if the injection technique is applied to small or very young mice, shortening the needle length has to be considered.
4. Mice that underwent intracisternal injection will lose some weight during the forthcoming 2 d, probably because of reduced food intake.

5. After intracisternal injection, we were able to demonstrate a transient local inflammatory reaction, with an increase of the blood–brain barrier permeability, lasting less than a week. Neuropathological signs of inflammation are, however, absent.
6. Intracisternal injection can be repeated, but, in our experience, at least 5 d apart.
7. Although liquoral circulation goes from the choroid plexi, where it is produced, to the cauda, the intracisternal injection will transiently revert the flux direction allowing viral vector particles to reach the whole ventricular system.
8. Usually, several cotton swabs have to be used in order to tear muscles apart and wipe the blood until it stops. Because avoiding blood contamination is a crucial issue, be very careful in this step.
9. A blood vessel runs exactly along the midline of the dura mater above the cisterna magna. Insertion of the glass syringe at the midline will therefore result, almost invariably, in a blood contamination of the CSF sample.

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