Neurotropism of herpes simplex virus type 1 in brain organ cultures

Efrat Braun,1,2,3 Tal Zimmerman,3 Tamir Ben Hur,2 Etti Reinhartz,2 Yakov Fellig,4 Amos Panet3 and Israel Steiner1,2

1,2,4Laboratory of Neurovirology1, Department of Neurology2 and Department of Pathology4, Hadassah University Hospital, PO Box 12000, Jerusalem 91120, Israel
3Department of Virology, The Hebrew University, Hadassah Medical School, Jerusalem, Israel

The mechanism of herpes simplex virus type 1 (HSV-1) penetration into the brain and its predilection to infect certain neuronal regions is unknown. In order to study HSV-1 neurotropism, an ex vivo system of mice organotypic brain slices was established and the tissue was infected with HSV-1 vectors. Neonate tissues showed restricted infection confined to leptomeningeal, periventricular and cortical brain regions. The hippocampus was the primary parenchymatous structure that was also infected. Infection was localized to early progenitor and ependymal cells. Increasing viral inoculum increased the intensity and enlarged the infected territory, but the distinctive pattern of infection was maintained and differed from that observed with adenovirus and Vaccinia virus. Neonate brain tissues were much more permissive for HSV-1 infection than adult mouse brain tissues. Taken together, these results indicate a complex interaction of HSV-1 with different brain-cell types and provide a useful vehicle to elucidate the mechanisms of viral neurotropism.

INTRODUCTION

Many viruses use ubiquitous receptors present on most cells of the body (Gosztonyi & Koprowski, 2001; Schweighardt & Atwood, 2001), yet viral infections in vivo with their resultant clinical diseases are typically confined to a single or several tissues and cell types. This viral 'tropism' may be a consequence of anatomic barriers, a unique transport mechanism or three-dimensional tissue–virus specific interactions. Herpes simplex virus type 1 (HSV-1; Human herpesvirus 1) is defined as a neurotropic virus, as it is the source of recurrent mucocutaneous disease that stems from its ability to establish latency in peripheral sensory ganglia (PSG) and reactivate. Rarely, it can also infect the brain, leading to fatal encephalitis (Steiner, 2003; Whitley & Roizman, 2001). In addition, the virus is a promising vector for gene therapy to the nervous system, and targeting herpes-derived vectors to selective brain regions is an important aspect of successful therapy (Kennedy & Steiner, 1993; Latchman, 2003). Therefore, uncovering the cellular and molecular aspects of its neurotropism is an important step not only in providing rational preventive and active therapeutic measures against encephalitis, but also for HSV-1 gene delivery to the nervous system.

Following infection of peripherally exposed tissues such as mucosal epithelia, HSV-1 can enter nearby nerve endings and either is transported to neuronal cell bodies in PSG by axonal transport or, in rare cases, enters the central nervous system (CNS) directly via the olfactory tract. In the nuclei of trigeminal ganglia neurons, the viral genome can enter a latent state (Steiner et al., 1989) or, rarely, initiate a productive replication cycle. Why is the CNS disease caused by HSV-1 so infrequent compared with the peripheral disorder? How does the virus enter the nervous system? Why is it attacking only selected brain regions? What determines a productive (versus latent) outcome of the CNS infection? How does the virus spread throughout the nervous system? Why is active brain infection not associated with extension of infection to other systemic organs? A number of experimental systems were developed to address these issues, including animal models and tissue cultures. Unfortunately, both have major limitations. In experimental animals, factors that can influence the severity of the disease and the affected areas include strain of the mouse, virulence of the virus strain (Lopez, 1975; Mao & Rosenthal, 2003), its mode of propagation (Jensen & Norrild, 2000) and inoculation route (Ben-Hur et al., 1988; Bergstrom et al., 1994). Intranasal HSV-1 inoculation mimics acquisition of herpes encephalitis (HE) from the periphery, but is associated with viraemia and infection of other organs such as lungs and liver (Kern et al., 1982). Intracerebral inoculation is less relevant to the pathology of HE and induces localized infection around the site of virus injection and virus spread via retrograde axonal transport to cell bodies located in distant brain areas in the rat (Maidment et al., 1996; Shiraki et al., 1998).

A supplementary figure showing the pattern of HSV-1 infection of neonate rat brain slices is available in JGV Online.

Received 22 January 2006
Accepted 6 June 2006

DOI 10.1099/vir.0.81850-0
Likewise, cell-culture systems are unsatisfactory. HSV-1 can infect and replicate productively in primary cultured cells established from most tissues or in cell lines, although neurons are relatively non-permissive for HSV-1 infection compared with other cell types (Kennedy et al., 1983). Therefore, the mechanisms underlying viral restriction to a specific organ or tissue cannot be studied in cultured cells.

To address the fundamental question of HSV-1 tissue tropism, independent of the immune system and systemic cytokines, we have applied an ex vivo organ-culture system that enables studies of virus tropism to specific cell populations and brain areas in the context of a normal architectural tissue organization. Previous studies have demonstrated the applicability of the organ system to the analysis of virus tropism (Havenga et al., 2002; Reinhardt et al., 2003; Taylor & Moffat, 2005) and gene therapy (Banin et al., 2005; Brill-Almon et al., 2005; Hasson et al., 2005). Brain organ cultures have been used extensively to study neuronal signal transmission and to examine pharmacological effects (Stoppini et al., 1991, 1997). Yet, there are only a few reports of application of brain organ-culture systems to study viral tropism (Bergold et al., 1993; Mayer et al., 2005; Sato et al., 2004), including cytomegalovirus (CMV), another herpesvirus (Kawasaki et al., 2002).

The present study aimed to explore the feasibility of the organotypic culture system for the assessment of HSV-1 neurotropism and the mechanisms involved in virus infection of a three-dimensional brain tissue.

**METHODS**

**Organotypic brain-slice cultures.** Brains from neonate (1–2 days) and adult (28 days old) BALB/c mice and neonate SABRA rats were removed surgically after decapitation and embedded in 5 % agarose (type IX; Sigma). Brains were cut coronally at 400–500 μm thickness with a tissue sectioner TC-2 (Sorvall) and transferred to Millipore membrane according to Stoppini et al. (1991). Slices were kept in growth medium until infection. Growth medium consisted of minimal essential medium (50 %), Hank’s balanced salt solution (25 %), horse serum (25 %), 6-5 mg dl-glucose ml⁻¹, 20 mM HEPES and streptomycin/penicillin (5 g ml⁻¹ and 5000 units ml⁻¹, respectively), pH 7.2. Tissue slices were incubated with virus in 1 ml serum-free adsorption medium for 2 h with occasional shaking. Then, the culture medium was replaced with growth medium containing serum. Slices were maintained at 37 °C in a humidified atmosphere of 95 % air and 7.5-5 % CO₂ for 8 h followed by X-Gal staining.

**Viruses.** Two HSV-1 viruses containing the reporter β-galactosidase (β-gal) gene were used: (i) tkLTRZ1, originating from HSV-1 KOS, includes the reporter β-gal gene under the control of the MuLV long terminal repeat (LTR) promoter inserted at the thymidine kinase gene locus (Davar et al., 1994), referred to as HSV-(MuLV-β-gal). (ii) HSV 17+ /pR20.5/5 contains the β-gal gene under the control of the RSV promoter and the green fluorescent protein (GFP) gene under the control of the CMV promoter, referred to as HSV-(RSV-β-gal). Both genes were inserted at the US3 gene locus (Thomas et al., 1999). Viruses were propagated in CV-1 cells.

Adenovirus Ad5CMVlacZ, containing the β-gal gene under control of the immediate-early CMV promoter and deleted of the E1A gene (Prevec et al., 1991), is replication-defective in cells that do not express the E1A gene and was propagated in 293 cells.

Vaccinia vsC9 virus, encoding the β-gal gene under control of the vaccinia TK early promoter, was propagated in human BSC-1 cells (Chakrabarti et al., 1995).

Viruses were purified by ultracentrifugation on 10–20 % sucrose cushions and titrated on mouse NIH-3T3 cells.

**MTT cell-viability assay.** The MTT cell-viability assay (Miller & McDevitt, 1991) is based on the ability of mitochondria to convert MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] (Sigma) into a blue formazan product and adapted to organ culture (Connelly et al., 2000). Brain slices were incubated with MTT reagent for 45 min at 37 °C, followed by the addition of 100 % ethanol to solubilize the coloured crystals. Samples were analysed by using an ELISA plate reader (Organon Teknika) at a wavelength of 540 nm in reference to 650 nm (Connelly et al., 2000). The amount of colour produced is proportional to the number of viable cells.

Brain slices were homogenized with 0.1 % Triton X-100 in PBS and the MTT values were normalized by quantity of protein in the extracts, as assessed by Bradford assay.

**Caspase viability assay.** The caspase viability assay measures caspase-3 activity as an indication of apoptotic death. It is based on hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp (Ac-DEVD) labelled with the chromophore p-nitroaniline (pNA) by caspase-3, resulting in release of pNA (Nicholson et al., 1995; Talanian et al., 1997). Free pNA produces a yellow colour, proportional to the amount of caspase activity present, and is measured by a spectrophotometer at 405 nm. Brain slices, 0, 8 and 24 h post-implantation with and without addition of 50 μM etoposide, were lysed with lysis buffer (0-1 % Triton X-100, 50 mM HEPES, 1 mM dithiothreitol) and homogenized by using an electric homogenizer. Lysates were then frozen and thawed three times at −20 °C, sonicated twice for 2 min and centrifuged for 10 min at approximately 2000 g. Protein amount was assessed by Bradford assay and 5, 20 and 50 μg protein was assayed by the CaspACE colorimetric assay system (Promega). Ninety-six-well plates were incubated at 37 °C and read by an ELISA reader every 30 min for 210 min. Results of 50 μg protein at 210 min are presented.

**X-Gal staining of tissues.** Brain slices or cells were fixed for 5–10 min in 0-2 % glutaraldehyde, 2 % formaldehyde, 2 mM MgCl₂ in PBS (pH 7.4), washed in PBS and incubated for 2 h at 37 °C in 0-05 M NaPO₄ buffer (pH 7.4), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂ and 20 mg X-Gal substrate (5-bromo-4-chloro-3-indolyl β-d-galactopyranoside) ml⁻¹, followed by fixation with 4 % formaldehyde. Two hours incubation was within the linear phase of the blue-colour accumulation, whilst a non-specific staining in mock-infected tissue was absent during this time frame.

**Histological examination.** Brain slices were fixed in 7-4 % formaldehyde, 80 % ethanol for 10 days. Sections (5 μm) of paraffin blocks were stained with haematoxylin and eosin (H&E). Slides were screened with a light microscope (Axioplan 2; Zeiss) and photographed with a CCD camera (Axiscam; Zeiss). For identification of brain areas, the Mouse Brain Atlas: C57BL/6J Coronal (www.mbl.org/atlas170/atlas170_frame.html) was used.

**Immunofluorescence.** After X-Gal staining, slices were washed in PBS, fixed with 2 % paraformaldehyde for 20 min at room temperature and washed three times in PBS. Slices were incubated in blocking buffer (1 % BSA, 10 % normal goat serum, 0-3 % Triton X-100, 1 % Triton X-100, 1 % Triton X-100, 1 % Triton X-100, 1 % Triton X-100).
in PBS) for 1 h at room temperature, followed by 2 days incubation with mouse anti-nestin mAb (Wiese et al., 2004) (IgG diluted 1:130; Chemicon) or with rabbit anti-cow glial fibrillary acidic protein (GFAP) antibody (diluted 1:130; Dako). As neuronal markers, we used the anti-β-tubulin isotype III mAb (diluted 1:600; Sigma) and the anti-human neurofilament protein mAb (diluted 1:60; Dako). Following washing, slices were incubated with Cy5-conjugated goat anti-mouse IgG antibody (diluted 1:150; Jackson ImmunoResearch) or with Texas red-conjugated goat anti-rabbit IgG antibody (diluted 1:150; Jackson ImmunoResearch) and mounted with a Vectastain ABC kit (Vector Laboratories Inc.). Immunostained slices were analysed with a laser-scanning confocal microscope at 2 μm increments through the z axis and sequential images were collected by using Zeiss supplied analysis software.

### RESULTS

**HSV-1 infection of neonate brain slices**

Brain tissue obtained from 1-day-old neonate mice was sliced coronally and 500 μm consecutive slices in a face-to-face orientation were infected with 1 × 10^6 p.f.u. HSV-(MuLV-β-gal) ml^{-1}, carrying the β-gal gene under control of the LTR promoter of MuLV. Infected slices were cultured for 8 h and stained for β-gal expression (Fig. 1a, d, g). Mock-infected brain slices did not show a non-specific staining and served as control. In slices taken from septo-striatal (bregma 0–74), septo-diencephalic (bregma –2–92) and the rostral mesencephalon (bregma –0–82) areas (Fig. 1a, d, g), infected blue cells were observed in the periphery of the brain tissue, corresponding to leptomeningeal and cortical cells. More pronounced infection was observed in cells located around the ventricle (Fig. 1a, d, arrows). Another area showing infection corresponded to the dentate gyrus of the hippocampus in slices taken from the rostral mesencephalon (Fig. 1j, arrowhead). In the olfactory bulb (bregma 4–64), the infection was mainly confined to the region surrounding the olfactory ventricle (Fig. 1j, arrow) and the meninges (Fig. 1j, arrowhead).

Brain tissues obtained from neonate rats were also infected with HSV-(RSV-β-gal). The infection pattern was similar to that observed in neonate mouse brain (see Supplementary Fig. S1a, b, available in JGV Online), namely, infection was confined to leptomeningeal, periventricular and cortical brain regions.
Is HSV-1 infection of neonate brain slices virus titre-dependent?

As cultured cells of neuronal origin are relatively non-permissive to HSV-1 infection at low m.o.i. (Kemp et al., 1990; Mador et al., 1998), we compared the infection of neuronal tissue of two HSV-(MuLV-β-gal) titres: $1 \times 10^6$ p.f.u. ml$^{-1}$ (Fig. 1a, d, g) and $3 \times 10^7$ p.f.u. ml$^{-1}$ (Fig. 1b, e, h). Infection with the low viral titre gave a localized pattern as described above, confined mainly to the meninges and to regions adjacent to the lateral ventricles (Fig. 1a, d, g). In contrast, infection with a 30-fold higher titre (Fig. 1b, e, h) showed a more extensive infection pattern in the meninges and the lateral ventricles, including cells in the parenchyma (Fig. 1b, white arrow), cortical cells (arrow), cells located in the periventricular region (Fig. 1b, e, arrowheads) and a vast territory of the hippocampus (Fig. 1b, arrow). Nevertheless, the basic pattern of infection observed with the low viral titre was maintained also with the high viral titre of infection, namely, HSV-1 infection was largely restricted to periventricular and perimeningeal regions. Therefore, it can be concluded that the pattern of HSV-1 infection ex vivo is specific and is basically preserved, even at high viral titres.

Viability of brain slices cultured ex vivo

The experiment illustrated in Fig. 1 also demonstrates, in part, the viability of the brain slices during 8 h culture. As only particular cells were infected when lower titres of virus were used, it could be argued that the metabolic state of specific cells in the tissue determined the pattern of infection. However, the fact that the increase in HSV-1 inoculum resulted in infection of many more cells within the tissue suggests that the pattern of infection is probably not affected by the viability of the tissue.

Two independent methods were used to verify tissue viability within the time frame of the experiments (Fig. 2). We examined tissue viability by the MTT assay at 8, 24 and 48 h post-explantation and compared it with a non-viable tissue treated with 4 % formaldehyde that served as a negative control. Brain slices immediately after explantation served as positive controls (zero time). Slices were incubated with MTT for 45 min, followed by extraction of the MTT colour product with ethanol.

In order to compare the viability among the tissue groups, protein content was determined and results of the MTT assay were corrected for protein content. The results shown in Fig. 2(a) indicate only a slight reduction in tissue viability during the first 24 h culture.

We also examined viability by using the caspase-3 colorimetric assay. Brain slices at 8 and 24 h post-explantation were lysed and caspase enzyme activity was measured by amount of Ac-DEVD-pNA substrate cleaved by caspase-3 (Fig. 2b). Fresh (zero time) slices (viable tissue) and etoposide-treated slices at 8 and 24 h served as negative and positive controls, respectively. During the first 24 h post-explantation, a gradual and slow increase (up to threefold) in caspase activity was observed. Nevertheless, as expected, etoposide increased caspase activity at 8 and 24 h by 4.5- and 8.7-fold, respectively.

As we observed a distinct reduction in viability (MTT assay) 24–48 h post-explantation, we conducted the experiments within the first 8–24 h post-explantation.

Comparison of HSVs containing a reporter gene controlled by different promoters

In order to investigate whether the specific pattern of tissue infection was HSV-1 strain-dependent or a consequence of

![Fig. 2. Viability of organotypic brain slices. Quantitative viability assessment of tissue was performed at 8, 24 and 48 h post-culturing by the MTT (a) and caspase-3 (b) assays. For the MTT assay: positive control, slices stained immediately after sectioning (zero time); negative control, slices incubated in 4 % formaldehyde for 2 h. For the caspase-3 assay: positive control, slices induced for apoptosis with 10 mM etoposide; negative control, slices stained immediately after sectioning (zero time). For both assays, standard errors from three different slices are provided.](image)
the regulatory elements controlling β-gal expression, we compared HSV-1 infection of neonate brain slices using two different β-gal-expressing HSV-1 mutants: HSV-(RSV-β-gal), derived from HSV-1 strain 17+, containing the reporter gene under control of the RSV promoter, and HSV-(MuLV-β-gal), a KOS strain virus, where the reporter gene is controlled by the MuLV LTR promoter (Fig. 3). Consecutive slices from the rostral mesencephalon (Fig. 3a, c) and the septo-diencephalon (Fig. 3b, d) were infected with similar titres (1.2 × 10^7 p.f.u. ml^{-1}) of the viruses. Infection of both viruses was located in similar areas of the slices: in the rostal mesencephalon, it was concentrated in the leptomeninges (Fig. 3a, c, arrows) and the hippocampus (Fig. 3a, c, arrowheads), and in the septo-diencephalon, infection was observed in the leptomeninges (Fig. 3b, d, arrows) and in the lateral ventricles (Fig. 3b, d, asterisks). Mock-infected brain slices did not show a non-specific staining (data not shown). The similar infection pattern observed with both HSV-(MuLV-β-gal) and HSV-(RSV-β-gal) indicated that the particular pattern of infection stemmed from HSV-1 tropism to specific cells in the tissue and was not the outcome of the reporter-gene promoter or virus strain specificities.

Comparable results were obtained with neonate brain tissue of rats. Both HSV-(RSV-β-gal) and HSV-(MuLV-β-gal) gave similar infection patterns when compared with mouse brain (see Supplementary Fig. S1a–c, available in JGV Online).

**Cells in the neonate brain permissive for HSV-1 infection**

In order to histologically define the cells permissive for HSV-1 infection in neonate brain, slices were infected with 3.3 × 10^6 p.f.u. HSV-(MuLV-β-gal) ml^{-1}, stained with X-Gal, and 5 μm wide histology sections were stained with H&E (Fig. 4). In a section obtained from the rostral diencephalon, infection was restricted to cortical cells within

---

**Fig. 3.** Patterns of infection with two HSV-1 strains. Brain slices were infected for 8 h with HSV-(RSV-β-gal) (a, b) or HSV-(MuLV-β-gal) (c, d) at equal titres of 1.2 × 10^7 p.f.u. ml^{-1} and X-Gal-stained. Original magnification, ×5. The anatomical location of sections is indicated.

**Fig. 4.** Delineation of regions permissive for HSV-1 infection. (a) H&E-stained 5 μm sections of neonate brain infected for 8 h with 3.3 × 10^6 p.f.u. HSV-(MuLV-β-gal) ml^{-1} and stained with X-Gal. (b) Photo of the section (original magnification, ×100) showing insets (i–iv) (original magnification, ×1000): (i) parenchyma; (ii) cortical cells; (iii) leptomeninges; (iv) ependymal and subependymal cells.
brain parenchyma [Fig. 4b(i, ii)], leptomeningeal cells [Fig. 4b(iii)] and ependymal and subependymal germinal matrix cells within the periventricular region [Fig. 4b(iv)].

In order to define cell types infected by HSV-1 in the periventricular area, immunofluorescent analysis was performed using antibodies against nestin, GFAP, β-tubulin and neurofilament (Fig. 5). Nestin is an intermediate filament protein that serves as a marker for progenitor cells in the nervous system (Frederiksen & McKay, 1988; Wiese et al., 2004) and GFAP is expressed in mature astrocytes, whilst β-tubulin (intracellular microtubules) and neurofilament (intermediate filaments of the cytoskeleton) are expressed in mature neurons (Katsetos et al., 2003; Liu et al., 2004).

Slices were infected with $1 \times 10^6$ p.f.u. HSV-(RSV-β-gal) ml$^{-1}$, expressing the reporter GFP gene, and immunofluorescent analysis was carried out (Fig. 5). The majority of cells harbouring HSV-1 that were also immunostained were nestin-positive (red cells) (Fig. 5a). In contrast, only a few of the infected cells stained for GFAP (Fig. 5b) or β-tubulin (Fig. 5c) and neurofilament (Fig. 5d). Slices that were incubated only with the second antibody (Cy5-conjugated goat anti-mouse IgG antibody or Texas red-conjugated goat anti-rabbit IgG antibody) showed no fluorescence (data not shown), and neither did the mock-infected tissue (Fig. 5e).

**Comparison of HSV-1 neurotropism with that of adeno- and vaccinia viruses**

Being a neurotropic virus responsible for human encephalitis, the question of specificity of HSV-1 infection of brain tissues *ex vivo* compared with that of non-neurotrophic viruses is an important issue. Therefore, we compared the infection pattern of HSV-1 with that of two other, non-neurotropic viruses, adenovirus and *Vaccinia virus*. Infection of neonate brain slices was performed by using adenovirus Ad5CMVlacZ (where the β-gal gene is controlled by the

![Fig. 5. Immunohistochemistry analysis of HSV-1-infected brain-cell types. Slices infected with $1 \times 10^6$ p.f.u. HSV-(RSV-β-gal) ml$^{-1}$ (stained green for GFP) and immunostained (red) for nestin (a), GFAP (b), β-tubulin (c), neurofilament (d) or mock-infected (e). Original magnification, × 40.]

![Fig. 6. HSV-1 neurotropism compared with that of adenovirus and *Vaccinia virus*. Neonate brain slices were infected for 8 h with equal titres of $5 \times 10^5$ p.f.u. ml$^{-1}$ of HSV-(MuLV-β-gal), vaccinia vsC9 or adenovirus Ad5CMVlacZ and stained with X-Gal. Olfactory bulb (i); caudal mesencephalon (ii).]
CMV immediate-early promoter), the vaccinia vsC9 virus (in which the β-gal gene is controlled by the vaccinia TK early promoter) and HSV-(MuLV-β-gal).

In order to infect the brain slices with equal amounts of the three viruses, titration was first carried out using mouse fibroblasts (NIH-3T3 cells). Tissue slices from two representative brain regions, the olfactory bulb [Fig. 6a(i), b(i), c(i)] and the caudal mesencephalon, were infected with $5 \times 10^5$ p.f.u. ml$^{-1}$ of the three viruses [Fig. 6a(ii), b(ii), c(ii)]. This comparison revealed that HSV-1 infection is more restricted and more intensive. Whilst the pattern of vaccinia and adenovirus infection was diffuse and distributed all over the brain, HSV-1 infection was restricted to the olfactory ventricle (Fig. 6a) and the meninges (illustrated in the region of the caudal mesencephalon; Fig. 6a).

**Neonate brain tissue is more permissive to HSV-1 infection than adult brain**

Neonatal HSV infections occur at a higher frequency than adult infections. We therefore compared the ex vivo infection in the neonate mouse brain with that of brain slices obtained from 6–8-week-old mice. Tissue slices were infected with $5 \times 10^5$ p.f.u. HSV-(RSV-β-gal) ml$^{-1}$ and stained for X-Gal 18 h post-infection (Fig. 7). This time point was used because, at 8 h post-infection, the reporter-gene signal in the adult tissue was not strong enough to appreciate. However, no difference in the pattern of infection was observed in the neonate brain at 18 h compared with 8 h, ruling out the possibility that viral spread occurred. The adult brain showed a very restricted pattern of infection, whereas infection of neonate brain-tissue slices with the same titre of virus was much more extensive. In the adult brain, the parenchyma was more resistant to HSV-1 infection, whereas the brain meninges [Fig. 7b(i, ii), arrows] and the periventricular regions [Fig. 7b(i), arrowhead] were infected.

**DISCUSSION**

Despite effective therapy, HE remains a devastating condition (Steiner & Biran, 2002) and patients, even if treated immediately, frequently develop severe cognitive deficits (Gordon et al., 1990). The neurotropism of HSV-1 is a crucial issue in the pathogenesis of HE and the present study aimed to examine the tropism of HSV-1 mouse organotypic cultures in order to elucidate the mechanisms responsible for herpes infection of neuronal tissue.

Organotypic brain cultures are in use to study the interaction between infective pathogens and brain tissue and the response of neuronal cells to infection in the context of the entire tissue ex vivo. Yet, whilst only a few reports have utilized HSV-1 to infect the brain or the spinal cord and explore pathogenesis (Chen et al., 2004), most studies employed the virus as a vehicle for gene transfer to neuronal tissue (Bahr et al., 1994; Casaccia-Bonnefil et al., 1993; Marsh et al., 2000). The advantages of the system are numerous. It maintains the three-dimensional brain architecture and the interaction of neuron and glial cells with the extracellular matrix. It also lacks the interference of the systemic immunity and the anatomical boundaries. However, although the tissue can be maintained ex vivo for several days, astrocytes start to divide and spread after several days and the tissue begins to grow and change its morphology (Raineteau et al., 2004). Whilst long-term culturing of brain slices for over a week is useful to study neuronal physiology (Corner et al., 2005; Crain, 1998) and neurodegenerative processes (Stoppini et al., 1997; Toni et al., 1997), we limited the cultures to 8–24 h to avoid changes that may influence the pattern of the infection. Indeed, using two independent methods to estimate tissue viability, the MTT and caspase-3 enzyme activity assays, we observed only minor changes in the brain slices within the first 24 h culturing.

**Pattern of infection**

The study aimed at following initial HSV-1 infection and not replication or spread of the virus in the tissue. For this purpose, we employed recombinants carrying reporter genes under the control of exogenous, non-HSV promoters and measured gene expression at a time frame (8 h post-infection) prior to the ability of HSV-1 to spread.

HSV-1 infection of brain-tissue slices was mostly localized at specific regions: (i) the periphery of the brain, consistent with leptomeningeal and cortical cells; (ii) around the ventricles (Figs 1, 3, 4); and (iii) in the hippocampus.
(Fig. 1). The findings were obtained in neonatal mouse brain tissue and verified in the neonate rat brain. This distribution differed distinctly from that observed with the two other, non-neurotropic viruses that were examined in the present work, *Vaccinia virus* and adenovirus (Fig. 6). A similar pattern of infection was observed when HSV-1 was inoculated intracerebrally into mice, initially infecting the meninges and the ependymal cells and then spreading to cells directly adjacent to the ventricles (Chrisp *et al.* 1989) or the hippocampus, cerebrum, brainstem and meninges (Thomas *et al.* 2001). Likewise, intraventricular inoculation resulted in infection of cells lining the ventricles and the subarachnoid space (Sundaresan *et al.*, 2000).

The distribution of the infection coincided with the localization of cells in the neonate and adult brain capable of division and regeneration. Neurogenesis has been well documented in *vivo*, both in the rodent (Frederiksen & McKay, 1988; van Praag *et al.*, 2002) and human (Eriksson *et al.*, 1998; Gage, 2002) mature brain. Cells located in the subventricular zone (SVZ) retain the ability to proliferate beyond birth in the mouse neonate brain (Frederiksen & McKay, 1988). Indeed, by using cellular antigen markers, we were able to demonstrate (Fig. 5) that a significant portion of cells infected with HSV-1 also stained for nestin, a protein that is expressed in undifferentiated cells, including neuronal stem cells (Frederiksen & McKay, 1988; Wiese *et al.*, 2004). HSV-1 infected only a few differentiated cells, as the proportion of GFAP- or β-tubulin/neurofilament-positive cells was relatively small.

Neuronal stem cells produce neuroblasts that migrate from the SVZ along a discrete pathway into the olfactory bulb, where they form mature neurons. Another neurogenic region is the subgranular layer of the hippocampal dentate gyrus, where neurons migrate only a short distance and differentiate into hippocampal granule cells. In addition, mouse hippocampal organotypic brain cultures contain neurons capable of neurogenesis (Kamada *et al.*, 2004; Raineteau *et al.*, 2004). Mapping HSV-1 infection to these regions suggests that most of the infected cells are precursors located in the SVZ that migrate to their target. The ability of dividing and metabolically active cells to sustain HSV-1 replication has also been observed in cultured cells. Thus, HSV-1 was able to replicate better in cultures of dividing cells obtained from neuronal tissues (Davido *et al.*, 2003; Schang *et al.*, 2002).

HE in humans is mainly a disease of the frontal and temporal lobes of the brain (Schmutzhard, 2001; Tyler, 2004). In the present *ex vivo* system, although the olfactory bulb, pyriform cortex and striatum were also infected, the infection was largely confined to the meninges, the periventricular areas and the hippocampus. The pattern observed in the neonate mouse brain was verified in the rat, giving credence to the validity of the findings. Whilst the explanation for these differing patterns is currently elusive, it might be attributed to the way in which the virus accesses the tissue: in encephalitis, it may get preferentially to the fronto-temporal lobes through the olfactory route or the trigemino-vascular pathway, whilst in the *ex vivo* system, the entire organotypic culture is exposed evenly to HSV-1.

This *ex vivo* infection pattern of HSV-1 also correlates with findings obtained with murine CMV, another herpesvirus, during infection of mouse brain organotypic cultures (Kawasaki *et al.*, 2002). Immature glial cells in the subventricular and cortical regions were most susceptible to murine CMV infection.

**Age**

Neonatal HSV infections occur at a relatively high frequency (approx. 1 in 3000 births in the USA), with up to two-thirds of those infected developing encephalitis, a more severe and devastating condition than in the adult (Steiner & Biran, 2002). Indeed, in the *ex vivo* system, HSV-1 infection of neonate brain was much more extensive than that observed in the adult tissue (Fig. 7). This was also observed following intracerebral inoculation of HSV-1 into mice (Kristensson, 1976). The decrease in viral neurovirulence with age correlates with a decline in brain thymidine kinase activity as a marker of cell proliferation (Ben-Hur *et al.*, 1983). The *ex vivo* model, lacking a systemic immune system, excludes the possibility that this observation is related to immune competence and favours the likelihood that an increased number of immature, undifferentiated cells in the neonate tissue and/or an augmented metabolic rate of the neonate brain tissue is responsible for the intense infection there. The observation that, in the adult brain, the infection is confined to the areas where nestin marker is expressed supports this explanation.

Thus, the finding that the adult brain tissue is relatively non-permissive for HSV-1 infection correlates with the very low incidence of HSV-1 encephalitis in adults and emphasizes the potential of the present *ex vivo* system to identify the causes that underlie herpes neurotropism and pathogenesis.

**Impact of infection multiplicity**

One explanation for the predilection of HE for the temporal and frontal brain lobes may relate to local factors rendering a relatively non-permissive tissue more susceptible to HSV-1 infection. The finding that increasing the amount of virus for infection of the tissue also enlarged the infected territory and increased the number of infected cells (Fig. 1) may support a repressive mechanism for HSV-1 infection that is overcome by a large multiplicity of virus infection. Increasing m.o.i. improves efficiency of HSV-1 infection of neuronal cells (Mador *et al.*, 1995). This is due to the neuronal intracellular-restriction mechanisms that block early stages of HSV-1 transcription and direct the viral genome towards establishment of a latent infection (Kemp *et al.*, 1990; Steiner *et al.*, 1990; Wheatley *et al.*, 1991). In this study, the reporter genes were under the control of exogenous promoters and expression was measured 8 h post-infection; therefore, we assume that viral ability to infect the cells and possibly to replicate was examined. Thus,
increasing the m.o.i. might have enabled the virus to overcome restriction at an early infection stage or a later stage associated with initiation steps of DNA replication.

**HSV-1 receptors, viral entry mechanisms and viral spread**

Several cell-surface molecules serve as receptors for HSV entry into cells (Campadelli-Fiume et al., 2000). Whilst heparan sulphate glycosaminoglycans mediate the attachment of the HSV virion to cells, the repertoire of HSV entry receptors include HveA (herpesvirus entry mediator), members of the nectin family and 3-O-sulfated heparan sulphate (Spear, 2004). As the present study examined only the initial HSV-1 infection and not viral spread, transition of the tissue from a non-permissive into a permissive state may be due to changes in receptor expression on the surface of cells. The expression of certain heparan sulphate molecules in rodent brain correlates with the pattern of HSV-1 infection observed in this work. In the developing brain, glypican-4 is expressed specifically in the ventricular zone and is restricted to cells that retain stem-cell properties. Moreover, glypican-4 expression is also found in the adult dentate gyrus, where neural stem cells are replicating continuously during adult life (Hagihara et al., 2000).

HSV-1 is a lytic virus that is also capable of entering latent neuronal infection. What governs the occurrence of one or the other? In contrast to the *in vivo* model, the present *ex vivo* system allows examination of the infection and its effects on the three-dimensional tissue in a controlled manner. HSV-1 spread in the brain *in vivo* may either be per continuum/regional or via neuronal circuits. There are experimental examples for both routes: virus inoculated intracerebrally disseminated either around the site of injection or spread via retrograde transport to distant brain areas (Maidment et al., 1996; Shiraki et al., 1998), whilst in other cases, the virus first infected the meninges and ependymal cells and subsequently spread to cells directly adjacent to ventricles (Chrisp et al., 1989). Intracerebro-ventricular injection (Sundaresan et al., 2000) causes spread of virus into the lining of the ventricles and the subarachnoid space. With this *ex vivo* system and using a reporter gene, one may follow sequentially the process of viral spread in the neuronal tissue.

**ACKNOWLEDGEMENTS**

The research was supported by grants from the European Commission, program number 6 – The Clinigene Consortium, and from the Israel Science Foundation and the Philip Morris USA and international external research program, and by the Israeli Cancer Research Fund fellowship to E. B. We would like to thank Dr Nurith Mador and Haya Falk for their scientific counsel.

**REFERENCES**


type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J 8, 505–511.


Tyler, K. L. (2004). Herpes simplex virus infections of the central nervous system: encephalitis and meningitis, including Mollaret’s. Herpes 11 (Suppl. 2), 57A–64A.


