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Targeting and gene expression in spinal cord motor neurons following intramuscular inoculation of an HSV-1 vector

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One problem in devising strategies of gene transfer to the nervous system is targeting specific neuronal populations. To evaluate the potential for using herpes simplex virus (HSV) as a vector for gene transfer to spinal cord motor neurons, the HSV-1 mutant LAT-LTR in which the E. coli β-galactosidase gene is expressed under control of the HSV LAT core promoter (LAT) and the Moloney murine leukemia virus long terminal repeat (LTR) was inoculated unilaterally into the gastrocnemius muscle. Infectious virus was isolated from the spinal cord on days 3–7 post inoculation (PI). Immunocytochemical labeling of HSV antigen was detected in ipsilateral ventral horn neurons in the spinal cord at day 3 PI and had spread to contiguous spinal cord regions by day 6 PI. No viral antigen was detected at 14 or 28 DPI. β-galactosidase expression (driven by the LAT-LTR promoter) was detected in neurons of the ventral horn on days 3, 6, 14, and 28 PI. Histological analysis showed mild lesions in the ventral horn on day 3 PI which progressed through days 6, 14 and 28 PI. This study demonstrates the feasibility of gene delivery into spinal motor neurons after injection of an HSV vector at a peripheral muscular site. This approach should prove useful in neurobiological investigations and it suggests a possible application to development of gene therapy for heritable diseases affecting motor neurons.

Keywords: herpesviridae; genetic vectors; gene expression; motor neurons

Introduction

The development of methods for the introduction and expression of genes in the central nervous system (CNS) is important because of the potential for functional studies in neurons and for gene therapy of neurological disorders. Goals for gene transfer to the CNS include the selected targeting of specific neuronal subpopulations and stable, long term expression in post mitotic neurons.

Herpes simplex virus (HSV) is a candidate viral vector for gene transfer to the nervous system (for reviews see, Kennedy and Steiner, 1993, Glorioso *et al*, 1994). It is neurotropic (Hill, 1985), has a large genome which can carry large amounts of foreign DNA (Knipe *et al*, 1978), and can establish a latent infection in neurons (Stevens *et al*, 1971; McLennan and Darby, 1980; Kennedy *et al*, 1983). During latency, expression of the viral genome is limited to the latency associated transcripts (LATs) (Deatly *et al*, 1987; Stevens *et al*, 1987; Spivack and Fraser, 1987; Wagner *et al*, 1988; Mitchell *et al*, 1990, Fraser *et al*, 1992). Since the LATs accumulate in the nucleus of latently infected neurons in both mice and humans (Stevens *et al*, 1987; Croen *et al*, 1987; Deatly *et al*, 1987; Steiner *et al*, 1988) it has been assumed that the LAT promoter remains active over a long period. While the classic latent HSV infection occurs in ganglionic sensory neurons, HSV DNA and LAT RNA have been detected in the brain (Fink *et al*, 1992) and in one study LAT RNA was detected in sensory neurons of the CNS (Deatly *et al*, 1988). The HSV LAT promoter may thus permit long term expression of genes in CNS neurons.

Recently, an HSV vector containing a chimera of the LAT core promoter of HSV and the long terminal repeat (LTR) of the Moloney murine leukemia

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virus has been shown to be transcriptionally active during the latent stage of infection in dorsal root ganglia (DRG) neurons of mice (Lokensgard *et al*, 1994).

The aims of the present study were to investigate whether the HSV-1 LAT-LTR vector could be used to achieve transfer and expression of genes in spinal cord motor neurons. These motor neurons, like DRG neurons, have axons which terminate in peripheral tissues. Viral uptake at axonal terminals in skeletal muscle, and subsequent retrograde axonal transport can target the innervating motor neurons in the spinal cord (Rotto-Percelay *et al*, 1992). In earlier studies, we have found that following intracerebral inoculation, HSV LAT expression can be detected in latently infected lower motor neurons of the spinal cord and brainstem (JR Martin, unpublished). Thus transfer and expression of foreign genes in motor neurons after peripheral inoculation with an HSV vector appeared possible. This strategy could prove useful for neurobiological investigations and perhaps also for the treatment of neurological diseases in which motor neurons are affected.

Here, an HSV vector that has been shown to direct long term expression of a foreign gene in sensory neurons of the DRG (Lokensgard *et al*, 1994) was injected at a peripheral intramuscular site; expression of a β-galactosidase reporter gene was examined to determine whether this approach may be used to target spinal motor neurons. We show that this vector was transported to motor neurons in the spinal cord following intramuscular injection and that a foreign reporter gene was expressed in spinal motor neurons of the ventral horn.

Results

Seventy-five mice were inoculated intramuscularly. Clinical signs of infection were first evident on day 5 PI (post inoculation) and consisted of ruffled fur and right hind limb weakness. While the majority of animals recovered and appeared healthy at the time of sacrifice, four developed hind limb paralysis and died. All deaths occurred between days 10–14 PI. Three of 15 mice with corneal inoculations died.

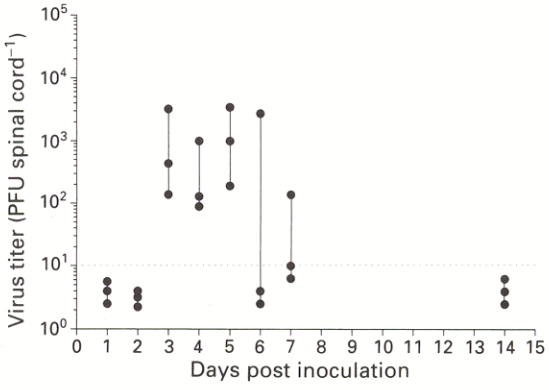

Figure 1 Titers of HSV-1 LAT-LTR in spinal cord after intramuscular inoculation.

Virus isolation

HSV-1 was detected in gastrocnemius muscle samples from the vicinity of the injection site at days 1–6 PI. The titer was highest on day 1 PI (10^4 PFU sample^{-1}) and declined thereafter until day 6 after which no virus could be isolated (data not shown). Virus was recovered from the ipsilateral sciatic nerve in two animals at day 3 PI and in one animal at day 5 PI with a titer ranging from 10^1 to 10^2 PFU sample^{-1}. Virus was not isolated from the sciatic nerves of any animals on days 1, 2, 4, 6, 7 or 14 PI. Virus was found in the spinal cord on day 3 PI and was last isolated on day 7 PI (Figure 1). The peak for virus isolation was observed on days 3–5 PI, with titers ranging from 10^3 to 10^4 PFU sample^{-1}. No virus was recovered from the spinal cord at day 14 PI.

Viral antigen localization

At day 3 PI, HSV-1 antigen was detected in large (approximately 30–50 μm in diameter) round and pyramidal shaped cells and their dendritic and axonal processes in the ventral horn gray matter on the inoculated side (Figure 2A and B). These cells were similar in size, location and configuration to motor neurons identified on adjacent H&E stained sections. Other slightly smaller round cells in the same area, which were similar in size to neuronal

Table 1 Presence of viral antigen and lesions in the spinal cords of mice inoculated intramuscularly with HSV-1 strain LAT-LTR

Days post infection	*Lesions*	*HSV-1 antigen*
3	5/5	5/5
6	5/5	4/5
14	3/3	0/3
28	3/3	0/3

The numerator is the number of animals which were positive and the denominator is the number of animals which were tested

Table 2 β-galactosidase expression in neurons in the ventral horn of the spinal cord following peripheral inoculation with HSV-1 LAT-LTR

Animal number	*Days post infection*			
	3	*6*	*14*	*28*
1	40	54	1	0
2	18	5	3	5
3	8	33	7	1
4	18	22	0	0
5	18	75	58	2

The numbers represent the total number of positive neurons, for each animal, in 150 sections of the ventral horn of the spinal cord

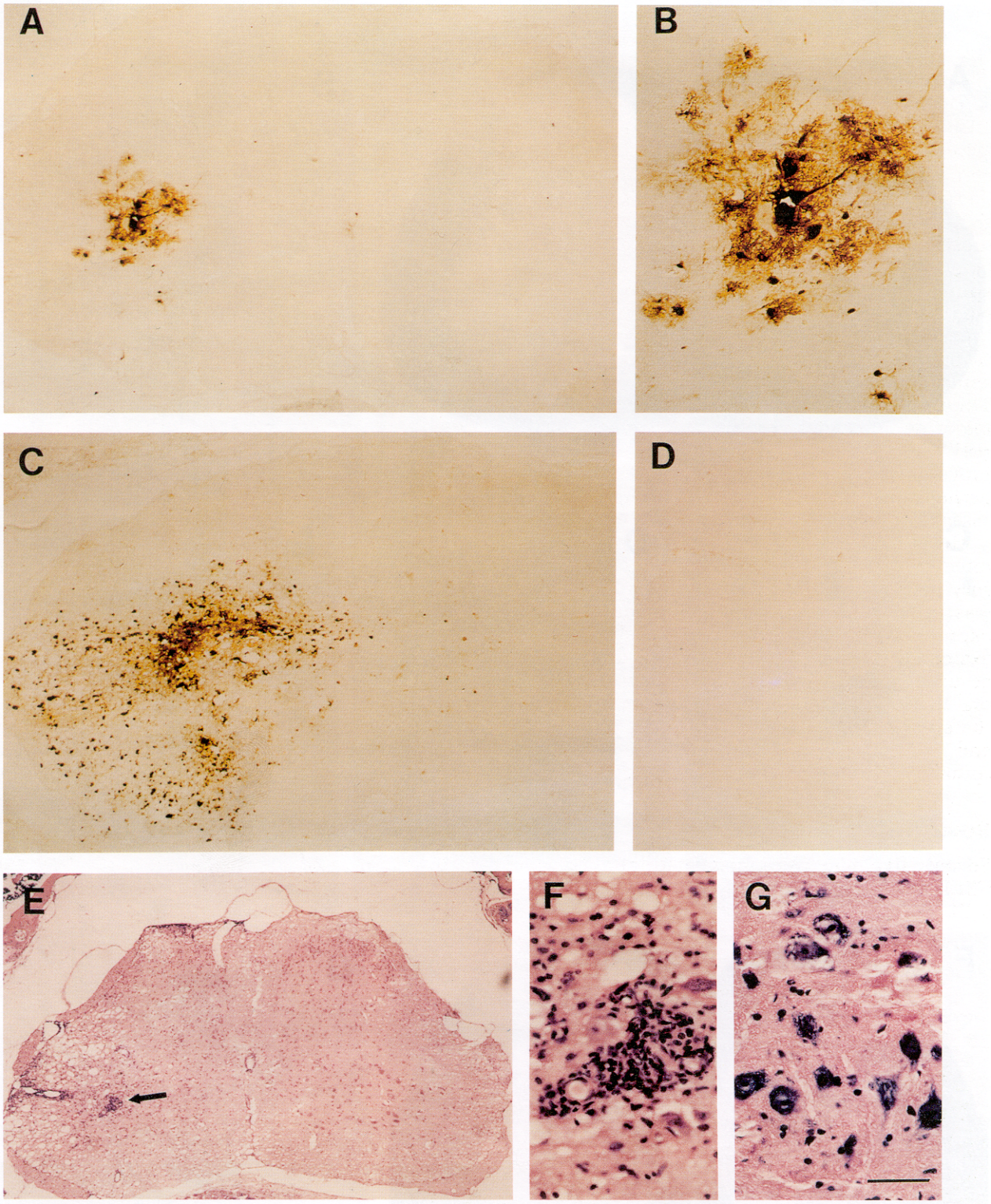

Figure 2 Viral antigen and histological lesions in transverse sections of the lumbar spinal cords of mice inoculated with the HSV-1 mutant LAT/LTR. (A) HSV antigen in ventral horn, 3 days following ipsilateral gastrocnemius muscle inoculation. Note that ipsilateral dorsal horn, top, and contralateral gray matter are antigen-negative. (B) Higher magnification of region shown in (A). Note two large and several smaller antigen-containing motor neurons, including labeled neuronal processes. (C) HSV antigen in spinal cord, 6 days PI. Note that antigen is present in larger numbers of cells, including motor neurons of ventral horn. (D) Ventral horn of lumbar spinal cord, uninfected control mouse. (E) Histological lesion in lumbar spinal cord ipsilateral to IM inoculation of HSV, 28 days PI (H&E stain). Arrow indicates focal inflammation in the ventral horn; motor neurons are much reduced in number (also shown in F), compared to the contralateral side. Note also that the adjacent lateral column white matter appears vacuolated, and that the dorsal root entry zone contains focal inflammation. (F) Higher magnification of region shown at arrow in E. Note lymphocytes and reduced numbers of remaining neurons. (G) Motor neurons in ventral horn, uninfected control mouse. The bar shown in Figure 2G corresponds to: 400 μm for Figures 2A, 2C and 2D; 160 μm for Figure 2B; 630 μm for Figure 2E; 100 μm for Figures 2F and 2G.

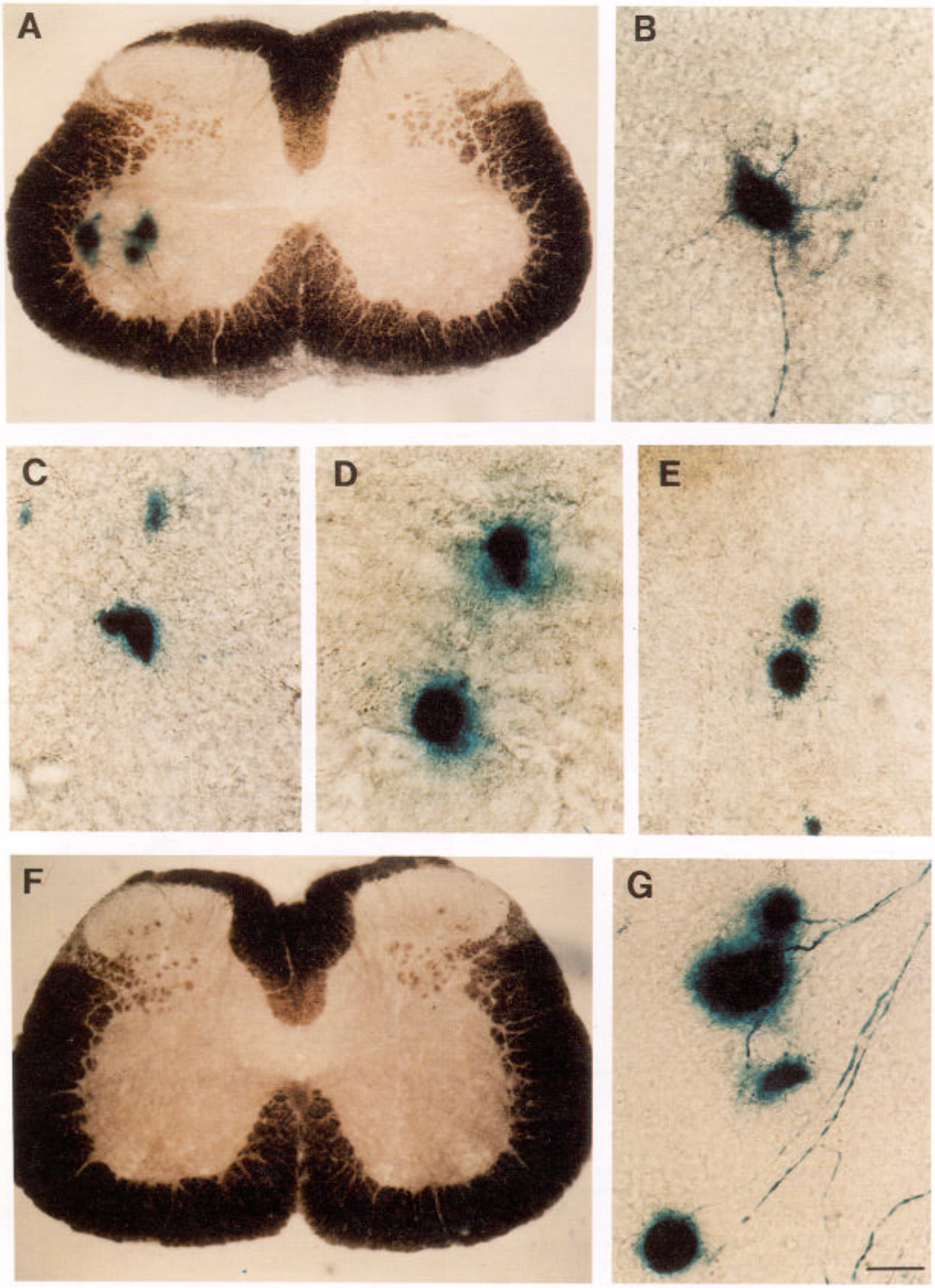
A
B
C
D
E
F
G

Table 3 β-galactosidase expression in sensory neurons of the trigeminal ganglia following corneal inoculation with HSV-1 LAT-LTR

Animal number	*Days post infection*			
	3	*6*	*14*	*28*
1	12	44	32	33
2	3	90	28	26
3	17	32	35	13

The numbers represent total numbers of positive neurons in two trigeminal ganglia from each animal

nuclei identified on H&E, were also labeled. In some animals antigen labeling in small round or oval structures was seen in white matter near the ventral horn gray matter; these cells were similar in size, configuration and location to glial cells observed in H&E sections.

On day 6, viral antigen labeling was similar to that observed on day 3; however virus spread was detected in one animal with labeled cells resembling neurons present in both the dorsal horn gray matter on the ipsilateral side and in the gray matter on the contralateral side (Figure 2C). This corresponded to areas of inflammation seen in the same animal on the adjacent H&E section. Viral antigen was not detected in any animals at days 14 or 28 PI (Table 1). Uninfected control mice and infected mice reacted with pre-immune serum were always negative (Figure 2D).

Detection of β-galactosidase

At day 3 PI, β-galactosidase labeling was seen in both the nucleus and cytoplasm of large round or pyramidal shaped cells in the gray matter of the ventral horn. These cells were similar to the cells in which viral antigen was immunolocalized. The label was distributed in the neuronal nucleus, cytoplasm and dendritic and axonal processes (Figure 3A and B). Positive cells were 30–50 μm in diameter and their labeled processes could be traced for 60–220 μm in a section. Groups of labeled cells were frequently observed. In addition, label was seen in isolated structures, 1–3 μm in diameter and up to 200 μm in length which appeared similar to neuronal processes. Eight to 40 positive neurons per animal were observed in the ventral horn gray matter at day 3 PI (Table 2).

By day 6 PI the labeling pattern was more complex. Labeled cells were seen in the gray matter of both the ventral and dorsal horns on the inoculated side and also, but to a lesser extent in contralateral gray matter. As with viral antigen, β-galactosidase labeling was also seen in small round or oval cells in the white matter.

At later time points the label appeared to be chiefly found in cell nuclei making identification of cells more difficult. For days 6, 14 and 28 those cells with a nucleus > 10 μm and located in the ventral horn were counted (Figure 3C, D and E). This nuclear size corresponds to that of motor neurons in the ventral horn, as measured in H&E sections and as reported by others (Rotto-Percelay *et al*, 1992). Smaller structures < 10 μm in diameter were present in the ventral and dorsal horn associated with motor and sensory root entry zones and in dorsal and ventral white matter columns. These were difficult to identify and were not quantitated but were thought to include glial cells, very small neurons and axonal processes.

At days 14 and 28 PI β-galactosidase containing neurons were found in the ventral horn despite the absence of viral antigen or infectious virus; however the numbers of positive neurons were greatly reduced compared with earlier times. Occasional labeled neurons were found in the dorsal horn on the inoculated side but these were excluded from the counts. A few small unidentified labeled structures were present elsewhere in gray and white matter. No labeling was observed in any sections from uninfected mice which were assayed in the same experiments (Figure 3F).

As a positive control for β-galactosidase activity, sensory neurons of the trigeminal ganglia were examined after corneal inoculations with the HSV vector LAT/LTR. β-galactosidase was detected on day 3 PI and was still detectable at day 28 PI (Table 3). The maximum number of β-galactosidase positive neurons were present at day 6 PI (Figure 3G). Expression was detected in fewer neurons on days 14 and 28 PI than on day 6.

Histopathology

Sections adjacent to those labeled for viral antigen were stained with hematoxylin and eosin and examined for lesions. At day 3 PI scattered necrotic neurons with pale eosinophilic, or fragmented cytoplasm and pyknotic nuclei were seen in ventral horns ipsilateral to the inoculation site in some animals. Neutrophils, scattered and in small aggregates and a few activated microglia were seen in the ventral horn gray matter on the inoculated side of all mice.

On day 6 PI necrosis and inflammatory infiltrates were present in the ipsilateral ventral horn, the surrounding white matter and in the gray matter of the

Figure 3 β-galactosidase labeling in lumbar spinal cords and trigeminal ganglia following peripheral inoculation of HSV-1 LAT/LTR. (A) β-galactosidase reaction product in three motor neurons of ventral horn, ipsilateral to gastrocnemius muscle inoculation; 3 days PI. (B) Labeled motor neuron in ventral horn. Note labeling in nucleus, cytoplasm and processes; 3 days PI. Labeled neurons were likewise found in ventral horns on days 6 (C), 14 (D) and 28 (E) post inoculation, ipsilateral to the inoculated gastrocnemius muscle. (F) Lumbar spinal cord section, uninfected control mouse. (G) Labeled neurons in trigeminal ganglion of positive control mouse, 6 days after corneal inoculation of HSV-1 vector. The bar shown in Figure 3G corresponds to: 100 μm for Figures 3A and 3F; 35 μm for Figure 3B; 22 μm for Figures 3C, 3D and 3E; 50 μm for Figure 3G.

contralateral ventral horn and ipsilateral dorsal horn. On day 14 PI mild lesions consisting of neutrophils, macrophages, activated microglia and lymphocytes were present in the ventral horns and also in the adjacent white matter and dorsal horns.

By day 28 PI lesions contained moderate to severe multifocal inflammatory infiltrates in the ipsilateral ventral gray matter (Figure 2E and F). Inflammatory foci were present in the white matter and sometimes extended into the contralateral gray matter; the inflammatory cells consisted mostly of lymphocytes and macrophages. In many sections, neuron numbers were visibly reduced in the ventral gray matter on the inoculated side (Figure 2F). In one animal the architecture of the spinal cord including the ventral gray matter and extending into the white matter had undergone necrosis and the tissue was cavitated. No evidence of inflammation was seen in uninfected control animals (Figure 2G).

Discussion

The conclusions of this study are primarily twofold. First, it is possible to target spinal cord neurons by a peripheral inoculation route with an HSV-1 vector. Second, expression of a foreign gene from the LAT-LTR promoter can be achieved in a limited number of spinal cord neurons for at least 1 month. In addition, the finding by Lokensgard *et al* that the LAT/LTR vector promotes long term gene expression in sensory neurons of lumbar dorsal root ganglia were extended to trigeminal ganglia.

Upon peripheral inoculation into the gastrocnemius muscle, HSV-1 LAT-LTR was isolated from the muscle, sciatic nerve and spinal cord. In the spinal cord the virus was located within motor neurons of the medial gastrocnemius motor column, as detected by immunolocalization of viral antigen, and expression of β-galactosidase from the LAT-LTR promoter. These findings are consistent with the hypothesis that HSV is taken up at axonal terminals with subsequent retrograde axoplasmic transport of the virus within the sciatic nerve to the cell bodies of the innervating motor neurons (Ugolini, 1992).

The number of neurons expressing β-galactosidase from the LAT-LTR promoter was greatest during the acute phase of infection (days 3 and 6 PI). While the number of cells expressing β-galactosidase markedly decreased at later time points (days 14 and 28 PI), it was still detectable after the acute infection had been cleared suggesting that the LAT-LTR promoter is capable of providing expression in at least some neurons of the ventral horn of the spinal cord for 28 days. The decrease in the number of labeled cells may be due to the destruction of target cells by virus induced cell lysis or destruction of neurons by the host inflammatory response. The presence of substantial lesions including neuronal loss in the ipsilateral ventral horn at 28 DPI, would be consistent with this explanation. It is also possible that down-regulation of the promoter in this population of neurons may also contribute to the decrease in β-galactosidase expression after the acute infection is cleared.

The presence of lesions within the spinal cord suggests that the virulence of the LAT/LTR HSV vector will limit its utility in achieving long term expression in motor neurons and that modifications of this model will be necessary. Several strategies have been tried to reduce the pathogenesis associated with HSV. We have inoculated KOS-1, an HSV vector derived from the relatively avirulent KOS laboratory strain (Margolis *et al*, 1992), into the gastrocnemius muscle. Similar titers of innoculum resulted in 10–100 fold less virus isolated from the spinal cord, and detection of only occasional β-galactosidase positive neurons (S Keir, unpublished observations). Another strategy includes the use of the HSV-1 amplicon, a defective virus genome assembled in virons whose proteins are derived from a helper virus (Spaete and Frenkel, 1982). However significant mortality levels and pathology have been reported for such vectors (During *et al*, 1994; Wood *et al*, 1994) probably due to the presence of wild type helper virus. It may also be possible to use replication incompetent viruses (Glorioso *et al*, 1994) which are deleted in one or more of the genes essential for herpes virus replication.

In HSV vectors a number of cellular and viral promoters have been used to direct gene expression. Cellular promoters include those for neuronal specific enolase, neurofilament (Glorioso *et al*, 1994) murine metallothionein and phosphoglycerol kinase (Lokensgard *et al*, 1994). Expression using these promoters has been transient, although long term, site specific expression has been reported with the preproenkephalin promoter (Kaplitt *et al*, 1994). Viral promoters used include the human cytomegalovirus major immediate early promoter (Glorioso *et al*, 1992), the Moloney murine leukemia virus long terminal repeat (Dobson *et al*, 1990; Lokensgard *et al*, 1994) and promoters from the LAT (Wolfe *et al*, 1992; Lokensgard *et al*, 1994), immediate early (Ho and Mocarski, 1988), early (Geller and Breakefield, 1988) and late (Fink *et al*, 1992) class genes of HSV. While CNS expression in most causes has been transient, long term expression has been reported in the brainstem of mice using the LAT promoter (Wolfe *et al*, 1992) and recently in the rat striatum using immediate early promoters of HSV in an HSV amplicon (During *et al*, 1994).

In these experiments we showed that after IM inoculation of an HSV vector the LAT-LTR promoter yielded gene expression in neurons of the spinal cord for up to 28 DPI. While refinement of the model is needed to reduce CNS pathology and increase the level of long term gene expression, it should prove useful in evaluating gene expression

in motor neurons and could be important in developing strategies for gene therapy of neurological disorders involving motor neurons.

Materials and methods

Virus stocks and animal inoculations

Viral stocks were replicated in Vero cells which were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum and virus stocks were prepared as described previously (Mitchell and Martin, 1992). Viral stock titers of 7.5×10^7 PFU ml^{-1} for the HSV-1 mutant LAT/LTR (Lokensgard *et al*, 1994) were used in all experiments.

Mice were 6-week-old, female, inbred Balb/C strain. Unilateral intra-muscular (IM) inoculations were carried out under Metofane anesthesia by a modification of a previously described method (Henken and Martin, 1991). The hind limb was shaved and extended and 50 µl (3.75×10^6 PFU) of virus stock or 50 µl of saline was injected into the gastrocnemius muscle, using a 1 ml syringe and a 26 gauge needle.

For studies in the trigeminal ganglia, which served as a positive control for detection of β-galactosidase, the corneal inoculations were performed as described previously (Mitchell *et al*, 1994). Each animal received either 10 µl (7.5×10^5 PFU) of virus, or 10 µl of MEM on each cornea following corneal scarification.

Virus titration

Virus titers were determined after IM inoculation, on days 1–7 and on day 14 PI, by standard plaque assay. Three animals at each time were sacrificed by CO_2 overdose, and gastrocnemius muscle, sciatic nerve and spinal cord were removed, snap frozen and stored at –70°C. Tissues were subsequently homogenized using a Tissumizer (Tekmar, Cincinnati, OH) in 1 ml media and serial 10-fold dilutions were plated onto Vero cells, overlaid with media containing 2% methylcellulose, and incubated at 37°C for 4 days. Cells were stained with cresyl violet and assayed for plaque formation.

Histopathology and immunocytochemistry

On days 3, 6, 14 and 18 PI mice were sacrificed and fixed by cardiac perfusion with 5% formalin. The spine was removed and post-fixed for 5 days in the same solution, rinsed in PBS and decalcified in a saturated solution of EDTA for 5 days (Henken and Martin, 1991). The portion of the spine containing the lumbar enlargement of each spinal cord (including lumbar segments 4 and 5 and their associated root entry zones) was cut transversely into four segments of approximately 1 mm length. These were embedded in the same paraffin block to obtain transverse sections of the spinal cord. Seven µm sections were cut (two per slide) and mounted on silanated slides (Digene Diagnostics, Beltsville, MD). Hematoxylin and eosin stained sections were evaluated by light microscopy for the presence of lesions, while adjacent deparaffinized sections were stained for viral antigen by the avidin-biotin-peroxidase method (Vector, Burlingame, CA) using HSV-1 immunoglobulin (Dako, Carpinteria, CA) at a 1:1000 dilution (Martin *et al*, 1988; Mitchell and Martin, 1992). Negative controls consisted of non-HSV infected animals and sections from HSV infected animals treated with non-immune rabbit Ig in the same assays.

β-galactosidase histochemistry

Sections of spinal cords and trigeminal ganglia were assayed for β-galactosidase activity as described previously (Mitchell *et al*, 1994). Animals inoculated as described above were sacrificed and the spinal cord (from IM inoculations) or trigeminal ganglia (from corneal inoculations) were removed, snap frozen and stored at –70°C. Before sectioning 1 mm cross-sections of spinal cord from the lumbar region were sliced and embedded in Tissue-Tek O.C.T. embedding medium (Miles Inc, Elkhart, IN). Serial frozen sections, 40 µm thick were cut through the spinal cord samples (approx. 150 sections/animal) and post-fixed with 4% paraformaldehyde, washed in phosphate buffered saline and incubated overnight in the substrate solution at 37°C. Trigeminal ganglia were embedded and processed as above. The substrate solution consisted of 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal, Life Technologies, Gaithersburg, MD), 20mM-potassium ferrocyanide, 20mM-potassium ferrocyanide, 2mM-$MgCl_2$, 120 µl of 10% Nonidet P-40, 200 µl of 1% sodium deoxycholate per 20 ml.

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