A neuroattenuated ICP34.5-deficient herpes simplex virus type 1 replicates in ependymal cells of the murine central nervous system

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Introduction

Herpes simplex virus type 1 (HSV-1) variant 1716 is deleted in the gene encoding ICP34.5 and is neuroattenuated after intracranial inoculation of mice. Although the mechanism of attenuation is unclear, this property has been exploited to eliminate experimental brain tumours. Previously, it was shown that infectious 1716 was recoverable for up to 3 days after intracranial inoculation suggesting that there may be limited replication in the central nervous system (CNS). Here it is demonstrated that 1716 replicates in specific cell types (predominantly CNS ependymal cells) of BALB/c mice, using immunohistochemical, immunofluorescence, in situ hybridization and virus titration studies. While 1716-infected mice exhibited no overt signs of encephalitis, histological analysis showed a persistent loss of the ependymal lining. Thus, although ICP34.5-deficient viruses are neuroattenuated, they do retain the ability to replicate in and destroy the ependyma of the murine CNS. A detailed understanding of the mechanism(s) of neuroattenuation and limited replication could lead to the rational design of safe HSV vectors for cancer and gene therapy in the CNS.

Herpes simplex virus type 1 (HSV-1) is a large neurotropic DNA virus which infects a wide range of cell types in different animals. Following primary infection, HSV-1 establishes a lytic or latent infection in neurons of the central nervous system (CNS) and peripheral nervous system (PNS). During lytic HSV infection, the macromolecular machinery of the infected cell is redirected toward the transcription, replication and assembly of virus progeny (reviewed by Roizman & Sears, 1996). The entire process of virus entry, replication, assembly and egress takes between 18 and 20 h, resulting in cell death (although neurons may survive acute infection; Simmons & Tscharke, 1992) and release of progeny virus. During this interval, viral genes are expressed through a tightly regulated transcriptional cascade and are designated the α (immediate early, e.g. ICP4); β (early, e.g. TK); and γ (late, e.g. VP5, gC, ICP34.5) genes (Honess & Roizman, 1974, 1975). During latent infection, only a small set of latency associated transcripts (LATs) are expressed (for review see Fraser et al., 1992). In humans, the natural host, HSV-1 causes a wide range of diseases including fever, cold sores, keratitis, blindness and encephalitis (reviewed by Whitley & Gnann, 1993). Most (60–90%) of the population is seropositive for HSV-1 by early adulthood, and HSV-1-induced disease is usually limited in immunocompetent hosts. However, when the immune system is compromised, HSV-1 can disseminate to cause severe and life-threatening infections (Whitley, 1996). For instance, in AIDS patients HSV causes chronic active systemic infections and brainstem encephalitis (Hamilton et al., 1995; Laskin et al., 1987).

HSV-1 encephalitis is the most serious consequence of CNS infection, and develops from primary or reactivated infection (Nahmias et al., 1982; Whitley et al., 1982a). The clinical manifestations of encephalitis reflect the site of infection in the brain and may include memory loss, speech deficits, hallucinations and seizures (Whitley et al., 1982b). Although the nature of HSV-1 tropism for specific regions of the brain is poorly understood, studies of the biology of HSV-1 isolates and mutants thereof in animal models have provided insights into how viral and cellular factors may affect CNS pathogenesis in humans (Chrisp et al., 1989; Valyi-Nagy et al., 1992, 1994a, b). More significantly, these studies have also led to the use of HSV-derived vectors for cancer and gene therapy of CNS disorders (Kesari et al., 1995, 1996; Lawrence et al., 1995; Martuza et al., 1991).

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There are many cellular and viral factors that determine the outcome of infection. Although many viral proteins are not required for the production of infectious virus in cultured cells, these ‘non-essential’ genes play important roles in the life cycle of the virus in animals (Roizman & Sears, 1996). One viral gene that is dispensable in vitro but essential in vivo is RLI, encoding infected cell protein 34.5 (ICP34.5 or p34.5) (Chou et al., 1990; McGeoch et al., 1991), a protein which has homology to the cellular genes MyD116 and GADD34 (Chou et al., 1990; Chou & Roizman, 1994; McGeoch & Barnett, 1991). ICP34.5 is thought to act by inhibiting the cessation of protein synthesis associated with programmed cell death in some non-permissive, dividing cells (i.e. in SK-N-SH human neuroblastoma cells) by regulating the interferon-inducible PKR pathway (Chou et al., 1995; Chou & Roizman, 1992, 1994). A more recent tissue culture study (Mohr & Gluzman, 1996) suggests that other HSV genes are involved in the PKR pathway and the action(s) of ICP34.5 are likely to be complex. Another study has shown that the inhibitory action of ICP34.5 can be separated from the virulence phenotype (Markovitz et al., 1997). However, the studies of ICP34.5 function to date have focused on in vitro tissue culture characterization, and the specific mechanism by which the absence of ICP34.5 confers neuroattenuation in vivo is not clear.

Variants of HSV in which RLI is deleted or mutated have been reported to be incapable of replicating in the CNS of mice and causing encephalitis (Chou et al., 1990; MacLean et al., 1991; Taha et al., 1990). From these and other studies, it was speculated that the replication of HSV-1 ICP34.5 mutants may be cell cycle dependent, thereby rendering these mutants incapable of replication in post-mitotic and/or quiescent cells in the brain (Brown et al., 1994a; Chou et al., 1990; Kesari et al., 1995). Previously, we showed that HSV variant 1716, which has a 759 nucleotide deletion in the RLI, was attenuated following intracranial (i.c.) inoculation, but infectious virus was recoverable for up to 3 days after i.c. inoculation (Kesari et al., 1995, 1996). The present studies demonstrate that 1716 replicates in specific cell types (predominantly ventricular ependymal cells), using immunohistochemistry (IHC) for the detection of de novo viral proteins, immunofluorescence for identification of infected cells, in situ hybridization (ISH) for the detection of viral transcripts and titration of infectious virus from brain. These results suggest that although ICP34.5-deficient viruses are neuroattenuated, they do replicate in ependymal cells of the CNS.

Methods

**Viruses stocks.** To produce virus stocks, subconfluent monolayers of baby hamster kidney 21 clone 13 (BHK) cells were infected with HSV variants 1716 (stock titre 1 × 10⁶ p.f.u./ml), 1771, 1771R or parental 17+ (stock titre 2 × 10⁸ p.f.u./ml). Variant 1716 has a 759 bp deletion which deletes parts of the genes encoding ICP34.5, LAT and orfP (MacLean et al., 1991). Variant 1771 has a single stop codon 9 bp downstream of the ATG for the ICP34.5 ORF, and 1771R is a revertant of 1771 which behaves identically to parental 17+ (McKie et al., 1994). Virus was concentrated from the culture, titrated on BHK cells by plaque assay, stored at −70 °C in 0.5 ml aliquots of virus culture medium and thawed rapidly just prior to use as described (Spivack & Fraser, 1987; Valyi-Nagy et al., 1992). Ultraviolet light-inactivated 17+ virus (17+UV) was prepared from stock 17+ (titre 2 × 10⁸ p.f.u./ml) as described previously (Notarianni & Preston, 1982) and inactivation was confirmed by plaque assay. Serum-free medium (SFM) was used for control (mock) inoculation studies as negative controls.

**Virus inoculation.** BALB/c mice (4–6 weeks old) obtained from HSD (Indianapolis, Ind., USA) were anaesthetized (87 mg/kg ketamine, 13 mg/kg xylazine). Using a Hamilton syringe with a 30 gauge removable needle, the appropriate amount of virus (5 × 10⁶ p.f.u. in 5 µl) was injected into the right lateral ventricle of the brain using a small animal stereotactic apparatus (Kopf Instruments). Virus stocks were serially diluted in culture medium for low dose inoculation (100 p.f.u. in 5 µl). The ventricular injections were performed over 3 min, the needle was inserted 2.5 mm into the right hemisphere and the inoculum was injected along the needle tract for 1 mm and then slowly withdrawn over 1 min as described previously (Kesari et al., 1995). The parenchymal injections were performed over 3 min, the virus (1 × 10⁶ in 2 µl) was injected into the right frontal lobe (less volume was used to reduce the chance of leakage). Experiments with 17+ and 1716 were performed in parallel.

**Immunohistochemical procedures.** Mice were transcardially perfused with PBS and fixed with 4% paraformaldehyde (0.1 M PBS pH 7.4), and the brain and trigeminal ganglia were dissected for histological and IHC analysis. The methods for tissue processing and light microscopic IHC analysis were similar to those described elsewhere (Trojanowski et al., 1993, 1994). Rabbit polyclonal antiserum to HSV-1 which detects the major glycoproteins present in the virus envelope and at least one core protein (Dako) was used at a dilution of 1:2000 to detect replicating virus (Adams et al., 1984). Although this antiserum had the ability to detect both structural and non-structural proteins, it did not detect the small amount of input protein following 17+UV inoculation. Antigen-expressing cells were detected by the indirect avidin–biotin immunoperoxidase (Vectastain ABC Kit, Vector Laboratories) and 3', 3'-diaminobenzidine as the chromogen. Sixty mice were inoculated with 1716 into the ventricle and sacrificed at different times (five each at days 1, 2, 3, 4, 7, 10, 15, 21, 27, 30, 44 and at month 7). Six mice were parenchymally inoculated with 1716 and three mice each were sacrificed at days 3 and 30. Six 17+ ventricle-inoculated mice were sacrificed at days 1, 2 and 3; two mice were parenchymally inoculated with 17+ and sacrificed at day 2.

**Immunofluorescence detection of double-labelled cells.** For immunofluorescence studies, anti-HSV (1:500), anti-GFAP (1:10) and anti-MAP2 (1:100) ascites were used alone and in combination for co-localization studies. The secondary antibodies were conjugated with either fluorescein isothiocyanate (FITC) or Texas red probes. A Nikon microphot FXA photomicroscope equipped with epifluorescence and FITC and Texas red filters was used for photomicrography. Bright-field as well as fluorescent photomicrographs were taken using a Sony 3CCD colour video camera and Northern Exposure Image analysis program (Empix Imaging, Release 2.9d). Sections from uninfected mice and primary antibody omission on infected sections were used as controls.

**ISH for HSV-1-specific gene expression.** Sections of perfused and fixed tissue were mounted on slides and ISH was performed to detect viral gene expression as previously described (Valyi-Nagy et al., 1994b). The LATT probe BstEII–BstEII subfragment of BamHII B, the BamHII Y fragment from plasmid pKB113 containing the ICPl4 probe, the
**CNS that were permissive for 1716, we performed IHC**

immunopositive cells were not observed in uninfected, SFM-rounding-up and the presence of inclusion bodies. HSV-subependymal region (Fig. 1C). Histologically, these cells had immunopositive for HSV with some positive cells in the (Fig. 1B). At day 2 almost all the ependymal cells were detected only in the ependymal cells lining the ventricles (Fig. 1A). The ependymal

were sacrificed by lethal injection of anaesthesia. The brains were dissected from mice sacrificed at different times post-inoculation (p.i.) (1, 4, 8, 12, 24, 48 and 72 h; two to five animals per time-point), quick frozen in liquid nitrogen and stored at −70 °C. For the ventricular injections, the whole brain was saved as one sample and titred as one sample. For the parenchymal injections, the frontal lobe from the posterior border of the injection site to the anterior border of the olfactory bulb (called anterior portion) and the rest of the brain posterior to the injection site (called posterior portion) were saved as separate samples and titred separately. The samples from the different time-points were quickly thawed in a 37 °C water bath, and the tissue was homogenized in culture medium at 10% w/v using a Pyrex Ten Broeck tissue grinder. The homogenates were centrifuged at 3000 g for 10 min at 4 °C, the supernatant was diluted exponentially in media and the virus titre of each sample was determined by plaque assay on BHK cells (Spivack & Fraser, 1987).

**Results**

**IHC localization of infected cells after ventricular and parenchymal inoculation**

In order to assess whether there were any cells in the mouse CNS that were permissive for 1716, we performed IHC analyses on brains of BALB/c mice infected via i.c. inoculation into the ventricle at different times (5 × 10^3 p.f.u./5 µl; days 1, 2, 3, 4, 7, 10, 15, 21, 27 and 30). Except for the first 5–7 days when piloerection was observed, these mice did not show any obvious signs of disease or encephalitis. However, on gross dissection and histology some of the mice infected with 1716 had enlarged ventricles, and this was not observed in uninfected mice. Some SFM- and 17+UV-inoculated mice were seen by histology to have slightly dilated ventricles at day 1 (Fig. 1A).

Using a polyclonal HSV antibody which detects late viral proteins, sections from animals were screened for virus-infected cells. At day 1 after 1716 inoculation, immunopositive cells were detected only in the ependymal cells lining the ventricles (Fig. 1B). At day 2 almost all the ependymal cells were immunopositive for HSV with some positive cells in the subependymal region (Fig. 1C). Histologically, these cells had the characteristic features of herpes-infected cells, including rounding-up and the presence of inclusion bodies. HSV-immunopositive cells were not observed in uninfected, SFM-

inoculated and 17+UV-inoculated mice at days 1, 2, 7 and 30 p.i. (Fig. 1A), suggesting that most of the immunopositivity is due to detection of de novo synthesis of viral proteins in infected cells and not due to detection of input virion proteins on the surface or on extracellular virus. Since most of the positive cells lined the lateral ventricles with some around the third and fourth ventricles, this may reflect the spread of virus by the flow of cerebrospinal fluid from the lateral to the third and fourth ventricles (Fig. 1E). By day 4, ependymal cells throughout the ventricular system were positive for virus antigens. Immunopositive cells were also detected in the subependymal region (Fig. 1D, E, G), choroid plexus (Fig. 1H) and the parenchyma (Fig. 1I). By day 7, only a few immunopositive cells were detected in the ependyma and adjacent brain parenchyma. From day 10 (Fig. 1F) through to day 30, no HSV-immunopositive cells were detected in 1716-inoculated mice. In contrast, in mice ventricularly inoculated with parental 17+ (10^6 p.f.u./5 µl, and also at lower doses) both ependymal and widespread parenchymal cells were positive as early as day 1 (Fig. 1, K, L).

In contrast to ventricle inoculation, parenchymal inoculation with 1716 (5 × 10^3 p.f.u.) showed immunopositive cells at the site of inoculation but not in the ependyma (day 2; Fig. 2A, B). Parenchymal inoculation with 17+ (10^6 p.f.u.) resulted in infection of parenchyma at the site of inoculation (Fig. 2D) and also widespread infection of ependyma and other parenchymal sites (Fig. 2C). Inoculation of mice with 17+ (doses ranging from 10^2 to 10^6 p.f.u.) produces encephalitis, and 100% of the mice die by 3–10 days p.i., the time depending on the dose (unpublished observations). The lethal dose (50%) is less than 10 p.f.u. for 17+ (McKie et al., 1994). Inoculation with 17+ induced a perivascular inflammatory response and in some cases enlarged ventricles were present (data not shown).

Similar findings to those for 1716 were observed in mice infected with 1771, which has a point mutation in the ORF of ICP34.5 (McKie et al., 1994). Thus, the properties of 1716 and 1771 are probably due to the specific absence of ICP34.5 and not to changes in other unidentified, overlapping genes in the region of ICP34.5. The behaviour of the revertant of 1771 (called 1771R) was indistinguishable from 17+. The ependymal inoculation and loss, as well as ventricular enlargement, were dose-dependent. For example, a low dose ventricular inoculation of 1716 (10^2 p.f.u.) did not result in the loss of ependyma and enlargement of ventricles, and analyses at early time-points showed fewer infected cells compared to the results of a high dose of 1716 (10^5 p.f.u.) (Fig. 1).

**Determination of the cellular phenotype of 1716-infected cells by morphology and immunofluorescence co-localization**

HSV has been shown to infect both neuronal and non-neuronal cells during productive and latent infections (Tenser...
Fig. 1. For legend see facing page.
et al., 1991). But different strains or mutants of HSV can have a different host-cell range (in the same host) which is dependent on a number of viral, immune and cellular factors (Fraser & Valyi-Nagy, 1993). Thus, to determine the cellular phenotype of HSV-infected cells, morphological characterization and double-immunofluorescence co-localization experiments were performed using markers for astroglial (GFAP) and neuronal (MAP2) cells (Fig. 3). Only a few HSV-immunopositive parenchymal cells (< 5 per section) were positive for GFAP in both 1716- and 17\(^+\)-infected mice; HSV is red, GFAP is green and cells that co-localize (arrows) are yellow (Fig. 3A–F). In 1716-infected mice (at day 4 when parenchymal cells were positive for HSV), most of the HSV-immunopositive cells were also MAP2-positive and had neuronal morphology; HSV is red, MAP2 is green and cells that co-localize (arrows) are yellow (Fig. 3G–I). Likewise, in 17\(^+\)-infected mice (at day 2), most of the HSV-positive cells also were MAP2-positive with neuronal morphology (Fig. 3J–L). Although 1716 predominantly infected ependymal cells (which are glial-derived), the small number of infected parenchymal cells were mostly neurons. Thus, similar to 17\(^+\), 1716 infects both ependymal and parenchymal cells, but the degree of infection of parenchymal cells is significantly less in the case of 1716. This suggests either that spread of 1716 from ependyma to parenchyma is inefficient, or that infection of parenchymal cells is reduced in relation to 17\(^+\).

**Virus productive cycle transcripts are detectable during 1716 infection**

HSV genes are expressed through a tightly controlled transcriptional cascade during productive infection and are termed immediate early, early and late genes in order of their appearance (Honess & Roizman, 1974). To determine whether the HSV 1716-immunopositive cells were undergoing a productive infection, ISH was performed to detect these different classes of viral transcripts. Serial sections were probed for RNA of LAT, ICP4 (an immediate early gene), gC (a late gene) and VP5 (a late gene). Tissue sections from 1716-infected mice were used as positive controls (Fig. 4A, C, E). For 17\(^+\), in both ventricle- and parenchyma-infected brains, both ependymal and parenchymal cells were

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**Fig. 1.** Detection of HSV-infected cells after ventricle inoculation with 1716 and 17\(^+\). BALB/c mice (4–6 weeks old) obtained from HSD (Indianapolis, Ind., USA) were stereotactically inoculated into the right ventricle with either 5 \(\times 10^5\) p.f.u. 1716; 1 \(\times 10^6\) p.f.u. 17\(^+\); or 1 \(\times 10^6\) p.f.u. 17\(^+\)-UV and sacrificed at different times p.i. as described under Methods. Representative photomicrographs are shown from some sections of brains after IHC for HSV antigens. No HSV-positive cells were detected in the ependyma of the right lateral ventricle from a 17\(^+\)-UV (1 \(\times 10^6\) p.f.u.)-infected mouse, and the ventricles were slightly dilated at day 1 (A). Ventricles of 1716 (5 \(\times 10^5\) p.f.u.)-infected mice at different days p.i.: days 1 (B); 2 (C); 4 (D) and 10 (F) showing the lateral ventricle, and day 4 (E) showing the third ventricle. During the acute stage of 1716 infection (< 10 days at 5 \(\times 10^5\) p.f.u.), HSV-immunopositive cells were found in the ependyma (day 4, G), choroid plexus (day 4, H) and subependymal parenchyma (day 4, I) and no antigen-positive cells were detectable after 7–10 days (F). Sections from 17\(^+\) (1 \(\times 10^6\) p.f.u.)-infected mice were used as positive controls and show infection of ependymal and subependymal cells (J), brainstem neurons (K) and parenchymal cells (L) as early as day 1. Scale bar: (A–D, J), 296 \(\mu\)m; (E, F, K), 118.5 \(\mu\)m; (G–I, L), 59.3 \(\mu\)m.

**Fig. 2.** Detection of HSV-infected cells after parenchymal inoculation of 1716 and 17\(^+\). Mice were stereotactically inoculated into the parenchyma (frontal lobe) with either 5 \(\times 10^5\) p.f.u. 1716 (three mice each sacrificed at days 2 and 30) or 1 \(\times 10^5\) p.f.u. 17\(^+\) (two mice sacrificed at day 2). HSV-positive cells in the parenchyma are localized at the site of inoculation of 1716 at day 2 (A). Note that both neurons and non-neuronal cells are infected in 1716-infected mice (B). No HSV-positive cells were detected at day 30 in 1716-infected mice (Fig. 7 and corresponding text). In contrast, 17\(^+\) spreads extensively from site of inoculation (D) to distant locations away from the inoculation site including the cortex (C), brainstem and ependyma. Scale bar: (A, C), 118.5 \(\mu\)m; (B, D), 59.3 \(\mu\)m.
positive for ICP4 (Fig. 4C), LAT (Fig. 4E), gC and VP5. In 1716 ventricle-infected mice, a subset of the immunopositive ependymal cells (Fig. 4B) was also positive for ICP4 (Fig. 4D), LAT (Fig. 4F), gC and VP5 transcripts. Also, in both 1716 ventricle- and parenchyma-inoculated brains, transcript-positive cells were detected in the parenchyma. The detection of late RNA and protein expression in these infected ependymal cells suggests that there is no block in the characteristic
productive gene and protein expression and that these cells may be undergoing a full productive infection (see below).

**After ventricular inoculation with 1716, infectious virus is recovered**

Although there is no block to viral gene and protein expression in 1716-infected cells, infectious virus may not be produced. To determine whether the infection observed by IHC and ISH resulted in the production of infectious virus, titrations were performed after i.c. inoculation at two different sites: ventricular versus parenchymal inoculations (Fig. 5A). The results of recovery of infectious 1716 or 17+ after i.c. inoculation into the ventricle is shown in Fig. 5(B). The titre of 1716 (Fig. 5B) drops at 8 h and then increases over time, levelling off between 24 and 48 h, and virus is not detectable after 72 h. The rapid drop and increase in the first 24 h is characteristic of a single-step growth curve. The flattening of the curve between 24 and 48 h and the decrease thereafter suggest that at least one round of replication and no more than 2–3 replication cycles are occurring, assuming 18–20 h per cycle as seen in tissue culture (Roizman & Sears, 1996). In contrast, the recovery of 17+ from ventricular inoculations showed a higher slope and no decrease after 24 h (Fig. 5B). Furthermore, no infectious virus was recoverable after i.c. inoculation of 17+UV or from uninfected mice (data not shown). Thus, infection of ependymal cells and/or parenchymal cells must lead to the production of infectious virus.

To determine whether the infectious virus recovered was from ependymal and/or parenchymal infection, a modified protocol was used (see legend to Fig. 5A). Fig. 5(C) shows the recovery of 1716 and 17+ after parenchymal inoculation. Virus was inoculated into the parenchyma and the brain was separated into two parts, anterior (containing the inoculation site) and posterior (rest of the brain posterior to the inoculation site) (Fig. 5A), and titred separately. At time 0 (input), 1716 was recovered in the anterior portion, but was not detected thereafter in either the anterior or posterior portions (Fig. 5C). This suggests that either there is no production of infectious virus in the parenchyma or there is a low level which is below the detection limit of the assay. Although immunopositive cells were detected in the parenchyma in both the ventricular
The long-term consequences of ependymal infection are ependymal loss, ventricular enlargement and inflammation. Although detailed behavioural studies have not been performed, the mice seem normal except for some hyperactivity. The enlarged ventricles persisted from early days (days 2 and 10; Fig. 6 B, C) to 7 months (Fig. 6 D) after 1716 inoculation. This was not observed in SFM- and 17+UV-inoculated mice (day 30; Fig. 6 A). Also, in all 1716-infected mice sacrificed after 10 days, there were no detectable HSV-immunopositive cells, but in most of these cases the ependyma was almost completely denuded (Fig. 6 D, E). The choroid plexus was preserved in all mice (Fig. 6 C, F). Perivascular inflammation was present in both 1716- and 17+.infected mice. In mice where the virus was inoculated into the parenchyma without leakage into ventricles, the ependyma was not denuded and there was no enlargement of the ventricles, suggesting that the ventricular enlargement is due to ependymal infection and not to another effect of HSV infection of non-ependymal cells.

To determine whether 1716 established a latent infection after ventricle inoculation, ISH was performed for the detection of LATs in the absence of other productive viral transcripts (ICP4, gC and VP5) and viral proteins. In ventricle-inoculated mice, at day 30, surprisingly few LAT-expressing cells were detected in the brainstem (Fig. 7 A, B). This is in contrast to a previous study which showed widespread latent infection of the CNS after parenchymal inoculation (Kesari et al., 1996). This may be due to reduced infection of the parenchyma after ventricle inoculation and/or to secondary effect of ependymal loss/ventricle enlargement on the expression of LATs.

Discussion

The data presented here suggest that although ICP34.5-deficient viruses are neuroattenuated, they do retain the ability to replicate in ependymal cells. In the CNS there is a variety of cell types having diverse functions, including neurons, oligodendrocytes and astrocytes as well as ependymal, subependymal, endothelial and microglial cells. Differences in the molecular phenotype of these cells in different areas of the brain may determine the contrasting patterns of infection, spread and pathogenicity of different viruses and of different strains or variants of viruses. Although the reason for the greater vulnerability of the ependyma than the parenchyma to 1716 infection is unclear, it may be related to the proliferative ability or maturational state of different CNS cell types. It is clear that there are dividing progenitor cells in the subependymal region of the adult CNS of mammals, including mice and humans, which give rise to neurons and glia (Reynolds & Weiss, 1992). There are also dividing cells in the hippocampus (Gage et al., 1995). How the proliferative capacity of different populations of adult CNS cells affects the ability of different strains of HSV to replicate is unknown, but this may be important in understanding the vulnerability of specific brain areas to certain diseases, such as the limbic system in HSV encephalitis. In the present studies, almost 100% of the ependymal cells were infected by 1716, suggesting that
factor(s) specific in ependymal cells (i.e. expression of complementing and/or inhibiting factors such as GADD34/MyD116 homologues) complement the defect in 1716. Thus, these data show that the ependyma is the major site for 1716 replication in the CNS in immunocompetent mice. Also, it would be of interest to study the biology of this virus in an immuno-incompetent mouse to determine if and how the immune system plays a role in restricting replication in the CNS.

Although the ependyma plays an important homeostatic role in the foetal CNS, the function of the ependyma in the adult CNS is not clear (Del Bigio, 1995). Over the course of these studies there was no lethality associated with CNS infection by 1716, confirming that the ependyma in the adult mouse is not essential for survival. But the enlarged ventricles (hydrocephalus) suggest that there is pathology associated with this loss. Hydrocephalus has been shown to be produced in foetal and neonatal animals by a variety of viruses (Johnson, 1967, 1975) and has been shown to be produced in adult mice by influenza virus (Johnson & Johnson, 1972) and HSV (Hayashi et al., 1986). Hydrocephalus often develops as a sequelae to ependymal damage in humans (Del Bigio, 1993, 1995; Sarnat, 1995). Hydrocephalus is characterized by accumulation of cerebrospinal fluid within the ventricle.
associated with an elevated i.c. pressure. However, the crania of mice (unlike those of humans) are capable of expanding with elevated pressure, as seen in animals with i.c. tumors (Kesari et al., 1995). Thus, several processes may be involved in the ventricular enlargement seen here, such as hydrocephalus, cell death, demyelination, inflammation or a combination of these processes.

The replication of herpesviruses is an extremely inefficient process; less than 10% of the viral DNA is assembled into virus particles and a large excess of viral proteins is produced (Johnson, 1982). The complex interaction of many viral and cellular proteins is involved in determining the final fate of the infected cell and the output of virus. The products of some genes are dispensable in certain situations (i.e. in vitro), or are needed only for efficient production of virus. The RL1 (ICP34.5) gene was identified genetically as a neurovirulence gene in mice (Chou et al., 1990; MacLean et al., 1991). Subsequent in vitro studies have been performed in cultured cells to study the function of this gene product (ICP34.5) (Brown et al., 1994a, b; Chou et al., 1994; Chou & Roizman, 1992, 1994). However, it is not clear if these in vitro studies will lead to the elucidation of the role of ICP34.5 in neurovirulence, as neurovirulence is an in vivo phenotype. In contrast, the in vivo studies described here using ICP34.5-deficient viruses show that limited replication of these mutant viruses can be detected in ependymal cells after direct ventricular injection but not after parenchymal injection. In light of the in vivo data documenting a large complement of HSV gene and protein expression in the CNS of 1716-infected mice, it is speculated that ICP34.5 is needed either for efficient virion production and/or for the cell-to-cell propagation or spread of HSV in vivo, or that factor(s) specific to ependymal cells complement the defect in 1716.

The further elucidation of the CNS cell-type-specific replication and gene expression of these and other attenuated herpesviruses in the CNS is important in understanding the natural biology and pathogenesis of HSV in humans. With increasing interest in using viral vectors for therapy of CNS diseases, study of the biology of HSV and mutants in the CNS will be important in evaluating its pathogenicity and will suggest ways to circumvent potential problems that may arise in the clinical setting. More significantly, such information is critical for the design of HSV vectors for use in cancer and gene therapy in the CNS.

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