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Asymptomatic herpes simplex type 1 virus infection of the mouse brain

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An asymptomatic and transitory brain infection takes place in adult Swiss CD-1 mice after intranasal inoculation of HSV-1 strain SC16. Time course and distribution of the infection in the brain are demonstrated, (i) by titration of the nasal tissue and olfactory bulbs for 16 days post-infection (p.i.), showing a maximum production yield on day 7 p.i. and no replicating virus on day 16 p.i.; (ii) expression in the brain of the *lac Z* reporter gene of HSV-1 strain SC16-ΔUS5-*lac Z* consistent with a central spread of the virus through the central olfactory pathways and the trigeminal system as described in acute HSV-1 encephalitis models; (iii) PCR amplifications of a segment of the thymidine kinase gene (*HSV-tk*) showing the persistence of viral genome in the nasal tissue and olfactory bulbs after clearance of infectious virus. The asymptomatic character of the infection is demonstrated over 2 months p.i. (i) by normal body weight; (ii) a neurological survey which excludes motor, sensory, balance and postural signs; (iii) two behavioral tests, the *open-field* test for exploratory activity and the *cookie-finding* test for olfactory search. On the other hand, intracerebral inocula cause encephalitis and death in a few days (LD_{50} ca. 14 p.f.u.). Intracranial, surgical transection of one olfactory nerve does not prevent infection of the corresponding bulb nor does it modify virus distribution, suggesting multiple entry routes from the nasal cavity to the brain. In conclusion, HSV-1 strain SC16 reaches the brain of CD-1 mice from the nasal cavity and replicates without neurological or behavioral signs. *Journal of NeuroVirology* (2000) **6,** 303–313.

Keywords: olfactory; β-galactosidase; reporter gene; encephalitis

Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotropic virus whose pathology depends on two biological properties, neurovirulence, as a result of gaining access and reproducing in the brain, and latency, namely, the capacity to remain in a non-reproducing but reactivable state in sensory ganglia, e.g., Roizman and Sears (1996). Neurovirulence can result in a disease with severe brain devastation as in acute encephalitis, whereas latency is a silent infection of the sensory ganglion cells which does not cause significant disease but provides a reservoir for the virus and its transmission to other subjects upon reactivation (Whitley, 1996). In mice, HSV-1 susceptibility depends on age, immunocompetence, previous immunization with herpes antigens and genetic background, as well as on the virus strain (Sabin, 1938; Slavin and Berry, 1943; Lopez, 1975; Dix *et al*, 1983; Hatano, 1989; Morrison and Knipe, 1997, and for a review Enquist *et al*, 1998). The model of intranasal inoculation has been widely used to study acute herpetic encephalitis in suckling mice or the inbred Balb/C strain, a receptive strain (Esiri, 1982; Tomlinson and Esiri, 1983; McLean *et al*, 1993; Drummond *et al*, 1994). However, less attention has been paid to HSV-1 infections in non-inbred mice such as CD-1 mice. The presence of an HSV-1 brain infection occurring without overt signs after peripheral inoculation has been occasionally reported in the literature (Magrassi, 1936; Balan *et al*, 1994). The present study focuses on an experimental model of asymptomatic herpesvirus brain infection in CD-1 mice. It is based on a systematic study of brain infection and on the exclusion of neurological and behavioral signs. This

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model may help explain a mechanism which protects the brain from acute herpesvirus infection and lends evidence to the possibility that humans may also suffer subclinical infections which remain unnoticed.

Results

Virus titer in the nasal tissue and olfactory bulbs
This experiment aimed at demonstrating a herpesvirus infection after intranasal inoculation. Forty-eight female albino Swiss CD-1 mice were divided according to intranasal inoculation of HSV-1 laboratory strain SC16 (6×10^6 p.f.u./mouse) in the right nostril and transection of the right olfactory nerve. *Virus* group (20 mice) had virus inoculation, *OFX+virus* group (20 mice) also had the transection of the right olfactory fibers 3 days before the virus, *OFX* group (four mice) had the transection but not the virus and *CTRL* group (four mice) had no virus and no transection. The dependent variable was the viral titer measured in the nasal tissue, in the right and in the left olfactory bulb. Five mice for each of the virus-inoculated groups, and one mouse for each of the non-inoculated groups were sacrificed on days 3, 7, 10 and 16 p.i. Virus titer was observed in eight out of 10 virus-inoculated mice on day 3 p.i., in eight out of 10 mice on day 7 p.i., in 10 out of 10 mice on day 10 p.i.; no titer was present on day 16 p.i., nor was it present in any of the non-inoculated mice. There was a peak of titer on day 7 p.i., Figure 1. There was no difference between the experimental *virus* and *OFX+virus* groups. Furthermore, the titer in the right and left olfactory bulb was similar. No lag of the titer in the olfactory bulb as compared to the nasal mucosa was observed, probably because it was too short compared to the sampling rate (3rd, 7th, 10th and 16th day p.i.). The complete retrograde degeneration of the olfactory receptor cells in the nasal neuroepithelium was checked through histological stainings after transection of the olfactory nerve.

Expression of the lac Z *reporter gene in the brain*
This experiment aimed at visualizing herpesvirus distribution in the brain. Twenty-four CD-1 female mice were equally divided into the four groups of the previous experiment, but 1.7×10^5 p.f.u./mouse of the HSV-1 strain SΔUS5-*lac Z* was used. This strain had been derived from strain SC16 by inserting the *lac Z* reporter gene under the CMV promoter and interrupting the reading-frame of the US5 gene (Balan *et al*, 1994). The glycoprotein gJ of the viral membrane, coded by the US5 gene, is not important for the proliferation rate and pathogenicity. As a control of expression, infected monolayers of VERO cells stained for β-galactosidase gave a strong reaction which allowed cell visualization of the infected cells under the microscope.

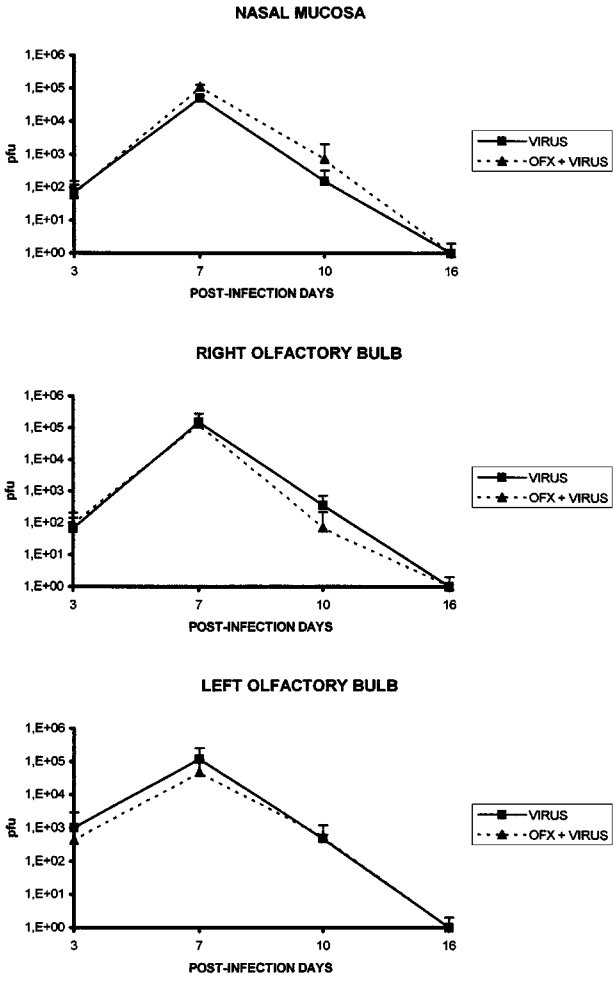

Figure 1 Virus titer in adult CD-1 mice inoculated with HSV-1 strain SC16 in the right nostril. Time course of HSV-1 titer in the nasal mucosa, right and left olfactory bulb expressed in p.f.u. per sample. Points are geometric means and bars+s.e.m. over five mice. Wet weight of nasal mucosa was about 90 mg and each olfactory bulb about 4 mg. Mice were infected by inoculation of 6×10^6 p.f.u./mouse of HSV-1 strain SC16 in the right nostril. Mice with the right olfactory fila cut (*virus+OFX* group) and normal mice (*virus* group) are compared in the same diagram. Zero titers are arbitrarily reported on the X axis. Non-inoculated mice (groups *OFX* and *CTRL*) had no virus in the samples (not reported).

β-galactosidase activity was observed in the brain of experimental mice on days 3 and 7 but not on day 10 p.i. The signal was appreciable on gross structures upon visual inspection but was too weak to be traced at the cellular level on histological sections. It was stronger in the brains of mice on day 7 p.i. than on day 3 p.i. Figure 2 shows examples of the signal seen in brains of mice sacrificed on days 3 and 7 p.i. Positive structures were the olfactory bulbs, the olfactory cortex, the entorhinal cortex, the hippocampus and lateral regions of the brainstem. In some cases the entire brain appeared positive. There was no obvious difference between the experimental groups and, within a group,

between the two brain hemispheres. The reaction was negative on the ganglia of the trigeminal nerves. There was no endogenous activity in the brain of non-infected mice, see control E in Figure 2, but there was endogenous β-galactosidase activity in the olfactory neuroepithelium of all mice. In this tissue, expression of the *lac Z* reporter gene could not be differentiated from endogenous activity.

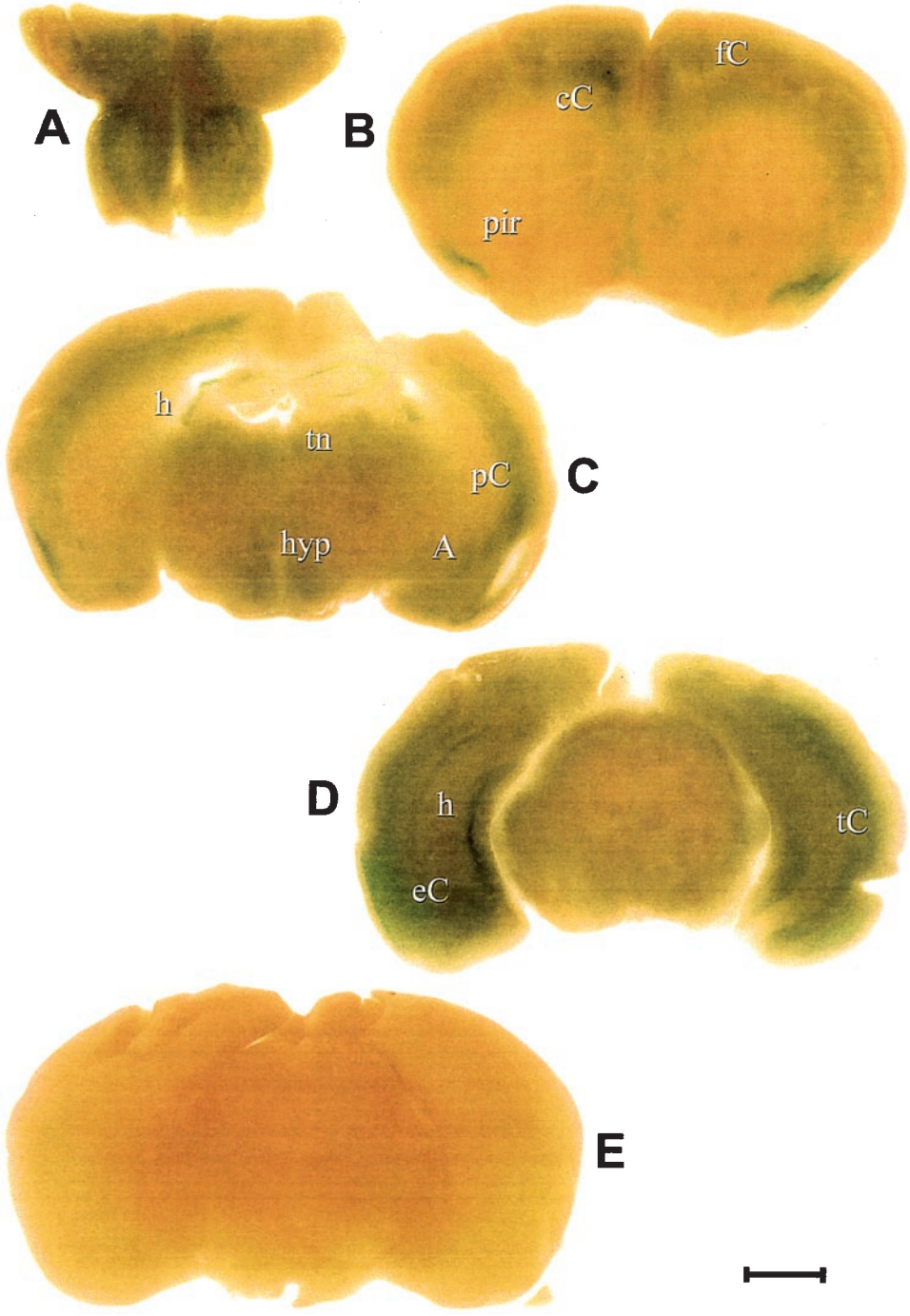

Figure 2 Reporter gene expression in the brain of mice infected with HSV-1 strain SΔUS5-*lac Z* by nasal inoculation. Photographs of paraffin-embedded blocks of the brain. The blue-green color is the product of the β-galactosidase reaction. (**A**) Horizontal section of the olfactory bulbs (OB) and of the forebrain (3rd day p.i.). (**B**) Coronal section behind the anterior commissure (3rd p.i. day) showing reaction in the piriform cortex (pir) and a weak reaction in the cingulate (cC) and frontal (fC) cortices. (**C**) Coronal section at the level of the thalamus (7th day p.i.) showing reaction in the piriform cortex, amygdala (A), parietal cortex (pC), hippocampus (h), hypothalamus (hyp) and thalamic nuclei (tn). (**D**) Coronal section at the level of the mesencephalon (7th day p.i.) showing reaction in the temporal (tC) and entorhinal (eC) cortices, and hippocampus. (**E**) Coronal section at the level of **D** of a non-infected mouse. The rule bar is 1 mm.

Nevertheless, neurons in the olfactory epithelium, trigeminal ganglia and brain as well as epithelial cells of the ethmoidal sinus and Bowman glands were stained in suckling mice infected by intranasal inoculation of HSV-1 and displaying encephalitis signs (not shown). A test for infection was made on eight CD-1 female mice equally divided in the above groups and sacrificed on days 3 and 10 p.i. The virus titer in the nasal tissue and brain specimens (olfactory bulbs, anterior brain, posterior brain, brainstem and cerebellum) of virus-inoculated mice confirmed the presence of HSV-1. The trigeminal ganglia were also positive. The titers (mean+s.e.m.) determined in four mice on day 3 p.i. are reported in Figure 3. Four control CD-1 female mice, kept alive, did not display any neurological nor behavioral sign during the period of the experiment. In conclusion, the distribution of herpesvirus was visualized within the brain.

PCR for HSV-1 DNA in the nasal tissue and olfactory bulbs

This experiment aimed at demonstrating the persistence of herpesvirus genome after infectious virus clearance. The 15 extracts taken for the titration of the left olfactory bulb on the 16 p.i., five from *OFX+virus* group, five from *virus* group and five from *OFX* group, were used. All the titrations for infectious virus were negative, as reported. The nasal tissue and the olfactory bulbs of three mice, one for each of the above groups, kept alive for 2 months after instillation of the virus were also used. The result was a 237 bp DNA segment of the gene coding for thymidine kinase (*HSV-tk*) amplified from DNA extracted from the olfactory bulbs and from the nasal tissue of all virus-instilled mice, Figure 4. It was concluded that herpesvirus genome persists in nasal tissue and in olfactory bulbs after infectious virus clearance for at least 2 months.

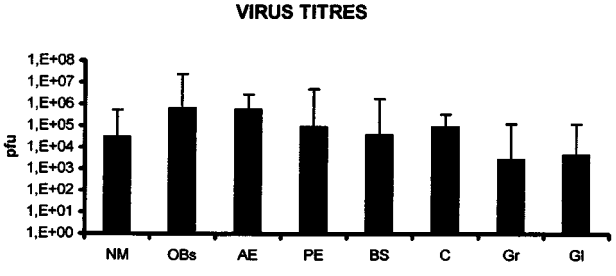

Figure 3 Histogram of titer of HSV-1 SΔUS5-*lac Z* (geometric mean+s.e.m., *n*=4) in different samples determined on day 3 p.i. NM nasal mucosa, OBs olfactory bulbs, AE anterior encephalon, PE posterior encephalon, BS brain stem, C cerebellum, Gr right trigeminal ganglion and Gl left trigeminal ganglion. Approximate wet tissue weight of the samples (in mg): NM (90), OBs (8), AE (100), PE (250), BS (50), C (100), G (2).

Body weight, neurology and behavior

This experiment aimed at excluding encephalitis signs in herpesvirus-instilled mice. Twenty CD-1 female mice were equally divided into the *virus*, *OFX+virus*, *OFX* and *CTRL* groups. The instilled virus was HSV-1 strain SC16 (6×10^6 p.f.u./mouse). The *open-field* and the *cookie-finding* tests were carried out for 2 weeks before inoculum and for about 2 months p.i. afterwards. The dependent variables of the *open-field* test were the number of squares, on the floor of the test box, crossed going from one side to another (crossed squares), of raisings on the box walls, of conversions as well as the number of feces and urine depositions during 3 min. Data concerning the number of crossed squares, raisings, and conversions were analyzed separately. The single data, inspected as a function of time, did not show any critical period nor evident trend. Time dependence was analyzed statistically by pooling the data taken over six sequential periods, two before virus inoculum (pool-2 and -1) and four after inoculum (pools 1–4). Mixed design

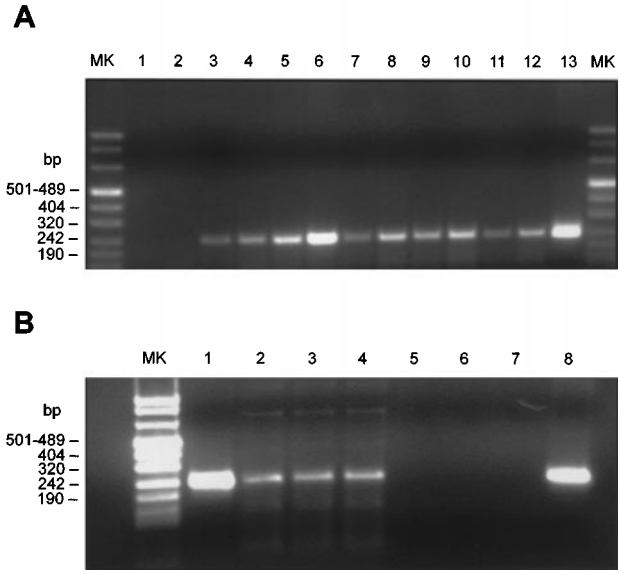

Figure 4 Agarose electrophoresis of DNA amplified by PCR. (**A**) Left olfactory bulb on p.i. day 16. Lane 1, blank reaction without template DNA (negative control); lane 2, PCR of DNA extracted from a non-infected mouse (group *OFX*); lanes 3–7, PCR product of DNAs extracted from five mice intranasally inoculated with HSV-1 (group *OFX+virus*); lanes 8–12, the same for the group, *virus*; lane 13, PCR of plasmid pUC-*tk* containing the thymidine kinase gene of HSV-1 (positive control), MK-basepair (bp) marker DNA. (**B**) Nasal mucosa (NM) and olfactory bulbs (OB) at 2 months p.i. Lanes: MK-basepair marker DNA as above; lanes 1 and 2, PCR product from DNAs extracted from nasal mucosa and pooled olfactory bulbs in a mouse intranasally inoculated with HSV-1 (*OFX+virus* group); lanes 3 and 4, the same for the *virus* group; lanes 5 and 6, PCR product of DNA from a non-infected mouse (*OFX* group); lane 7, reaction blank without template DNA; lane 8, PCR product of the plasmid pUC-*tk*. Observe the experimental bands of DNAs with the migration velocity of the 237 bp DNA segment of the positive control.

ANOVAs for the factors Group and Pooled data did not show any difference among the groups. For the crossed squares, factor Group and interaction were not significant: Group, $F(3,16)=1.885$, $P=0.172$; Group × Pooled data, $F(15,80)=1.527$, $P=0.115$. For Pooled data, $F(5,80)=2.543$, $P=0.034$, there was a statistically significant decrease ($P=0.019$) in the number of crossed squares between the first and the second pool of data, both taken before inoculum. For the raisings, similar statistics were observed: Group, $F(3,16)=1.638$, $P=0.220$, Group × Pooled data, $F(15,80)=1.302$, $P=0.220$, Pooled data, $F(5,80)=3.578$, $P=0.005$, with only a statistically significant decrease in the number of raisings between the first and the second pool of data ($P=0.039$) both taken before inoculum. Also for the conversions, statistics were similar: Group, $F(3,16)=0.369$, $P=0.775$, Group × Pooled data, $F(15,80)=0.702$, $P=0.774$, and Pooled data, $F(5,80)=4.133$, $P=0.002$, with a statistically significant decrease between the first and second ($P=0.001$) and between the first and fourth ($P=0.007$) pool of data. Figure 5 reports the four histograms of the pooled data for the *open-field* test as well as body weight. No change was observed among groups in the number of fecal pellets or urine depositions.

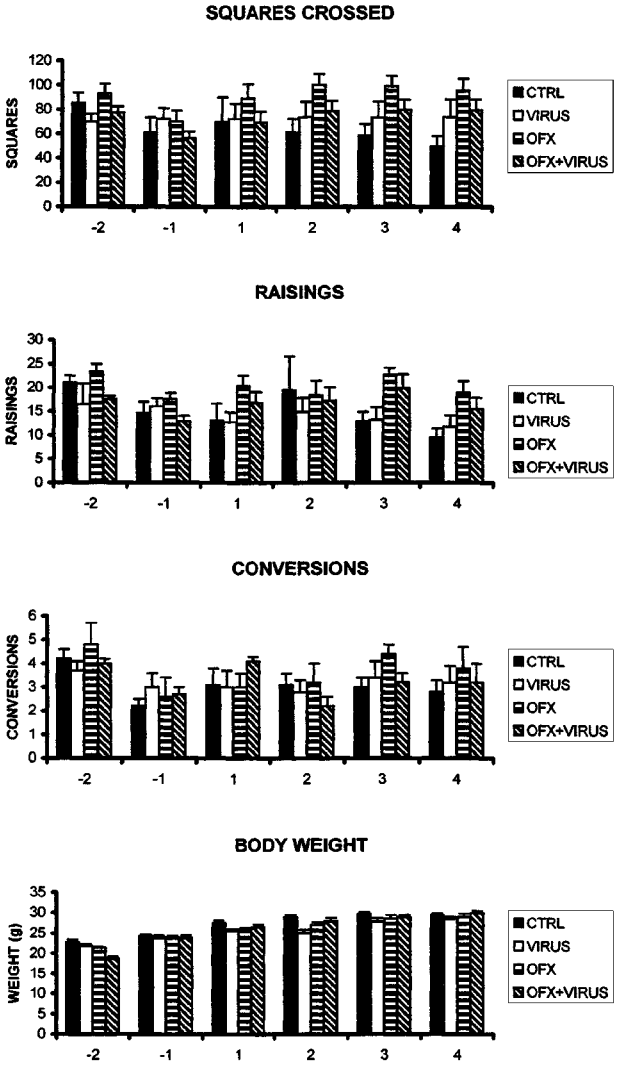

Figure 5 Histograms of the *open-field* test and body weight. Squares crossed, raisings on the walls and conversions in a 3-min period as well as body weight as a function of time. Means and s.e.m. are reported for each experimental group as shown in the insets. Data were pooled over six periods: -2 and -1 covering the 2 weeks before inoculation and 1–4 covering 2 months p.i.

Figure 6 shows the histograms of pooled data from the *cookie-finding* test. A mixed design ANOVA for the factors Group and Pooled data showed that while Groups did not differ, $F(3,16)$, $P=0.739$, Pooled data, $F(6,96)=12.670$, $P<0.001$, showed a minimum latency on the third pool of data, the one immediately after the inoculum, and an increase afterwards. The interaction Group × Pooled data, $F(18,96)=2.483$, $P=0.002$, did not show any consistent pattern among groups, in particular, the group that underwent axotomy and inoculum did not show any significant difference between each pool of data.

Body weight was analyzed with a mixed design ANOVA. Groups did not differ, $F(3,16)=1.905$, while body weight increased throughout the observation period, $F(5,80)=256.528$, $P<0.001$. This increase was present in all groups, $F(15,80)=6.083$, $P<0.001$, Figure 5 lower histogram. The neurological examination gave a score which was zero for all mice thus excluding neurological signs (not shown). To sum up, the analysis did not show any significant change relevant to the experiment.

A control test for infection was made on 18 CD-1 female mice, six from the *virus* group, six from the *OFX-virus* group, three from the *OFX* group and three from the *CTRL* group. Mice, two from each one of the *virus* and *OFX+virus* groups and one from the *OFX* and *CTRL* groups were sacrificed on days 3, 7 and 10 p.i. The virus titer was determined in the nasal tissues, in the right and left olfactory bulb. All the samples from virus-instilled mice were positive on day 3 and 7 p.i. (not shown). In conclusion, there was no overt sign despite a likely ongoing or overcome herpesvirus infection.

Intracerebral inoculum

This experiment aimed at verifying the neurovirulence of herpesvirus after intracerebral inoculation. HSV-1 strain SC16 was inoculated into the right olfactory bulb at serial doses of 1.4×10^4 (n=5), 1.4×10^3 (n=5), 1.4×10^2 (n=4), 1.4×10 (n=4), and 1.4 (n=3) p.f.u./mouse in 21 adult male CD-1 mice. The survival time was recorded for 20 days p.i., Figure 7. It depended on virus dilution, and encephalitis signs preceded death. One of the surviving mice remained alive after displaying neurological signs. The 50% lethal dose (LD_{50}) was

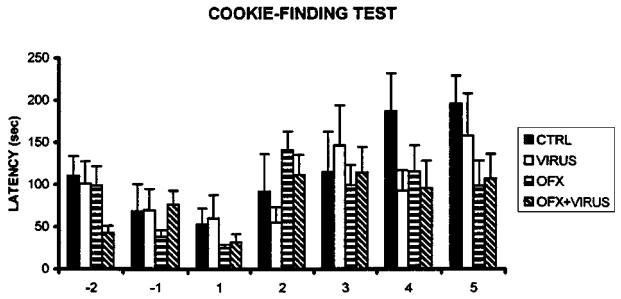

Figure 6 Histogram of the *Cookie-finding* test. Time to discover a buried food pellet reported as a function of time as in Figure 5. Data were pooled over seven periods: -2 and -1 covering the 2 weeks before inoculation and 1 to 5 covering 9 weeks p.i.

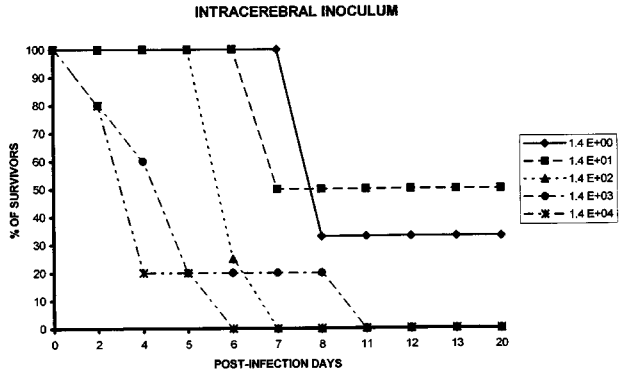

Figure 7 Plot of the survival of intracerebrally inoculated adult CD-1 mice. Survival is expressed in per cent of tested mice. Mice were inoculated into the right olfactory bulb with serial concentrations of HSV-1 SC16 as reported in the inset in p.f.u./mouse.

estimated at about 14 p.f.u./mouse. It was concluded that intracerebrally, inoculated herpesvirus was neurovirulent.

Discussion

Asymptomatic infection

Our results indicate that intranasally inoculated HSV-1 does not cause death of the albino Swiss CD-1 mouse strain (LD_{50} greater than 6×10^6 p.f.u.), whereas the LD_{50} is ca. 10^5 p.f.u. for inbred Balb/C mice (Field *et al*, 1984). Nevertheless, the HSV-1 titer determined in the brain of CD-1 mice shows an ongoing infection which is resolved in about 2 weeks' time. During this period, the clinical follow-up comprehensive of body weight determination, of a neurological score and of two behavioral tests for motor, olfactory and autonomic signs, was negative. The normal body weight excludes bad general conditions, often associated with brain degeneration and cognitive function loss in humans (Aronson *et al*, 1993; Gallagher and Rapp, 1997); the neurological survey does not show any postural, balance or motor deficits; the *open-field* test does not show changes in locomotor activity (Herro, 1993); the deposition of feces and urine gives no indication of a change in autonomic activation; the *cookie-finding* test does not bring into evidence any change in the drive for food or olfactory performance. It should be observed that the *cookie-finding* test is not a very sensitive test for olfactory performance and a discrimination- or memory-based test might perhaps detect modifications in the experimental conditions. In conclusion, observation cannot distinguish experimental CD-1 mice from their control partners. At variance, Balb/C mice and rats develop a highly productive infection with neurological signs of encephalitis in a few days (Tomlinson and Esiri, 1983; MacLean *et al*, 1993; Drummon *et al*, 1994). The Balb/C is a strain selected by inbreeding whereas the genetic background of the CD-1 line is similar to wild mice, a relatively resistant species. The genetic factors that confer resistance to the CD-1 strain are not known.

An asymptomatic HSV-1 brain infection was first demonstrated by Magrassi (1936) with peripheral inoculation of a non-neurovirulent HSV-1 strain in the rabbit, a very susceptible animal. In the mouse, an asymptomatic HSV-1 infection of the medulla and hindbrain after inoculation in the ear-pinna with a syringe needle has been confirmed by virus titration (Balan *et al*, 1994). The present model by nasal inoculation, a more natural infection route, extends this observation to the higher part of the brain. The occurrence of a short-term asymptomatic HSV-1 infection in the mouse brain after intranasal inoculation of the virus seems particularly interesting. It emphasizes the utility of non-genetically selected animals as appropriate models to study HSV-1-related neuropathological phenomena that may also take place in humans. Earlier researchers were well aware of the complexity of mechanisms reducing virulence in the brain (Doerr and Seidenberg, 1936) and some of these mechanisms have recently been described. Antibodies directed against viral antigens as well as B-cells play an important role in protecting the brain (Levine *et al*, 1991; Beland *et al*, 1999). MHC antigens on the microglia are induced by herpesvirus (Weinstein *et al*, 1990) but the susceptibility of inbred-mouse strains does not depend on MHC haplotype (Lopez, 1975). There is a production of γ-interferon during HSV-1 infection (Cantin *et al*, 1995). Natural killer cells play an important role in the natural defense of murine HSV-1 encephalitis (Adler *et al*, 1999). NO-synthase induction mediated by cytokines seems to play a role in controlling viral infections through the nasal route by means of non-cytotoxic mechanisms, and is constitutively elevated in the olfactory bulb (Karupiah *et al*, 1993; Komatsu and Shoshkes Reiss, 1996; Carr *et al*, 1997; Shoshkes Reiss and Komatsu, 1998). The level of HSV-1 immediate-early genes could be determined by cellular

transcription factors such as octamer-binding proteins and could be further modified by the interaction with viral factors such as VP16 (Dawson *et al*, 1998; Quinn *et al*, 2000). Yet, the precise mechanisms which reduce neurovirulence in the brain are still unknown.

Virus DNA persistence

The observation that viral DNA persists in the brain when asymptomatic encephalitis is overcome, confirms and extends a previous report on the acute encephalitis model of Balb/C mice (Drummond *et al*, 1994). In humans, HSV-1 DNA sequences have been amplified from nervous structures of the olfactory system taken from brain autopsies of subjects with no report of encephalitis in their history, by PCR (Liedtke *et al*, 1993; Baringer and Pisani, 1994; Sanders *et al*, 1997) and nucleic acid hybridization (Efstathiou *et al*, 1986), the number of positive cases increasing with age. Our results lend evidence to the possibility that also humans may suffer subclinical herpesvirus infections which remain unnoticed.

A new and surprising result is the persistence of viral DNA in the nasal mucosa. We have no evidence of the cellular localization for this DNA. However, in the model of acute encephalitis in newborn mice, a primary infection with SΔUS5-*lac Z* is detectable in regions of the olfactory neuroepithelium (A Cavaggioni, unpublished data). It is well known that HSV-1 can persist in a latent state in somato-sensory neurons of trigeminal and dorsal spinal cord ganglia. It will be interesting to find out whether latency also occurs in olfactory-sensory neurons.

Virus distribution

In the present asymptomatic infection model, reporter gene expression confirms the presence of the virus in the central olfactory structures and the related nervous centre. Virus titer in the ganglia and reporter gene expression in the central trigeminal system also shows that these structures are infected. The pattern of virus distribution in the higher brain corresponds to the pattern seen in acute encephalitis caused by nasal instillation of HSV-1 in susceptible mice, with immunohistochemical methods (Burnett and Lush, 1939; Slavin and Berry, 1943; Tomlinson and Esiri, 1983; McLean *et al*, 1993).

Virus access to the olfactory bulb

Our experiments demonstrate an infection of the nasal tissue but fail to localize the infection to olfactory neurons. Venezuelan equine encephalitis virus (Charles *et al*, 1995), vesicular stomatitis virus (Shoshkes Reiss *et al*, 1998), rabies virus (Lafay *et al*, 1991), mouse hepatitis virus (Perlman *et al*, 1990; Barnett *et al*, 1993) seems to replicate first in the olfactory-sensory neurons and to travel to the bulbs along the olfactory fibers. The present experiments show that HSV-1 gains access from the nasal cavities to the right olfactory bulb when the right olfactory nerve has been cut. The virus inoculated in the right nostril is likely to spread into both nasal cavities. The entry route may be through the left olfactory fibers and the interbulbar extensive nervous connections. From the nasal cavity, however, a virus may propagate along different nerves, e.g., swine pseudorabies virus propagates along the trigeminal, sympathetic and parasympathetic nerves of the mouse and only rarely along the olfactory nerve (Babic *et al*, 1994). Also for HSV-1 the olfactory nerve is not the only possible entry route, as shown by the early observations of Burnett and Lush (1939) in mice and Levaditi *et al* (1935) in rabbits. The entry may be through the trigeminal and autonomic nerves that innervate the nasal mucosa (Martin *et al*, 1991), from the brain stem the virus could reach the olfactory bulbs by means of the fibers of the raphe serotoninergic system originating in the pons (Paivarinta *et al*, 1993). Olfactory mucosa is leaky and passage of a number of substances from the nasal cavity to the olfactory bulb has been described (Illum, 1991). The virus could also reach the olfactory bulbs bypassing nervous routes, carried by cells shuttling across the lamina cribrosa (Boerman *et al*, 1992).

Nasal instillation versus intracerebral inoculation

Mice in whose olfactory bulbs the virus titer peaks at about 10^4-10^5 p.f.u. on the seventh day p.i., do not release neurological nor behavioral signs whereas mice with an intracerebral inoculum in the olfactory bulb of about 14 p.f.u. run a 50% risk dying (see also Field *et al*, 1982). This observation confirms the reduced neurovirulence (100–100 000-fold) of a variety of HSV-1 laboratory-adapted strains and clinical isolates when inoculated by the intranasal as opposed to the intracerebral way (Magrassi, 1936; Dix *et al*, 1983). The physical barrier of the mucous nasal secretion has been shown to limit access of HSV-1 to olfactory neurons in nasal epithelium (McLean *et al*, 1989). The barrier set up by the immune system involves precipitous clearance of infectious virus from the inoculation site by T-cells (Speck and Simmons, 1998). However, the high viral titers determined in the olfactory bulbs would tend to exclude an impediment to the spread of the virus from the nasal cavity to the brain. Intracerebral inoculation may be more pathogenic because of the local trauma of inoculation. Another possibility is a slow surge of brain mechanisms which attenuate the virus pathogeny (Levaditi *et al*, 1935; Doerr and Seidenberg, 1936; Magrassi, 1936; Hatano, 1989) in the days following intranasal inoculation, but which are too slow to cope with a sudden intracerebral inoculum. Further studies are needed to solve this problem.

Finally, considering the applications of this study, olfactory neuroblastoma would seem a plausible target for intranasal applications of herpesvirus-based vectors (Chambers *et al*, 1995; Roizman, 1996; Fink *et al*, 1996; Slack and Miller, 1996). Neuroblastoma is a rare, but locally aggressive tumor arising from the olfactory neuroepithelium in the nasal cavity (McElroy *et al*, 1998) and prognosis is poor despite *cis*-platinum-based chemotherapy. More extensive investigations, which exploit the model we are presenting, are needed to properly address this subject.

Materials and methods

Animals and surgery

Albino Swiss CD-1 mice (30–40 g in body weight, 6–10 week old) were used. Mice were kept in cages $27 \times 42 \times 15$ cm^3 with wood-shavings as bedding, at a density of four to six mice per cage. The temperature in the room was 26°C and relative humidity 65%. The mice were kept under artificial 12 : 12 h light and darkness schedule, light on at 06.00 h. Mouse chow and water were *ad libitum*.

For surgery the mice were deeply anesthetized with xilazine and ketamine i.p. (20 mg/kg and 75 mg/kg respectively). Intracranial transection of the olfactory nerve was done by drilling a hole 1.5 mm in diameter on the exposed frontal bone, breaking the dura with a fine needle thus exposing the olfactory bulb, and cutting the olfactory fila with a properly shaped spatula. The spatula was passed through the hole and pushed carefully under the extension of the olfactory bulb. Experiments were carried out in conformity with the EEC laws on animal experiments and handling.

Virus and inocula

HSV-1 strain SC16 and the derived vector SΔUS5-*lac Z*, kindly provided by Dr Minson (Department of Pathology, Cambridge University, UK) were used. In virus strain SΔUS5-*lac Z* the reading-frame of gene US5 was interrupted by the insertion of the reporter gene *lac Z*. Virus was grown on VERO cells. Infected cells were scraped from the flask, frozen and thawed three times, sonicated to disrupt the cells, and stored at −70°C. Stocks used to infect mice had titer of $1.4 \times 10^7 - 6 \times 10^9$ plaque forming units (p.f.u.) per ml. For virus instillation, a 1 μl droplet of virus suspension was applied to the opening of the right nostril of conscious mice, and was rapidly taken up by the mouse in the nasal cavity. Inoculation into the right olfactory bulb was performed in deeply anesthetized mice by delivering 1 μl of viral suspension with a 10 μl syringe into the surgically exposed bulb.

Virus titration and lac Z *gene expression*

Viral titer was determined by tissue homogenization assay. Briefly, mice were sacrificed on different post-inoculum days with an overdose of anesthetic drug and the tissues rapidly taken and frozen at −70°C in 1 ml of Dulbecco's modified Eagle's medium (DMEM) for a storage shorter than 15 days. The tissues were then thawed, homogenized within an Eppendorf test tube by 20 passages through the tip of a pipette, sonicated for 20 min in a bath sonicator and sedimented ($320 \times$ g, 5 min). The liquid phase was serially diluted and used to infect three VERO cell monolayers per dilution. The mean number of plaques in the three monolayers, corresponding to a dilution yielding between 30 and 100 plaques, was scored and the titer computed accordingly. Some plaques of SΔUS5-*lac Z* were stained with the β-galactosidase reaction (see below).

The *lac Z* gene expression was visualized by staining for β-galactosidase activity as described by Lachmann and Efstathiou (1997). The samples were the nasal tissue together with the turbinates and septum and the brain divided in four parts with transverse cuts. Cuts were at the level of the anterior commissure dividing the anterior and the posterior brain, at the level of the mesencephalon separating the hindbrain, and of the cerebellar pedunculi for the cerebellum. Specimens were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde. The specimens were then 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 X-Gal, 4.5 mM K-ferrocyanide and 4.5 ferricyanide at 37°C in the dark, overnight. The specimens were then dehydrated, embedded in paraffin, sectioned for macroscopic and microscopic observation. Control histology was made on formalin-fixed, paraffin embedded 7 μm sections with Nissl or Hematoxilin/Eosin stainings.

PCR assay

Polymerase chain reaction (PCR) was performed basically according to Newton (1995). Briefly, the tissues were homogenized and sonicated as described for virus titration. The proteins were solubilized with SDS-proteinase K treatment and DNA precipitated in isopropanol. After washing in 70% ethanol, the DNA was resuspended in 70 μl of water. PCR was carried out with 35 amplification cycles using the primers 5′-CTGCGGGTTTATATAGACGG-3′ and 5′-CATTGTTATCTGGGCGCT-3′ expected to amplify a 237 bp HSV-1 thymidine kinase gene (*HSV-tk*) segment comprised between base-pairs 47655 and 47418 according to the map of strain 17 by McGeoch *et al* (1988). The test was carried out and repeated with the suggested precautions for contamination and a positive and a negative control were always included. The amplified DNA was visualized with 0.5 mg/ml ethidium bromide after standard 2% agarose gel electrophoresis.

Body weight, neurological score and behavioral tests

The variables were determined regularly for 2 weeks before surgery, and about 2 p.i. months. The body weight was determined once a week. Neurological testing was based on the work of Wolf *et al* (1996). A score was made ranging from 0 to 8 assigning one point when one of the following observations was not normal: rightening on the side, rightening in flight, hindlimb placing reaction, geotactic reaction, edge avoidance, balance on a horizontal shaft, posture and deambulation. The *open-field* test (Herro, 1993) was performed twice a week by placing a mouse in the center of a plastic cage with 15 squares drawn on the floor (55×33 cm^2) and recording its behavior for 3 min with a video camera. The variables of locomotor activity were counted on replay. They were the squares crossed, namely, passed in walking from one side to another, the rightenings on the hindlimbs raising the forelimbs on the walls of the cage and the conversions, namely, going out and coming back to a square. The number of defecations and urine depositions was also scored. The *cookie-finding* test was done twice a week with mice deprived of food for 24 h. Mice were placed in a housing cage with a piece of food buried about 1 cm deep under clean wood shavings. The time to discover the food was taken with a maximum of 3 min. The statistical analysis ANOVA was performed on the variables among and within the groups. Data were pooled over time, so as to obtain two pre-inoculum and four p.i. pools over 2 months p.i. observation for the *open-field* test and body-weight data, and two pre-inoculum and five p.i. pools over about 9 weeks for the *cookie-test*. *Post-hoc* Newman-Keuls tests were conducted whenever appropriate.

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