Restrictions that control herpes simplex virus type 1 infection in mouse brain \textit{ex vivo}

Meytal Cohen,1,2† Efrat Braun,1,2† Yael Tsalenchuck,1,2 Amos Panet2 and Israel Steiner1,3

\textsuperscript{1}Laboratory of Neurovirology, IMRIC, The Hebrew University-Hadassah Medical School, Jerusalem, Israel
\textsuperscript{2}Department of Biochemistry, IMRIC, The Hebrew University-Hadassah Medical School, Jerusalem, Israel
\textsuperscript{3}Department of Neurology, Rabin Medical Center, Campus Beilinson, Petach Tikva, Israel

Elucidating the cellular and molecular factors governing herpes simplex virus type 1 (HSV-1) neurotropism is a prerequisite for understanding HSV-1 encephalitis and for targeting HSV-1-derived vectors for gene transfer to the brain. Earlier we had described an \textit{ex vivo} system of mouse brain slices and demonstrated a selective and unique infection pattern, mostly around the ventricles. Here, we examined tissue factors controlling HSV-1 infection of brain slices. We demonstrated that heparan sulphate, while an important factor, does not determine the infection pattern. Hyaluronic acid, but not collagen, appears to enhance HSV-1 brain infection. To investigate whether tissue distribution of viral receptors determines the infection pattern, we examined transcription of herpes virus entry mediator and nectin-1 receptor genes in infected and uninfected brain regions. Both the infected and the uninfected regions express the receptors. We also explored the influence of intra-cellular factors. HSV-1 does not preferentially infect proliferating cells in the brain slices, despite its predilection to the ventricular zones. To delineate the step at which the HSV-1 infection cascade is restricted, mRNA was isolated following tissue infection, and transcription of the immediate-early and late viral genes was evaluated. The results indicated that HSV-1 genes are not expressed in regions that do not express a viral reporter gene. Therefore, we conclude that tissue resistance to infection is associated with a block at or prior to the immediate-early mRNA synthesis. Taken together, using the \textit{ex vivo} system of organotypic culture we describe here extra-cellular and intra-cellular restriction levels of HSV-1 brain infection.

\section*{INTRODUCTION}

Herpes simplex virus type 1 (HSV-1) is a human pathogen responsible for severe encephalitis (Steiner, 2003). The infection is mainly localized to the brain temporal and frontal lobes and unless treated is either fatal or associated with significant neurological deficit in survivors. This virus is also a promising vector for gene therapy to the nervous system (Berges et al., 2007). For both aspects, disease and therapy, understanding the viral predilection to certain cells in the brain (neurotropism) is of paramount importance. Studying HSV-1 neurotropism might provide means to prevent the disease and to improve the use of HSV-1 for gene therapy to the brain.

There are two main models to study the cellular and molecular aspects of HSV-1 neurotropism: the use of animal models and cell cultures. In animal models, the ability of virus to infect particular cells or tissues is dependent mainly on the route of infection (Ben-Hur et al., 1988; Bergström et al., 1994) and on other factors such as the immune system, extra-cellular matrix (ECM) and tissue architecture. Animal models have been used extensively to explore HSV-1 neurotropism (Chrip et al., 1989; Roizman et al., 2007; Thomas et al., 2001). However, both anatomical barriers and immunological parameters, which sometimes govern the outcome of infection, hamper the ability to examine the effect of tissue structure upon HSV-1 infection of brain tissue. The interaction between a virus and a single cell has been studied effectively \textit{in vitro} (Immergluck et al., 1998; Kennedy et al., 1983; Li et al.,
It enables important issues to be addressed that are related to entry receptors and intra-cellular mechanisms of viral replication (reviewed by Heldwein & Krummenacher, 2008). However, due to the ubiquitous receptors it employs (Spear, 2004), HSV-1 tends to be relatively non-selective in infection of a variety of cells in culture, while in vivo it shows discriminative neurotropism (Taylor et al., 2005).

We have developed a unique ex vivo organ culture system of mouse brain that, while lacking the immune system, enables issues to be addressed that are related to viral tropism and the interaction between HSV-1 and the cell in the context of a solid tissue. We use mice brain organotypic culture (Stoppini et al., 1991) that maintains the three-dimensional structure and the neuron–glia cells interaction with the tissue ECM. This ex vivo system complements the in vivo and in vitro models used to study HSV-1. Using this brain slices system, we have shown that HSV-1 has a restricted and specific primary infection pattern as opposed to non-neurotropic viruses, i.e. adenovirus-5 (AD5) and vaccinia (Braun et al., 2006). HSV-1 infection in the organ culture of newborn mouse brain is mostly confined to leptomeningeal, periventricular, hippocampal and cortical brain regions. Most of the infected cells are early progenitor cells rather than mature neurons or astrocytes. A similar pattern of infection was observed in older mice brain, albeit fewer cells were infected in the adult tissue compared with the newborn tissue (Braun et al., 2006).

What mechanisms govern this pattern of HSV-1 primary infection? What distinguishes the HSV-1-infected brain areas (permissive) from those that are resistant (non-permissive) to infection? Various factors, either extra-cellular or intra-cellular, could play a role in herpes neurotropism in the ex vivo model. The extra-cellular factors may include the tissue architecture that restricts viral penetration, ECM molecules, or receptor distribution and polarity. In the present work, we systematically examined early events of infection in brain areas, permissive and restrictive of HSV-1. These include virus adsorption, the role of ECM molecules and receptor distribution in the tissue. Since the HSV-1 infection pattern is mainly confined to cells in neurogenesis areas, we investigated whether cell proliferation affects the HSV-1 infection pattern. We also examined whether intra-cellular factors have a role in the unique pattern of HSV-1 brain infection. Hereby, we sought to reveal whether restriction to HSV-1 infection in the non-permissive areas of the tissue occurs before or after viral RNA early transcription.

RESULTS

Involvement of ECM components in HSV-1 tropism

Since cells in a three-dimensional solid tissue are embedded in ECM, as opposed to cells in culture, we investigated whether two major components of the ECM, collagen and hyaluronic acid, affect the pattern and extent of HSV-1 brain infection. Pre-treatment of brain slices with collagenase-II affected neither the extent of HSV-1 infection (Fig. 1a), nor the unique tissue pattern of infection (data not shown). In contrast, collagenase-II enhanced HSV-1 infection of the skin tissue ex vivo (Fig. 1a). Thus, it appears that collagen distribution in the ECM of brain tissue is not a significant factor in HSV-1 neurotropism, while it reduces viral infection in the skin tissue. As opposed to collagen, hyaluronic acid is one of the most abundant molecules in the brain ECM, especially during the developing stages (Novak & Kaye, 2000). To examine the possible role of hyaluronic acid in HSV-1 neurotropism, brain slices were pre-treated with hyaluronidase, and infected with HSV-1, or with vaccinia virus as a control. While hyaluronidase enzyme treatment did not significantly change vaccinia brain infection, HSV-1 infection was reduced by 33%. Hyaluronidase enzyme treatment of the skin tissue also resulted in a significant reduction of HSV-1 infection (70% reduction), as well as a reduction of vaccinia infection (~50% reduction) (Fig. 1b). Therefore, hyaluronic acid in the ECM appears to contribute to HSV-1 infection of both the brain and the skin tissues.

Heparan sulphate involvement in HSV-1 neurotropism

HSV-1 adsorbs to cells mostly through membrane heparan sulphate molecules before internalization by specific receptors (Spear, 2004). Since certain heparan sulphate molecules have a unique brain distribution, e.g. glypican-4 is present mainly around the ventricles (Braun et al., 2006; Karthikeyan et al., 1994; Ybot-Gonzalez et al., 2005), we studied the role of heparan sulphate in HSV-1 neurotropism using several independent approaches.

Pre-incubation of HSV-1 with high molecular-mass heparin (100 μg ml⁻¹) reduced brain infection by sevenfold, as indicated by the quantitative Beta-Glo assay (Fig. 2d). Yet infection was not completely eliminated, especially in areas of the brain most susceptible to HSV-1 infection, i.e. the meninges and lateral ventricles (Fig. 2a, b, c – brain tissue architecture of adult mouse). To control for a possible effect of heparin on the brain tissue architecture heparin was pre-incubated with brain slices for 1 h, washed away and virus was added. The infection pattern and extent were not affected by the heparin pre-treatment (data not shown).

The involvement of heparan sulphate in HSV-1 neurotropism was further investigated by comparing infection with another virus that uses heparan sulphate as a receptor. AD5.RGD.pK7 is an adenovirus vector genetically modified to contain a seven lysine residue stretch (pK7) fused to the C terminus of the fiber protein, and also an RGD motif (Wu et al., 2002). As a result, the capsid-modified AD5.RGD.pK7 binds to heparan sulphate and integrins, rather than the adenoviral coxsackievirus and adenovirus receptor (CAR) receptor. While HSV-1 infection is relatively localized, the
infection of AD5.RGD.pK7 was dispersed in the entire brain parenchyma and was not confined to certain areas (Fig. 3a, b). Comparison of brain infection pattern of AD5.RGD.pK7 and the wild-type AD5 indicated that both adenoviruses infected the brain tissue in a dispersed and non-specific fashion (data not shown). As a control, we evaluated the contribution of heparan sulphate to HSV-1 and AD5.RGD.pK7 infection. Viruses were pre-incubated with heparin before addition to brain slices (Fig. 3d). A substantial though not complete abolition of AD5.RGD.pK7 infection was observed after pre-incubation with heparin (50 % reduction of AD5.RGD.pK7 infection compared with 80 % inhibition of HSV-1 infection). The residual AD5.RGD.pK7 infection was probably due to binding of the RGD motif to integrin molecules (Schmid & Anton, 2003).

Is the tissue pattern of HSV-1 infection determined at the adsorption phase? To address this issue we used HSV-1/VP26–GFP, where the capsid protein VP26 is fused to GFP (Hoppe et al., 2006). Thus, localization of GFP-virus fluorescent particles can be observed directly following binding to the tissue. To synchronize adsorption and block viral entry and gene expression, HSV-1/VP26–GFP was incubated with the brain slices at 4°C for 30 min (Fig. 4b, c). In comparison, the infection pattern was demonstrated by incubation of slices at 37°C for 24 h (Fig. 4d, e). While HSV-1 infection was specifically abundant in the brain ventricles and meninges, viral particle adsorption was widespread in the brain, and was not copious in specific anatomical areas. HSV-1/VP26–GFP adsorption appeared as a net-like shape, around the cell bodies (Fig. 4b, c – arrowheads), and on the axons (Fig. 4c – arrows). Taken together, these findings indicate that while the presence of heparan sulphate is critical for infection, it does not determine the unique pattern of HSV-1 brain infection.

Expression of HSV-1 receptors in viral permissive and non-permissive brain areas is similar

As adsorption of HSV-1 to the tissue is not restricted to the permissive zones (ventricles and meninges), we next explored whether tissue expression of receptors for HSV-1 is confined to the permissive regions. To this end,
Modified adenovirus AD5.RGD.pK7 and HSV-1 have different brain infection patterns. Neonate brain slices were infected with AD5.RGD.pK7 (1.2×10^8 IU ml^-1) (a) and mirror slices were infected with HSV-(GFP) (1.2×10^7 p.f.u. ml^-1) for 18 h (b) (magnification ×2.5). Arrows mark the GFP signal in HSV-1-infected areas around the lateral ventricles as opposed to the diffuse GFP signal of AD5.RGD.pK7 infection. (c) Corresponding sections from adult; Mouse Brain Atlas: C57BL/6J Coronal (www.mbl.org/atlas170/atlas170_frame.html). (d) Following pre-incubation (30 min) of AD5.RGD.pK7 (1.2×10^6 IU ml^-1) with heparin (100 µg ml^-1), brain slices were infected for 18 h, while their mirror slices were infected with AD5.RGD.pK7 without heparin pre-exposure. As control, slices were infected with HSV-(β-gal) (3×10^6 p.f.u. ml^-1) under similar conditions. Extent of AD5.RGD.pK7 infection was evaluated in tissue extracts by the luciferase assay (0.17 RLU 10^-5 µg protein^-1 is 100%), and HSV-(β-gal) infection by Beta-Glo assay (0.25 RLU 10^-5 µg protein^-1 is 100%). Statistical analysis (Student’s t-test) was obtained by a comparison between the experimental and control groups: (*) P<0.05. LV, Lateral ventricle; CC, corpus calosum.

Fig. 3. Modified adenovirus AD5.RGD.pK7 and HSV-1 have different brain infection patterns. Neonate brain slices were infected with AD5.RGD.pK7 (1.2×10^8 IU ml^-1) (a) and mirror slices were infected with HSV-(GFP) (1.2×10^7 p.f.u. ml^-1) for 18 h (b) (magnification ×2.5). Arrows mark the GFP signal in HSV-1-infected areas around the lateral ventricles as opposed to the diffuse GFP signal of AD5.RGD.pK7 infection. (c) Corresponding sections from adult; Mouse Brain Atlas: C57BL/6J Coronal (www.mbl.org/atlas170/atlas170_frame.html). (d) Following pre-incubation (30 min) of AD5.RGD.pK7 (1.2×10^6 IU ml^-1) with heparin (100 µg ml^-1), brain slices were infected for 18 h, while their mirror slices were infected with AD5.RGD.pK7 without heparin pre-exposure. As control, slices were infected with HSV-(β-gal) (3×10^6 p.f.u. ml^-1) under similar conditions. Extent of AD5.RGD.pK7 infection was evaluated in tissue extracts by the luciferase assay (0.17 RLU 10^-5 µg protein^-1 is 100%), and HSV-(β-gal) infection by Beta-Glo assay (0.25 RLU 10^-5 µg protein^-1 is 100%). Statistical analysis (Student’s t-test) was obtained by a comparison between the experimental and control groups: (*) P<0.05. LV, Lateral ventricle; CC, corpus calosum.

we used brain tissues of adult mice, since at this age the difference between the infected brain areas [β-galactosidase (β-gal) positive] and the resistant areas (β-gal negative) are more pronounced than in the neonate brain (Braun et al., 2006).

Adult brain slices were infected with HSV-1 and 6 h later small portions of brain tissue (around 2–5 mm in diameter) were taken, by pinching using a sharp spatula, from both the permissive (ventricles) and the resistant (parenchyma) regions. RNA was extracted from the permissive (stained for β-gal) and resistant areas (β-gal negative) and subjected to RT-PCR using primers for nectin-1 and herpes virus entry mediator (HVEM) mRNA. Primers for β-actin mRNA were included with every RT-PCR analysis to correct for differences related to amounts of RNA (Fig. 5). In parallel, X-Gal staining of some of the infected brain slices confirmed the permissiveness and resistance to HSV-1 of the corresponding areas (Supplementary Fig. S1, available in JGV Online). The results indicated a similar expression of nectin-1 and HVEM mRNAs in the permissive and non-permissive regions of the brain (Fig. 5a, b). Thus, the unique pattern of brain infection by HSV-1 does not correlate with the distribution of its receptor tissue.

HSV-1 infection in the brain is not restricted to proliferating cells

As was previously reported, the infection pattern of HSV-1 in neonate mouse brain is confined to leptomeningeal, periventricular, hippocampal and cortical brain regions (Braun et al., 2006), areas that are also characterized by neurogenesis (Frederiksen & McKay, 1988; van Praag et al., 2002). Indeed, we showed that HSV-1-infected cells were positive for nestin, a marker of early progenitor cells (Braun et al., 2006). Therefore, we investigated if cells in the permissive areas are proliferating. Infected brain slices were immune-stained for the proliferation protein marker Ki67 and for 5-bromo-2'-deoxyuridine (BrdU) incorporation to DNA (Fig. 6). Using confocal microscopy, a correlation was observed between anatomical areas of HSV-1 infection and areas of proliferating cells. Notwithstanding, only a few cells in these areas showed co-localization (yellow cells) of HSV–(GFP) infection (green) and a proliferation marker (red) after merging the two fluorescent colours (Ki67 – Fig. 6b, d, BrdU – Fig. 6c, e).

To further investigate a possible correlation of cell proliferation and HSV-1 infection, we compared the infection pattern of HSV-1 with that of murine leukemia virus (MuLV), a gammaretrovirus. MuLV infects only dividing cells and has been considered a functional proliferation marker (Mozdziak & Schultz, 2000; Namba et al., 2005). The results shown in Fig. 7 clearly indicate different patterns of tissue infection by the two viruses. While MuLV infection was widespread across the tissue with no specific pattern, except for an intense infection of the meninges, HSV-1 infection was concentrated in the lateral ventricles and meninges of the brain tissue. Therefore, HSV-1 infection is not restricted to proliferating cells, and other factors must govern its tissue distribution. We next explored whether infection was restricted to the parenchyma area due to an intra-cellular block.

Restriction of HSV-1 infection in the parenchyma occurs prior to viral gene expression

The infection pattern of the brain has been determined by the use of a recombinant HSV-1 that express GFP and β-gal under the constitutive Rous sarcoma virus (RSV) and cytomegalovirus (CMV) promoters, respectively (Figs 2–7). The purpose of the following experiment was to determine the step at which the infection is blocked at in the non-permissive areas of the brain, while confirming that the pattern of brain infection seen by the reporter β-gal gene is the same as the expression of the viral genes.
Therefore, we tested the expression of HSV-1 immediate-early gene (ICP0) and a late gene (UL15) in the permissive and non-permissive areas of the brain, following HSV-1 infection. Adult brain slices were infected with HSV-1, and 6 h later, small sections of brain tissue from the permissive and restrictive areas were taken by pinching using a sharp spatula, as described earlier for Fig. 5. RNA samples extracted from the infected peri-ventricles and areas in the parenchyma that are not stained for β-gal (Supplementary Fig. S1) were subjected to RT-PCR using primers specific for HSV-1 immediate-early gene ICP0 and the late gene UL15. Primers for β-actin mRNA were included with every RT-PCR analysis to correct for differences related to amounts of RNA. We first tested for expression of β-gal mRNA, to verify that the high activity of the β-gal enzyme in the permissive areas is consistent with its mRNA expression. Indeed, β-gal mRNA level was significantly higher in the permissive areas in accordance with β-gal enzyme activity (data not shown). Next, expression of a late gene (UL15) was compared in the permissive and the non-permissive areas at 6 h post-infection (Fig. 8a). Level of late UL15 mRNA, normalized to β-actin mRNA, was significantly higher in the permissive ventricle regions compared with the parenchyma. The expression of an immediate-early gene ICP0 (Fig. 8b) was also higher in the ventricle regions as compared with the non-permissive parenchyma tissue. This result correlates well with the differences seen in the reporter β-gal and UL15 mRNA expression. Taken together, these results verify that the tissue distribution of HSV-1 viral genes expression is similar to the activity of the reporter gene. Moreover, the results suggest that restriction to HSV-1 infection in parenchyma areas of the brain takes place at an early stage during or prior to the expression of the immediate-early mRNA.

DISCUSSION

The aim of this study was to delineate the mechanisms that govern HSV-1 infection in an ex vivo system. The ultimate goal is to understand HSV-1 neurotropism, namely the predilection of the virus to infect and replicate in specific brain regions and cells. The ex vivo brain system, that has extensively been used in physiological studies of the central nervous system (Corner et al., 2005; House et al., 1998), lacks aspects of complete neuronal circuits and the immune response; both are known to be important factors in HSV encephalitis (Barnett et al., 1994; Conrady et al., 2010, Norgren & Lehman, 1998; Wuest & Carr, 2008). This experimental system, on the other hand, with its normal tissue architecture, facilitated the study of factors, such as extracellular molecules, that affect virus–tissue interactions, and thus it complements previous in vivo and tissue culture studies.

We therefore examined the contribution of the extracellular tissue factors, the distribution of HSV-1 receptors,
the proliferative state of the cells and the intra-cellular restriction to viral replication on the unique infection pattern of HSV-1 in mice brain slices maintained ex vivo.

Extra-cellular factors

ECM components. The contribution of ECM molecules to HSV-1 infection in a three-dimensional solid tissue was previously studied, using tissues such as colon and skin (Kolodkin-Gal et al., 2008). These studies have shown that collagen acts as a barrier for HSV-1 infection. Treatment of muscle with hyaluronidase in vivo was shown to improve spreading of adeno-associated virus vectors following co-injection with the vector (Favre et al., 2000; Molnar et al., 2004).

We found that collagenase pre-treatment of brain tissue did not affect HSV-1 infection while enhancing the infection of skin tissue. This may be attributed to the fact that collagen is not a prevalent component of the brain ECM (Hubert et al., 2009; Novak & Kaye, 2000). In contrast, hyaluronic acid is a major constituent of the brain ECM, especially in the developing brain (Margolis et al., 1975; Novak & Kaye, 2000). Indeed, newborn brain slices pre-treated with hyaluronidase showed some reduction in HSV-1 infection. If hyaluronic acid acts as a physical barrier, we would expect a positive effect on infection following hyaluronidase treatment. This result indicates that the presence of hyaluronic acid in the tissue may assist HSV-1 infection. The treatment did not affect brain infection by vaccinia, ruling out a toxic effect of hyaluronidase on the tissue. Hyaluronic acid, a glycosaminoglycan found in the ECM, is in close interaction with chondroitin sulphate proteoglycans (Deepa et al., 2006; Novak & Kaye, 2000), which are known to serve as adsorption/receptor molecules for HSV-1 (Banfield et al., 1995). Digestion of hyaluronic acid from the tissue ECM may damage the aggregations of these two proteoglycans molecules and reduce the capacity of HSV-1 to productively interact with the tissue receptors. Taken together, these observations may indicate a role of hyaluronic acid but not collagen in HSV-1 neurotropism.

Receptor distribution. Heparan sulphate mediates infection of many viruses, acting as a non-specific adsorption molecule (Liu & Thorp, 2002). For HSV-1 however, heparan sulphate, in its 3-O-sulphated form, is also a specific entry receptor (Spear, 2004). In the brain, especially in the developing brain, specific derivatives of heparan sulphate are highly distributed (Esko & Lindahl, 2001; Liu & Thorp, 2002). We studied the contribution of heparan sulphate to HSV-1 neurotropism by several approaches. Firstly, we observed that soluble heparin, a molecule closely related to heparan sulphate, significantly inhibited HSV-1 brain infection, though not completely, as was previously shown in vitro (Yura et al., 1992). Secondly, we compared patterns of brain tissue infection by HSV-1 and AD5.RGD.pK7; both employ heparan sulphate as a receptor (Wu et al., 2002). We observed that while HSV-1 had a specific infection pattern, restricted to the ventricles, AD5.RGD.pK7 infection was dispersed over the entire tissue and thus was non-specific. Thirdly, analysis of virion particle HSV-1/VP26–GFP adsorption to the brain tissue

![Fig. 5. Analysis of HSV-1 receptor gene expression in permissive and non-permissive regions of the brain. Adult mice brain slices were infected with HSV-1 (7.5×10⁶ p.f.u. ml⁻¹) for 6 h. RNA samples from permissive areas (n=6) and non-permissive areas (n=10) were subjected to RT-PCR with primers specific for HSV-1 receptors (a) nectin-1 and (b) HVEM (for primer sequences see Supplementary Table S1, available in JGV Online). Relative HSV-1 mRNA values were calculated by dividing the PCR DNA band density (shown below each graph) of viral products by the band density of β-actin DNA for each sample. The last two columns in each graph present the mean of each experimental group. The value for mock infection served as a background and was subtracted from sample values. Statistical analysis (Student’s t-test) was obtained by a comparison of the experimental and control groups: P>0.05.](https://www.microbiologyresearch.org/article/doi/10.1099/mic.0.000000)
indicated widespread adsorption with no specific pattern. Our findings suggest that although heparan sulphate is important for infection, its distribution in the tissue is not a determinant of HSV-1 infection pattern. Likewise, we examined the distribution of two HSV-1 receptors, nectin-1 and HVEM, in areas of HSV-1 infection (which expressed the β-gal reporter gene) as compared with areas resistant to infection. The results did not show a significant difference in mRNA expression of these receptors in permissive and non-permissive brain regions. Thus, we conclude that receptor tissue distribution is not responsible for the unique pattern of HSV-1 infection.

The proliferative state of cells in brain tissue does not determine the pattern of infection

Several observations might have supported the hypothesis that HSV-1 infection pattern of the brain is determined by

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**Fig. 6.** Correlation between HSV-1 infection and cell proliferation in the brain. Neonate brain slices were infected with HSV–(GFP) \((3 \times 10^6 \text{ p.f.u. ml}^{-1})\) with acyclovir \((20 \text{ μM})\) for 24 h, or were mock infected (a). Slices were fixed and immunostained (Red Cy5) for cell proliferation markers: (b) Ki67 and (c) BrdU. For BrdU incorporation, slices were pre-incubated overnight with BrdU \((10 \text{ μM})\) at 37 °C before HSV-1 infection. Virus infection (green) and proliferation cell markers (red) were viewed by a confocal microscope [magnification ×40 (a–c), ×100 (d, e)]. Arrows indicate a few co-localizations of HSV-1 infection and cell proliferation (yellow cells). Parts (d) and (e) are magnifications (×2.5) of the squares in (b) and (c), respectively.

**Fig. 7.** Patterns of infection of MuLV and HSV-1 in the brain tissue are different. Neonate brain slices were mock infected (a) or mirror slices were infected with HSV-(β-gal) \((6 \times 10^6 \text{ p.f.u. ml}^{-1})\) for 8 h (b) or infected with MuLV-(β-gal) \((6 \times 10^6 \text{ p.f.u. ml}^{-1})\) for 3 days (c). Slices were stained by X-Gal (magnification ×2.5). Arrows mark the specific intense HSV-1 infection around the lateral ventricles (LV) as opposed to the diffuse MuLV infection and the meninges.
cellular proliferation: (i) the extent of infection is higher in the neonate brain, where cell proliferation is more extensive compared with the mature adult brain (Braun et al., 2006); (ii) brain cells that are sensitive to HSV-1 infection also stained positive for nestin, an early progenitor cells marker (Braun et al., 2006); (iii) another herpesvirus, murine cytomegalovirus, has a preference to infect immature proliferating glial cells in the subventricular and cortical regions (Kawasaki et al., 2002); and (iv) HSV-1 favours dividing cells over quiescent cells, since they express cyclin-dependent kinase (CDK)-1 and -2 and other cellular factors that are needed for HSV-1 transcription and replication (Diwan et al., 2004; Roizman et al., 2007). On the other hand, HSV-1 establishes latent infection in postmitotic neurons that lack CDKs (Kawasaki et al., 2002).

We therefore examined whether the proliferation state of brain cells contributes to HSV-1 neurotropism using three independent approaches. In fact, most of the HSV-1 infection was localized to regions that were also stained for the nuclear proliferation marker Ki67 and for DNA synthesis (BrdU incorporation). Nevertheless, only a few cells showed co-localization of HSV-1 infection and the two proliferation markers. To further investigate this point, we infected consecutive brain slices with HSV-1 and MuLV, the latter virus is capable of infecting only dividing cells (Mozdziak & Schultz, 2000; Namba et al., 2005). Comparison of MuLV and HSV-1 infection patterns showed no similarity. While MuLV infection was dispersed over the entire tissue, HSV-1 infection was restricted to the ventricle areas. Taken together, these findings indicated that cell proliferation is not an exclusive factor for HSV-1 brain infection. Indeed, HSV-1 is able to infect mature neurons in culture and to establish latent infection in postmitotic neurons (Roizman et al., 2007).

**Intra-cellular restriction**

Our previous study revealed that certain brain areas are non-permissive for HSV-1 infection as indicated by the absence of β-gal reporter activity (white areas) (Braun et al., 2006). Yet, expression of the reporter gene in the HSV-1 vector may not indicate the expression of viral genes in a productive infection cycle. In this study, we correlated the cascade of viral gene expression with that of the reporter gene, in both the permissive and the non-permissive brain areas. Expression of the immediate-early and late genes was largely confined to areas also positive for the reporter gene. We therefore conclude that HSV-1 infection was restricted in the non-permissive areas, particularly in the parenchyma, at an early stage of infection either prior or during the synthesis of immediate-early mRNA.

It should be noted that the earlier steps of infection, such as internalization, uncoating and nuclear transport are difficult to delineate even in cultured cells, and cannot be analysed in the organ culture system. It will also be of interest to investigate whether the virus is maintained in its latent state in the non-permissive tissue areas. Further studies, both in vivo and ex vivo, to identify additional factors that determine HSV-1 neurotropism would contribute to our understanding of HSV-1 encephalitis and to the application of HSV-1 as a vector to target the nervous system.

**METHODS**

**Organotypic brain slice cultures.** Brains of neonate (1–2 days) and adult (28 days old) BALB/c mice were removed, cut coronally at 300–500 µm thickness with a tissue sectioner and kept in growth media as described previously (Braun et al., 2006; Stoppini et al., 1991) Tissue...
viability ex vivo was tested as described previously (Braun et al., 2006). Viability of brain slices is maintained for over a week post-explantation, as was shown before (Cho et al., 2007). In the experiments above, brain slices were maintained in culture for 6 h to 3 days post-explantation.

Organotypic skin cultures. Skin slices of neonate BALB/c mice were prepared essentially as described by Hasson et al. (2005). In brief, dorsal skin tissues were washed in Dulbecco’s modified Eagle’s medium (DMEM; Biological Industries), cut with a tissue sectioner into thin slices (300 μm) and incubated in DMEM.

Virus infection. Slices were incubated with the virus in 1 ml serum-free ‘adsorption-media’ for 2 h with occasional shaking, and the medium was replaced with growth medium containing 10% FBS (Biological Industries), for 6–8 h or overnight and infection was evaluated as described below.

HSV-1. Two strains of HSV-1 were used: (i) HSV 17+/pR20.5/5 with the β-gal gene under the control of the RSV promoter, and the GFP gene under the control of the CMV promoter, referred to as HSV-(β-gal) or HSV-(GFP), respectively; and (ii) HSV-1/VP26–GFP, a recombinant HSV-1 strain 17 that expresses the capsid protein VP26 fused to GFP. With regard to HSV-(β-gal) and HSV-(GFP) both genes (β-gal and GFP, respectively) were inserted at the U65 gene locus and had no effect on the virus replication capacity (Thomas et al., 1999). With regard to HSV-1/VP26–GFP the virus was constructed originally in KOS strain (Desai & Person, 1998) and reconstructed in strain 17 by Dr G. Elliott (Marie Curie Research Institute, Oxford, UK). The virus was provided by D. Knebel-Mörsdorf Cologne, Germany (Hoppe et al., 2006). HSV-1 viruses were propagated in Vero cells.

Adenoviruses. Adenovirus-5 (AD5) and AD5.RGD.PK7 are E1-deleted, replication-defective viruses encoding luciferase and GFP reporter genes in the E1-locale controlled by the constitutive CMV promoter (Wu et al., 2002). AD5.RGD.PK7 has seven residues of lysine fused to the exon protein enabling binding to cells through heparan sulphate molecules. The viruses were propagated in 293T cells, purified and concentrated as described before (Alian et al., 2000). Virus infectious units (IU) were determined in 293T cells.

Vaccinia. vSC9 virus, encoding the β-gal gene under the control of the Vaccinia TK promoter was propagated in BSC-1 cells (Chakrabarti et al., 1985).

All viruses were purified by ultracentrifugation at 25 000 g on a 10–20% sucrose cushion and titrated on Vero cells.

MuLV. MuLV vectors were produced by cotransfection of 293T cells as described previously (Naldini et al., 1996), using pCL packaging constructs expressing the amphotropic envelope protein and pCL expression vector (pCLMFG-LacZ) expressing the LacZ under the constitutive LTR promoter. Viruses were harvested from the media at 48 and 72 h post-transfection, and titrated on 293T cells.

X-Gal staining of tissues. Brain slices or cells were stained for X-Gal as described previously (Braun et al., 2006). In a separate experiment HSV–(GFP) (3 × 10^6 p.f.u. ml^-1) was pre-incubated with 100 μg heparin (Komada) ml^-1 prior to being used to infect neonate brain slices. Slices were examined for GFP expression or homogenized for the Beta-Glo assay.

Beta-Glo quantitative assay for the β-gal enzyme. Slices infected with the virus were extracted with lysis buffer (100 μl PBS, 0.1% Triton X-100), homogenized, freeze-thawed three times, sonicated and the extract was clarified by centrifugation (3000 g for 10 min). Supernatant extracts were mixed with 20 μl Beta-Glo assay reagent (Promega), and the β-gal enzyme product level, measured by luminescent activity expressed in relative luciferase units (RLU), was examined in a luminometer (Mithras-LB940; Berthold). Enzyme specific activity was determined by correction to protein content of the extracts, using the Bradford assay (Bradford, 1976). Under conditions of the Beta-Glo assay, β-gal enzyme activity was linear with respect to time and the amount of extract protein added.

Luciferase enzyme assay. Infected slices were extracted as described above for the Beta-Glo assay. Supernatant extracts (15–20 μl) were mixed with 50 μl luciferase assay reagent (Promega) and the level of reporter luciferase activity was examined in a luminometer (Mithras-LB940; Berthold) (Honigman et al., 2001; Wu et al., 2002). Enzyme specific activity was determined by correction to protein content in the extracts.

RT-PCR. RNA was extracted from brain tissues using the RNeasy Mini kit (Qiagen) and treated by RQ1 DNase (Promega). RNA was subjected to reverse transcription with AMV reverse transcriptase (Promega) in a reaction volume for 1 h at 48 °C (Supplementary Table S1 summarizes the primers used in the RT-PCR and is available in JGV Online). PCR was done with the GoTaq DNA polymerase (Promega). DNA products were collected at several PCR intervals: for β-actin mRNA at 20, 25 and 30 cycles; for UL15, ICP0 and β-gal at 38, 42 and 47 cycles; and for nectin-1 and HVEM at 30, 33 and 36 cycles. The results presented are from the linear phase of the amplification reaction. Analysis of the PCR DNA product was done by MiniBisPro (Bio-Imaging systems) using the TINA20 software. Relative HSV-1 mRNA values were calculated by dividing the PCR DNA band density of viral products by that of β-actin DNA in each sample.

Whole-mount immunofluorescence. Brain sections (300 μm) were fixed in 2% fresh paraformaldehyde for 15 min at room temperature, washed twice with PBS and stored in 70% ethanol at 4 °C. The tissues were transferred to 30% methanol for 1 h at room temperature, washed twice with PBS, permeabilized with 1% Triton X-100 in PBS for 1 h and blocked in Cas-Block (Zymed Laboratories) for 2 h at room temperature. Primary antibodies were diluted with 0.5% Triton in Cas-Block and incubated with the tissue at 4 °C overnight. The following antibodies were used: rabbit monoclonal anti-Ki-67 diluted 1:100 (Sp6; Thermo Scientific), or mouse monoclonal anti-BrdU diluted 1:200 (B44; Becton Dickenson). Tissue sections were washed three times with 0.1% Tween 20 in PBS, and incubated for 2 h at room temperature with secondary antibodies: Cy5-conjugated goat anti-rabbit diluted 1:200 (Jackson Lab.), or Cy5-conjugated goat anti-mouse diluted 1:200 in 1% BSA in PBS (Jackson Lab.). Tissue sections were washed three times with PBS and placed on a microscope slide with mounting buffer. All immunofluorescence images were captured with a Zeiss 510 confocal microscope.

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REFERENCES


