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Cyclosporin A reduces the inflammatory response to a multi-mutant herpes simplex virus type-1 leading to improved transgene expression in sympathetic preganglionic neurons in hamsters

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Herpes simplex virus type 1 (HSV-1) based vectors hold great promise for gene transfer to CNS neurons. Problems such as loss of transgene expression, vector-associated cytotoxicity and the immune response to the vector or encoded transgene still remain obstacles to success. We used a replication-defective, HSV-1 vector (14HΔ3vhsZ) that was engineered to have reduced cytotoxicity and express recombinant β-galactosidase. A previous study in our laboratory showed no evidence for cytotoxicity in infected neurons although an inflammatory infiltrate occurred around infected cells and transgene expression was lost between 5 and 8 days. The immune response consisted of a primary response at the site of inoculation (adrenal gland), and a secondary immune response in the spinal cord around infected adrenal sympathetic preganglionic neurons due to retrograde transport of the vector. We tested whether conventional immunosuppressants could reduce the secondary immune response, leading to improved transgene expression at the secondary CNS site. 14HΔ3vhsZ was injected into the adrenal gland in hamsters 1 day after immunosuppressant treatment began. Non-drug treated, 14HΔ3vhzZ-infected hamsters were used as controls. Cyclosporin A administration led to the most persistent β-galactosidase activity in neurons at 5 and 8 days. Methylprednisolone treatment resulted in the greatest reduction in the inflammatory cell infiltrate but the numbers of infected neurons did not increase concomitantly. This suggested no direct relationship between extent of the inflammatory cell infiltrate and level of transgene expression. These data demonstrate the potential of cyclosporin A as an immunosuppressant adjunct treatment for HSV-1 vector-mediated gene transfer from a peripheral site to neurons in the spinal cord.

Keywords: cyclosporin A; inflammation; methylprednisolone; recombinant herpes simplex virus type 1; spinal cord; transgene expression

Introduction

Recombinant herpes simplex type 1 viruses (rHSV-1) have elicited considerable interest for their potential as a gene transfer vector to neurons of the central nervous system (CNS). HSV-1 has many features well suited for this task. The genetic organisation of this large human virus and the properties of replication are well characterised. Large portions of the HSV genome (up to 30 kb) can be replaced with genes of interest (Huard *et al*, 1997). HSV-1 is naturally neurotropic, able to infect a wide range of mitotic and postmitotic cells, and maintain a lifelong latent or 'quiescent' infection in sensory neurons (Fink *et al*, 1996; Glorioso *et al*, 1995a, b; Kennedy and Steiner, 1993). Moreover, HSV and replication defective rHSV, with the

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appropriate helper cell lines, can generate the high titer viral stocks ($>10^8$ p.f.u.) necessary to deliver a gene efficiently and specifically to a large number of target neurons (Fink *et al*, 1996; Glorioso *et al*, 1995a, b). Herpes viruses have been used successfully by several investigators to express potentially therapeutic or reporter genes in the mammalian brain (Bloom *et al*, 1995; Wolfe *et al*, 1996; Glorioso *et al*, 1994) and spinal cord (Hong *et al*, 1995; LeVatte *et al*, 1997b; Mabon *et al*, 1997). The goal of HSV-1 vector systems is to permit short or long term regulated gene expression at therapeutic levels in the absence of immune or virus-mediated cytotoxicity. Presently, however, these criteria have not been satisfied fully. The immunological constraints, in particular, remain a significant obstacle to success.

Sympathetic preganglionic neurons (SPNs) are the last site for integration of synaptic inputs that regulate systemic arterial pressure. Ultimately, gene transfer to SPNs may allow better control of blood pressure after spinal cord injury, for example, and strategies to promote longer expression of potentially therapeutic genes by rHSV-1 vectors in SPNs are important. SPNs are not easily accessible by intraspinal injection since they are organised in a narrow column along the length of the thoracolumbar spinal cord. Axons of SPNs converge in peripheral organs and ganglia that can be used as sites for vector inoculation. Peripheral inoculation of the vector at a primary site for retrograde neuronal transport is necessary for selective targeting of these neurons (secondary site; Figure 1).

To utilise HSV vectors for gene delivery to the CNS, both direct and indirect virus-induced cytotoxic effects must be addressed. In this study we used the 14HΔ3vhsZ rHSV-1 vector (Johnson *et al*, 1994) to infect adrenal SPNs. This vector was engineered to produce less cytotoxicity than the first generation replication-defective recombinant herpes simplex viruses (thymidine kinase deficient rHSV-1 (LeVatte *et al*, 1997a)) while maintaining strong neurotropism. Deletion or inactivation of three of the major genes responsible for cellular cytotoxicity, rendered 14HΔ3vhsZ replication-deficient in neurons. LeVatte *et al* (1998) demonstrated a lack of cytotoxicity of 14HΔ3vhsZ in SPNs *in vivo* and minimal toxicity in differentiated PC12 cells *in vitro*. Indeed, *in vivo* there was no loss of SPNs or alteration in the levels of the important cellular enzymes, choline acetyltransferase or nicotinamide adenine dinucleotide phosphate-diaphorase activity at 14 days after infection with 14HΔ3vhsZ. The greatest number of β-galactosidase (β-gal) expressing SPNs (approximately 300) in the thoracic spinal cord occurred at 3 days post inoculation with 14HΔ3vhsZ into the adrenal gland (LeVatte *et al*, 1998). This represents a large percentage of the total population of adrenal SPNs since studies using conventional tracers showed a range of 300–1000 neurons on a single side of the cord (Schramm *et al*, 1975; Pyner and Coote, 1994). Further improvements are still required, though, since an inflammatory infiltrate appeared around infected SPNs by 5 days and expression of the marker gene *lacZ* was essentially lost by 8 days post-infection with 14HΔ3vhsZ (LeVatte *et al*, 1998). Immune responses to the vector or transgene product may be responsible for driving the vector into latency (Wood *et al*, 1994). Thus, strategies of immunomodulation must be developed to promote longer expression of virally transferred genes.

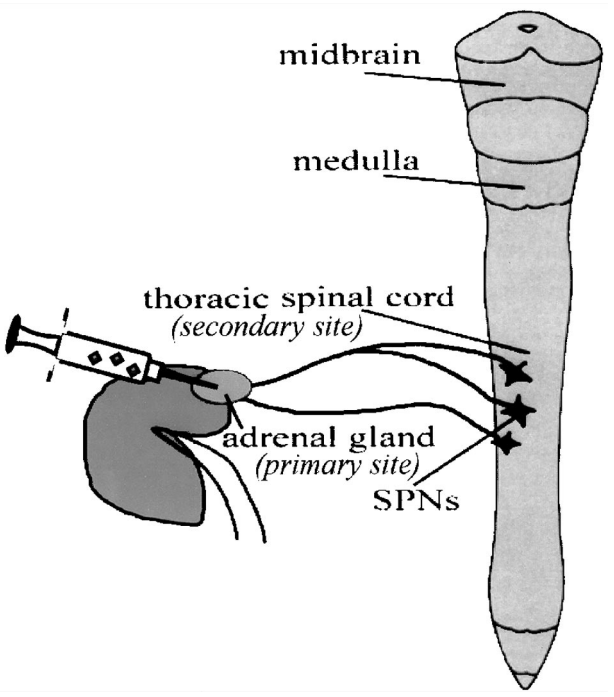

Figure 1 Schematic diagram of the thoracic spinal cord (secondary site) and innervation of the adrenal gland (primary site) by sympathetic preganglionic neurons.

The characteristics of inflammation in the CNS and ganglia due to peripheral infection with HSV-1 have been outlined in several reports (Liu *et al*, 1996; Wood *et al*, 1994). Both wildtype HSV-1 and recombinant HSV-1 vectors evoke similar immune responses consisting of a primary response at the site of inoculation (i.e. adrenal gland) and a secondary immune response in the spinal cord due to retrograde transport of the vector. Briefly, this secondary response in the spinal cord is characterised by perivascular infiltration of macrophages, neutrophils, T lymphocytes (both $CD4^+$ and $CD8^+$), induction of MHC class I and II molecules and production of interferon gamma (IFNγ) and tumour necrosis factor-alpha (TNF-α) (Wood *et al*, 1994). A similar response pattern is also observed in the animal model of multiple sclerosis, experimental allergic encephalitis (EAE). Injection of ence-

phaletogenic antigen at a peripheral or primary site leads to perivascular inflammation of the CNS (secondary site; Zamvil and Steinman, 1990). In HSV-1 infection of ganglia and in EAE, TNF-α and macrophages appear to be major participants in the immune response at the secondary site (Johnson *et al*, 1994; Liu *et al*, 1996; Martin *et al*, 1995).

In addition to its proven benefit in organ transplantation (Perico and Remuzzi, 1997), Cyclosporin A (CsA) is a potent immunosuppressive compound that has been shown to be protective in the chronic EAE model (Calder *et al*, 1987). CsA is able to prevent EAE and the concomitant inflammatory response in the CNS, if it is administered at or before immunisation with encephaletogenic antigen and adjuvant, despite its inability to cross the blood brain barrier (Branisteanu *et al*, 1995; Huitinga *et al*, 1995; Mustafa *et al*, 1993). Two other efficacious immunosuppressants, methylprednisolone (MP) and azathioprine (AZA) are also widely used in clinical settings and animal experiments for their ability to suppress globally the immune response of the host. In addition, these drugs have been shown to ameliorate EAE (Bolton, 1995).

These conventional immunosuppressants have prevented the induction of EAE or reduced the severity of the immune response in the CNS (secondary site) in the chronic EAE model. We hypothesized that they also may prevent or reduce the vector or transgene-associated immune response in our model of retrograde transport of 14HΔ3vhsZ from the adrenal gland (primary site) to the spinal cord (secondary site). We assessed the level of gene expression by counting numbers of SPNs expressing the marker gene product, β-gal, at 5 and 8 days. We assessed extent of inflammation by calculating areas of inflammatory infiltrate around SPNs and areas of perivascular cuffing in drug-treated and non drug-treated animals.

Results

Effect of immunosuppressants on the morphology and number of SPNs exhibiting β-gal activity at 5 days post-infection with 14HΔ3vhsZ

In the absence of immunosuppressant treatment, an average of 45 ± 9 SPNs expressing β-gal was observed throughout the entire length of the thoracic spinal cord in four hamsters (Figure 2). The majority of these SPNs showed incomplete filling of neuronal cell bodies with reaction product and few visible dendrites as revealed by X-gal histochemistry (Figure 3A and B), although some SPNs exhibited β-gal reaction product entirely filling kite-shaped somata and elaborating several major dendrites. CsA treatment led to a significant improvement in the persistence of β-gal expression in SPNs averaging 126 ± 23 in the thoracic region of the cord in six hamsters (Figure 2). Moreover, in CsA treated animals, robust β-gal activity in infected SPNs revealed normal kite-shaped somata and exhibited many elaborate dendritic processes (Figure 3C and D). No appreciable difference in quality of transgene expression (represented by number and morphology of SPNs expressing β-gal) was evident at a lower dose (1 mg/kg/day) of MP tested in three animals compared to control values. Immunosuppression by MP at a higher dose (3 mg/kg/day, i.p.) tended to increase the numbers of SPNs expressing β-gal (95 ± 30 SPNs; Figure 2) in six hamsters, although these values were not significantly different than control animals. Expression of the marker enzyme in neurons of MP treated hamsters revealed the morphology of the neuron somata and extensive dendritic arbors in great detail (Figure 3E and F). Daily injections of AZA (10 mg/kg/day; i.p.) did not increase the number of SPNs (30 ± 11 SPNs) expressing the transgene in four hamsters compared to control animals (Figure 2). Some SPNs expressing β-gal in AZA treated hamsters exhibited kite-shaped somata and blue reaction product in dendritic processes, but many SPNs exhibited less β-gal activity in smaller areas of the cell body and fewer dendritic processes than in CsA or MP treated hamsters (data not shown). In addition, in later studies we increased the dose of AZA to 20 mg/kg/day, i.p., but still found no improvements in any of the measured parameters (data not shown). Daily immunosuppression by a combination therapy with a lower dose of CsA (35 mg/kg; i.m.) and the higher dose of MP (3 mg/kg; i.p.) led to no significant improvement in the number of SPNs exhibiting β-gal activity (62 ± 3

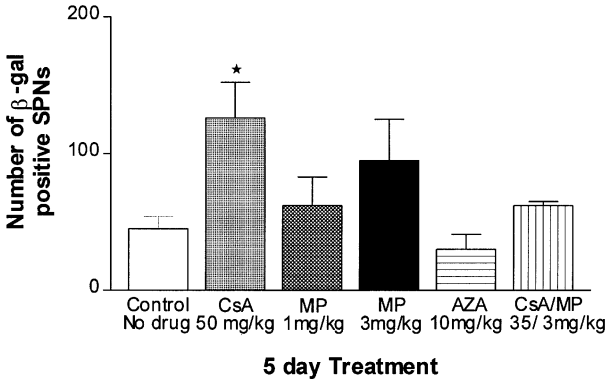

Figure 2 Mean numbers of SPNs visualised by X-gal histochemistry for β-galactosidase activity in the intermediolateral cell column (IML) of spinal cord segments T1-13 at 5 days post-infection with 14HΔ3vhsZ. From left to right, bars represent non-drug treated control hamsters (n=4), CsA treated (50 mg/kg) (n=6), MP 1 mg/kg (n=3), MP 3 mg/kg (n=6), AZA 10 mg/kg treated hamsters (n=4) and CsA/MP 35/3 mg/kg (n=3). Values represent the mean $\pm$ standard error of the mean (s.e.m.). The * indicates a significant difference ($P < 0.05$) compared to non-drug treated control animals.

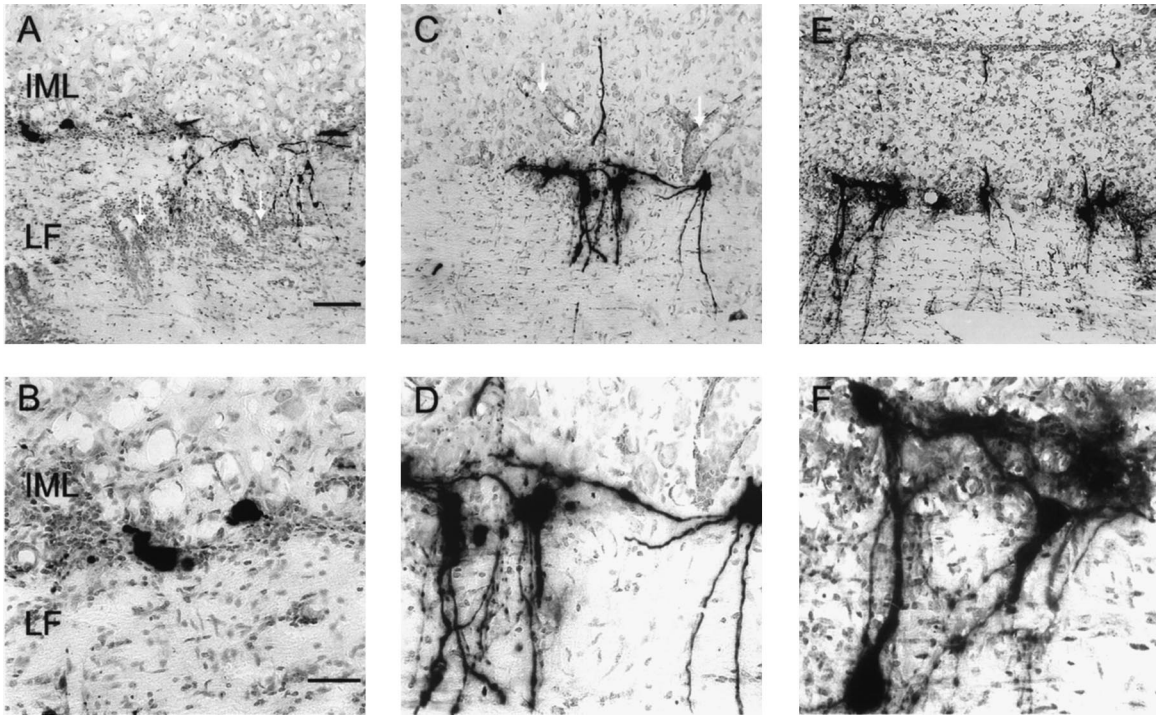

Figure 3 Photomicrographs of infected SPNs in horizontal sections of thoracic spinal cord 5 days post-infection with 14HΔ3vhsZ. Non-drug treated control animals (**A**,**B**) exhibit β-gal reaction product incompletely filling the cell bodies and dendrites. An extensive inflammatory infiltrate and perivascular cuffing (white arrows) are present. CsA-treated (**C**,**D**) and MP-treated (**E**,**F**) animals exhibit normal cellular morphology with elaborate dendritic processes extending from kite-shaped somata. There is indication of an inflammatory infiltrate around infected SPNs and perivascular cuffing (white arrows) on the infected side of the cord but it is markedly reduced compared to control animals. Calibration bar in **A** is 50 μm and pertains to **A**, **C** and **E**. Calibration bar in **B** is 20 μm and pertains to **B**, **D** and **F**.

SPNs; Figure 2) in three hamsters compared to control animals.

Effect of immunosuppressants on the immune response at the secondary site at 5 days post-infection with 14HΔ3vhsZ

In non-immunosuppressed control hamsters, a robust inflammatory infiltrate occurred around infected SPNs (Figure 3A and B) with a mean area of inflammation per neuron of 0.024 ± 0.002 mm^2 (Figure 4A). Perivascular cuffing was evident on the infected side of the spinal cord (Figure 3A, white arrows) with a mean area of 6.12 ± 1.28 mm^2 (Figure 4B). Treatment with CsA reduced significantly the inflammatory infiltrate surrounding infected SPNs to 0.009 ± 0.002 mm^2 (Figures 3C, D and 4A) and tended to reduce the area of perivascular cuffing compared to control hamsters (4.17 ± 0.88 mm^2; Figure 3C and D arrows, Figure 4B). The lower dose of MP (1 mg/kg/day) did not alter the area of inflammation around infected SPNs (0.021 ± 0.002 mm^2) compared to control values. However, the area of perivascular cuffing of immune cells was significantly reduced to 2.79 ± 1.16 mm^2 (Figure 4B). Treatment with MP (3 mg/kg/day) significantly reduced the average area of inflammation around infected SPNs to 0.009 ± 0.003 mm^2 (Figure 4A). Moreover, the area of perivascular cuffing also was significantly reduced to 1.06 ± 0.36 mm^2 (Figure 4B). Treatment with AZA (10 mg/kg) however, did not reduce inflammation around infected SPNs (0.032 ± 0.009 mm^2; Figure 4A). AZA reduced the area of perivascular cuffing to 2.940 ± 0.848 mm^2 (Figure 4B) compared to non-immunosuppressed hamsters. Combination drug therapy with CsA and MP (35 mg/kg; 3 mg/kg) resulted in no significant reduction in the inflammation surrounding infected SPNs (0.071 ± 0.003 mm^2; Figure 4A), or area of perivascular cuffing (4.19 ± 0.80 mm^2; Figure 4B) compared to non-immunosuppressed control values.

Effect of immunosuppressants on the morphology and number of SPNs exhibiting β-gal activity at 8 days post infection with 14HΔ3vhsZ

Daily administration of CsA (50 mg/kg; i.m.) increased significantly the average number of SPNs expressing β-gal to 19 ± 7 at 8 days post-inoculation with 14HΔ3vhsZ in four hamsters compared to no expression at this time in our previous study (LeVatte *et al*, 1998). SPNs in the thoracic spinal cord still exhibited a robust reaction product elaborating the

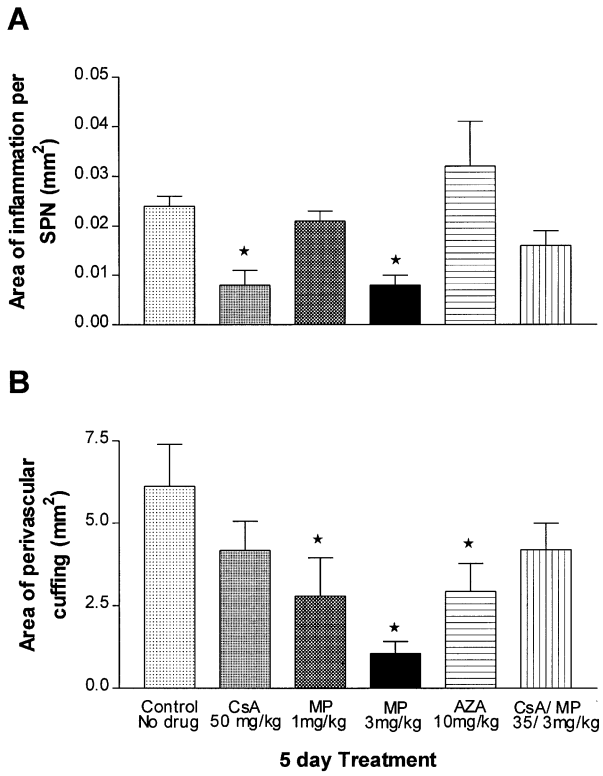

Figure 4 (**A**) Histograms depict mean areas of inflammation per SPN infected with 14HΔ3vhsZ at 5 days in the five treatment groups (format as in Figure 2). An MCID system was used to trace the extent of inflammation around SPNs and the area of inflammation was calculated per animal. This value was converted to area of inflammation per SPN by dividing this area by the total number of SPNs labelled by X-gal histochemistry for that animal. (**B**) depicts mean areas of perivascular cuffing (PC) on the infected side of the spinal cord. Areas of PC were traced using the MCID system and summed per animal. Mean areas were calculated per treatment group and compared to non-drug treated control values. Numbers are means $\pm$ standard error. The * indicates a significant difference ($P<0.05$) compared to non-drug treated control animals.

neuronal somata and dendritic process (Figure 5A and B). Daily administration of MP (3 mg/kg; i.p.) resulted in 9 ± 2 SPNs exhibiting β-gal activity following adrenal injection of the 14HΔ3vhsZ vector in six hamsters.

Effect of immunosuppressants on the immune response at the secondary site at 8 days post-infection with 14HΔ3vhsZ

In non-immunosuppressed control hamsters, we could not calculate the area of inflammatory infiltrate surrounding infected SPNs since none were evident at 8 days post-infection with the rHSV-1 vector. Daily injection of CsA led to an average area of inflammation per neuron of 0.007 ± 0.003 mm^2. Treatment with MP (3 mg/kg/day) resulted in a mean area of infiltrates around infected SPNs of 0.013 ± 0.003 mm^2. Perivascular cuffing was evident on the side of the cord ipsilateral to injection with 14HΔ3vhsZ with mean area of 0.92 ± 0.21 mm^2 in non-immunosuppressed hamsters. Treatment with CsA or MP did not significantly reduce the area of perivascular cuffing at this time point compared to control values. Mean areas were 2.05 ± 1.05 mm^2 and 0.79 ± 0.09 mm^2 respectively.

Discussion

Viral vectors have great potential for transfer of therapeutic genes to various tissues to ameliorate certain neurologic conditions. Despite good initial success with high infection efficiencies and high initial expression levels, many groups have reported a quick decline in vector-mediated transgene expression in targeted tissues (Yang *et al*, 1994; Herz and Gerard, 1993; Keir *et al*, 1995). At the same time, experimental studies have demonstrated the best success with either newborn or immunodeficient animals (Davidson *et al*, 1993; Hermens and Verhaagen, 1997). Since these animals lack a mature or properly functioning immune system, the immune response could be at least partly responsible for loss of transgene expression by the vector. Clinically useful gene transfer protocols for treatment of various neurological disorders will require stable and prolonged expression of therapeutic genes. Other studies have reported success in improving the length of vector-mediated marker gene expression with various immunosuppressants. Kaplan and Smith (1997) reported, for example, improvement in the longevity of transgene expression and ability to readminister adenoviral vector in mouse lung, resulting from transient immunosuppression with deoxyspergualin (Guerette *et al*, 1995). Similarly, Durham *et al* (1997) used FK506 to prolong transgene expression in the brain following adenovirus-mediated gene transfer. However, these studies were carried out using various adenovirus vectors to deliver transgenes to organs or were injected directly into the brain. In certain conditions, specific populations of spinal neurons may require the transfer of therapeutic genes in the absence of invasive techniques such as stereotactic injection that results in injury and a concomitant inflammatory response at the injection site. Gene transfer to specific populations of spinal cord neurons would be attained effectively via HSV vectors that naturally undergo retrograde transport from a defined peripheral inoculation site. Thus, HSV vectors can be targeted to specific sites in the spinal cord and other CNS areas without direct CNS injection. While stereotactic injection and the use of adeno, adeno-associated vectors or lentiviral vectors with neuron-specific promoters will allow a certain degree of targeting of neuronal populations, the biology of these viruses does not normally include

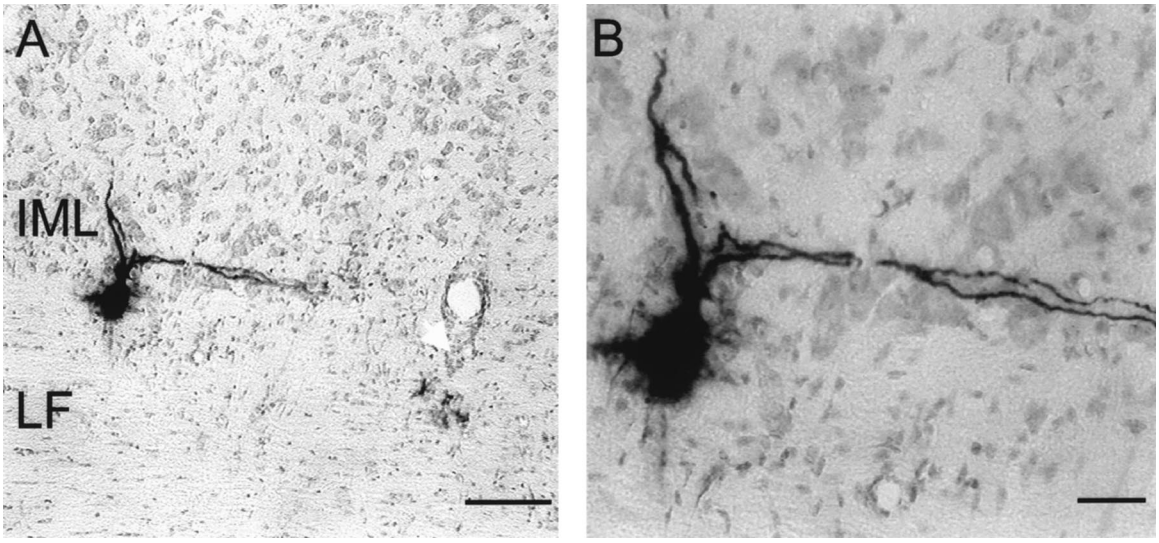

Figure 5 Photomicrographs of an SPN in the IML of a horizontal section of thoracic spinal cord taken from a hamster at 8 days post-infection with 14HΔ3vhsZ and receiving daily injections of CsA (50 mg/kg, i.m.). White arrow indicates residual perivascular cuffing. Calibration bar in **A** is 50 μm, calibration bar in **B** is 20 μm.

efficient retrograde transfer from a peripheral site to neurons in the CNS. For example, various adenovirus vectors have been used for gene transfer to the CNS by direct injection (Davidson and Bohn, 1997; Peltekian *et al*, 1997; Hermens and Verhaagen, 1997). Previous work in our laboratory, however, reported that adenoviruses were not efficient in retrograde transport from the adrenal gland to SPNs in the spinal cord (LeVatte *et al*, 1998). HSV-1 vectors, then, may prove to be more useful for gene delivery to specific sets of neurons from a defined peripheral site outside the CNS and thus an immunomodulation system designed to prolong transgene expression in this model will be important.

This study is, to our knowledge, the first to report the use of immunosuppression to prolong transgene expression from a rHSV-1 vector at a secondary site in the CNS following injection at a peripheral site outside the CNS. In our study, the potential of conventional immunosuppressants was examined for their ability to prolong transgene expression by the 14HΔ3vhsZ vector in SPNs. A clear advantage of immunosuppression by CsA was found as twice as many SPNs expressed β-gal at 5 days post-infection with 14HΔ3vhsZ than were found in non-immunosuppressed animals. Thus, it was possible at 5 days post-infection to have recombinant gene expression in 100–150 SPNs out of a possible 300–1000 adrenal SPNs on one side of the spinal cord (Schramm *et al*, 1975; Pyner and Coote, 1994). Further, daily CsA increased the length of time β-gal positive SPNs could be detected to 8 days, the time when β-gal activity had been lost in this system in a previous study (LeVatte *et al*, 1998).

The mode of action of CsA is well known since it is the most widely used drug in organ transplantation and neural transplant studies (Borlongan *et al*, 1996). It selectively reduces the synthesis of IL-2 by activated helper T-cells (Th T-cells) and thus inhibits activation of resting T-lymphocytes (Lemstrom *et al*, 1996). Macrophages, as antigen presenting cells, have been shown to play a critical role in this process (Benson and Ziegler, 1989; Palay *et al*, 1986). In addition to blocking the proliferative response of T-cells and induction of IL-2 synthesis, it inhibits the ability of cytotoxic T-cells to respond to IL-2. Moreover, CsA has been shown to abrogate both the recruitment and activation of macrophages (Berkowitz-Balshayi *et al*, 1995). Other groups have reported the involvement of CsA in the regulation of T-cell-epithelial cell adhesion by altering leukocyte-function associated antigen-1 (LFA-1) and its counter-receptor intercellular adhesion molecule-1 (ICAM-1) expression (Frishberg *et al*, 1996). Due to CsA's known inhibition of resting T-lymphocyte activation (Lemstrom *et al*, 1996), our results suggest that an important component of the vector-induced and/or transgene-induced immune response is cell-mediated and likely involves T-cells and macrophages. CsA's suppressive effect on the macrophage's ability to present antigen is likely the critical event at both the primary and secondary sites of HSV vector infection in our model. Normally, as described for other models of virus infection (Kasaian and Biron, 1990), macrophage-mediated antigen presentation leads to Th T-cell and cytotoxic T-cell activation and IL-2 mediated proliferation at 3–5 days post-infection. This response peaks at 7 days and diminishes by 9 days.

This time course closely reflects the time course of the vector and/or transgene induced inflammatory response in the spinal cord in the absence of immunosuppression based on the results presented previously and in this report (LeVatte *et al*, 1997a, 1998). The early antiviral macrophage and T-cell response triggered in immunocompetent hamsters and blocked by CsA could indeed be limiting the duration of vector-encoded gene expression. The residual inflammatory response that we observed around infected SPNs could be due to the arrival of activated NK cells and neutrophils which usually arrive before activated T-cells at 3 days post-infection (Kasaian and Biron, 1990). CsA does not block the activation of NK cells (Kasaian and Biron, 1990). As mentioned previously, a study involving an adenoviral vector found that adenoviral vector-mediated gene expression was prolonged following direct injection into the CNS in T-cell deficient nude rats compared to immunocompetent Wistar rats (Hermens and Verhaagen, 1997), further emphasising the importance of cell-mediated immunity on expression of vector-encoded genes.

Methylprednisolone significantly reduced inflammation around infected SPNs and the area of perivascular cuffing, an effect which tended to coincide with more pronounced expression of β-gal at 5 days. Corticosteroids, such as MP, inhibit several aspects of T-cell immunity mostly via inhibition of cytokine expression at the transcriptional and posttranscriptional level (Almawi *et al*, 1996; Chaudhary and Avioli, 1996). They also are toxic to lymphocytes when given systemically and they inhibit the inflammatory activity of macrophages and other phagocytic cells, further hindering the immune response to pathogens (Almawi *et al*, 1996). In addition, steroids reduce the ability of macrophages to respond to lymphocyte-derived signals (Lemstrom *et al*, 1996). The success of MP in enhancing transgene expression may have resulted from a reduction in number and action of macrophages, which have been demonstrated to suppress HSV gene expression (Wu *et al*, 1993).

Daily administration of AZA at both doses tested did not improve transgene expression or decrease the inflammatory response around infected SPNs. Azathioprine is a powerful anti-metabolite that inhibits the development of both humoral and cellular immunity by interfering with the proliferation of activated lymphocytes (Lemstrom *et al*, 1996). Reports indicate that the bioavailability of AZA can be low and very mutable with highly variable time to peak, maximum concentration, clearance and half-life (Daar, 1995). Moreover, low penetration into the CNS and/or lack of conversion to its active metabolite in tissue might explain why the immune response is seemingly unaffected or only slightly affected by its antimitotic effects. We were hesitant to increase the dose of AZA further due to potential negative side effects of the anti-metabolite drug.

Recent studies have shown much improved survival of xenografted tissue in animals receiving a combination therapy of immunosuppressants as compared to CsA use alone (Pedersen *et al*, 1997). A similar combination therapy of CsA and MP in our study did not significantly alter the transgene expression or immune response. This result is somewhat surprising in light of the many studies indicating better success with combination drug therapies. A possible explanation is that CsA is only proficient at prolonging transgene expression at the targeted site in the CNS at high doses (>50 mg/kg/day). We reduced the dose of CsA to 35 mg/kg/day in combination with MP in accordance with other multiple-drug protocols for use in allo- and xeno-transplantation studies (Pedersen *et al*, 1995; Haberal *et al*, 1995).

It is interesting to note that, although MP significantly reduced the area of inflammation and perivascular inflammation proximal to SPNs, it did not significantly prolong transgene expression in SPNs, as did CsA. This finding suggests that the relationship between the extent of inflammatory cellular infiltrate and level of transgene expression by the 14HΔ3vhsZ is not direct. Simply reducing the number of immune cells present in the spinal cord does not lead to prolonged transgene expression in SPNs infected with the vector. The better effects of CsA treatment may relate to its actions on cytokine expression or other factors normally involved in the immune response to the vector or the encoded transgene. The *in vivo* effect of CsA on cytokine expression has remained mostly uncharacterised despite extensive investigation (Shin *et al*, 1998). The success of CsA in this system could be related to its ability to block cytokine synthesis through blocking calcineurin phosphatase, an enzyme required in signalling activation of IL-2 and other genes (Daar, 1995). The better performance of CsA in this model, moreover, may be the result of a direct action on the inflammatory macrophages or due to indirect effects like inhibition of release of substances from infiltrating T-cells.

Neither CsA nor MP were able to halt the progression of HSV-1 latency associated termination of the transgene expression in infected SPNs. Thus, although immunosuppression with CsA proved advantageous at early stages by improving transgene expression by the vector in SPNs, this effect was not long-lived. The eventual loss of transgene expression may still be overcome through the employment of tissue-specific promoters used alone or in conjunction with the HSV-1 latency associated promoter (Andersen *et al*, 1992). The utilisation of immunosuppression in conjunction with vector application, as outlined in this study,

may prove to be useful in determining which promoters will enable optimal levels of transgene expression.

As part of the immune response, antibodies directed against the vector or encoded heterologous transgene are likely to be produced, although appearing late relative to the inflammatory process. They may not be playing an active role in reducing expression of the transgene but would be more active following a second challenge with the same vector. This obstacle is significant, as the vector may require repeat administrations to ameliorate diseases or neuropathic conditions. To provide flexibility of repeat administration, immunosuppression would be necessary to prevent active removal of the vector by the host immune system. Further, individuals to be treated may have already been exposed to HSV-1 and therefore would react faster and with a more robust immune response to vector administration, leading to reduction in successful therapeutic gene transfer. Thus, an immuno-modulation system in conjunction with an HSV-1 vector system would be vitally important to increasing the potential success of the gene transfer system. Moreover, immune responses of the host directed against viral proteins and/or immunogenic transgene products expressed by SPNs would potentially limit the persistence of gene expression (Kaplan and Smith, 1997). For example, despite the fact that β-gal is a very effective marker enzyme that is expressed early after infection of SPNs and easily detected using simple histochemistry, it may be a major factor contributing to the immunogenicity of the vector. Reports indicate β-gal is likely a potent immunogen responsible for activating the cellular immune response (Christ *et al*, 1997; Juillard *et al*, 1995; Brubaker *et al*, 1996). Therefore, finding an alternative non-immunogenic reporter gene may be an important improvement to this vector. Some studies have reported more successful gene expression using host-specific marker enzymes. For example, Tripathy *et al* (1996) found more stable gene expression in mice using 'self' murine erythropoeitin and only transient gene expression with human erythropoeitin adenoviral vectors. Potentially therapeutic transgenes used in the future also may be immunogenic.

In this study we did not characterise the cells involved in the immune response to this modified, replication-defective triple mutant viral vector. In future studies we will use monoclonal antibodies to identify the cells involved in the immune response to 14HΔ3vhsZ or expressed transgene product, β-gal, in the rat since such markers are not readily available for hamsters. In doing so, we can gain knowledge regarding the time course and components of the response and use this information to develop a more selective immunomodulation strategy aimed at enhancing the therapeutic potential of this vector for gene transfer.

This study is, to our knowledge, the first to report improved efficacy of transgene expression in spinal cord neurons infected with a replication defective, triple-mutant HSV-1 vector. This is an important gene transfer system since it may prove useful for delivering genes to SPNs and other spinal neurons that are targeted most effectively by retrograde transport from a peripheral administration site. CsA, which has been shown to be a good immunosuppressant for transplantation also is a good immunosuppressant for HSV-1-mediated gene transfer. It is likely that each new gene transfer protocol will require experimentation to determine the best vector dose and mode of immunomodulation. Important goals are to achieve strong gene expression for periods long enough to produce a positive outcome and to permit possible readministration of the vector. And, while new generation transduction-efficient, non-immunogenic vectors are in development, we can utilise immunosuppression with currently available vectors to enable us to express genes of interest and examine the outcome of this expression in different physiological systems.

Materials and methods

Characteristics and preparation of recombinant HSV-1 (14HΔ3vhsZ)

The vector, 14HΔ3vhsZ, obtained from Dr Theodore Friedman and Dr Paul Johnson (University of California, San Diego, USA) has both copies of infected cell protein 4 (ICP4) deleted and the transcriptional transactivation domain of virus protein 16 (VP16) inactivated by mutation. In addition, the virus host shut-off (vhs) gene has been inactivated by insertion of the *lacZ* gene, driven by an immediate early human cytomegalovirus (CMV) promoter (Johnson *et al*, 1994). Stocks of the replication defective 14HΔ3vhsZ were prepared as previously described (LeVatte *et al*, 1998).

Immunosuppression with cyclosporin A (CsA), azathioprine (AZA), or methylprednisolone (MP)

From 1 day before adrenal injection with 14HΔ3vhsZ until the time of terminal anaesthesia, hamsters were immunosuppressed with daily injections of either CsA (50 mg/kg i.m.; Sandimmune, Novartis), AZA (10 mg/kg, 20 mg/kg i.p.; Imuran, Glaxowelcome), or MP (1 mg/kg or 3 mg/kg i.p.; Solu-Medrol, Upjohn), or a combination of CsA (35 mg/kg i.m.) and MP (3 mg/kg i.p.). Doses of the above-mentioned drugs were slightly higher than average doses used in xenograft transplantation (Pedersen *et al*, 1995, 1997; Murase *et al*, 1993) or aortic allograft arteriosclerosis studies (Lemstrom *et al*, 1996) in the rat.

Adrenal virus injection
All protocols for these experiments were approved by the University of Western Ontario Animal Care Committee in accordance with the policies established in the 'Guide to the Care and Use of Experimental Animals' prepared by the Canadian Council on Animal Care. All efforts were made to minimize animal suffering and to minimize the number of animals used. During the study, all hamsters ate and drank normally and exhibited normal activity and behaviour. Experiments were performed on 31 male Syrian golden hamsters weighing 90–110 g (Charles River, St. Constant, Quebec, Canada). The animals were first sedated with diazepam (3.5 mg/kg, i.p.; Sabex International Ltd., Boucherville, Quebec, Canada) 10 min prior to anaesthesia with sodium pentobarbital (40 mg/kg i.p.; M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada). The virus was injected into the left adrenal gland (primary site of infection) of each animal as previously described (LeVatte *et al*, 1997b). We used 6 μl of the vector at 8.75×10^8 plaque forming units per ml (p.f.u./ml). Control animals were injected with 14HΔ3vhsZ in the same way as described previously without accompanying immunosuppressant. Animals survived 5 or 8 days. After 5 or 8 days, the hamsters were deeply anaesthetised with urethane (3 g/kg i.p.; Aldrich Chemical Company, Inc., Milwaukee, WI, USA), perfused transcardially with a 4% formaldehyde fixation solution. Following perfusion with fixative, the entire thoracic (T) spinal cord was removed and divided into three portions including: T1-5, T6-9, T10-13 as described previously (Mabon *et al*, 1997). The spinal cord portions were then cut into 30 μm, horizontal sections on a cryostat.

Histochemistry for detection of β-galactosidase activity
Histochemistry with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was performed on the horizontal sections of spinal cord to assay for β-gal activity (Mabon *et al*, 1997). All sections of spinal cord were mounted onto gelatin coated slides, counterstained with cresyl violet for cytoarchitectural analysis using standard procedures, and coverslipped with DPX mountant (BDH Laboratory Supplies, Poole, UK).

Quantitation of infection
SPNs labelled by X-gal histochemistry to detect 14HΔ3vhsZ were characterised and identified as described previously (Mabon *et al*, 1997). Upon injection into the adrenal medulla of the left adrenal gland, 14HΔ3vhsZ is taken up and transported retrogradely to cell bodies of adrenal SPNs in the intermediolateral cell column (IML) or lateral funiculus (LF) of the thoracic spinal cord (Figure 1). Sympathetic preganglionic neurons exhibiting β-gal activity were not evident on the contralateral, uninfected side of the cord. The total number of SPNs exhibiting β-gal activity was counted in the IML or LF of the thoracic spinal column ipsilateral to adrenal inoculation. The equation of Abercrombie was applied to correct for the possibility of counting the same neuron more than once (Abercrombie, 1946). In all analyses, data were collected under blinded conditions. A qualitative assessment of gene expression was done by examining cell morphology and extent of arborization of dendrites. Greater β-gal activity revealed more dendrites and more complete filling of the neuronal somata with blue reaction product as revealed by X-gal histochemistry, whereas less blue reaction product led to reduced elaboration of dendrites and incomplete filling of somata.

Quantitation of inflammatory response to 14HΔ3vhsZ
An inflammatory infiltrate occurred around infected SPNs in the thoracic spinal cord. To quantify this inflammatory response we used a microcomputer imaging device (MCID) system to trace the extent of inflammation around SPNs and converted this to an area of inflammation (mm^2) using preprogrammed calibration standards. We normalised these values by dividing the area measurement by the total number of infected SPNs (detected by X-gal histochemistry) to obtain the area of inflammation per SPN in each animal. Infected animals also had evidence of perivascular cuffing on the infected side of the spinal cord whereas the contralateral side had none. We traced areas of perivascular cuffing using the MCID system and summed the areas per animal. Mean areas were calculated per treatment group and compared to control values (non-drug treated). Inflammatory infiltrates and perivascular cuffing were not observed in the corresponding regions of the uninfected, contralateral side of the spinal cord (data not shown). Statistical analysis of cell counts were done by the Tukey's *t*-test after completely random measures analysis of variance (Sokol and Rohlf, 1981). A probability value of less than 0.05 was used to indicate statistically significant differences. Values are given as means $\pm$ standard errors of the mean (s.e.m.).

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