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Differential effects on the survival of neuronal and non-neuronal cells after infection by herpes simplex virus type 1 mutants

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Replication-defective mutants of herpes simplex virus type 1 (HSV-1) are powerful tools to transfer genes into postmitotic neurons and show promise for gene therapy protocols *in vivo*. To evaluate the efficacy and safety of these vectors for the treatment of deafness we infected dissociated cochlear ganglia with HSV mutants defective in the immediate early genes IE 2 (5dl1.2) or IE 3 (d120). Our results reveal striking differences in the survival of neuronal and non-neuronal cells caused by these mutants. Surprisingly, cochlear neurons infected with 5dl1.2 at various concentrations show a significant increase in survival after 2 days in culture. In contrast, many non-neuronal cells undergo apoptosis reducing cell number to less than 50%. In both neuronal and non-neuronal cell types we also observe a population of cells with important changes in morphology. Analysis of dissociated cochlear ganglia infected with d120 reveals a decrease of neuronal survival, whereas non-neuronal cells were almost unaffected. To further characterize and compare the effects of 5dl1.2 and d120 we transduced central nervous system-derived cell types including cortical neurons and astrocytes. Similarly, as observed for cochlear neurons, infection with 5dl1.2 results in increased survival of cortical neurons, whereas d120 shows cytotoxic effects. Survival of astrocytes is equally reduced by both HSV deletion mutants. We conclude that HSV-1 mutants defective in immediate early genes cause very distinct cytopathic phenotypes depending on the cellular context. Possible reasons for these differences, like various patterns of cellular and viral gene expression, and the implications for the use of HSV-1 vectors for gene transfer are discussed.

Keywords: cochlear neurons; immediate early genes; amplicon vector; gene transfer; cytotoxicity

Introduction

Herpes simplex virus type 1 (HSV-1), a neurotropic virus has many unique features, like a wide host-cell range, the ability to infect non-dividing cells and long-term persistence, which make it suitable as a gene transfer vector for the nervous system (Geller, 1993; Glorioso *et al*, 1995; Fink *et al*, 1996). On the other hand, fundamental obstacles like the elimination of lytic viral gene expression and cytotoxicity must be overcome to create safe and efficient HSV-1 based vectors specifically designed for individual types. The principal strategy for eliminating cytotoxicity of HSV-1 vectors is to block viral gene expression at an early stage, thus aborting the lytic cycle. The more than 75 genes of HSV-1 are expressed in a regulated and sequential manner and are classified as immediate early (IE), early (E) or late (L). There are five IE genes (IE 1–IE 5) encoding the proteins infected cell polypeptide 0 (ICP0), ICP27, ICP4, ICP22 and ICP47, respectively. Two of these genes, IE 2 and IE 3 are absolutely essential for replication and have profound effects on viral gene expression, initiating and controlling expression of E and L genes through transcriptional and posttranscriptional mechanisms (McCarthy *et al*, 1989; Smith *et al*, 1992; DeLuca

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and Schaffer, 1985; Sacks *et al*, 1985). A deletion mutant of the IE 3 gene, is associated with the overexpression of the four remaining IE genes, yet is defective in the synthesis of E and L proteins (DeLuca *et al*, 1985; Paterson and Everett, 1990). In contrast, IE 2 mutants maintain the expression of IE and the majority of E genes and are primarily affected in the synthesis of L gene products (McCarthy *et al*, 1989). Both types of IE mutants have been employed to generate replication-incompetent HSV-1 vectors that can be propagated in the respective cell lines needed to complement the deleted gene in trans (Johnson and Friedmann, 1994; Lim *et al*, 1996). There are two types of vectors: those based on recombinant viral genomes (Dobson *et al*, 1990; Fink *et al*, 1992; Johnson and Friedmann, 1994) and defective HSV-1 vectors, which are composed of a plasmid, or amplicon, which is packaged into virus particles in the presence of a helper virus (Spaete and Frenkel, 1982; Geller and Breakefield, 1988; Ho, 1994). Defective HSV-1 vectors have been used to efficiently express genes in neurons from the PNS and CNS, both in primary cell cultures and *in vivo* (Wolfe *et al*., 1992; Battleman *et al*, 1993; Geller *et al*, 1993; Ho *et al*, 1993; During *et al*, 1994; Kaplitt *et al*, 1994; Goodman *et al*, 1996; Lawrence *et al*, 1996; Starr *et al*, 1996). However, in spite of the inability of replication-incompetent viruses to progress through the lytic cycle, cytotoxic effects have been observed in PNS and CNS derived neurons and non-neuronal cells (Johnson *et al*, 1992a,b; Ho *et al*, 1995). Therefore, it is essential to monitor potential side-effects produced by HSV-1 mutants, before performing gene transfer experiments *in vivo*. Our laboratory is interested in developing a gene therapy protocol for deafness which uses replication defective HSV-1 vectors to deliver growth factors into cochlear sensory neurons *in vivo*. Forms of deafness caused by aging, noise, mechanical injury, infections or therapeutic agents are amenable to treatment with electronic cochlear implants, whose functioning is directly dependent on the survival of cochlear neurons (Hartshorn *et al*, 1991). Differential viral vector systems are presently being tested for their efficiency and safety to deliver genes into cochlear neurons (Geschwind *et al*, 1996; Raphael *et al*, 1996; Dazert *et al*, 1997; Weiss *et al*, 1997; reviewed in Kelly, 1997). Here we examine the consequences of infecting dissociated cochlear ganglia with the HSV-1 deletion mutants IE 2 (5dl1.2) or IE 3 (d120) (DeLuca *et al*, 1985; McCarthy *et al*, 1989). Our results reveal striking differential effects on the survival of neuronal and non-neuronal cells derived from this peripheral ganglion. Infection and analysis of central nervous system-derived primary cell cultures confirm the different behaviour of HSV-1 IE gene mutants, which apparently results from the specific interplay of viral and cellular parameters.

Results

Differential effects of IE deletion mutants on cell survival in cochlear ganglia

To evaluate the capacity of HSV-1 deletion mutants for gene transfer into the PNS of the cochlea we examined the potential cytopathicity of two HSV IE mutants in cultures of dissociated cochlear ganglia (see Materials and methods). The HSV mutant 5dl1.2 and d120 contain deletions in the immediated early (IE) genes IE 2 and IE 3, respectively (DeLuca *et al*, 1985; McCarthy *et al*, 1989). Effects of infection with these mutant viruses on cochlear neurons and non-neuronal cells were analysed after 48 h in culture. To examine cell survival we performed stringent counting of neuronal and non-neuronal cells after infection with various concentrations of 5dl1.2 and d120 (Figures 1 and 2). Infection with 5dl1.2 lead to a significant increase of neuronal survival at various concentrations tested (average of 29%) compared to uninfected control cultures (Figure 1). This survival-promoting effect of 5dl1.2 was also observed after 6 days when

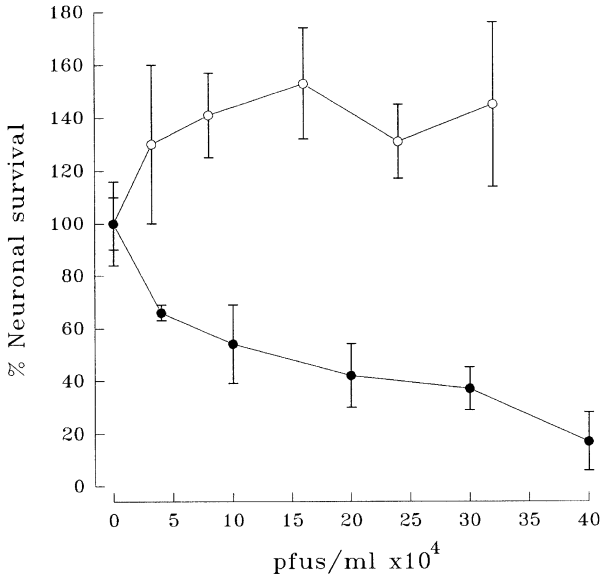

Figure 1 Survival of cochlear neurons infected with HSV-1 IE deletion mutants. Cultures derived from dissociated cochlear ganglia from developmental stage HH34 were infected with different concentrations of HSV-1 mutants 5dl1.2 or d120 and neuronal survival was analysed 48 h after infection. Note the increased neuronal survival response after infection with 5dl1.2 (white circles), compared with mock-infected control cultures. Neuronal numbers decline in cultures transduced with increasing concentrations of d120 (black circles). The percentage of surviving neurons is expressed as the mean $\pm$ s.d. of triplicate determinations at each concentration. Neuronal survival in 5dl1.2 infected cultures was significantly different from the control culture at 3.2×10^4, 8×10^4 and 1.6×10^5 pfu/ml ($P < 0.02$; *t*-test). Neuronal survival in d120 infected cultures was significantly different from the control culture at every concentration tested ($P < 0.05$; *t*-test).

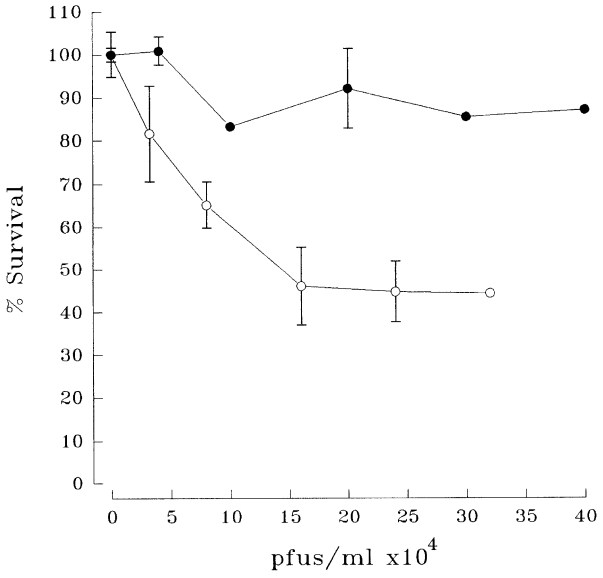

Figure 2 Survival of non-neuronal cells derived from cochlear ganglia after infection with HSV-1 IE deletion mutants. Cultures derived from dissociated cochlear ganglia from developmental stage HH34 were infected with different concentrations of HSV-1 mutants 5dl1.2 or d120 and survival of non-neuronal cells was analysed 48 h after infection. Note the reduction of cell number in 5dl1.2 infected cultures (white circles), whereas survival is almost unaffected in cultures transduced with d120 (black circles). Survival of non-neuronal cells was calculated by comparison with mock-infected control cultures. With the exception of 3.2×10^4 pfu/ml survival of non-neuronal cells in control cultures versus 5dl1.2 infected cultures was significantly different ($P<0.02$; *t*-test) at every concentration tested, whereas with the exception of 1×10^5 pfu/ml ($P<0.05$; *t*-test) no concentration of d120 produced significant differences. The percentage of cells is expressed as the mean $\pm$ s.d. of triplicate determinations at each concentration.

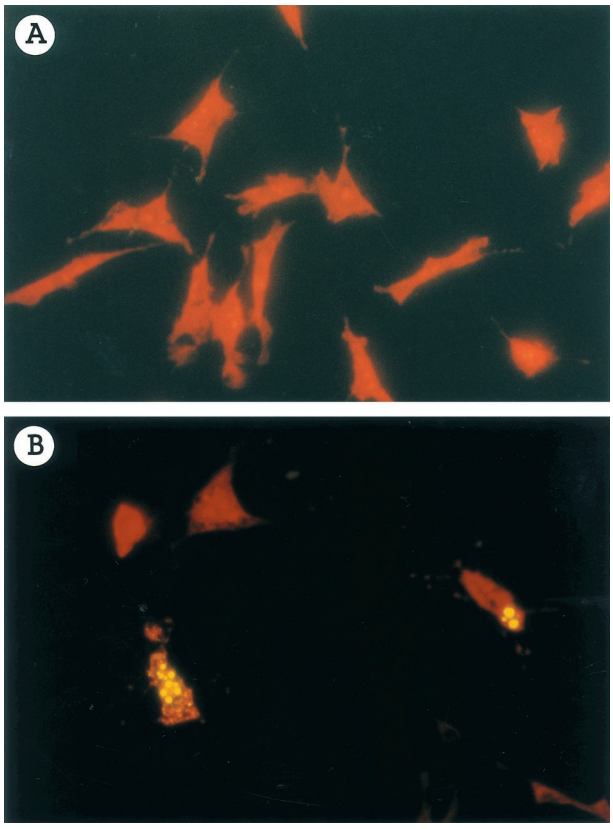

Figure 3 Apoptosis of non-neuronal cells derived from cochlear ganglia. Dissociated cultures derived from cochlear ganglia were mock-infected (**A**) or infected with the HSV-1 deletion mutant 5dl1.2 (**B**) and cells were analysed for apoptosis by TUNEL labelling in the presence of TdT enzyme and fluorescein-12-dUTP after 24 h. After cytoplasmatic counterstaining with propidium iodide (red) cells were viewed in a fluorescence microscope. Note the presence of apoptotic bodies (yellow) in the cell nuclei and the reduced cell number in 5dl1.2 infected cultures.

approximately 25% of neurons were maintained, whereas in control cultures survival dropped down to about 5%. In contrast, survival of non-neuronal cells declined upon addition of increasing quantities of 5dl1.2 reducing cell number below 50% after 2 days compared with control cultures (Figure 2). To gain further insight into the time course and mechanism of cell death of non-neuronal cells we performed a TUNEL assay, which allows the identification of apoptosis *in situ* by labelling of cleaved ends of chromatin DNA with terminal transferase (see Materials and methods). We observed no indication for increased cell death 14 h after infection compared with mock-infected cultures. However, 24 h after infection with 5dl1.2 we detected a dramatic increase of non-neuronal cells undergoing apoptosis (43% *versus* 5% in control cultures), characterized by the appearance of dense nuclear chromatin masses (Figure 3). Upon inspection of 5dl1.2 infected cell cultures at the light microscope level we also noticed a percentage of non-neuronal and neuronal cells with a strikingly distinct morphology (Figure 4). Up to 24% of non-neuronal cells showed a high number of cell processes and a tendency to condense their cell body leading to fragmentation (Figure 4D). This phenotype might be a consequence of an ongoing process of apoptosis in these cells. Additionally, up to 15% of neurons revealed an unusual morphology characterized by numerous branches extending from the neurites and the cell body (Figure 4C).

Infection of dissociated cochlear ganglia with increasing amounts of d120 resulted in a decline of the number of neuronal cells, reducing survival to only 17% at the highest concentration added (Figure 1). In contrast to the effects observed in 5dl1.2 infected cultures survival of non-neuronal cells was almost unaffected by the d120 mutant (Figure 2). At the light microscope level, neuronal and non-neuronal cells showed a normal cell morphology (Figure 4A and B). In summary, infection with viruses deleted in distinct IE genes

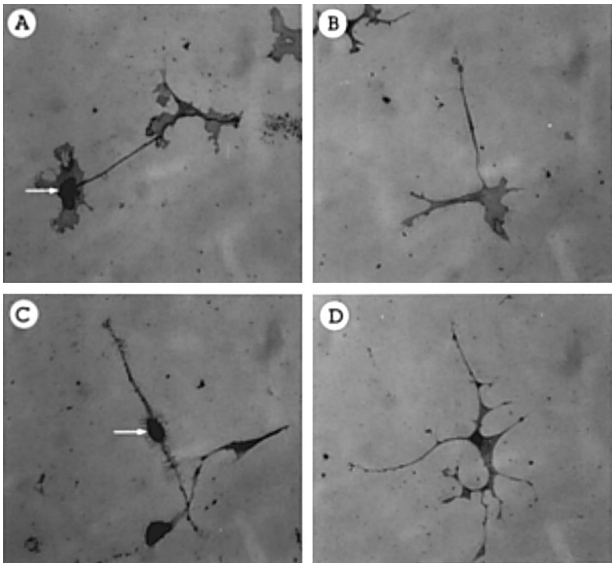

Figure 4 Morphology of cells derived from cochlear ganglia after infection with HSV-1 IE deletion mutants. Dissociated cochlear ganglia were infected with d120 (**A** and **B**) or 5dl1.2 (**C** and **D**), fixed after 48 h and stained with the A2B5 antibody to define neurons (dark brown stain). Cochlear neurons (**A**, arrow) and non-neuronal cells (**B**) in d120 infected cultures show a normal morphology. (**C**) Image of a cochlear neuron with numerous branches extending from the cell body (arrow) and neurites. (**D**) Photograph of a non-neuronal cell with a condensed cell body and signs of fragmentation at the cell periphery.

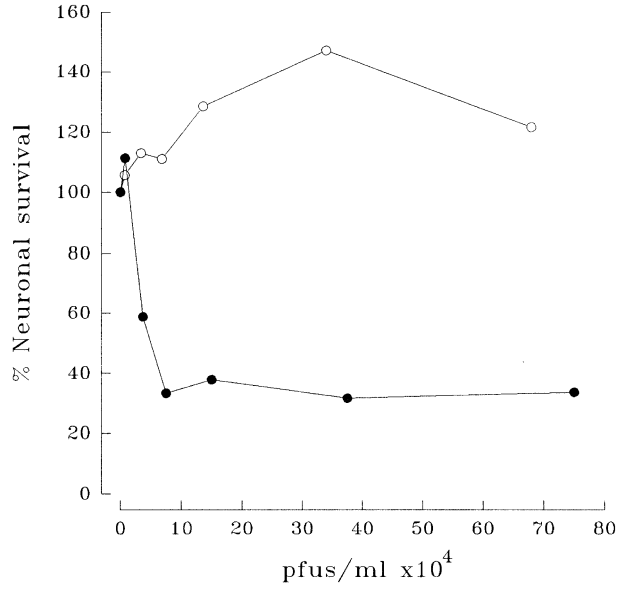

Figure 5 Survival of cortical neurons after infection with HSV-1 IE mutants. Cultures of cortical neurons were infected with different concentrations of HSV-1 mutants 5dl1.2 or d120 and neuronal survival was analysed 48 h after infection. Note the increased neuronal survival response after infection with 5dl1.2 (white circles), compared with mock-infected control cultures. Neuronal numbers decline in cultures transduced with increasing concentrations of d120 (black circles). The percentage of surviving neurons is expressed as the percentage of cells surviving compared with an uninfected control (100% survival).

cause strikingly different results on the survival and morphology of neuronal and non-neuronal cells derived from cochlear ganglia.

Cytotoxicity of IE deletion mutants in CNS-derived neurons

We were interested in comparing the sensitivity of PNS-derived cochlear sensory neurons to HSV-1 mutants with CNS-derived cells. To do so, we assessed damage of 5dl1.2 and d120 to CNS-derived primary cortical neurons and astrocytes (see Materials and methods).

Cultures of cortical neurons were infected with 5dl1.2 or d120 at various concentrations and analysed after 2 days. Surprisingly, at every concentration of 5dl1.2 tested stringent cell counts revealed an increase in survival of cortical neurons (average of 21%) compared with control cultures (Figure 5). On the contrary, infection with d120 generally led to a reduction in cell number. Microscopic inspection revealed the presence of many differentiated neurons with axonal elongations in 5dl1.2 infected cultures (Figure 6A). Preparations to which d120 had been added contained only neurons with very short or no axonal extensions (Figure 6B). Therefore, similarly to the PNS-derived neurons studied above CNS-derived neurons also differ in their survival response after infection with IE 2 or IE 3 HSV-1 deletion mutants.

We next examined cytotoxic effects of 5dl1.2 and d120 in cultures of primary astrocytes. Analysis of cell survival was performed 48 h after infection. Increasing amounts of both IE deletion mutants caused cytopathic effects as revealed by the presence of floating cells (data not shown). Stringent counts of cells with a normal morphology revealed a similar profile of survival at various virus concentrations of 5dl1.2 and d120 (Figure 7).

Discussion

HSV-1 IE genes show different effects on cellular survival

In the present study we have compared the cytopathic effects of HSV-1 mutants defective in the IE 2 or IE 3 gene in PNS- and CNS-derived neuronal and non-neuronal cells. Whereas d120 only expresses the remaining four IE genes, 5dl1.2 additionally produces E and some L gene products (DeLuca *et al*, 1985; McCarthy *et al*, 1989; Paterson and Everett, 1990). Although d120 shows a restricted phenotype in that it expresses few HSV-1 genes our data confirm a number of earlier studies demonstrating cytotoxic side-effects of this IE mutant. We observed a decrease of the survival of

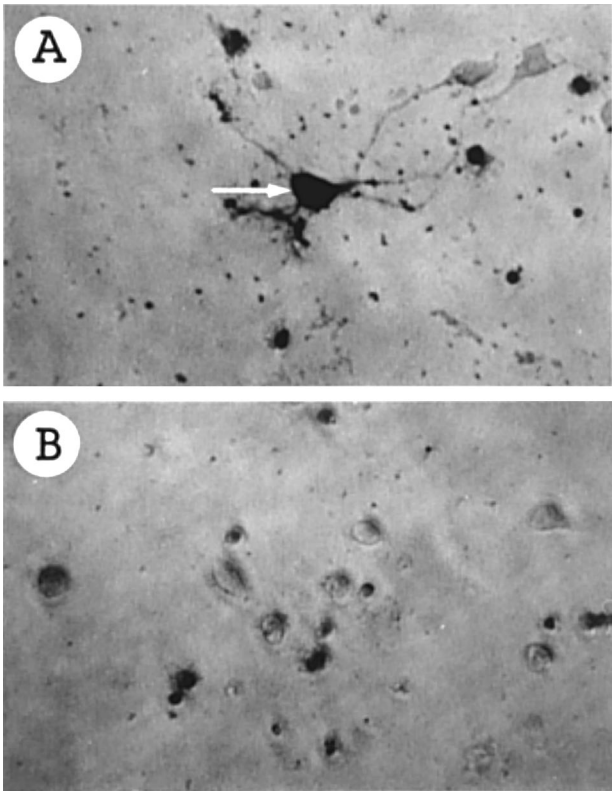

Figure 6 Morphology of cortical neurons after infection with HSV-1 IE deletion mutants. Cultures of cortical neurons were infected with 5dl1.2 (**A**) or d120 (**B**), fixed after 48 h and stained with crystal violet. (**A**) Image of a neuron with numerous axons extending from the cell body (arrow). (**B**) Photograph showing cortical neurons lacking axonal elongations.

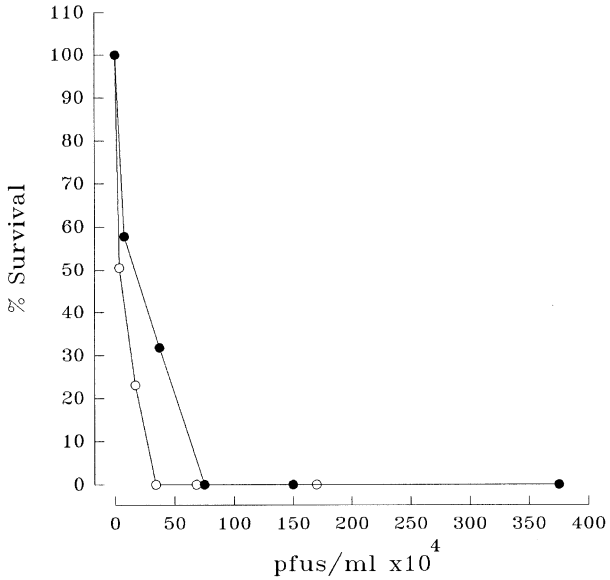

Figure 7 Survival of primary astrocytes after infection with HSV-1 IE mutants. Cultures were infected with various concentrations of 5dl1.2 (white circles) or d120 (black circles) and cell survival was determined after 48 h. Both viruses produced a similar reduction of cell survival. The percentage of surviving astrocytes is expressed as the percentage of cells surviving compared with an uninfected control (100% survival).

cochlear and cortical neurons and astrocytes after infection with d120. Similar results have been obtained in neurons derived from dorsal root ganglia or hippocampus (Johnson *et al*, 1992a; Ho *et al*, 1995) and in cortical neurons and astroglia (Johnson *et al*, 1992b) after infection with IE 3 mutants. Non-neuronal cells derived from cochlear ganglia appear more resistant to d120 but less so to the IE 2 mutant 5dl1.2 (see Figure 2). This differential resistance to cytotoxicity appears not to be caused by a reduced infectivity of d120 in non-neuronal cells, since we found that similar amounts of non-neuronal cells (more than 70%) were infected in cochlear cell cultures transduced with d120 or 5dl1.2 (see Materials and methods). The most likely cause for the increased cytopathic effects observed in 5dl1.2 infected non-neuronal cells is the expression of HSV-1 early or late genes which are not expressed by d120. Unexpectedly however, survival of 5dl1.2 infected cochlear and cortical neurons is not reduced but even increased compared with control cultures (see Figure 1). This striking behaviour of the 5dl1.2 mutant is not limited to the avian embryonic sensory neurons examined in this study, but is also found upon infection of postnatal mammalian cochlear neurons (JJ Garrido and T Schimmang, unpublished observations). This surprising finding might be explained by the expression of HSV-1 genes which suppress apoptosis (Leopardi and Roizman, 1996; Koyama and Miwa, 1997; Leopardi *et al*, 1997). Interestingly, the capacity of wild-type HSV-1 to suppress apoptosis has been detected by studying apoptotic effects of the IE 3 deletion mutant d120 (Leopardi and Roizman, 1996). A recent study identified a secondary mutation in d120 which affects the HSV-1 early gene U_s3 and demonstrates its requirement for protection from apoptosis induced by the virus (Leopardi *et al*, 1997). Since in contrast to d120 the IE 2 mutant 5dl1.2 maintains the expression of early genes (McCarthy *et al*, 1989) apoptosis of cochlear neurons might be suppressed in 5dl1.2 infected cultures. However, why would this antiapoptotic mechanism only work in neurons and not in non-neuronal cells? A possible explanation might be that in natural infection HSV-1 invades sensory neurons of the PNS where it can persist throughout the life of the host in a latent state. Therefore, the cellular machinery of PNS-derived cochlear sensory neurons probably resists the cascade of cytotoxic events triggered by IE genes via interaction with early genes like U_s3 which block apoptosis and allow establishment of HSV latency. On the other hand, no latency is established

in CNS-derived neurons during natural infection and therefore the expression of antiapoptotic gene(s) by 5dl1.2 appears to be the major cause for the survival effects observed. In contrast, a non-neuronal cell is apparently unable to interact with HSV-1 genes in such a manner and the cell will enter apoptosis.

Implications for HSV-1 vectors

We are interested in using defective HSV-1 mutants as gene-transfer vectors to introduce therapeutic genes into the PNS of the inner ear *in vivo*. Therefore, one of the major purposes of this study was to test the pathogenicity of HSV-1 mutants deleted in different IE genes in cochlear neurons. Our results revealed that after infection with IE 2 or IE 3 mutants cochlear neurons show very distinct phenotypes. The IE 2 mutant causes an increased survival response, which might even be conceived as an advantage for gene transfer studies. In contrast, injection of amplicon stocks containing 5dl1.2 as a helpervirus cause cell damage when injected in adult rat brain (Fraefel *et al*, 1996). Our results show that the same deletion mutant also causes a severe reduction of the survival of non-neuronal cells derived from cochlear ganglia (Figure 2). Non-neuronal cells have been shown to promote survival and differentiation of PNS neurons (Verdi *et al*, 1996; Pfrieger and Barres, 1997; Riethmacher *et al*, 1997). Although, we have not observed any negative consequences on neuronal survival in 5dl1.2 infected cultures, the integrity of monitored during non-neuronal cells will have to be carefully monitored during *in vivo* experiments. In this context, it will also be important to analyse the possible consequences on normal cellular functions accompanied by the striking morphological changes in a percentage of non-neuronal and neuronal cells (Figure 4).

Analysis of dissociated cochlear ganglia infected with the IE 3 mutant d120 showed a severe reduction of neuronal survival whereas non-neuronal cells appeared almost unaffected (Figures 1 and 2). Therefore, employment of an IE 3 mutant for the infection of cochlear neurons *in vivo* appears to bear the risk of causing cytotoxic effects. Nevertheless, many *in vivo* studies in the CNS or PNS have been performed with HSV-1 vectors containing mutations in the IE 3 gene (Federoff *et al*, 1992; Ho *et al*, 1993; During *et al*, 1994; Kaplitt *et al*, 1994). A careful study of the cytopathic effects of the IE 3 deletion mutant d120 in the CNS *in vivo* has shown no damage after infection of the hippocampus (Ho *et al*, 1995). On the other hand, the same study reveals cytotoxic effects 2 days after infection of hippocampal neurons with d120 *in vitro*. Consequently, cells might be more resistant to pathogenic effects in the context of the whole animal compared to dissociated cell cultures. In summary, although both IE 2 and IE 3 mutants cause specific cytopathic effects on dissociated cochlear ganglia *in vitro*, a systematic analysis of cochlear ganglia infected *in vivo* will be necessary to clarify the risk of cytotoxic side-effects. In any case, the production of HSV-1 vectors with a reduced potential to affect cellular functions is desirable. Since it is known that nearly all of the HSV-1 IE genes cause cytopathic effects (Johnson *et al*, 1992a, 1994), a systematic deletion of these genes has been performed. Cytotoxicity studies of HSV-1 mutants deleted in various combinations of IE genes have indeed shown an improvement or complete recovery of cell survival compared to single mutants (Marconi *et al*, 1996; Wu *et al*, 1996; Preston *et al*, 1997; Samaniego *et al*, 1998). An additional way to reduce cytotoxicity may be the use of HSV-1 vectors deleted in various IE genes as helpervirus in the context of the HSV-1 amplicon system. Cell lines complementing the deleted IE genes would allow the production of a mixed population of particles containing non-toxic plasmid concatemeres or the defective helper genome which will have a severely reduced potential to cause cellular damage. An alternative route to completely circumvent the side-effects by the helpervirus has recently been accomplished by using a plasmid-based transfection system to provide the helper functions (Fraefel *et al*, 1996). Therefore, progress in developing HSV-1 vectors with reduced cytotoxicity may eventually allow safe gene transfer with therapeutic applications.

Materials and methods

Preparation of viral stocks

Cell lines were grown in DMEM containing 10% foetal calf serum. The IE 2 deletion mutant 5dl1.2 (McCarthy *et al*, 1989) virus was grown and titered on 2-2 cells (Smith *et al*, 1992), a VERO cell line which contains the IE 2 gene. The IE 3 deletion mutant (DeLuca *et al*, 1985) was grown and titered on E5 cells, a VERO cell line containing the IE 3 gene (DeLuca and Schaffer, 1987). Packaging and titering of 5dl1.2 and d120 was performed as described previously (Lim *et al*, 1996, 1997). Two viral stocks of each deletion mutant containing 8×10^6 and 3.4×10^7 plaque forming units (pfu)/ml of 5dl1.2 and 1×10^7 and 7.5×10^7 pfu/ml of d120 were used.

Preparation, infection and analysis of cell cultures

Cochlear ganglia Primary cultures of dissociated cochlear ganglia were prepared as described previously (Garrido *et al*, 1998). Briefly, cochlear ganglia from chicken embryos of stage HH 34 (according to Hamburger and Hamilton, 1951) were isolated, incubated with 0.05% trypsin (Gibco) in HBSS without Ca^{2+} or Mg^{2+} for 20 min at 37°C and dissociated using a fire polished Pasteur pipette. Cells were resuspended in insulin-free medium (DMEM/F12 with Glutamax-I (Gibco) supplemented

with 10% foetal calf serum, 50 U/ml penicllin, 50 μg/ml streptomycin, 6 mg/ml glucose, 0.5 mM sodium pyruvate, 100 mg/ml transferrin, 60 ng/ml progesterone, 16 μg/ml putrescine and 30 nM sodium selenite) and seeded in poly-D-lysine (50 μg/ml) and laminin (10 μg/ml) coated culture dishes at a final density of 1000 cells in 0.5 ml per well. After 2 h at 37°C in a 5% CO_2 atmosphere, medium was changed to reduce the serum concentration to 1% and viruses were added. After 48 h, primary cultures containing neurons and non-neuronal cells were fixed in 4% paraformaldehyde in PBS, washed in PBS and endogenous peroxidase was inhibited with 0.03% H_2O_2. Cells were washed in PBS, blocked with 10% goat serum for 1 h at room temperature (RT) and incubated with the supernatant from the A2B5 hybridoma diluted 1 : 1 in PBS at 4°C for 12 h. Cultures were washed in PBS and incubated with a biotinylated IgM antibody (Sigma) diluted 1 : 100 for 2 h at RT. Finally cells were washed in PBS and immunoreactivity was detected using the ABC method (Vectastain Kit, Vector Laboratories). The A2B5 monoclonal antibody recognizes a cell surface glycolipid on chicken neurons (Eisenbarth *et al*, 1979; Vogel and Weston, 1988; purchased from the American Type Culture Collection). Cells were classified as A2B5 positive neurons, which composed approximately 10% of the total amount of cells. Neurons with neurites four times longer than cell bodies were scored as surviving neurons (Davies *et al*, 1986). This number was expressed as the percentage of the total number of neurons plated. Infection of neurons was assayed by staining with a rabbit antibody raised against HSV-1 glycoproteins (DAKO; diluted 1 : 1000). The following percentages of infection for 5dl1.2 were found: $81.6 \pm 0.1\%$ at 3.2×10^4 pfu/ml, $97.2 \pm 1.3\%$ at 8×10^4 pfu/ml, $98.2 \pm 0.3\%$ at 1.6×10^5 pfu/ml and 100% at 2.4×10^5 pfu/ml and 3.2×10^5 pfu/ml. Prior to immunocytochemistry viability of neurons was confirmed by Trypan blue staining and loading with Fura-2, indicating the intactness and normal function of the cell membrane. Survival of non-neuronal cells, the vast majority of which are Schwann cells was expressed as the percentage of cells surviving compared to the control culture. Statistics were performed using Student's *t*-test.

Morphology of neuronal and non-neuronal cells was analysed at various concentrations of the 5dl1.2 mutant. Morphology was altered in $4.9 \pm 1.2\%$ of neurons at 3.2×10^4 pfu/ml, $14.6 \pm 1.6\%$ at 1.6×10^5 pfu/ml and $12.6 \pm 2.2\%$ at 3.2×10^5 pfu/ml and $7.5 \pm 1.0\%$ of non-neuronal cells at 3.2×10^4 pfu/ml, $12.4 \pm 1.6\%$ at 1.6×10^5 pfu/ml and $24.4 \pm 1.1\%$ at 3.2×10^5 pfu/ml. Immunocytochemical staining revealed that all cells with abnormal morphology were infected by the 5dl1.2 HSV-1 deletion mutant. At 1.6×10^5 pfu/ml 80.0% of neurons and 72.9% of non-neuronal cells were infected by 5dl1.2. At 3×10^5 pfu/ml 59.7% of neurons and 74.9% of non-neuronal cells were infected by d120.

Cortical neurons Cortical neurons were isolated from day 18.5 post coitum rat embryos and 7.5×10^4 neurons were seeded per well in 0.5 ml of culture medium (Ruiz *et al*, 1998) and infected with different concentrations of 5dl1.2 ($6.8 \times 10^3 - 6.8 \times 10^5$ pfu/ml or d120 ($7.5 \times 10^3 - 7.5 \times 10^5$ pfu/ml). After 48 h cultures were stained with crystal violet to visualize neurons with axonal elongations which were scored as surviving neurons. Stringent cell counts of random fields were performed. Cell survival is expressed as the percentage of neurons compared with uninfected control cultures. Viability of neurons was confirmed by Trypan blue staining. Percentages of infection for 5dl1.2 were 17.6% at 6.8×10^3 pfu/ml, 34.5% at 3.4×10^4 pfu/ml, 34.2% at 6.8×10^4 pfu/ml, 40.9% at 1.4×10^5 pfu/ml, 71.5% at 3.4×10^5 pfu/ml, and 80.9% at 6.8×10^5 pfu/ml.

Astrocytes Primary cultures of astrocytes derived from cortex of newborn rats were prepared as described by McCarthy and de Vellis (1980). Briefly, 2000 cells were seeded per well in 24-well dishes in 1 ml of culture medium (DMEM containing 10% serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine). After 3 h cultures were infected with various concentrations of 5dl1.2 ($3.4 \times 10^4 - 1.7 \times 10^6$ pfu/ml) or d120 ($7.5 \times 10^4 - 3.75 \times 10^6$ pfu/ml) and 48 h later cells fixed with 4% paraformaldehyde. To score survival of cells stringent cell counts of random fields were performed and compared with uninfected control cultures.

In situ *analysis of apoptosis*
Cultures from dissociated cochlear ganglia were prepared as described above and seeded on coverslips coated with poly-D-lysine (0.5 mg/ml). Two hours later cells were infected with 8×10^6 pfu/ml and after 14 or 24 h cells were washed twice with PBS and fixed with 4% paraformaldehyde for 25 min at 4°C. Apoptotic cells were detected using the apoptotic detection system purchased from Promega. Briefly, cells were washed twice with PBS at RT and permeabilized with 0.2% Triton for 5 min on ice. After washing twice for 5 min at RT in PBS, cells were equilibrated in equilibration buffer for 8 min followed by an incubation of 1 h at 37°C in the presence of terminal deoxynucleotidyl transferase enzyme and dNTPs including fluorescein-12-dUTP in equilibration buffer. Coverslips were washed in 2 × SSC for 15 min and three times for 5 min in PBS at RT. After counterstaining of cells with propidium iodide at 1 μg/ml in PBS for 15 min, coverslips were washed in distilled water and mounted using Vectashield (Vector Laboratories). Cells were viewed by epifluorescence using standard fluorescein excitation.

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