HSV1 Latency Sites after Inoculation in the Lip: Assessment of their Localization and Connections to the Eye

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PURPOSE. To localize the sites of HSV1 latency in mice after a primary infection induced by injection into the lip and to assess their connection to the eye.

METHODS. The SC16 strain of HSV1, or a recombinant virus containing the HSV1 latency-associated transcript (LAT)-promoter driving expression of the LacZ reporter gene, was injected into the left upper lip. Tissues from animals killed at 6, 28, 180, and 720 days postinoculation (dpi) were analyzed for LATs, either by in situ hybridization (ISH) or by identifying LAT-promoter–driven transgene expression. HSV1 antigens were detected by immunohistochemistry.

RESULTS. At 28 dpi, all the neurologic structures that were acutely infected at 6 dpi exhibited a pattern of virus gene expression consistent with HSV1 latency—that is, LATs with no detectable HSV1 antigens. LAT staining differed among structures: intense and widespread within trigeminal neurons, intermediate within the sympathetic intermediolateral cell group of the spinal cord and the facial motor nucleus, and weak in other sites. Long-term expression of LATs (positive at 180 and 720 days) was observed only in tissues where the staining was intense or intermediate at 28 dpi.

CONCLUSIONS. After inoculation into the upper lip of mice, HSV1 established latency in several nervous system structures that have direct or indirect connections with ocular tissues. These results suggest that after an oral primary infection, the most frequent in humans, HSV1 may establish latency in several sites connected to the eye and may finally result in herpetic ocular disease involving the cornea, the iris, or even the retina. (Invest Ophthalmol Vis Sci. 2005;44:217–225) DOI:10.1167/iovs.02-0464

Hermes simplex virus type (HSV)-1 induces recurrent oral or ocular disease in humans. Primary infection generally occurs after contact with infected lesions or saliva, resulting in symptomatic primary infections which usually involve the oropharyngeal tract and rarely the eyes.1 Conversely, herpetic ocular disease is frequent, mostly in the cornea, affecting 21 of 100,000 persons per year in Western countries.1–4 This suggests that herpetic ocular disease may result from an oropharyngeal primary infection much earlier in life.1 In addition, the mechanisms underlying several types of recurrent herpetic ocular disease—keratitis, anterior uveitis, and retinitis—remain largely unresolved. The cornea is the most frequent site of ocular herpetic disease, and it is probable that most clinically significant HSV1 reactivations occur within trigeminal pathways. In contrast, herpes infections that involve the anterior uvea (iris and ciliary body) or the retina are generally not associated with simultaneous or past keratitis,5–7 suggesting that these clinical events result from virus reactivation within the autonomic or the visual pathways rather than within the trigeminal system. Clinical observations therefore suggest that primary HSV1 infection within the oral mucosa may lead to latency in different kinds of neurons, which in turn may explain the several types of ocular herpetic diseases observed in man.

This hypothesis is supported by experimental data showing that inoculation of the lower lip10 or snout of mice11–13 with HSV1 leads to latent infection in trigeminal (TG) and superior cervical ganglia (SCG), because virus can be readily recovered from these ganglia by ex vivo reactivation. In addition, postmortem human studies have shown evidence of herpetic latency in the human central and autonomic nervous systems, including the SCG.14–17

To gain further understanding of the propagation pathways of HSV1 from the oral mucosa to the eye, we recently described the acute phase of infection in a mouse model of primary herpetic infection into the upper lip. HSV1 infection was detected in the anterior uvea (iris and ciliary body) 6 days after inoculation with the virus.18 Some mice also exhibited keratitis 2 days later. Histologic examination revealed that ocular infection was probably the consequence of viral transfer in the sympathetic and sensory nervous pathways (including the superior cervical and trigeminal ganglia) which supply both the oral mucosa and the anterior segment of the eye. Moreover, acute infection was also observed in several brain nuclei, some of which were connected to the cornea, the iris or the retina.

In the present study, we have investigated sites of HSV1 latency by using the expression of latency-associated transcripts (LATs) as a marker of latent virus in serial histologic sections from mice inoculated in the lip and sampled at 6, 28, 180, and 720 days postinoculation (dpi). In addition to the wild-type HSV1 strain SC16, we used the SC16-derived recombinant LBA, which contains the LacZ gene of Escherichia coli inserted within the major LAT locus of HSV1.19,20 This construct allows for the straightforward detection of LAT pro-
moter activity by histochemical detection of β-galactosidase activity.

We observed that most, if not all, the nervous system structures infected during the acute phase of the disease became latently infected, including those connected to the eye.

**MATERIALS AND METHODS**

Two strains of HSV1 were used in the study, the wild-type SC16 strain of HSV15–7 and the recombinant SC16-LβA strain, which contains an encephalomyocarditis virus internal ribosomal entry site (IRES) linked to the LacZ gene of *Escherichia coli* inserted into a 168-bp *HpaI* deletion (nucleotides 120301 to 120469) within the major LAT locus of HSV1 strain SC16, as previously described.19,20 Viruses were cultured in infant hamster kidney cells, concentrated, and kept at −80°C until use.22 Concentrates were thawed, diluted and titrated on Vero cells (/H9252/).

Six-week-old inbred BALB/c female mice were used. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Sixty-two mice were inoculated with either SC16 or SC16-LβA (31 mice in each group). Under microscopic observation, 1 μL of HSV1 suspension containing 10^6 plaque-forming-units (PFU) was injected into the left upper lip, as previously described.19 Each animal was examined daily to detect ocular infection (blepharitis, conjunctivitis, keratitis, or iritis) or clinical signs of a disseminated viral infection (ruffling of fur, abnormal gait, weight loss). The number of clinical events, including death, was noted everyday until 21 dpi. Only mice that survived the primary infection and that developed clinical ocular disease beginning during the first 8 dpi were used for studies of HSV1 latency. Mice infected with either SC16 or SC16-LβA were randomly killed in groups of three at 28, 180, and 720 dpi, and tissues were fixed and prepared for cryosections, as previously described.19 In addition, six mice infected with SC16 or SC16-LβA and two control mice (sham infected) in an independent experiment were killed at 6 dpi to compare the acute phase of the disease between both strains. Tissues were processed using the same protocol as described earlier.

Frontal cryosections (10 μm) of the whole brain (decalcified skull), spinal cord, and SCG were collected into three parallel series, each one containing every third section. For SC16-infected mice, the first series was analyzed for HSV1 antigens using an immunoperoxidase assay,18 and the second series was analyzed for LAT production with an in situ hybridization (ISH) method using a LAT-specific probe.20 For SC16-LβA-infected mice, the first series was analyzed consecutively for β-galactosidase expression with 5-bromo-4-chloro-3-indolyl-β-D-galacto-sidase (X-gal),23 and then for HSV1 antigens with an immunoperoxidase assay. The second and third series were kept in reserve. The sections were counterstained with Giemsa blue or neutral red, washed, dried, and mounted.22

Acute or latent HSV1 infection was quantified during two independent microscopic evaluations (performed by ML). For each animal, between 250 and 500 sections of a total of 750 were examined. Each slide was analyzed independently at random to limit the subjective nature of the evaluation. Levels of high interest were also examined (by FL and SM).

**RESULTS**

Sham-infected mice showed no clinical signs of infection, and their tissues were negative for both HSV1-antigens and β-galactosidase activity.

**Pathogenesis of Wild-Type SC16 and LβA**

No striking difference in the pathogenesis of wild-type SC16 and LβA was observed during the first 3 weeks of infection. The survival curves after either SC16 or SC16-LβA inoculation (Fig. 1) were similar (log rank test; P > 0.90), as were the rates of surviving mice at 21 dpi that had developed a transient clinical ocular disease (13 among 21 animals in each group).

Immunohistochemical staining using polyclonal anti-HSV1 antibodies confirmed that the spread of recombinant LβA was similar to that observed with the wild-type viral strain, because there was no difference in the distribution of HSV1-positive cells at 6 dpi in the brain, eye, or spinal cord (the distribution of acutely infected structures at 6 dpi is shown schematically in Fig. 2A). Moreover, we detect no difference between the two strains in the level of acute infection—that is, the number of immunostained cells, in the visual and nervous systems, with the exception of the left trigeminal ganglion (Table 1) in which the number of acutely infected neurons was four to eight times higher in mice inoculated with the wild-type SC16 strain than in mice inoculated with the recombinant SC16-LβA virus (P < 0.05, Wilcoxon test). Preliminary evaluation showed that double staining (X-gal and immunoperoxidase) did not lead to an underestimate of the number of HSV1-antigen–positive neurons. For example, we found 7 and 11 acutely infected neurons on two consecutive series of 50 left TG sections from mouse 408 when a double staining or a single immunoperoxidase assay was performed. Whatever the viral strain, HSV1-antigen–positive cells were only found during the acute infection (6 dpi). Immunohistochemical staining was negative when clinical signs of infection had resolved (i.e., 28, 180, and 720 dpi), as expected when HSV1 becomes latent. Indeed, the latent state was confirmed with the detection of LAT or β-galactosidase activity in all the mice killed at 28 days or later (described later).

**Detection of LAT Promoter Activity during Acute Infection**

LAT-promoter–driven β-galactosidase activity was detected in SC16-LβA–infected mice as soon as 6 dpi in most of the acutely infected neurologic structures—that is, in those positive for HSV1 antigens. The β-galactosidase signal was strong and frequent in the left TG: 1.2 to 2.4 positive neurons per section, that is four times more than neurons acutely infected (Table 2). In contrast, direct detection of LAT by ISH was observed in rare TG neurons from SC16-infected mice at 6 dpi and were detectable only when a high probe concentration (10×) was used.

In nervous tissues other than the TG from SC16-LβA–infected mice, the β-galactosidase signal was weak (light blue) and rarely present in comparison to the number of acutely infected neurons. The percentage of infected neurons was systematically four times less than neurons acutely infected (Table 2).

![Image](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933224/ on 11/25/2018)
infected cells (Figs. 3, 4). For example, only a few blue cells were detectable among hundreds of acutely infected cells in the left SCG or within the brain nuclei. In addition, detection of LAT by ISH in the same tissues of SC16-infected mice was consistently negative, despite the strong acute infection at 6 dpi. Both these results suggest that LAT-promoter activity is low but not absent in these tissues during the acute stage of infection. Furthermore, LAT expression was detected neither in noninfected neuronal tissues nor in nonneurologic tissues (e.g., lip, iris, and cornea), even when clearly positive for acute infection and whatever the viral strain. These results suggest that the expression of LATs begins very early and mostly in structures that will later contain latently infected cells.

Activity of the LAT Promoter during the Latent Phase

Systematic examination of every third section of brain, spinal cord, and SCG of mice infected with SC16-LβA and killed at 28 dpi showed β-galactosidase expression (i.e., LAT promoter activity) in all neurologic structures that were acutely infected at 6 dpi (Fig. 2B). Cells expressing β-galactosidase did not contain detectable HSV1-antigens, indicating that HSV1 infection had already become latent. Labeled neurons were detected almost exclusively on the side of the inoculation (left) with the exception of the hypothalamic nuclei, the area postrema, and the locus coeruleus, which were labeled bilaterally.

TABLE 1. Detection of Acutely HSV1-Infected Neurons in the Left Trigeminal Ganglion

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mouse</th>
<th>Positive Neurons*</th>
<th>Average Positive Neurons per Section (n)</th>
<th>Average in the Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC16</td>
<td>401</td>
<td>197 (86)</td>
<td>2.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>402</td>
<td>77 (38)</td>
<td>2.03</td>
<td>4.82</td>
</tr>
<tr>
<td></td>
<td>403</td>
<td>426 (42)</td>
<td>10.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>407</td>
<td>57 (66)</td>
<td>0.86</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>408</td>
<td>9 (61)</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>LβA</td>
<td>406</td>
<td>42 (73)</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>407</td>
<td>57 (66)</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>408</td>
<td>9 (61)</td>
<td>0.15</td>
<td></td>
</tr>
</tbody>
</table>

Six mice were inoculated in the left upper lip with 10⁶ PFU of either HSV1 strain SC16 or SC16-LβA (three in each group). Every three frontal cryosections (10 μm) of the left trigeminal ganglia were analyzed for HSV1 antigens with an immunoperoxidase assay. Data were recorded at 6 dpi.

* Data are expressed as number of positive neurons, with number of sections examined shown in parentheses.
a distribution similar to the one observed during the acute phase. Also some LAT-positive neurons were found in the right TG, contralateral to the inoculation site, but these labeled neurons were very rare (see the Discussion section).

A similar number of neurons was positive for LAT-promoter activity (β-galactosidase assay) at 6 and 28 dpi in SC16-LβA-infected mice within the left TG (respectively, 2.36 and 1.86 neurons per section on average, \( P > 0.2 \), Wilcoxon test, Table 2). In contrast, an increase in the number of β-galactosidase-positive cells between 6 and 28 dpi was detected in other tissues, such as the left SCG or motor nucleus of the facial nerve (\( P < 0.05 \), Wilcoxon test, Table 3, Fig. 3). Moreover, the intensity of β-galactosidase staining varied among different types of neurons: intense in numerous neurons within the left TG, intense but found in only few neurons within the left intermediolateralis (IML) sympathetic column of the spinal cord and within the left motor nucleus of the facial nerve, weak (light blue) and rare in the SCG and the brain nuclei other than those of the facial nerve (Table 3, Fig. 4).

As with the recombinant virus LβA at 28 dpi, the intensity of LAT signal revealed by ISH in SC16-infected mice depended on the type of neuron. The signal was intense in numerous neurons in the TG (Fig. 4), high but found in only a few neurons within the motor facial nucleus and the spinal cord, and undetectable in other brain nuclei or in autonomic ganglia.

The results with recombinant virus LβA and wild-type SC16 suggest that the dynamics of LAT-promoter activity differs between tissues, because the level of LAT expression depended on the type of neuron. High levels of expression were detected in sensory neurons, intermediate levels in motor or medullar neurons, and low levels in other kinds of latently infected neurons.

**Connection to the Eye of Several HSV1-Latency Sites after Inoculation in the Lip**

The detection of HSV1 in cells by using the reporter gene at 28 dpi in SC16-LβA-infected mice showed that HSV1 latency, revealed by both the activity of the LAT-promoter and the absence of viral antigen expression, occurred in several nervous system structures that are connected to the eye. More precisely, HSV1 latency was found in the peripheral ganglia and/or the central nuclei connected to one of the main three kinds of ocular tissues (i.e., cornea, uvea, or retina).

In mice inoculated in the left upper lip, LAT-promoter activity was detected in the ipsilateral TG (left) which supplies the cornea, or in its connections (zona incerta [ZI], superior colliculi, or posterior thalamus). β-Galactosidase staining was also found in the left SCG, IML, ciliary ganglion, and Edinger-Westphal nucleus.

**Table 2. Detection of Neurons Positive for LAT Promoter Activity in the Trigeminal Ganglion**

<table>
<thead>
<tr>
<th>dpi</th>
<th>Strain</th>
<th>Mouse</th>
<th>Positive Neurons</th>
<th>Average Positive Neurons per Section (n)</th>
<th>Average in the Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>LβA*</td>
<td>406</td>
<td>154 (73)</td>
<td>2.11</td>
<td>2.36</td>
</tr>
<tr>
<td>6</td>
<td>SC16</td>
<td>407</td>
<td>162 (66)</td>
<td>2.45</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>408</td>
<td>154 (61)</td>
<td>2.52</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>SC16</td>
<td>411</td>
<td>446 (65)</td>
<td>6.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>412</td>
<td>175 (55)</td>
<td>3.18</td>
<td>4.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>413</td>
<td>174 (73)</td>
<td>2.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LβA*</td>
<td>416</td>
<td>157 (70)</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>417</td>
<td>50 (77)</td>
<td>0.39</td>
<td>1.86</td>
</tr>
<tr>
<td>180</td>
<td>SC16†</td>
<td>418</td>
<td>256 (73)</td>
<td>3.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>421</td>
<td>43 (73)</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>422</td>
<td>196 (73)</td>
<td>2.68</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>423</td>
<td>46 (65)</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LβA*</td>
<td>426</td>
<td>27 (91)</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>427</td>
<td>60 (87)</td>
<td>0.69</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>428</td>
<td>22 (97)</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>720</td>
<td>SC16†</td>
<td>452</td>
<td>131 (47)</td>
<td>2.79</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>453</td>
<td>151 (79)</td>
<td>1.91</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>454</td>
<td>53 (47)</td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LβA*</td>
<td>457</td>
<td>6 (72)</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>458</td>
<td>6 (99)</td>
<td>0.06</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>459</td>
<td>41 (95)</td>
<td>0.43</td>
<td></td>
</tr>
</tbody>
</table>

Mice inoculated with \( 10^6 \) PFU of either HSV1 strain SC16 or SC16-LβA were killed in groups of three at 6, 28, 180, and 720 dpi. Every three frontal cryosections (10 \( \mu m \)) of the trigeminal ganglia were analyzed for LAT production with either ISH using a LAT-specific probe in SC16-infected mice, or a \( \beta \)-galactosidase assay in SC16-LβA-infected mice. The number of sections examined for positive neurons is shown in parentheses.

* Detection of positive neurons with \( \beta \)-galactosidase activity (X-gal staining).
† Detection of positive neurons with ISH.

**Footnotes:**
- *: The average numbers of labelled neurons at 6 and 28 dpi are significantly different (\( p < 0.05 \), Wilcoxon test)
- †: The average numbers of labelled neurons at 28 and 720 dpi are significantly different (\( p < 0.05 \), Wilcoxon test)
Westphal nucleus, which supply the left iris and the ciliary body, and in connected brain nuclei (solitary tracts, area postrema, paraventricular nuclei, locus coeruleus, and amygdaloid nuclei). Finally, LAT-promoter activity was found in the suprachiasmatic nuclei that are directly connected to the retina (Fig. 2B). As detailed earlier, LAT expression varied between different types of neurons (high expression in many neurons in the TG, and low expression in few neurons in the SCG for example), and this could be related to the higher rate of herpetic recurrences in some tissues (see the Discussion section).

**Long-Term Expression of LATs in the Nervous System of Latently Infected Mice**

ISH revealed long-term expression of LATs in the nervous system of mice infected with SC16 sampled at 180 and 720 dpi, particularly in the left TG in which no statistically significant decrease in the number of LAT-positive cells was found between 28, 180, and 720 dpi ($P > 0.1$, Wilcoxon test). As in the TG, no significant decrease in the number of LAT-positive neurons was found in the left motor nucleus of the facial nerve from 28 to 720 dpi ($P > 0.5$, Wilcoxon test), whereas a...
A recombinant containing an \( \beta \)-galactosidase protein encoded by a chimeric LAT-LacZ transcript was used in inbred BALB/c mice and the wild-type SC16 strain of HSV1 to compare the results with other animal models.\(^{20,24,25}\) In the present study, we used inbred BALB/c mice and the wild-type SC16 strain of HSV1 to compare the results with other animal models.\(^{20,24,25}\) In the present study, we used inbred BALB/c mice and the wild-type SC16 strain of HSV1 to compare the results with other animal models.\(^{20,24,25}\)

### Table 3. Detection of Neurons Positive for LAT Promoter Activity in Structures Other Than the Trigeminal Ganglion

<table>
<thead>
<tr>
<th>dpi</th>
<th>Strain</th>
<th>Mouse</th>
<th>Left SCG</th>
<th>Spinal Cord</th>
<th>Motor Nucleus of the Facial Nerve</th>
<th>Paraventricular Nucleus</th>
<th>Zona Incerta</th>
<th>Suprachiasmatic Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>L( \beta )^{a}</td>
<td>406</td>
<td>1 (18)</td>
<td>0 (77)</td>
<td>0 (12)</td>
<td>0 (16)</td>
<td>1 (15)</td>
<td>0 (15)</td>
</tr>
<tr>
<td>28</td>
<td>SC16(^{\dagger} )</td>
<td>411</td>
<td>0 (11)</td>
<td>10 (53)</td>
<td>18 (14)</td>
<td>0 (11)</td>
<td>0 (10)</td>
<td>0 (15)</td>
</tr>
<tr>
<td>180</td>
<td>SC16(^{\dagger} )</td>
<td>421</td>
<td>0 (5)</td>
<td>5 (82)</td>
<td>0 (14)</td>
<td>0 (11)</td>
<td>0 (9)</td>
<td>0 (13)</td>
</tr>
<tr>
<td>720</td>
<td>SC16(^{\dagger} )</td>
<td>452</td>
<td>0 (12)</td>
<td>0 (47)</td>
<td>2 (9)</td>
<td>0 (11)</td>
<td>0 (10)</td>
<td>0 (11)</td>
</tr>
</tbody>
</table>

The procedures are the same as those described in Table 2. Results are given for the six most-consistent sites of LAT-promoter activity. For each region of the nervous system, the first column of data indicates the number of labeled neurons (with the number of sections examined shown in parentheses), and the second column indicates the number of positive neurons per section for each group of three animals.

* Detection of positive neurons with \( \beta \)-galactosidase activity (X-gal staining).

\(^{\dagger} \) Detection of positive neurons with ISH.

...decrease was found in the spinal cord (\( P < 0.05 \), Wilcoxon test, Table 3).

\( \beta \)-Galactosidase assays confirmed a decreasing level of LAT-promoter activity from 28 to 180 and 720 dpi in some tissues latently infected with SC16-L\( \beta \). The light blue signal observed at 28 dpi in the hypothalamic nuclei and the SCG were not found at 180 and 720 dpi (\( P < 0.05 \), Wilcoxon test). The decrease also was significant within the left IML and motor nucleus of the facial nerve, but not in the left TG (Tables 2, 3; Fig. 3).

### DISCUSSION

We localized the sites of LAT expression during acute and latent stages of HSV1 infection on serial histologic sections after inoculation of virus in the mucocutaneous border of the left upper lip of mice.

In the present study, we used inbred BALB/c mice and the wild-type SC16 strain of HSV1 to compare the results with those from our previous study concerning the propagation pathways of HSV1 after primary infection of the lip.\(^{18} \) In addition, we used the SC16-L\( \beta \) recombinant containing an IRES-linked LacZ gene inserted within both LAT regions (one within each inverted repeat). This construction results in the expression of a chimeric LAT-LacZ transcript that does not accumulate in the nucleus as does the wild-type LATs and is sensitive than the direct detection of LATs with ISH in our experiments. Moreover, the difference in sensitivity between the two methods gave us semiquantitative data concerning the relative levels of LATs in neurons—that is, abundant when both methods were positive, as within the left TG, and low when only the \( \beta \)-galactosidase assay was positive, as within the left SCG. In the latter case only a light blue signal was observed. Other methods such as quantifying LATs or the amount of HSV1 DNA in tissues homogenates does not give information as to the precise histologic localization of latently infected neurons nor any indication as to the level of LAT expression within individual neurons.\(^{26-30} \) and in situ PCR cannot readily be performed on large numbers of serial whole brain and spinal cord sections.

In SC16-L\( \beta \)-infected mice, LAT-promoter activity was detected as early as 6 dpi in all the neurologic tissues positive for HSV1-antigens. This suggests that the onset of LAT expression begins very early after neuronal infection. This is consistent with the detection of LATs during acute HSV1 infection of cultured neurons and nonneuronal cells.\(^{30} \) and in vivo in other animal models and/or with other HSV1 strains.\(^{20,25,27-51,52} \)
However, production of LATs at 6 dpi was poorly low, because it was only detectable by measuring β-galactosidase activity, ISH being negative, with the exception of some rare positive TG neurons when very large amounts of riboprobe were used.

LAT-positive neurons with no HSV1 antigens were detected at 28 dpi in all the neurologic structures that were acutely infected at 6 dpi. In contrast, LAT-promoter activity was not detected in neurologic structures free of acute infection at 6 dpi, or in nonneurologic tissues, even those severely infected at 6 dpi, such as the iris, the ciliary body, or the cornea. This suggests that, in our model, herpetic latency was widely distributed in the nervous system after inoculation into the lip, but LAT expression was restricted to nervous system tissues that were involved during primary HSV1 infection. Other experiments have suggested that LATs may have been detected in the cornea of latently infected rabbits, but this result remains controversial. Even if several types of neurons were latently infected, significant differences were observed: First, the left TG was the only structure where the number of LAT-producing neurons at 28 dpi was in the same order of magnitude as the number of acutely infected cells at 6 dpi (4.14 and 4.82 neurons per section respectively with the SC16 strain). In comparison, LAT-promoter activity was detected only in a few neurons of the left SCG (7–27 cells) or the paraventricular nuclei (2–14 cells) at 28 dpi, despite a severe acute infection. Second, ISH or β-galactosidase staining showed a similar number of positive cells within the left TG, the left IML column of the spinal cord, and the left motor nucleus of the facial nerve, suggesting that LAT-promoter activity was high in these latently infected neurons (Table 3). In contrast, the light blue signal in β-galactosidase assays, besides the negative results of ISH, in other latently infected structures such as the SCG, the PVN, or the ZI suggests that the accumulation of LAT in these latently infected neurons was probably much lower. Third, the number of LAT-expressing neurons did not decrease between 28 and 720 dpi in the left TG and the left motor nucleus of the facial nerve from SC16-infected mice, whereas only one positive neuron was found in the spinal cord from three mice at 720 dpi. This is consistent with a previous study that reported a decreasing number of LAT-producing neurons between 4 and 21 days after ocular inoculation of the KOS strain of HSV1 in the sympathetic and parasympathetic neurons, whereas the number was stable in the TG during the same period. LAT-promoter activity also decreased with time in SC16-LβA-infected mice, even in the TG (Tables 2, 3). This difference, in comparison to the wild-type SC16 strain suggests that the LβA recombinant strain may be impaired in its ability to persist over long periods in the central nervous system (CNS). This lack of persistence could result from the presence of chimeric LAT transcript, the absence of natural LATs, and/or an immune-mediated clearance of neurons producing E. coli β-galactosidase, a foreign protein.

Whatever the differences between latently infected tissues, our results clearly show that HSV1 became latent in most, if not all, nervous tissues associated with acute infection in mice. This suggests that an enhanced viral spread during primary infection may result in an increased number of latently infected cells, which could correlate with an increased risk of reactivation.

Because several HSV1 latency sites are connected to the eye, our results give a possible explanation to the seeming paradox that many ocular herpetic diseases follow primary oral infection. Corneal recurrences could result from reactivation of HSV1 within the opthalmic part of the TG or within tissues connected to the latter, such as the ZI, the superior colliculus, or the posterior thalamus. Moreover, the fact that the TG (i.e., the main site of HSV1 latency within the sensory system) was almost exclusively positive for LAT expression ipsilateral to the inoculation site could be related to the clinical observation that HSV1 keratitis is essentially a unilateral disease. Herpes simplex latency within the autonomic pathways (SCG, intermediate column of the spinal cord, Edinger-Westphal nucleus, ciliary ganglion) or the brain nuclei that are connected to them (solitary tract, area postrema, paraventricular nuclei, locus coeruleus, and amgyldoid nuclei) could explain iris or ciliary body infection. Because some LAT-positive neurons were found in the left superior cervical and ciliary ganglia, reactivation of virus within these ganglia could result in pupil abnormalities, as has been suggested for Varicella-Zoster virus in some cases of Holmes-Adie and Horner syndrome. Last, retinitis could occur after reactivation within the suprachiasmatic nuclei or the paraventricular nuclei, the latter being connected to the former, or even within the Edinger-Westphal nucleus after virus has spread through the afferent pathways of the pupil reflex.

Because of the role of LATs in the establishment of HSV1 latency and viral reactivation, it is likely that the low accumulation of LATs in autonomic or hypothalamic structures, as discussed earlier, explains the lower frequency of HSV1 reactivation in these neuronal pathways (leading to an infection of the anterior uvea or the retina, respectively) than the frequency observed in the sensory system (leading to a keratitis). Indeed, experimental reactivation has been shown to be difficult to induce in mouse nonsensory neurons. Using our animal model, future experiments will be attempted to overcome this difficulty to obtain further information on the kinetics of HSV1 reactivation in the different types of latently infected neurons.

Our results provide an explanation as to the mechanisms by which HSV1 reactivation can result in viral production in the three ocular tunics (i.e., the cornea-sclera, the uvea, and the retina) and why recurrences within the cornea, richly connected to the TG, are far more frequent than those involving the iris, the ciliary body, or retina.

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