Comparative Efficacy of Expression of Genes Delivered to Mouse Sensory Neurons With Herpes Virus Vectors


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ABSTRACT

To achieve gene delivery to sensory neurons of the trigeminal ganglion, thymidine kinase-negative (TK-) herpes simplex viruses (HSV) containing the reporter gene lacZ (the gene for E. coli β-galactosidase) downstream of viral (in vectors RH116 and tkLTRZ1) or mammalian (in vector NSE-lacZ-tk) promoters were inoculated onto mouse cornea and snout. Trigeminal ganglia were removed 4, 14, 30, and 60 days after inoculation with vectors and histochemically processed with 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal). With vector tkLTRZ1, large numbers of labeled neurons were observed in rostromedial and central trigeminal ganglion at 4 days after inoculation. A gradual decline in the number of labeled neurons was observed with this vector at subsequent time points. With vectors RH116 and NSE-lacZ-tk, smaller numbers of labeled neurons were seen at 4 days following inoculation than were observed with vector tkLTRZ1. No labeled neurons could be observed at 14 days after inoculation with vectors RH116 and NSE-lacZ-tk.

Immunocytochemistry for E. coli β-galactosidase and in situ hybridization to HSV latency-associated transcripts revealed labeled neurons in regions of the trigeminal ganglion similar to that observed with X-Gal staining. A comparable distribution of labeled neurons in trigeminal ganglion was also observed after application of the retrograde tracer Fluoro-Gold to mouse cornea and snout.

These data provide evidence that retrogradely transported tk- herpes virus vectors can be used to deliver a functional gene to sensory neurons in vivo in an anatomically predictable fashion.

Key words: trigeminal ganglion, herpes simplex virus, pain

The delivery of functional genes to the nervous system offers a potential form of treatment for a variety of neurologic conditions (Breakefield and Geller, '87; Geller et al., '91; Martuza et al., '91; Breakefield et al., '92). In the somatosensory system, it may be possible to model conditions of persistent pain in humans by delivering toxic genes to sensory neurons in experimental animals, thereby producing a cell-specific injury. An ability to produce a cell-specific injury in animals that is associated with a change in pain behavior could lead to a better understanding of human painful conditions such as post-herpetic neuralgia, which follows varicella zoster infection (Watson, '89), or painful neuropathy seen in association with the acquired immunodeficiency syndrome (AIDS; Cornblath and McArthur, '88).

In addition, cell-specific delivery of genes encoding analgesic peptides to sensory neurons involved in nociception might eventually be helpful for relieving the persistent pain that follows nerve injury in conditions such as causalgia (Livingston, '43), trigeminal neuralgia (Fromm, '89), and painful diabetic neuropathy (Thomas and Eliasson, '84).

Herpes simplex and other neurotropic virus vectors (Loewy et al., '91) offer one potential means of delivering functional genes to alter the physiology of sensory and
other neurons (Breakefield et al., '92). Herpes simplex virus (HSV) is known to be retrogradely transported from the peripheral terminals of sensory neurons to their sensory ganglia by a mechanism of fast axonal transport (Cook and Stevens, '73). HSV that reaches the nucleus of a sensory neuron is capable of either lytic replication or latent infection. Lytic replication is characterized by the production of mature virus particles and destruction of the cell. Latent infection is characterized by the long-term stable presence of viral DNA in the nuclei of infected cells, most likely in the form of an episomal unit (Rock and Fraser, '83; Efstathiou et al., '86; Mellerick and Fraser, '87), and transcription that is limited to a specific region of the HSV genome (Croen et al., '87; Rock et al., '87; Spivak and Fraser, '87; Stevens et al., '87; Javier et al., '88; Wagner et al., '88; Kosz-Vnenchak et al., '90).

Latent infection with HSV in the absence of lytic replication is possible by using replication-defective recombinant vectors mutated by deletion, or insertion, of foreign elements into HSV genes necessary for replication (Dobson et al., '90; Andersen et al., '92). Foreign genes stably expressed by these vectors have included lacZ (Ho and Mocarski, '89) or rabbit β-globin inserted downstream of the putative HSV LAT promoter (Dobson et al., '89), and lacZ inserted downstream of the Moloney murine leukemia virus long terminal repeat (MoMLV-LTR) retrovirus promoter into the HSV immediate early gene ICP4 (vector 8117/43) (Dobson et al., '90). Stable expression of foreign genes in the central and peripheral nervous system may also be possible by using vectors that are replication-defective because of a mutation in the thymidine kinase (tk) gene. These thymidine kinase deficient (TK-) mutants can replicate in the periphery in dividing cells, but are replication-defective in trigeminal ganglion neurons (Coen et al., '89; Efstathiou et al., '89). TK- viruses may preferentially enter latency in sensory neurons (Kosz-Vnenchak et al., '90) and, similar to the lacZ-containing vector described by Dobson et al. ('90), are unable to reactivate from latency to produce a lytic infection (Coen et al., '89; Efstathiou et al., '89).

In the present experiments, TK- HSV vectors were used to deliver the marker gene, lacZ, to mouse trigeminal ganglion neurons in vivo. The expression of lacZ by these vectors was under the control of different viral or mammalian promoters. The strength of these promoters and their ability to confer stable expression of lacZ on trigeminal ganglion neurons were compared at different time points after inoculation of these vectors onto the mouse cornea and snout. In addition, to histochemical staining for E. coli β-galactosidase in trigeminal ganglion, immunocytochemistry for β-galactosidase and in situ hybridization to latency-associated transcripts (LATs) were performed. The distribution of neuronal labeling after corneal and snout inoculation of the retrograde tracer Fluoro-Gold was also examined.

MATERIALS AND METHODS

Virus vectors

HSV vectors were constructed in a wild type HSV type 1 (KOS strain) DNA background (Smith, '64), and were TK- because of insertion of foreign elements into, or a deletional mutation in, the tk gene. The following HSV vectors containing lacZ were tested: RH116 (insertional mutant; lacZ downstream of the HSV beta 8 promoter; kindly provided by Drs. Ho and Mocarski, Stanford Univ.; Ho and Mocarski, '88), tkLTRZ1 (insertional mutant; lacZ downstream of the Moloney murine leukemia virus long terminal repeat promoter), and neuron-specific enolase (NSE)-lacZ-th (insertional mutant; lacZ downstream of the NSE promoter; Andersen et al., '92); (Fig. 1). To construct tkLTRZ1, a plasmid (ptkLTRZ1) was generated by subcloning the 3 kb lacZ BglII fragment from pJ3β-gal (a gift of C. Cepko, Harvard Medical School), into the polylinker 3' to the MoMLV-LTR in pJ4w (a gift of C. Cepko, Harvard Medical School). The 3.4 kb LTR-lacZ fragment from this plasmid was subsequently cloned into the PsiI site in a plasmid containing the tk coding sequences from the BglII site to the PsiI site (Coen et al., '89) in the same orientation as tk, thus utilizing the tk polyadenylation signals. Recombinant virus was generated by co-transfection of linearized ptkLTRZ1 and HSV-1 (KOS) infectious DNA in Vero cells, selected by acyclovir resistance as previously described (Coen et al., '89), screened by polymerase chain reaction amplification of the region of the tk gene encompassing the PsiI insertion site, and three times plaque purified (data not shown). Virus was grown and titered on Vero cells, and maintained in DMEM (Gibco) media at 70°C. Details of the construction of NSE-lacZ-th vector have been recently described by Andersen et al. ('92). In addition to these lacZ-containing vectors, the non-lacZ-containing TK- mutant dlsptk (deletion mutant; (Coen et al., '89) was also evaluated. All viruses used for inoculation procedures were kept in 15% glycerol or tissue culture media (DMEM [Gibco] with 10% fetal calf serum, 100 μm/ml penicillin, and 100 μm/ml streptomycin) at -70°C prior to use. Titration to obtain an estimate of the number of plaque-forming units per milliliter (PFU/ml) was performed in Vero cells.

Fig. 1. Diagram of the herpes simplex virus (HSV) genome. Upper: The location of the thymidine kinase (TK) gene at map coordinate 0.3 is shown. Long diagonal lines connect to an expanded view of the 3.6 kb BamHI Q fragment encompassing the TK coding region and upstream regions with selected restriction endonuclease sites indicated. Short diagonal lines indicate the locations of the insertion mutations within (vectors NSE-lacZ-th, tkLTRZ1, and tkLTRdyn) and outside (vector RH116) the TK protein coding region. The ATG and TGA marked above the line represent the initiation and termination codons of the TK coding region; the ATG and TGA marked below the line represent the initiation and termination codons of the adjacent UL24 coding region. Lower: The location of the deletion mutation in mutant dlsptk is shown.
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Inoculation procedures

Male Swiss Webster mice 10–16 weeks of age (30–50 g) were anesthetized with 0.001 ml/g of a 1:1 solution of ketamine (100 mg/ml) and xylazine (20 mg/ml). The corneal surface was lightly scarified with a 20 gauge needle; the mystacial vibrissae on the same side were clipped and the surface of the vibrissal pad shaved, and then lightly scarified with an emory board or 25 gauge needle. RH116 \[4 \times 10^6\] plaque forming units (PFU); n = 14), tkLTRZ1 \[3.8 \times 10^6\] PFU; n = 26), and NSE-lacZ-tk \(1 \times 10^6\) PFU; n = 12) were inoculated onto these surfaces in volumes ranging from 30 to 100 \(\mu\)l. The nasal openings were lightly covered with absorbent towelling in order to prevent the inoculum from reaching the nasal mucosae. Control mice were either inoculated with TK\(^-\) vector that did not contain the lacZ gene \(\text{dis}^{\text{tk}, \text{lac}Z}\); n = 4) or underwent corneal scarification and snout shaving and scarification without inoculation of vector (n = 4). Mice were allowed to recover from the effects of anesthesia and housed individually in covered Nalgene cages in a BL-2 facility under a 12 hour light-day cycle with water and food ad libitum. Animals were observed for signs of illness or infection daily or every other day.

Histochemistry

At 4, 14, 30, and 60 days (d) following corneal and snout inoculation with vectors or control procedures, mice were deeply anesthetized with 0.001 ml/g of a 60 mg/ml solution of sodium pentobarbital and then intracardially perfused first with 0.9% normal saline (50 ml) at room temperature followed by 75 ml of 2% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.2--7.4 (PB). Both trigeminal ganglia were removed, postfixed for 1–2 hours in 2% paraformaldehyde in PB at 4°C and then incubated overnight at 37°C in 1% 5-bromo-4-chloro-3-indolyl-beta-galactoside (X-Gal) in a solution containing 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, and 2 mM magnesium chloride in PB (Price and Cepko, '87). Histochemically treated ganglia were placed in 30% sucrose in PB at 4°C for 24 hours and sectioned in the coronal plane at 10–40 \(\mu\)m on a cryostat. Serial sagittal sections (40 \(\mu\)m) from the trigeminal ganglion of two additional animals (4d postinoculation) were used to examine the range of diameters of labeled neurons, or to calculate a correction factor for estimating neuronal cell counts (Abercrombie, '46; Burstein et al., '90; see below). Sections were mounted directly onto prepared glass slides (Fisher, Superfrost Plus) and then air-dried at room temperature. Slide-mounted sections were rehydrated in phosphate-buffered saline (PBS) for 2 minutes, H\(_2\)O for 1 minute and transferred to Neutral Red in acetic buffer (pH 4.5) for 3 minutes and then to H\(_2\)O for 30 seconds prior to being coverslipped with Crystal Mount (Biomeda, Inc.).

Permanently mounted sections were examined under brightfield in a light microscope. Trigeminal ganglion tissue was examined for the presence of infection, (neuronal cell swelling, chromatolysis, cytoplasmic vacuolation), inflammation (lymphocytic or plasma cell infiltrate), or degeneration of satellite cells (residual nodules of Nageotte; Mazza and Dixon, '72; Adams et al., '84; Yamamoto et al., '84). Cells positively stained for \(\beta\)-galactosidase (blue reaction product) were examined for neuronal morphology by comparison with adjacent trigeminal ganglion neurons counterstained with Neutral Red. Criteria for identifying labeled cells as trigeminal ganglion neurons included: a typical rounded or ellipsoid shape, size range 15–50 \(\mu\)m diameter (Sommier et al., '85), a visible neuronal nucleus or nucleolus, and the presence of perineuronal satellite cells. The number of labeled neuronal profiles present in all sections obtained from each animal was counted and classified according to staining pattern. In one representative case, a reconstruction of the distribution of labeled trigeminal ganglion neurons, observed 4 days after inoculation with vector tkLTRZ1, was performed with the assistance of a drawing tube attached to a light microscope.

Immunocytochemistry

In two additional animals inoculated with vector tkLTRZ1, trigeminal ganglia were analyzed immunocytochemically (with a polyclonal rabbit anti-\(E.\ coli \beta\)-galactosidase antibody (5 Prime to 3 Prime, Inc.) at 4d for the presence of \(\beta\)-galactosidase protein product. After removal of ganglia as above, coronal sections were cut (20 \(\mu\)m), mounted onto prepared slides (Fisher, Superfrost Plus), and kept at −21°C prior to immunocytochemical processing. Slide-mounted sections were thawed to room temperature, and treated with 0.3% H\(_2\)O\(_2\) and 50% methanol in PB for 20 minutes, followed by 10 × 3 minute washes in PB. Sections were then incubated with 10% goat serum in PB for 60 minutes prior to incubation in primary antisera (diluted 1:1,000) overnight at room temperature in a humidified chamber. The following day, sections were washed 10 × 1 minute in PB, 10 × 2 minutes in 5% blocking (goat) serum and then incubated for 60 minutes with secondary antibody (goat anti-rabbit; Vector Laboratories, Vectastain kit) in the humidified chamber. Sections were then washed 10 × 3 minutes in PB, followed by final incubation in avidin-biotin-peroxidase complex (Vector Laboratories, Vectastain kit) for 60 minutes. Ten 1 minute washes in PB were followed by 10 × 2 minute washes in 0.05 M Tris-buffered saline (TBS). The sections were incubated in diaminobenzidine (DAB) (5% in TBS) and 30% H\(_2\)O\(_2\)/10 ml DAB solution, dehydrated, coverslipped with Permount, and then examined by brightfield light microscopy for the presence of \(\beta\)-galactosidase-immunoreactive neurons.

In situ hybridization

Trigeminal ganglia from both sides were removed, as described above, 6d after corneal and snout inoculation with vector NSE-lacZ-tk. These trigeminal ganglia were postfixed for an additional 1–2 hours in 2% paraformaldehyde in PB, transferred to 30% sucrose in PB at 4°C overnight, and then sectioned in the coronal plane at 10 \(\mu\)m on a cryostat. Sections were mounted directly onto gelatin-coated slides prepared for in situ hybridization according to the method of Stroop et al. ('84). Hybridization was performed on sectioned tissue, as described by Stroop et al. ('84), using a \(^{35}\)S-labeled DNA probe (plasmid pPH; Leib et al., '89) containing a 1.4 kb fragment from within the LAT encoding region. This probe does not overlap transcripts of the adjacent immediate early gene (ICP0) (Leib et al., '89). After hybridization, tissue sections were dehydrated, dipped in Kodak NTB-2 liquid emulsion prior to development. After development, sections were counterstained with Giemsa, coverslipped with Permount, and examined by brightfield light microscopy for the presence of LAT-positive neurons.
Fluoro-Gold retrograde tracing

The fluorescent tracer Fluoro-Gold (Fluorochrome Inc.) was applied (20 μl of a 4% solution in 0.9% saline) to the scarified cornea and shaved/scarified snout of two animals. The ipsilateral and contralateral trigeminal ganglia were removed 4d after inoculation, postfixed in 2% paraformaldehyde in PB for 1–2 hours, and then placed in 30% sucrose in PB overnight prior to sectioning at 40 μm on a cryostat. Sections were mounted onto gelatin-coated slides and then dehydrated in Xylene for 5 minutes before being permanently mounted in DPX mounting medium (BDH Chemicals). Sections were examined for Fluoro-Gold labeling of neurons with fluorescence microscopy.

Data analysis

Quantification of labeled neurons was performed for sections treated with X-Gal as described above (see Histochemistry). Since neurons labeled might be counted more than once in overlapping sections, a correction factor (CF) was calculated and applied to the cell counts to compensate for this effect (Abercrombie, '46; Burstein et al., '90). This CF was obtained from a measurement (under 100× magnification) of the dorsoventral diameter of all X-Gal-labeled neurons (n = 9) containing a visible nucleus in sagittal sections of a trigeminal ganglion obtained 4 days after inoculation with tkLTRZ1. The mean of these cell diameters and the section thickness were used to calculate the CF (Abercrombie, '46; Burstein et al., '90); CF equals mean section (M) thickness divided by the mean section thickness plus the longitudinal diameter (LD) of the cell (CF = M/M + LD). The number of neurons present in a ganglion from any animal was estimated by multiplying the total number of neurons counted by the appropriate CF (calculated number of neurons = total neurons counted × CF). Since only small numbers of X-Gal-labeled neurons with a visible nucleus were found, the CF was also calculated by using the dorsoventral diameters of randomly selected, labeled neurons (n = 102) containing a visible nucleus in sagittal sections from trigeminal ganglia (n = 4) 4 days after inoculation of Fluoro-Gold onto the cornea and snout of mice.

Comparisons among the number of neurons labeled with each vector and among the different time points after inoculation were performed by using a one-factor analysis of variance (SuperANOVA, Abacus).

RESULTS

Histochemical labeling with X-Gal substrate

Light microscopy of histochemically processed coronal sections from the ipsilateral trigeminal ganglia of mice inoculated with lacZ-containing vectors (tkLTRZ1 (Fig. 2A–D), RH116, NSE-lacZ-th) revealed labeled neurons of varying size. In one representative case examined 4d following inoculation with vector tkLTRZ1, labeled neurons ranged from 8 to 46 μm in longitudinal diameter (Fig. 3). Labelling of non-neuronal cells was not observed in experimental animals at any time point tested. A benign infiltrate of cells with lymphocytic morphology, primarily localized to the border of ganglionic tissue, was occasionally observed in tissue sections from some mice (Fig. 2E). No evidence of degeneration or death of neurons, or satellite cells (residual nodules of Nageotte), was observed.

Four neuronal staining patterns were observed: 1) a dense diffuse blue stain that filled the cytoplasm (Fig. 2A–C); this staining pattern was also observed within single and branching peripheral processes that at times clearly originated from labeled neurons (Fig. 2C); 2) a lighter, speckled, diffuse blue stain (data not shown); 3) a granular, cytoplasmic staining pattern without (Fig. 2A) or with (Fig. 2D) a densely labeled nucleus; and 4) a light, speckled, diffuse stain, as above in the second pattern of staining, with granular cytoplasmic staining as well (data not shown). The third staining pattern occurred most frequently. The first staining pattern was rarely seen at time points beyond 4d, and nuclear labeling was rarely observed prior to 14d.

Within the trigeminal ganglion, labeled neurons were observed in a rostral distribution along the medial aspect and in the central region of the ganglion (Fig. 4). These regions of the trigeminal ganglion are represented primarily by neurons from the first and second divisions of the trigeminal nerve (Arvidson, '79; Bigotte and Olsson, '87; Kuwayama et al., '87; Margolis et al., '87).

When the trigeminal ganglion from the noninoculated side was examined, labeled neurons were only occasionally seen, possibly as a result of inadvertent inoculation of the contralateral snout by mice during normal face grooming behavior. When trigeminal ganglia from control mice were examined 4d after inoculation with vector dlspkt, or 4d after shaving and scarification alone, no labeled neurons were seen.

Labeled neurons were counted and the CF for section thickness (see Materials and Methods) was applied to obtain an estimate of the total number of labeled neurons per ganglion. The mean number of labeled neurons for each vector at each time point is shown in Table 1. Inoculation with vector tkLTRZ1 produced a significantly greater mean number of labeled neurons at 4d than inoculation with vectors RH116 or NSE-lacZ-th (F(2) = 5.686, P = 0.0136). Post hoc comparison revealed that the mean number of neurons labeled with tkLTRZ1 was significantly greater than the mean number of neurons labeled with RH116 (P = 0.0338) or NSE-lacZ-th (P = 0.0367) at this time point. There was no significant difference between the number of neurons labeled at 4d with vectors RH116 and NSE-lacZ-th. Since labeled neurons were not observed with either RH116 or NSE-lacZ-th at 14d after inoculation (Table 1), further evaluation was not done at 30 or 60d with these vectors. In contrast, with vector tkLTRZ1, neuronal labeling was observed at all time points, but the number of labeled neurons showed a gradual and significant (P(3) = 4.047, P < 0.0204) decline with time (Fig. 5). The analysis of results was not changed by the application of the CF obtained from dorsoventral diameters of Fluoro-Gold-labeled neurons in sagittal sections from trigeminal ganglia.

β-Galactosidase-like immunoreactivity

To confirm the presence and distribution of E. coli β-galactosidase in the trigeminal ganglion after corneal and snout inoculation with lacZ-containing vectors, sections from additional mice inoculated with tkLTRZ1 were processed for immunocytochemistry. Similar to the results with X-Gal histochemical staining, trigeminal ganglion neurons containing β-galactosidase-like immunoreactivity were observed in rostromedial and central regions of the trigeminal ganglion ipsilateral to the inoculation site. La-
Fig. 2. Brightfield photomicrographs of X-Gal-labeled neurons (A–D), or neurons showing positive hybridization signal with a DNA probe to latency-associated transcripts (LATs; E and F), in coronal sections from trigeminal ganglion obtained 4 days (X-Gal) or 6 days (LATs) after inoculation with vectors tkLTRZl and NSE-lacZ-B, respectively. A–D (counterstained with Neutral Red): Dense cytoplasmic labeling of neurons can be seen in A–C. Granular cytoplasmic labeling of neurons is also shown without (A; arrow) or with (D) nuclear labeling. A branching peripheral process, which originates from a densely labeled neuron, is seen in C. E, F (counterstained with Giemsa): Dense silver grain deposition is shown over the nuclei of neurons showing positive hybridization signal for LATs in E (long arrow). A mononuclear cell infiltrate can be observed along the margin of the ganglion in E (short arrow). Scale bars = 100 μm in A and E; 50 μm in B and C; 10 μm in D and F.

labeled neurons were not observed in sections obtained from the trigeminal ganglion contralateral to the inoculation site in the few animals examined here. The intracellular distribution of immunoreactivity resembled the first pattern of staining (see above, pattern 1) seen in trigeminal ganglia reacted with X-Gal; DAB reaction product completely filled the cytoplasm, partially or completely obscuring the nucleus (data not shown).
To confirm the presence of latent HSV in trigeminal ganglion neurons after corneal and snout inoculation, in situ hybridization to LATs was performed 6d after inoculation with vector NSE-lacZ-Δ4. Positive hybridization signal was observed over the nuclei of 3–4% of neurons in sections obtained from the trigeminal ganglion ipsilateral to the inoculation site (Fig. 2E,F). Although no direct comparison was made, labeled neurons were found in a distribution within the ganglion similar to that observed for β-galactosidase after inoculation with vector tkLTRZ1 and histochemical staining with X-Gal. Similar to the results with X-Gal staining, only occasional LAT-positive neurons were observed in sections obtained from the contralateral ganglion.

**Fluoro-Gold labeling**

The distribution of X-Gal-labeled neurons after inoculation of tkLTRZ1 was compared with the distribution of fluorescent-labeled neurons after inoculation of the retrograde tracer Fluoro-Gold onto the cornea and snout. With this technique, large numbers of labeled neurons were observed occupying the central and rostromedial regions of the trigeminal ganglion. In one experimental animal coinoculation of Fluoro-gold and vector RH116 resulted in the double labeling of several neurons within but not outside of the central and rostromedial trigeminal ganglion (data not shown). All X-Gal-labeled neurons observed were also Fluoro-Gold labeled.
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DISCUSSION

In these experiments, retrogradely transported TK- herpes virus vectors were used to deliver the gene lacZ to trigeminal ganglion neurons. Labeled neurons were of a size range comparable to that previously described for mouse trigeminal ganglion neurons (Sommer et al., '85), and showed several different patterns of intracellular labeling for β-galactosidase. The greatest number and longest duration of labeling was observed with a vector (tkLTRZ1) containing lacZ downstream of the MoMLV-LTR. The distribution of X-Gal-labeled neurons in trigeminal ganglion after inoculation of tkLTRZ1 was similar in location to neurons labeled with the retrograde tracer Fluoro-Gold. The significance of each of these points will be discussed below.

Why were different patterns of intracellular labeling observed in these neurons with X-Gal histochemistry?

Although a variety of intracellular labeling patterns were observed with X-Gal histochemistry, the predominant pattern was a granular cytoplasmic staining. This type of labeling has been suggested by Dobson et al. ('90) to be due to the association of histochemical reaction product with membrane-bound organelles. Since this pattern of intracellular labeling is similar to the distribution of lysosomal and nonlysosomal acid hydrolases observed in mouse sensory ganglion (Sommer et al., '85), granular cytoplasmic staining may represent histochemical reaction product that was being degraded by lysosomal or other cellular enzymes. Although the observed distribution of intracellular labeling could also have been the result of metabolism of X-Gal by endogenous lysosomal enzymes rather than by E. coli β-galactosidase (Zhang et al., '91), the inoculation of non-lacZ-containing virus (dlsptk) and scarring without inoculation, both of which might induce endogenous hydrolase activity, were not associated with neuronal labeling.

The other patterns of intracellular staining observed in these experiments may have reflected variable levels of expression of lacZ in trigeminal ganglion neurons. The presence of dense nuclear labeling in some neurons at 14d but not at 4d may have been the result of gradual accumulation and/or concentration of the histochemical reaction product within nuclei.

Why did vector tkLTRZ1 produce the largest number of labeled neurons and the longest duration of labeling?

The large number of neurons labeled, relative to the other vectors tested, and the presence of labeling for up to 60d with vector tkLTRZ1, were most likely effects of the MoMLV-LTR promoter (Price et al., '87; Dobson et al., '90) used in this vector. This strong promoter may be active in most cell types and should maximally drive the expression of a downstream foreign gene.

In contrast to the results obtained with vector tkLTRZ1, inoculation with vector RH116, which contains an HSV delayed-early promoter (β 8) of viral replication, resulted in small numbers of neurons being labeled at the earliest time point but not at subsequent time points. Labeling observed with this vector was likely an effect of the β 8 promoter, which may be able to drive expression of lacZ at low levels and for a short duration (Ho and Mocarski, '88). Longer duration expression is unlikely since promoters of lyric genes, such as β 8, should not be active during latency. Consistent with these results in the peripheral nervous system, Huang et al. ('92) and Andersen et al. ('92) have demonstrated only short-term labeling of neurons after inoculation of vector RH116 into the central nervous system.

Similar to the results obtained with vector RH116, vector NSE-lacZ-th, containing the NSE promoter (Forss-Petter et al., '90), labeled small numbers of neurons at the earliest, but not subsequent time points. Although this enzyme is expressed in most neurons, different levels of NSE promoter activity have been described in the nervous system. Frontal cortex and hippocampus reportedly demonstrate higher levels of promoter activity (as determined by reporter gene expression) than other regions of the nervous system (Forss-Petter, unpublished observations). In addition, radioimmunoassay methods have demonstrated low levels of NSE in the peripheral nervous system when compared with the central nervous system (Marangos and Schmechel, '87). The small number and limited duration of neuronal labeling with the NSE promoter in the present experiments may therefore reflect weak activity of this promoter in peripheral sensory neurons.

Although weak promoter function may have resulted in the absence of labeling at 14d after inoculation with vectors RH116 and NSE-lacZ-th, there may have been small amounts of E. coli β-galactosidase present that we could not detect with our histochemical methods.

Why did neuronal labeling seen with vector tkLTRZ1 decrease with time?

Although more labeled neurons were initially observed after inoculation with vector tkLTRZ1 than were seen with the other vectors tested, the number of labeled neurons gradually decreased over time, reaching lowest levels at 60d. This decline may have been the result of a gradual loss of promoter function, as has been observed with the LTR promoter (Palmer et al., '91) in some in vivo situations. Longer duration expression of lacZ has been described in an HSV vector containing the LTR promoter inserted into the HSV immediate-early gene ICP4 (Dobson et al., '90). However, this expression may have been partly driven by the adjacent HSV LAT promoter, which is active during latency (Dobson et al., '89). Alternatively, the site of insertion of the LAT promoter in vector tkLTRZ1, within the tk gene, may have resulted in a gradual loss of promoter function. For example, although the LAT promoter drives the expression of foreign genes in neurons (Margolis et al., '92), the use of a vector containing LAT-lacZ within the HSV glycoprotein G (gC) gene resulted in a gradual decrease in neuronal labeling (Sawtell and Thompson, '92). Therefore, the activity of a promoter used to drive the expression of a foreign gene may depend on its site of insertion within the HSV genome, and on its proximity to other adjacent promoter elements.

The gradual decrease in the number of labeled neurons after inoculation with vector tkLTRZ1 could have also been the result of degeneration or death of HSV-infected neurons in trigeminal ganglia. However, this TK- vector is known to be replication-defective in neurons, and its use in these experiments was not associated with signs of degeneration or death of trigeminal ganglion neurons at any time point after inoculation.
What accounts for the variability in the number of neurons labeled with vector tkLTRZ1?

The variability in expression of lacZ between animals seen with vector tkLTRZ1, which was greatest at 4d after inoculation, may have reflected variation in the efficacy of the MoMLV-LTR promoter during the early stages of infection. Other factors that might have contributed to the variability in lacZ expression between animals include: differences in the amount of eyelid and/or tear washing of the corneal surface after inoculation, differences in technique (e.g., different depth of incisions produced by needle scarification), and variation in the activity of the LTR promoter within subtypes of trigeminal ganglion neurons. Similar variation in the number of labeled neurons has been previously described by Dobson et al. ('90) in mouse dorsal root ganglia after sciatic nerve injection of a replication-defective, lacZ-containing HSV vector.

Why was labeling with HSV vectors observed in only in a small proportion of trigeminal ganglion neurons?

The small numbers of neurons labeled in comparison with the total number of neurons in trigeminal ganglion may have been due to the absence of replication and consequent spread of these TK− HSV vectors within trigeminal pathways. Coen et al. ('89) have shown that titters of TK− HSV in trigeminal ganglia are at least fourfold lower than titters of wild type HSV 3d after corneal inoculations in the mouse. The titters of TK− HSV at the corneal surface in these experiments were also much lower than wild type HSV 3d after infection, possibly resulting in reduced seeding of sensory neuron terminals by HSV. The regulation of viral entry into neurons, which involves the interaction of viral glycoproteins and sensory afferent terminals supplying the cornea and snout, may have been another factor limiting the number of viral particles delivered to trigeminal ganglion neurons.

What is the significance of the distribution of neurons labeled with lacZ-containing TK− HSV vectors?

In the present experiments, rostromedial labeling of neurons in trigeminal ganglion was observed with X-Gal have been due to the absence of replication and consequent spread of these TK− HSV vectors. A similar distribution of neuronal labeling was observed after inoculation of the retrograde tracer Fluoro-Gold onto these same facial regions. This distribution is consistent with the results of experiments in which replication-competent HSV and the retrograde tracer wheat germ agglutinin (WGA) (Margolis et al., '87), or horseradish peroxidase (HRP) (Arvidson, '79), were injected into the anterior chamber of the mouse eye. Degeneration of trigeminal ganglion cells in an anteromedial distribution was also observed by Bigotte and Olsson ('87) after injection of the toxin doxorubicin into the mouse cheek. The similarity between the results of these retrograde tracing and degeneration studies, and the distribution of X-Gal and Fluoro-Gold labeling in the present experiments, is evidence in favor of the specific uptake and axonal transport of both TK− HSV and Fluoro-Gold by neurons supplying the ophthalmic and maxillary divisions of the trigeminal nerve. The large number of neurons labeled with Fluoro-Gold in comparison with vector inoculation and histochemistry with X-Gal is consistent with known efficacy of this substrate as a retrograde axonal tracer (Burstein et al., '90). The results obtained with the TK− HSV vectors used in these experiments is evidence that replication-defective HSV mutants can be used to deliver functional genes to trigeminal ganglion neurons in an anatomically predictable manner.

CONCLUSIONS

These data demonstrate that TK− HSV vectors can be used to deliver the reporter gene, lacZ, to mouse trigeminal ganglion neurons in vivo. Although small numbers of trigeminal ganglion neurons were labeled overall, the largest number and longest duration of labeling was observed with a TK− vector containing lacZ downstream of the Moloney murine leukemia virus promoter. The distribution of labeled neurons was within regions of the trigeminal ganglion innervating the cornea and snout. Therefore, similarly constructed vectors may provide a method for delivery of functional genes to anatomic subsets of sensory neurons. Such genes might include toxic or killing genes, which would permit the development of experimental models of painful neuropathy (e.g., postherpetic neuralgia).

Alternatively, such vectors could be used to deliver "analgesic" neuropeptides, such as the genes for enkephalin or dynorphin, to sensory neurons in an effort to change the physiology of these neurons.

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LITERATURE CITED


