Journal of NeuroVirology, 8: 180–190, 2002
 ISSN 1355–0284/02 $12.00+.00
DOI: 10.1080/13550280290049633

Brain resistance to HSV-1 encephalitis in a mouse model

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Brain resistance to intracerebral superinfections develops after a peripheral inoculation of neurovirulent viruses. Superinfection resistance combines specificity, toward the virus used for the peripheral inoculum, and short-term duration *after* the inoculum. In order to study this unusual combination, neurovirulent superinfections were made on albino Swiss mice previously infected with a nasal inoculum. A herpesvirus strain SC16, or a homologue recombinant virus carrying the reporter *lac Z* gene or a vesicular stomatitis virus (VSV) (a virus taxonomically unrelated to Herpesviridae) were used. The mice underwent a neurological examination and their survival rate was recorded. The brains superinfected with the reporter virus were stained for the β-galactosidase reaction to trace the virus spread and the inflammatory infiltrates were characterized immunocytochemically. The results confirm and extend previous observations about virus specificity and short-term duration of superinfection resistance. They show, moreover, an enhanced brain inflammation with T-cells and macrophages infiltrating the tissue around microvessels, at a time when both neurovirulence and the spread of herpesvirus in the brain are reduced. The results suggest that the immune response to superinfection in the nervous tissue is enhanced by blood-brain barrier mechanisms that promote the timely extravasation of immune cells. *Journal of NeuroVirology* (2002) **8,** 180–190.

Keywords: HSV-1; VSV; encephalitis; superinfection; β-galactosidase

Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotropic virus known to cause a common, benign pathology when infecting the neurons of the sensory ganglia, where it can reside without showing signs for long periods until it occasionally reactivates (Whitley, 1996). It, however, does cause a rare and severe clinical encephalitis characterized by the productive replication of the virus and often death, when infecting the brain (McLean *et al*, 1993; Enquist *et al*, 1998). This simple distinction has recently been questioned and the possibility of a benign and asymptomatic encephalitis has been considered (Bergstrom *et al*, 1994) in order to explain that HSV-1 DNA is often found in autoptic brain samples of humans, surprisingly, without a history of herpesvirus encephalitis (Efstathiou *et al*, 1986; Liedtke *et al*, 1993; Baringer and Pisani, 1994; Sanders *et al*, 1997). It is conceivable that HSV-1 may occasionally infect the brain, but that clinical encephalitis shows up only when natural defenses have been defeated. In addition, HSV-1 asymptomatic infection of the brain seems to be a risk factor for senile dementia (Dobson and Itzhaki, 1999) and a cause of oxidative stress (Valyi-Nagy *et al*, 2000). Experimental studies of brain HSV-1 infection had been initially prompted by the outburst of pandemic encephalitis which started in January 1917. Although a great effort has been made since then in order to identify the genes responsible for neurovirulence (Chou *et al*, 1990), the study of the mechanisms of host brain resistance has lagged behind.

The efficiency and duration of the defense mechanisms which protect the brain from HSV-1 were

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This work has been supported by the Consiglio Nazionole delle Ricerche.

Received 22 October 2001; revised 11 January 2002; accepted 27 February 2002.

studied herein on an albino Swiss mouse model in which brain infection by means of nasal inocula of HSV-1 strain SC16 did not cause clinical encephalitis (Boggian *et al*, 2000), whereas a few virions inoculated into the brain caused encephalitis and death. To this aim, the defense mechanisms were first alerted by means of a nasal inoculum of the virus and then tested with a superinfecting (Flexner and Lewis, 1910) inoculum in the brain. The spread of HSV-1 (Tomlinson and Esiri, 1983; McLean *et al*, 1989; McLean *et al*, 1993) into the superinfected brain was studied using a reporter virus carrying the *lac Z* gene (Balan *et al*, 1994). The extravasation of inflammatory immune cells from blood into the nervous tissue was studied with immunocytochemical methods. Finally, the specificity of the antiviral response was tested with a superinfection of vesicular stomatitis virus (VSV), an RNA virus that causes encephalitis in mice (Barna *et al*, 1996) but is not taxonomically related to Herpesviridae. The experiments demonstrated that the brain infection through the nasal route, although lacking signs or pathology, primes the brain to trigger off an enhanced inflammatory reaction which protects brain functions from superinfection restricting virus spread along the nervous pathways.

Results

Resistance to superinfection of the olfactory bulb

The hypothesis of this experiment was that a nasal inoculum of HSV-1 renders the brain resistant to an inoculum in the olfactory bulb. The hypothesis was tested on four different groups of mice with inocula (HSV-1 strain SC16, 0.5×10^6 p.f.u.) in the olfactory bulbs on days 2, 7, 14, and 21 following a nasal inoculum (1×10^6 p.f.u.). Thus, 105 albino Swiss female mice were divided into four experimental and four control groups. The experimental groups were *n/ob2*, *n/ob7*, *n/ob14*, and *n/ob21*, where *n* stands for nasal inoculum and, e.g., */ob2* for the superinfection in the olfactory bulb on day 2. The control groups: −/−, for nontreated; *n*/−, for inoculated only in the nose; *n*/blank, with sham superinfection (to exclude an effect of the surgical trauma of inoculation); and finally −/*ob* inoculated only in the olfactory bulb.

Every mouse was tested for 21 days once a day for neurological signs and assigned a score ranging from 0 to 8. A score for ocular or cutaneous signs was also assigned ranging from 0 to 2. The survival rate of the mice was observed. The survival rate of each group, as a function of the days after superinfection, is reported in Figure 1. The control −/*ob* group did not survive day 7 postinfection, and the median survival period was 4 days, the *n*/− control group survived and so did the *blank* groups (not shown). The survival of the experimental groups depended on how long the superinfection was delayed. The survival rate was 0/8 in group *n/ob2* with a delay of 2 days, maximal (13/15) in group *n/ob7* with a delay of 7 days,

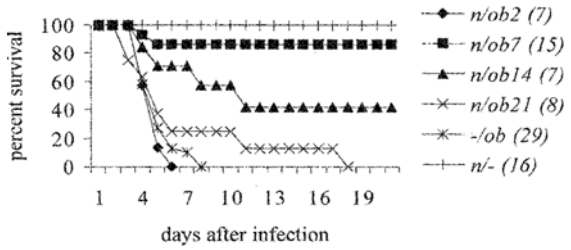


Figure 1 Survival of mice as a function of the days following the inoculation with HSV-1 in the olfactory bulb. Time course of survival. Day 0 is the day of inoculation in the olfactory bulb. Observe the survival of the group superinfected on day 7.

intermediate (3/7) in group *n/ob14* with a delay of 14 days, and 0/8 again in group *n/ob21* with a delay of 21 days, Figure 1. At the onset of the signs, the mice showed enhanced reactivity followed by hunched postures, lack of coordination, loss of placing reactions beginning from the hind limbs, unsteadiness, poor equilibrium on the shaft, and—shortly before death—absence of rightening reactions. The neurological score rose in the three days that preceded death. Some of the mice did, however, fully recover despite transient defects in placing reactions, Figure 2. Conjunctivitis and blepharitis as well as hair loss in some regions of the head were present in 2/16 of mice infected in the nose. These were resolved in 4 weeks' time after infection. The eye-and-skin score (not shown) had no relation to the neurological score.

Resistance to superinfection studied in different regions of the brain

The hypothesis of this experiment was that the resistance to superinfection extends to some regions of the brain in addition to the olfactory bulb. To test this hypothesis, 106 albino Swiss female mice were divided into three experimental groups, *n/ob7* for olfactory bulb, *n/crbll7* for cerebellar, and *n/ent7* for entorhinal superinfection (0.5×10^6 p.f.u.) on day 7 after nasal inoculation (1×10^6 p.f.u.), and in control groups *n*/−*, −/−*, −/*cbll*, −/*ent*, −/*ob**, with the obvious meaning, and finally groups −/*cbll-blank* and −/*ent-blank* with sham inoculation. The mice of the groups marked with an asterisk were only

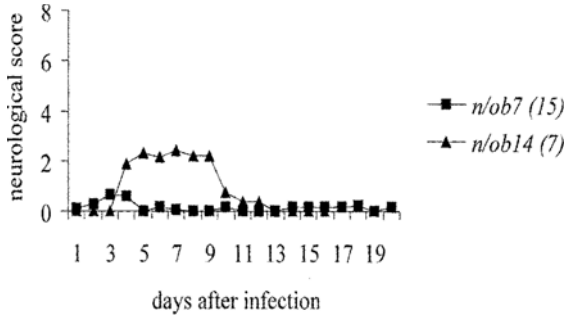


Figure 2 Mean neurological score as a function of the days following inoculation in the olfactory bulb. Group symbols as in Figure 1. The score of control group *n*/− (not reported) was 0 throughout the period of observation.

6 weeks old. The 29 control mice inoculated with HSV-1 in the brain did not survive day 8 postinfection with day 4 as median survival. On the other hand, the experimental group *n/ob7* and *n/crbll7* survived 8/14 and 10/16, respectively, and the experimental group *n/ent7* survived 4/8, as determined on day 21 after superinfection. The survival rate was thus higher in the experimental groups than in the control groups, which had been inoculated in the brain. There was very little difference among the experimental groups. The signs, however, of the mice infected in the cerebellum appeared earlier and were characterized by postural abnormalities (body asymmetries, distonies, and clones). The other control mice survived 6/6 in group $-/-^*$, 9/9 in the groups *−/crbll-blank* or *−/ent-blank*, and 7/15 in group $n/-^*$. This experiment demonstrated that the resistance induced by the nasal inoculum extends to brain structures other than the olfactory bulb.

Resistance and reporter gene expression

The hypothesis of this experiment was that during the period characterized by resistance to HSV-1, the neural spread of superinfecting HSV-1 is reduced. To this aim, superinfections were carried out with the recombinant replication-competent HSV-1 SΔUS5-*lac Z*, expected to express β-galactosidase activity in infected cells. Superinfections (0.5×10^6 p.f.u.) were either made in the olfactory bulb or in the cerebellum. Ten out of 19 experimental and control mice survived until day 3 when their sacrifice was planned. Four brains cut into four parts and stained for β-galactosidase are shown in Figure 3. The first two rows are brains with inoculum in the olfactory bulb and the lower rows with inoculum in the cerebellum. Parts a–d show a superinfected brain (group *n/ob7*), with the olfactory bulbs on the left and the medulla on the right; likewise, parts e–h show a control brain (group *−/ob*). The regions of the brain, which were positive for the reaction, are dark blue in color. They are the olfactory bulbs (*ob*), the piriform cortex (*pir*), the entorhinal cortex (*eC*) and the amygdala nuclear complex (*A*), the hypothalamus (*hyp*), the first field of Ammon's horn (*CA1*), and the region of the locus coeruleus (*lc*) and of the adrenergic neurons. Comparatively, the reaction in the superinfected brain appeared to be more restricted and confined mainly to one side as compared to that of the control group. Parts i–l of Figure 3 show the brain of a mouse superinfected in the cerebellum (group *n/crbll7*), and m–p a control brain (group *−/crbll*). In the control brain, the positive regions are the cerebellum (*crbll*), with the exclusion of the lateral parts of the lobes and paraflocculi, the brainstem starting from medulla (*m*) and extending rostrally to the basal prosencephalon, namely, pons and mesencephalon, thalamic nuclei (*tn*), and hypothalamus (*hyp*) including the preoptic area (*pa*) and the septal region (*sa*). A very weak and diffuse staining in the frontal cortex is barely visible in the image. In the superinfected brain, i–l, positive regions were restricted to the cerebellum close to the site of inoculation and locus coeruleus, with weakly positive regions in the medulla (*m*) and frontal cortex (*fC*).

The variability in these observations from mouse to mouse was considerable and appeared to depend on the severity of the neurological signs. Table 1 reports the reaction of different parts of the brain of experimental and control mice, with the intensity of the reaction ranked in four grades (−,+,++,+++). An average reaction score, (Σ_+/n), obtained giving 2 points to a bilateral + and 1 point to a unilateral +, is reported for each group. The reaction score of the mice superinfected in the olfactory bulb was lower than that of the control group in the piriform cortex, entorhinal cortex, and hypothalamus. The reaction score of mice superinfected in the cerebellum was lower than that of the control group in the thalamus and hypothalamus.

Inflammatory reaction to superinfection

This experiment tested the hypothesis that the infection through the nasal route primes the brain to trigger off an enhanced inflammatory reaction upon superinfection. Both β-galactosidase and normal histology were carried out on microtome sections of control and superinfected brains inoculated (0.5×10^6 p.f.u.) in the olfactory bulb or in the cerebellum and sacrificed 3 days later. Infection of group *n/−* mice did not produce appreciable signs of inflammation in the brain and the mice looked healthy (Boggian *et al*, 2000). Infection of mice group *−/ob* and *−/crbll* was associated with a mild pathology characterized by only a few reactive cells dispersed in the parenchyma, notwithstanding mice that had severe neurological

Figure 3 Reaction of β-galactosidase of the reporter virus inoculated in the brains of four mice. Inoculum site shown by an arrow. (**a**) Olfactory bulb in dorsal view; coronal sections of (**b**) anterior brain, (**c**) posterior brain, and (**d**) cerebellum and pons in frontal view; (**e**) olfactory bulbs in ventral view showing the lateral olfactory tracts; (**f–h**) sections and views as in (**b–d**). (**i**) Olfactory bulbs in dorsal view; (**j–l**) sections and views as in (**b–d**); (**m**) olfactory bulbs in ventral view showing the optic chiasm; (**n** and **o**) coronal sections of anterior and posterior brain in frontal view; (**p**) cerebellum and medulla in posterior view. The calibration bar corresponds to 1.5 mm (0.75 mm for the olfactory bulbs). Symbols are *ob* olfactory bulbs, *pir* piriform cortex, *eC* entorhinal cortex, *fC* frontal cortex, *A* amygdala nuclear complex, *tn* thalamic nuclei, *crbll* cerebellum, *lc* locus coeruleus, *m* medulla and pons, *sa* septal area, *pa* preoptic area, *hyp* hypothalamus, *CA1* first field of Ammon's horn. **Figure 4** β-Galactosidase and immunohistochemistry. β-Galactosidase (β-gal) reactive neurons (**a** and **b**); (**a**) cerebellar Purkinje cells localization with adjacent inflammatory infiltration; (**b**) brainstem neuron localization with perivascular lymphocyte cuffings; (**c**) immunohistochemical CD3+; and (**d**) CD43+ strong reactivity (brown staining). (H&E and β-gal: (**a**) and (**b**) 250×; H&E staining and peroxidase: **c** and **d**, 250×.)

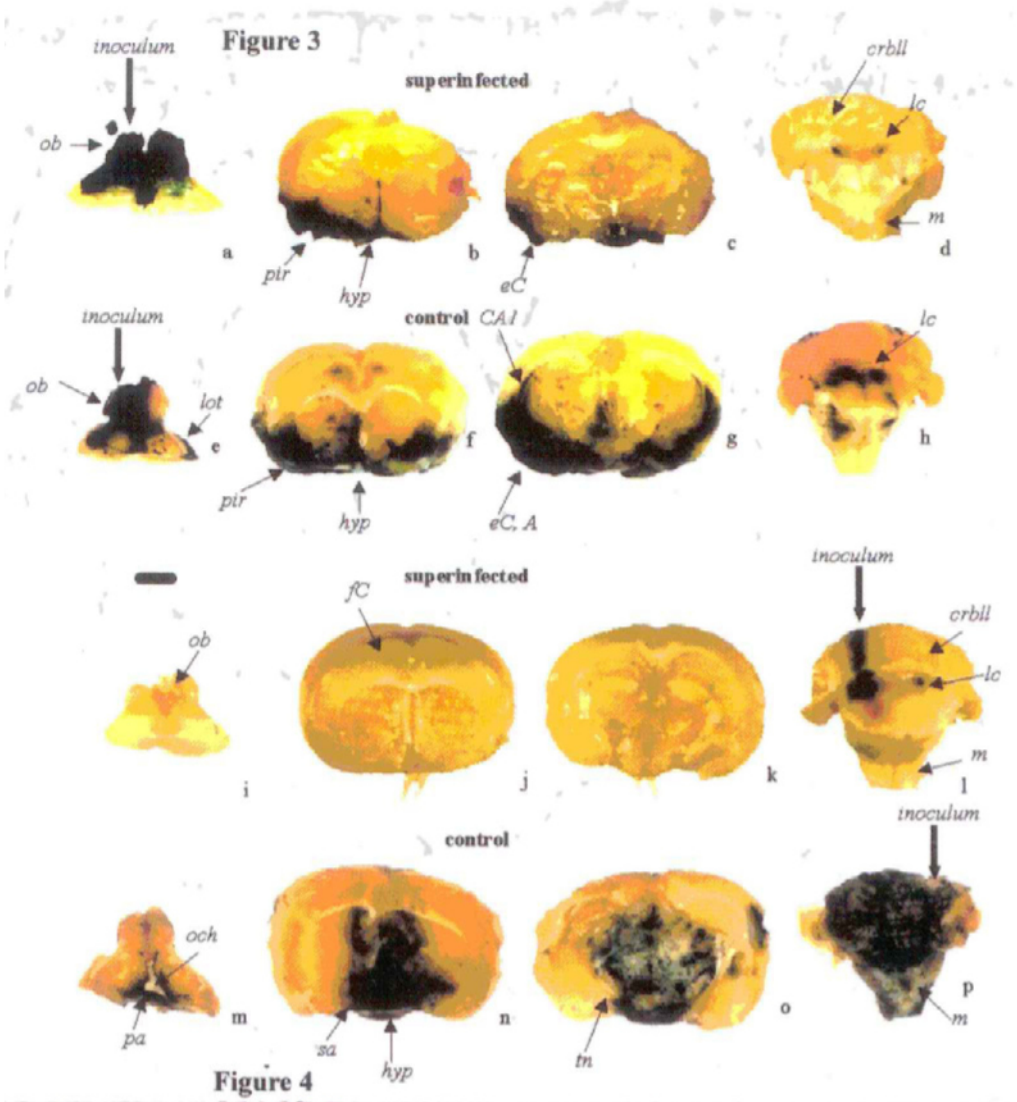
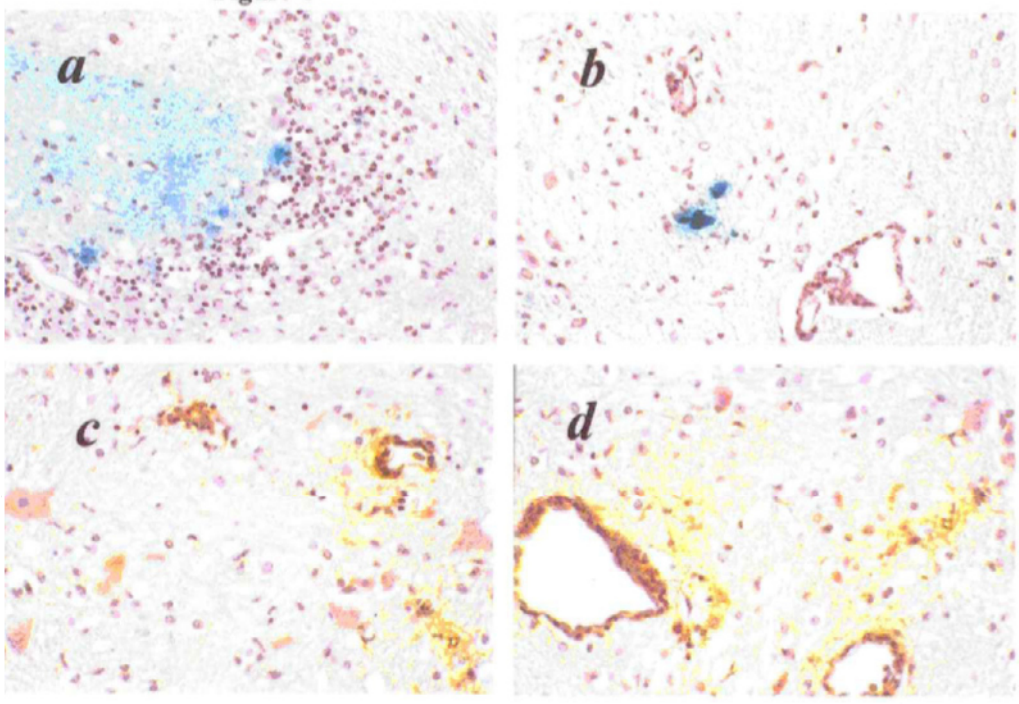


Figure 4



Figures 3–4

Table 1 Intensity (+, ++, +++) and average score ($\sum_+$/n) of the β-galactosidase reaction on day 3 p.i. in control (−/) and experimental (n/) brains inoculated with HSV-1 SΔUS5-*lacZ*

Group	*ob*	*pir*	*hyp*	*eC*	*hip*	*tn*	*Vth*	*Brain stem*	*crbll*
−/ob	+++	+++	−	+++r	+	+	+	+	−
−/ob	+++r	++	++	+++	+	−	+r	+	+r
−/ob	+++r	+	+	+++r+l	+	+	+	+	−
$\sum_+$/n	4	4[a]	2	4.3[b]	2	1.3	1.6	2	0.3
n/ob7	+++	+	+	+	+CA1	+v.w.	+	++	++
n/ob7	++	+r	+r	+r	+	−	n.d.	+	−
$\sum_+$/n	5	1.5[a]	1.5	1.5[b]	2	1	1	3	2
−/crbll	+	+	+++	+	+	+++	+	++	+
−/crbll	+l		+++	−	−	+	+	++	++
$\sum_+$/n	1.5	1	6[c]	1	1	4[d]	2	4	3
n/crbll7	++	−	+v.w.	−	−	+v.w.	−	++	+v.w.
n/crbll7	+	−	++	−	−	+++	−	+++	++
n/crbll7	−	−	−	−	−	−	+	+	−
$\sum_+$/n	2	0	2[c]	0	0	2.6[d]	0.6	4	2
−/−	−	−	−	−	−	−	−	−	−

+, ++, +++: weak, medium, strong staining; v.w.: very weak; crbll: cerebellum; eC: entorhinal cortex; hip: hippocampus; hyp: hypothalamus; ob: olfactory bulb; pir: piriform cortex; tn: thalamic nuclei; Vth: trigeminal ganglion; r: right, the side of inoculum; l: left; n.d.: not determined; [a,b,c,d]: differences brought into evidence.

signs or were moribund. In the superinfected brains, instead, there was an enhanced inflammatory reaction around β-galactosidase positive neurons, such as cortical neurons in olfactory areas, cerebellar Purkinje cells, Figure 4a, granule cells and brainstem neurons, Figure 4b; the greatest accumulation of reactive cells was around the blood vessels, the sheaths of which were surrounded by a thick collar of cells (perivascular cuffing). Regions with diffuse spongiotic degeneration disorganized the tissue texture with massive infiltration of reactive cells, some of which had a pycnotic nucleus. In a number of reactive cells the β-galactosidase reaction was positive. The enhanced inflammatory reaction was at variance with the lack of or the weak signs displayed by the mice.

The inflammatory cells of the superinfection

The hypothesis of this experiment was that leukocytes are recruited in superinfected brains. Microtome sections of superinfected brains in the cerebellum (group *n/crbll7*) sacrificed on the third post-superinfection day were characterized immunocytologically using antibodies directed towards CD antigens. The majority of reactive cells were T-lymphocytes CD3+, Figure 4c; CD43+ lymphocytes were also found, Figure 4d; CD68+ macrophages and rare CD20+ B-lymphocytes without the morphology of mature plasmacells were also detected. The T-cells were prevalently localized in the nervous tissue forming inflammatory perivascular cuffs whereas B-cells were almost exclusively found within the leptomeninges.

Ear pinna superinfection

The hypothesis of this experiment was that the nasal inoculum raises resistance to HSV-1 also outside the brain, that is, in the ear pinna tissue. Ten albino Swiss female mice were equally divided into two groups, an experimental group with a nasal inoculum 7 days before pinna inoculation (*n/pinna7*) and a control group (−/*pinna*). Mice of the control group had signs of ear inflammation, the ear pinna becoming pink in color and thicker in size, with enlarged vessels for 4 weeks with the greatest reaction on about day 14 p.i., whereas the experimental group showed only a modest reaction. The results of a post hoc Newmann-Keuls test on the group-ear thickness interaction on day 14 p.i. showed that the experimental ear pinna was thinner in the experimental group (*n/pinna7*) than in the control group (−/*pinna*), whereas in the control group the experimental (right) ear pinna was thicker than the left pinna, Table 2. The conclusions drawn were that the nasal inoculum protected the ear pinna from an inoculum made 7 days later.

Virus specificity of resistance

The hypothesis of this experiment was that the nasal inoculum of HSV-1 does not induce resistance to a superinfection with VSV. To test this hypothesis, 16 albino Swiss female mice were divided into one experimental group (*nHSV/ob7VSV*) inoculated in the nose with HSV-1 and superinfected in the olfactory bulbs with 0.6×10^6 p.f.u. of VSV 7 days

Table 2 Pinna ear thickness (mm/10²) measured on day 14 after inoculum in the pinna (m ± S.D.); experimental group with a nasal inoculum of HSV-1, 7 days before pinna inoculation, and control group without nasal inoculum

	Inoculated ear pinna	*Control ear pinna*
With nasal inoculum	18.7 ± 3.0[a]	17.0 ± 1.6
Without nasal inoculum	27.6 ± 0.5[a,b]	19.2 ± 2.6[b]

[a]$P = 0.0004$, [b]$P = 0.0005$.

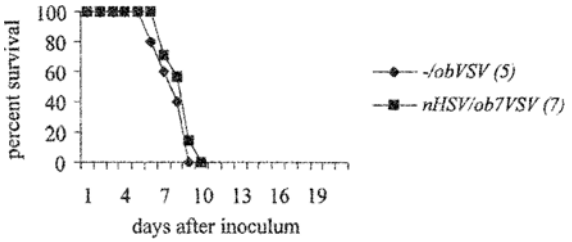


Figure 5 Survival as a function of the days following the inoculation of vesicular stomatitis virus (VSV) in the olfactory bulb. Day zero is the day of VSV inoculation. The superinfected and control groups (*nHSV/ob7VSV* and *−/obVSV*, respectively) are reported on the right-hand side together with the number of mice (N). Please note that survival and time course appear to be the same for the two groups.

later, and three control groups, inoculated in the olfactory bulb with VSV (*−/obVSV*), inoculated in the nose with HSV-1 (*nHSV/−*) and an untreated group (*−/−*). Both experimental and control mice infected with VSV started looking sick within three days, displaying progressive hypotonia with an occasional circling behavior and did not survive day 10 after the inoculum, with day 7 as the median survival, Figure 5. It was concluded that a nasal inoculum of HSV-1 does not raise resistance to an inoculum of 0.6×10^6 p.f.u. VSV.

Comments and discussion

The model The present model of asymptomatic herpesvirus encephalitis is based on strain properties. Mice have long been utilized as animal models (Slavin and Berry, 1943) but different mouse strains differ greatly in susceptibility to HSV-1 (Lopez, 1975; Kastrukoff *et al*, 1986). The albino Swiss mouse, with a similar genetic background to a wild-type mouse, is suitable because it is very susceptible to cerebral inocula, but particularly resistant to peripheral inocula. Wild-type, laboratory, and clinical isolates of HSV-1 differ by orders of magnitude in neurovirulence of peripheral as well as brain inocula (Dix *et al*, 1983). The HSV-1 strain SC16, originally a wild-type strain, had gone through many *in vitro* passages. The LD_{50} for cerebral inoculum in our model is less than the commonly used F strain (unpublished observations). Moreover, nasal instillation of HSV-1 suspensions is a well-established method of brain infection in rabbits as in mice (Levaditi *et al*, 1935; Sabin, 1938; Slavin and Berry, 1943; Tomlinson and Esiri, 1983; Field *et al*, 1984; Hatano, 1989; McLean *et al*, 1993).

Experiments one and two confirm that superinfected brains become resistant to HSV-1 following a nasal inoculum. The LD_{50} of superinfection, an estimate of resistance, is greater than 5×10^5 p.f.u., i.e., more than 3.3×10^4 times the LD_{50} in control mice (about 14 p.f.u. HSV-1). The time course of resistance is in keeping with the rise of the viral titre in the brain as previously shown by us (Boggian *et al*, 2000). The increase of the LD_{50} as well as the apparent overlap of resistance and virus cerebral titre are in agreement with early observations made by Magrassi (1936a) and Doerr and Seidenberg (1936) in rabbits and by Sabin in mice (1938). For the first time, however, resistance has been tested in three different regions of the brain, namely, in the olfactory bulb, the cerebellum, and the entorhinal cortex. It is pertinent that, in these regions, the virus was titred after nasal inoculation (Boggian *et al*, 2000). It should be pointed out, besides, that the survival of group *n/−** of experiment two was lower than that of *n/−* of experiment one. The difference can be ascribed to the younger age of the mice of experiment two (6 weeks old) as compared to experiment one (14 weeks old). This observation is in accordance with the effect of mouse age on virus susceptibility (unpublished results) and with Kintner and Brandt (1995).

Experiment three describes the anatomical restriction of the β-galactosidase reaction in superinfected brains. The expression of the *lac Z* gene of the recombinant HSV-1 SΔU5-*lac Z*, driven by the CMV promoter, is assumed as a faithful reporter of the presence of the virus. The recombinant virus, deleted in the unique *US5* locus in the viral genome, that has not been fully characterized as yet (Zhou *et al*, 2000), maintains the neurovirulence of the parent SC16 strain (Balan *et al*, 1994). Mice infected in the olfactory bulb showed the β-galactosidase reaction spread over the basal encephalon comprising the piriform and the entorhinal cortex, CA1, of hippocampus and a nucleus we identified with locus coeruleus. The locus coeruleus is a nucleus which projects diffusely into the brain with noradrenergic terminals. This β-galactosidase distribution was also seen macroscopically in mice inoculated in the nose only, although the reaction was weaker and the cellular localization was not possible. This distribution was first described in acute encephalitis with histological and immunocytological methods (Levaditi *et al*, 1922a, 1922b; Veratti and Sala, 1923; Stroop *et al*, 1989; Kristensson *et al*, 1982; Kuypers and Ugolini, 1990; Martin *et al*, 1991; Boerman *et al*, 1992; Barnett *et al*, 1993; McLean *et al*, 1993). It was ascribed as a retrograde transneuronal transport of the virus through the central olfactory and noradrenergic pathways. Mice infected in the cerebellum showed the β-galactosidase reaction in the brainstem extending rostrally to the hypothalamus, a distribution which is in agreement with the extensive projections of these structures to the cerebellum. Thus, the locus *US5* seems dispensable for axonal and transsynaptic transport.

The β-galactosidase reaction was weaker and anatomically more restricted in the experimental brains than in control brains. This was particularly evident in regions connected to the inoculum site by polysynaptic pathways such as the piriform cortex, entorhinal cortex, and hippocampus, structures that project to the olfactory bulb with polysynaptic

relays (McLean *et al*, 1993). Another example was the reduced signal on the side of the brain which is connected to the side of inoculum by an interhemispheric connection. It has long been known that virus reproduction is reduced in superinfected brains (Magrassi, 1936b). The present observations suggest that also the spread of the virus is reduced in superinfected brains. It is not known, however, whether the anatomical restriction is consequent to the reduced replication of the virus or whether other factors come into play.

Experiments four and five show that the inflammatory reaction is enhanced in the superinfected brains. The T-cells and macrophages infiltrating the brain, as determined by clonal determinants CD3, CD43, and CD68, while confirming the cellular nature of superinfection resistance (for poliovirus see Jugenblut, 1936), suggest a cell-mediated immune response (Weinstein *et al*, 1990). It is believed that T-cell-mediated responses are essential to anti-viral immunity whereas natural resistance mechanisms (natural killer cells, macrophages, interferons, nitric oxide) operate at very early stages of infection (Leung *et al*, 1984; Kintner and Brandt, 1995; Morrison and Knipe, 1997). Accordingly, mice lacking T-cells and natural killer cells undergo a severe necrotizing encephalitis after nasal HSV-1 inoculation (Adler *et al*, 1999). However, mice with severe combined immunodeficiency (SCID) do not die earlier (Hudson *et al*, 1991) and corticosteroid immunodepressant treatment fails to increase replication and dissemination of the virus in the brain (Thompson *et al*, 2000). HSV-1, like other proinflammatory stimuli, is a potent inducer of transcription factor NF-κB, a critical regulator of genes involved in inflammation and immunity (Karin and Delhase, 2000). Moreover, intercellular and vascular cell adhesion molecules (ICAM-I and VCAM-1) are strongly expressed in infected brain regions (Lewandowsky, 1997) and the interaction of these integrins with their receptors is important in facilitating a number of cell events including antigen-specific T-cell activation and leukocyte transendotelial migration (Warren, 1994). Blood cell extravasation into the nervous tissue could be prompted by cytokine and cytokine receptor expression (Halford *et al*, 1996; Ransohoff *et al*, 1998; Chen *et al*, 2000). The mechanisms controlling the extravasation of blood cells across the restrictive blood–brain barrier have recently attracted some attention (Andjelkovic and Pachter, 2000). The entry of activated leukocytes into the central nervous system is thought to be guided by a superfamily of structurally related chemotactic cytokines called chemokines such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and fractaline (Pan *et al*, 1997). Astrocytes, perivascular microglia, and infiltrating leukocytes have been identified as major cellular sites of chemokine production in the brain (Karpus and Ransohoff, 1998). A production of chemokines in the tissue and the expression of their receptors on brain microvessels for 2 weeks after nasal infection could play a role in directing leukocyte extravasation in the brain during superinfection. Most notably, the enhanced inflammation is associated to lower or even absent neurological signs, suggesting that the inflammatory reaction protects the nervous tissue and its functions from neurovirulence.

It is long known that an inoculum of HSV-1 induces immunity and resistance to peripheral inocula (Levaditi *et al*, 1922a, 1922b). Experiment six on edema of the ear pinna infection shows immunity present as early as on day 7 p.i., i.e., when brain resistance was first observed and is consistent with the immunological hypothesis. VSV, an RNA virus taxonomically unrelated to HSV-1, causes meningoencephalitis in mice after nasal or cerebral inoculation (Sabin, 1938; Bi *et al*, 1995; Barna *et al*, 1996). Experiment seven shows that the resistance induced in the brain by HSV-1 does not confer resistance to an inoculum of VSV and confirms the notion that superinfection resistance is virus specific (for pseudorabies virus see Sabin, 1934). This suggests that the resistance studied in the present model does not rely only upon natural resistance factors very efficient in controlling VSV meningoencephalitis such as interleukin 12, γ-interferon, and type I nitric oxide synthase (Komatsu *et al*, 1996; Reiss *et al*, 1998). To some extent, however, these factors may help build up resistance against herpesviruses as well (Cantin *et al*, 1995; Carr *et al*, 1997; Fujii *et al*, 1999). Intrinsic cell mechanisms of interference with the herpesvirus should also be considered.

Resistance to superinfection has also been noted in cells of neural origin *in vitro* (Doller *et al*, 1979; Su *et al*, 2000). Cell mechanisms could be based on cellular transcription factors such as octamer binding proteins and factors interacting with the viral factor VP16, thus limiting the expression of immediate-early viral genes and finally virus replication into neurons (Kriestie, 1997; Dawson *et al*, 1998; Quinn *et al*, 2000). Very little is known about the neuron-to-neuron transport of HSV-1 through the synapses (Enquist *et al*, 1998) but the downregulation of the herpesvirus receptors should not be dismissed (Campadelli-Fiume *et al*, 2000; Haarr *et al*, 2001).

In conclusion, the present experiment highlights the timely recruitement of immune cells, from blood, across the restrictive blood–brain barrier and into the nervous tissue in order to unfold resistance to superinfection.

Materials and methods

Animals and the virus

Albino Swiss CD-1 mice (30–40 g in body weight, 8–14 weeks old, unless otherwise stated) were used. Mice were kept in cages 27 $\times$ 42 $\times$ 15 cm^3 with wood-shavings as bedding, with 4–6 mice per cage.

The temperature in the room was 26°C with a relative humidity of 65%. The mice were kept under an artificial 12:12 hours light and darkness schedule. The lights went on at 6.00 a.m. Their food and water were *ad libitum*. Experiments were carried out in conformity with the EEC laws on animal experiments and handling.

HSV-1 strain SC16 and the derived vector SΔUS5-*lac Z*, kindly provided by Dr Minson (Dept of Pathology, Cambridge University, UK), were used. In the virus strain SΔUS5-*lac Z*, the reading-frame of gene US5 was interrupted by the insertion of the reporter gene *lac Z* driven by the cytomegalovirus (CMV) promoter. The viruses were grown and titred by plaque assay on Vero cell monolayers as previously described (Anderson *et al*, 1999; Boggian *et al*, 2000). Virus stocks had titre 5×10^8 and 6×10^8 plaque forming units (p.f.u.) per ml for HSV-1 and VSV respectively.

Inoculations
Virus instillation in the nasal cavities was carried out by applying to the opening of the right nostril of a conscious mouse a 2-μl droplet of virus suspension that was rapidly taken up in the nasal cavity. For intracranial inoculations, the mice were deeply anaesthetized with xylazine and ketamine i.p., 20 mg/kg and 75 mg/kg, respectively. Intracranial inoculation was done by drilling a hole 1.5 mm in diameter in the bone, breaking the dura with a fine needle, thus exposing the brain and slowly delivering 1 μl of viral suspension in the tissue with a 10-μl syringe. Inoculations were made in the right olfactory bulb, in the paravermian region of the cerebellum, or in the entorhinal cortex. In sham inoculations the virus was omitted. Peripheral inoculations were made in the left ear pinna subcutaneous tissue using a microsyringe and delivering 2 μl of the viral suspension slowly over 1 min.

β-galactosidase reaction
Staining for β-galactosidase (EC 3.2.1.23) was done according to Lachmann and Efstathiou (1997). Mice were sacrificed with excess anesthesia and perfused with saline solution (150 mM NaCl, 2 mM $MgCl_2$, M/150 phosphate buffer pH 7.4) for 15 min and then with phosphate-buffered 2% paraformaldehyde- 0.2% glutaraldehyde fixing solution for 30 min. The brains were dissected and placed for 2 h in fixing solution and then divided into four parts with coronal cuts at the level of anterior commissure, mesencephalon, and cerebellar pedunculi. Specimens were partially solubilized with a detergent solution in saline (0.01% deoxycholate and 2% NONIDET-P40) over ice for 30 min. The staining reaction was carried out with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 4.5 mM K-ferrocyanide, and 4.5 mM ferricyanide in a detergent solution at 37°C in the dark overnight. The specimens were postfixed in phosphate-buffered 10% formaline, dehydrated, paraffin embedded, and sectioned for microscopic observation. The histology was made with Meyer's hematoxylin and eosin (H&E) staining.

Immunoperoxidase staining (avidin-biotin-complex method)
The method of Hsu *et al* (1981), though slightly modified, was used for immunostaining. Sections (4 μ thick) were deparaffinized and an endogenous peroxidase activity was blocked by incubating the slides in methanol containing 0.3% hydrogen peroxide for 30 min at room temperature. After washing the slides with phosphate-buffered saline (pH 7.4), diluted normal horse serum (Vectastain ABC Kit PK-4002, Vector Labs, Burlingame, CA) was applied for 20 min and the sections were incubated overnight (18 h) at 4°C with primary antibodies with the following dilutions: Monoclonal mouse anti-CD3, 1:100 (DAKO); anti-CD43, 1:30 (CLONAB); anti-CD4 1:20 (NOVOCASTRA); anti-CD8, 1:50 (DAKO); anti-CD20, 1:100 (DAKO); anti CD68 1:100 (DAKO); and anti-CD19 1:50 (DAKO). Incubation with the primary antibody was followed by washing with phosphate-buffered saline and by applying a diluted biotinylated secondary antibody for 30 min. Brown staining was produced with a diaminobenzidine solution consisting of 20 mg of diaminobenzidine in 100 ml of 0.05 M Tris-HCl buffer (pH 7.4) and 0.01% hydrogen peroxide for 3 min. The slides were counterstained with methyl green or hematoxylin and eosin, and then mounted.

Neurological score, eye-and-skin score, and ear pinna thickness
The clinical signs of the infected mice were observed and scored for three weeks after inoculation. The body weight was determined once a week. The neurological tests were based on the work published by Wolf *et al* (1996). A neurological score, ranging from 0 to 8, was computed giving one point to each of the following tests which was not normal: righting on the side, rightening while falling, hindlimb placing reaction, geotactic reaction, edge avoidance, balance on a horizontal shaft, posture, and deambulation. An eye-and-skin score, ranging from 0 to 2, was assigned, giving 1 point when one of the following signs was present: conjunctivitis and/or blepharitis and hair loss and/or sores on the skin of the head. Pinna thickness was measured near the tip of the ear, with a digital caliper with 0.01-mm divisions. The pinna thickness was measured six times over 35 days. The data were analyzed with a three-way variance analysis with mixed design for the factors group (2), day (6), ear (2, experimental and control).

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