Oral-oesophageal inoculation of mice with herpes simplex virus type 1 causes latent infection of the vagal sensory ganglia (nodose ganglia)

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Herpes simplex virus type 1 (HSV-1) gingivostomatitis during childhood is known to result in a latent infection of the trigeminal ganglion neurons, which innervate the oral mucosa. During latency the viral genome is maintained in a non-infectious state. However, stimuli such as stress, fever or localized trauma can cause HSV-1 to reactivate in neurons and produce recrudescent disease in the peripheral tissues. Recently, HSV-1 proteins and nucleic acids have been detected in biopsies from human duodenal and gastric ulcers, raising the possibility that HSV-1 latency within the enteric nervous system is involved in this chronic recurrent gastrointestinal disorder. The studies in mice described here were done to determine whether HSV-1 latency could be established in neurons that innervate the murine gut. We found that after either intraperitoneal or oral-oesophageal inoculation of mice, HSV-1 establishes a latent infection in nodose ganglia of the vagus nerve, whose sensory neurons project to the gastrointestinal tract. This animal model of HSV-1 latency in the vagal sensory ganglia will be useful to examine the possible relationship between HSV-1 and recurrent gastrointestinal disease.

Introduction

Herpes simplex virus type 1 (HSV-1), which causes recurrent inflammatory lesions of the eye, mouth, skin and genitalia (Corey & Spear, 1986b; Whitley, 1990), is commonly first encountered as an acute primary infection of the oral mucosa (gingivostomatitis) during childhood (Corey & Spear, 1986b; Whitley, 1990; Buddingh et al., 1953; Hale et al., 1963). By adulthood, greater than 90% of some populations are sero-positive for herpes simplex virus (Buddingh et al., 1953; Corey & Spear, 1986a; Nahmias & Roizman, 1973). During primary mucosal infection, the virus enters nerve endings and is transported by axonal transport to the sensory ganglia that innervate the face and oral mucosa; here the viral genome remains in a non-replicative, latent form for the life of the host. The trigeminal ganglion of the fifth cranial nerve is the most often reported site of HSV-1 latency in humans (Baringer & Swoveland, 1973; Bastian et al., 1972; Warren et al., 1977). Recurrent disease of the mouth (herpes labialis) or eye (herpes keratitis) is believed to occur when the virus reactivates in the ganglion and travels down the branches of this nerve, resulting in a recrudescent peripheral infection with pathognomonic lesions (Spruance, 1984; Kaufman, 1978).

While lesions on the surface of the body are readily detected, other less apparent mucosal lesions have also been attributed to HSV-1. In the absence of oral lesions, this virus has been cultured from inflammatory lesions in the oesophagus, of both immunodeficient and normal patients (Buss & Scharyl, 1979; Deshmukh et al., 1984; Ginaldi et al., 1987; McBane & Gross, 1991). Furthermore, HSV-1 has also been associated with ulcerative lesions of the distal stomach and proximal duodenum; HSV-1-specific nucleic acids and proteins were found histologically in 18% of ulcer biopsies obtained from patients with peptic ulcer disease (Lohr et al., 1990). This suggests that HSV-1 may either cause or potentiate a subset of this recurrent inflammatory disorder. In the upper gastrointestinal tract, sensory innervation of the oesophagus and stomach is provided by bipolar neurons in the nodose ganglion, whose peripheral and central processes form a portion of the vagus nerve (Altschuler et al., 1989; Andrews, 1986; Berthoud et al., 1990; Berthoud & Powley, 1992). If recurrent gastrointestinal disease is caused, as are herpes labialis and keratitis, by HSV-1 reactivation, latent HSV-1 should be found in the sensory neurons innervating these tissues. Indeed, Warren et al. (1978) reported HSV-1 reactivation from the jugular portion of the vagus ganglion (ganglionic nodosum or nodose ganglion) of human cadavers. Latent infection
of this ganglion is likely to have been established during symptomatic primary oral infection, when a significant viral inoculum may be swallowed.

HSV-1 infection of the mouse cornea has been shown to produce a latent infection in the trigeminal ganglia with features comparable to HSV-1 latency in humans (Knotts et al., 1974; Roizman & Sears, 1987; Fraser et al., 1991). After inoculation in the peripheral tissues, HSV-1 replicates within the innervating sensory ganglia. By 7 to 10 days post-inoculation this acute infection has resolved in murine trigeminal ganglia; viral antigens and infectious virus are no longer present although viral genomes persist (Fraser et al., 1991; Valyi-Nagy et al., 1992; Steiner et al., 1990). When latently infected ganglia are removed intact and explanted onto susceptible cells, the HSV-1 genome becomes transcriptionally active, resulting in viral replication and eventually the release of infectious virions (Knotts et al., 1974; Deatly et al., 1988).

In the present study we wanted to determine whether a latent viral infection could be established in the sensory neurons innervating the murine gastrointestinal tract. We found that after either intraperitoneal (i.p.) or oraloesophageal (o.o.) inoculation of mice, HSV-1 establishes and maintains a latent infection within the sensory nodose ganglia of the vagus nerve. Latency in these ganglia occurred in a large percentage of animals, depending on the dose and strain of HSV-1 used. To our knowledge this is the first report of experimentally induced herpesvirus latency in these sensory ganglia. This model of herpes simplex virus latency in the extrinsic vagal sensory pathway should provide a valuable system with which to examine the possible relationship between HSV-1 and recurrent gastrointestinal diseases.

**Methods**

**Viruses.** The HSV-1 strains 17+ (Brown et al., 1973), F (Ejercito et al., 1968) and HFEM (Wildy, 1954; Stoker & Ross, 1958; McLennan & Darby, 1980) were propagated and titrated on baby hamster kidney (BHK-21) cells as previously described (Deatly et al., 1988).

**Infection of mice.** Female BALB/c mice (Jackson Laboratory), 4 to 10 weeks old, were infected with HSV-1 by either i.p. or o.o. inoculation. For i.p. inoculation, stock virus was diluted in serum-free medium and 0.2 ml was injected percutaneously into the peritoneal cavity using a 28 gauge insulin syringe (Becton Dickinson). For o.o. inoculation, animals were lightly sedated by metophone inhalation and 0.1 ml of diluted virus was placed into the oesophagus using a 24 gauge, 16 mm Tellon catheter (Critikon). Animals were examined daily for clinical illness and mortality. At various times after infection animals were sacrificed by pentobarbital overdose.

**Immunocytochemistry.** Animal tissues were fixed in paraformaldehyde–lysine periodate, embedded in paraffin and sectioned (5 to 6 μm thickness). Rabbit antiserum to HSV-1 (Dako) was used at a dilution of 1:1000 and viral antigen-expressing cells were detected by the indirect avidin–biotin immunoperoxidase method (Hsu et al., 1981; Vectastain ABC Kit, Vector Laboratories) as specified by the manufacturer with slight modification. Briefly, tissue sections were deparaffinized, rehydrated, quenched in H2O2 and blocked in 3.5% goat serum. Sections were reacted overnight with the primary antibody at 4 °C, then at room temperature with biotinylated goat anti-rabbit IgG, followed by the avidin–biotin horseradish peroxidase complex, and finally 3,3′-diaminobenzidine tetrahydrochloride substrate (Poly-sciences). Sections were counterstained with haematoxylin and examined under the light microscope. Controls consisted of uninfected tissues and acutely HSV-1-infected tissues processed as above, except that non-immune rabbit serum was substituted for the primary HSV-1 antiserum.

**Explant co-cultivation.** Viral latency was determined by the explant co-cultivation method for reactivating latent HSV-1 (Knotts et al., 1974; Deatly et al., 1988). Four weeks post-infection, when infectious virus was no longer present, animals were sacrificed. Under a dissecting microscope, the neck tissues were dissected to expose the vagus nerve and nodose ganglion at the exit from the jugular foramen. The ganglia were removed by severing the foramen, the superior laryngeal nerve and approximately 1 to 2 mm of the descending vagus nerve. Each intact ganglion was then explanted separately onto a monolayer of African green monkey kidney (CV-1) cells and assayed for reactivated virus as previously described (Deatly et al., 1988). Explanted cultures were examined daily for 30 days or until positive. Ganglia were scored positive when viral c.p.e. was first evident in the CV-1 reporter cells.
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Fig. 2. Acute infection in the enteric nervous system after o.o inoculation with HSV-1. Mice received HSV-1 strain 17+ via intraoesophageal canulation. At 2, 4 and 6 days post-infection, tissues were examined for HSV-1 antigens by indirect immunohistochemical analysis using polyclonal anti-HSV-1 antibody (Dako). Uninfected tissues and infected tissues tested with non-immune rabbit serum instead of the anti-HSV-1 antibody served as negative controls (data not shown). (a) Oesophagus, 4 days post-infection, demonstrating viral antigen-positive cells in the myenteric plexus between the circular and longitudinal muscle layers (arrow). (b) Area surrounding arrow in (a) enlarged to demonstrate infected myenteric plexus (arrow). (c) Nodose ganglion 4 days post-inoculation showing viral antigen-positive neurons and satellite cells. The central projections of the nodose ganglion neurons synapse on neurons within the nucleus of the solitary tract (NTS). (d) Rostral brainstem 6 days post-inoculation showing a viral antigen-positive neuron (arrow) within the NTS. The fourth ventricle is indicated (4V).
In situ hybridization. This method for the detection of HSV-1 latent gene expression, using \(^{35}\)S-labelled DNA probes, has been described previously (Deatly et al., 1988). Serial sections (5 to 6 μm) of paraffin-embedded ganglia were prepared. A 0.9 kb BsrEII-BsrEII probe, specific for the latency-associated transcripts (LATs), was obtained from a subcloned portion of the BamHI B fragment of HSV-1 (Valyi-Nagy et al., 1992) and nick-translated to a sp. act. of 3 × 10^6 c.p.m./μg DNA.

**Results**

**Nodose ganglia are latently infected after i.p. inoculation with HSV-1**

Infection (i.p.) of mice with lethal doses of HSV-1 results in acute viral replication in the intestinal and gastric myenteric nerve plexi, the nodose ganglia and vagal-associated areas in the brainstem (Krinke & Dietrich, 1990). Thus, we first determined that latent HSV-1 infection could be established in the nodose ganglion of mice following a non-lethal i.p. challenge.

As susceptibility to i.p. infection with HSV-1 is virus strain-dependent (Becker et al., 1986), we examined three different strains of HSV-1 with differing virulence potentials. Female BALB/c mice (aged 4 to 8 weeks) received a single i.p. inoculation with various doses of HSV-1 strains HFEM, F and 17+. Repeated experiments using individual groups of 4- and 8-week-old animals did not reveal significant age-related differences in mortality or latency reactivation frequency. As expected, strain 17+ was the most pathogenic, resulting in 60% mortality (nine out of 15 mice) with the lowest inoculum (1-3 × 10^2 p.f.u.) used. At the highest inoculum, both strains HFEM (6.5 × 10^6 p.f.u.) and F (1.0 × 10^7 p.f.u.) caused considerably less mortality (HFEM, three deaths out of 15 animals; F, two deaths out of 16 animals). Death from disseminated disease occurred within 2 weeks of the i.p. inoculation.

The explant co-cultivation assay was used to investigate nodose ganglion latency in mice surviving i.p. inoculation with HSV-1. Ganglia from animals infected by the i.p. route at least 28 days previously were explanted onto CV-1 cell monolayers (Fig. 1). No reactivations were noted in 17 ganglia from 12 animals surviving HFEM i.p. inoculation (6.5 × 10^6 p.f.u.). In contrast, nodose ganglia from survivors of strain F (1 × 10^7 p.f.u.) and strain 17+ (1-3 to 2 × 10^2 p.f.u.) i.p. inoculation both contained latent virus as determined by the explant co-cultivation assay. Surprisingly, the reactivation frequency of ganglia from strain F-infected animals (three out of 14 ganglia; 21%) was considerably less than that of strain 17+-infected animals (eight out of 11 ganglia; 73%) despite having received nearly 10^6-fold more virus. Thus, the likelihood of reactivating latent HSV-1 from these ganglia after i.p. inoculation is highly virus strain-dependent; strain 17+ is more virulent and more likely to establish latency in the nodose ganglion than either strain F or HFEM.

**Inoculation (o.o.) with HSV-1**

Symptomatic gingivostomatitis can involve a substantial portion of the oral mucosa and therefore it is likely that the oesophagus is exposed to a large virus inoculum when oral secretions are swallowed. Sensory innervation of the oesophageal mucosa, as well as the gastric and intestinal mucosa, is provided by the sensory fibres in the vagus nerve, whose cell bodies reside in the nodose ganglia (Altschuler et al., 1989; Andrews, 1986; Berthoud & Powley, 1990; Berthoud et al., 1990; Berthoud & Powley, 1992). Having determined that HSV-1 latency is established in this ganglion after experimental i.p. infection and presumably replicates within the myenteric plexus of the digestive organs, we wanted to determine whether oral inoculation (a more physiological route) would also cause viral latency in the nodose ganglion. The total length of the oesophagus in 6 to 8-week-old BALB/c mice is approximately 4 cm. Blind oral intubation with the 16 mm Teflon catheter placed the tip within the proximal one-third of the oesophagus. Transit through the gastrointestinal tract was rapid; in mock infections using 0.1 ml of 10% Trypan Blue, the inoculum could be identified in the proximal duodenum of animals sacrificed as early as 5 min post-inoculation. Occasionally, blind intubation would result in mainstem bronchus intubation, evident by unilateral pulmonary lobar Trypan Blue staining.

HSV-1 strains 17+ and F were used in these experiments. Strain 17+ was also more virulent than strain F when o.o.-inoculated (Table 1). Animals died of systemic illness between 3 and 12 days post-inoculation; surviving animals appeared well up to the time of sacrifice. Mock o.o. inoculation with serum-free medium alone resulted in no mortality (10 mice observed for 28 days). Paradoxically, whereas strain 17+ was less pathogenic by

<table>
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<tr>
<th>HSV-1 strain*</th>
<th>Inoculum (p.f.u.)</th>
<th>Mortality† deaths/total (%)</th>
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<tr>
<td>17+</td>
<td>2 × 10^6</td>
<td>5/5 (100)</td>
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<td></td>
<td>2 × 10^5</td>
<td>20/24 (83)</td>
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<td></td>
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<td>0/20 (0)</td>
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<tr>
<td>F</td>
<td>3 × 10^6</td>
<td>22/37 (60)</td>
</tr>
<tr>
<td></td>
<td>1 × 10^5</td>
<td>5/12 (42)</td>
</tr>
<tr>
<td>Mock infection</td>
<td>Serum-free medium</td>
<td>0/10 (0)</td>
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* Mice received either 0.1 ml of HSV-1 diluted in serum-free medium or a mock infection with medium alone by intraoesophageal cannulation.
† Mice were observed for 4 weeks.
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the o.o. than the i.p. route, o.o. infection with strain F caused considerably more mortality than i.p. infection.

**HSV-1 replication in the gastrointestinal tract and enteric nervous system after o.o. inoculation**

In order to determine the site of HSV-1 replication in the gastrointestinal tract, immunohistochemical analysis using polyclonal anti-HSV-1 rabbit serum (Dako) was carried out on tissue sections from animals o.o.-inoculated with HSV-1. The digestive organs (oesophagus, stomach and proximal duodenum) were fixed, divided into proximal, medial and distal segments, and embedded in paraffin. Multiple sections through all regions of these organs failed to demonstrate viral antigen-positive cells in the mucosa or submucosa of animals sacrificed at 2 and 4 days post-infection. We did not detect gross or histological evidence of ulcerative lesions in the oesophagus or stomach during acute infection either. However, virus-infected cells, most probably representing enteric ganglionic neurons, were located between the longitudinal and circular muscle layers of the stomach and the oesophagus 4 days post-infection, without evident overlying mucosal or submucosal infection (Fig. 2a, b). These viral antigen-positive ganglia were rare, present in only seven of over 200 sections studied. Each positive ganglion contained from one to three positive neuronal cells. At 4 days post-infection by the o.o. route, virus replication was also apparent within the nodose ganglia (Fig. 2c). By 6 days post-inoculation, virus-infected cells were seen within the vagal areas of the brainstem (Fig. 2d).

**Nodose ganglia are latently infected after o.o. infection with HSV-1**

Inoculation with both strains 17+ and F by the o.o. route resulted in latent HSV-1 infections of the nodose ganglia. Twenty-eight days after o.o. inoculation with either of these strains, explanted ganglia reactivated and released infectious virus when co-cultivated on CV-1 cells (Fig. 3). Ganglia from similarly infected animals that were immediately homogenized instead and plated on susceptible reporter cells did not produce viral plaques, indicating the absence of infectious virus at the time of sacrifice in these latently infected ganglia (none out of eight ganglia from four animals). Strain 17+, in addition to being less virulent when delivered by the o.o. route, required a larger inoculum to achieve the same reactivation frequency as that achieved with i.p. infection. Interestingly, o.o. inoculation with strain F proved more lethal and resulted in a higher frequency of reactivation than i.p. inoculation.

**Expression of the HSV-1 LATs occurs in latently infected nodose ganglia**

In addition to the absence of infectious virus and the ability to produce reactivated virus, latently infected sensory ganglia are characterized by expression of viral-encoded LATs (reviewed in Fraser et al., 1991). Using a LAT-specific probe, in situ hybridization of nodose ganglia from animals 4 weeks after o.o. HSV-1 inoculation demonstrated LAT-positive neurons with a nuclear-localized pattern typical of latently infected sensory neurons (Fig. 4). Neurons to which the LAT-specific probe hybridized constituted no more than 3% of the total 100 to 150 neurons present in each tissue section. Positive neurons were also seen with similar frequency in latently infected nodose ganglia from i.p.-inoculated animals. Nodose ganglia from uninfected and strain HFEM i.p.-infected animals did not hybridize with the LAT-specific probe.

**Discussion**

The work presented here demonstrates that oral acquisition of HSV-1 in mice results in viral latency in the sensory ganglia that innervate the upper gastrointestinal tract. Recently, Irie et al. (1992, 1993) described a method of oral immunization against subsequent lethal
herpesvirus challenge by directly placing (using a canula) infectious HSV-1 into the stomachs of mice. Mice tolerated the immunization procedure well and the authors found that the inoculum could no longer be detected in the stomach within minutes of gastric inoculation and had presumably been inactivated by gastric secretions. Despite the fact that infectious virus was necessary for effective immunity (u.v.- and heat-inactivated virus did not elicit effective protection) these investigators were unable to detect (by culture methods or immunohistochemical analysis) viral replication in the gastrointestinal tract after placement of HSV-1 in the stomach. In order to avoid the potential for rapid gastric inactivation of the oral inoculum, we chose instead to place HSV-1 directly into the oesophagus.

Like Irie et al. (1992, 1993), we did not detect replicating virus within the mucosa or submucosa of the digestive organs examined, including the oesophagus. Sampling errors might account for our inability to detect superficial foci of viral infection; alternatively, a major site of mucosal involvement may occur distal to the duodenum. In support of this latter possibility, it has been shown that children continue to shed infectious HSV-1 in their stools for weeks during and after the resolution of primary oral gingivostomatitis (Buddingh et al., 1953). These clinical data in humans suggest that HSV-1 can avoid permanent inactivation by gastric secretions and may infect distal sites in the small and large intestines.

HSV-1, like the other alphaherpesviruses, is neurotropic and able to pass trans-synaptically from one neuron to the next. These features of herpes simplex virus and the porcine herpesvirus, pseudorabies virus, have been utilized by neuroanatomists to map interconnected neuronal networks (Ugolini et al., 1989, 1992; Card et al., 1990). We have described here the movement of virus across the gut surface with subsequent acute replication within the enteric nervous system. Gastrointestinal HSV-1 replication appears to be minimal after o.o. inoculation. Since HSV-1 strain 17r established latency more efficiently after o.o. inoculation than strain F, and neither i.p. or o.o. inoculation with strain HFEM resulted in nodose ganglion latency, we presented data on acute gut and enteric nervous system infection with strain 17r only. Replicating HSV-1 was seen in the oesophageal and gastric myenteric plexi 4 days after oral inoculation. This was followed by replication within the nodose ganglia and finally within the vagal areas of the brainstem. Four weeks after o.o. inoculation, infectious virus was no longer present within the nodose ganglia; instead, LAT gene expression and reactivation by explant co-cultivation demonstrated latency. That this occurred without an identifiable focus of acute infection in the gut epithelium is not without precedent. The establishment of HSV-1 latency does not require acute viral replication; using replication-defective HSV-1 mutants in the mouse model of sensory ganglion latency, it has previously been
shown that latency can be established in trigeminal or dorsal root neurons in the absence of detectable viral replication in the ganglia or peripheral tissues (Steiner et al., 1990; Sederati et al., 1993). Our data show evidence of viral infection within neurons intrinsic to the gastrointestinal tract. Thus, it is plausible that HSV-1 can enter vagus nerve endings in the mucosa, submucosa and myenteric plexi of the digestive tract with minimal viral replication at the surface of the gut. Latency would subsequently be established in the corresponding sensory ganglia.

We have found that efficient establishment of vagal ganglion latency after i.p. or o.o. inoculation is virus strain-dependent. HSV-1 strains differ in their inherent virulence. These differences most probably reflect their ability to spread and be transferred trans-synaptically within the nervous system (Dix et al., 1983; Thompson et al., 1986; Sederati & Stevens, 1987; Henken & Martin, 1991). A single o.o. inoculation is probably a less efficient method of establishing sensory ganglion latency than infection of the traumatized cornea or oral mucosa; thus, differences in the abilities of virus strains to enter and spread within the nervous system are likely to be accentuated in this system.

In children, infectious HSV-1 persists in the saliva for weeks during acute primary gingivostomatitis (Buddingh et al., 1953). This prolonged exposure should increase the probability of establishing viral latency in the human vagal system. In addition, clinical isolates of HSV-1 may differ in their inherent ability to establish latency in human nodose ganglia. There is a single report of human vagal ganglion latency in the literature (Warren et al., 1953). This prolonged exposure should increase the probability of establishing viral latency in the human trigeminal ganglia. There is a single report of human nodose ganglia. There is a single report of human nodose ganglia. There is a single report of human nodose ganglia.

The animal model presented here demonstrates that o.o. exposure to HSV-1 readily results in viral latency in the sensory ganglia that innervate the entire gastrointestinal tract. It remains to be determined what role, if any, HSV plays in human gastrointestinal disease. Certain pathological features of these diseases and their periodicity suggest either a causative or potentiating association. The absence of mucosal lesions in mice after acute intra-oesophageal infection may reflect a requirement for prior epithelial breakdown or irritation in order for efficient HSV-1 replication to occur at the surface. This breach in the luminal surface would not be required during reactivated infection, where infectious virus is introduced from innervating neurons within the gastrointestinal tissues rather than intraluminally. Recently Fawl & Roizman (1993) described an effective method for inducing in vivo latent HSV-1 reactivation in mice by subcutaneous administration of the heavy metal salt, cadmium sulphate. We plan to utilize this technique in order to examine the extent of HSV-1 reactivation and resultant inflammation in the enteric nervous system and gastrointestinal tract of mice with latent HSV-1 nodose ganglion infection. Further studies using this animal model should provide useful information regarding the possible relationship between HSV-1 latency in the vagal sensory system and recurrent gastrointestinal diseases.

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