Two patterns of persistence of herpes simplex virus DNA sequences in the nervous systems of latently infected mice

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The number of herpes simplex virus (HSV) genome equivalents recovered from latently infected mouse spinal ganglia was compared with the proportion of neurons containing latency-associated transcripts (LATs). Two distinct patterns of HSV persistence were observed, depending on the anatomical location of ganglia with respect to the site of cutaneous inoculation. The location of the bulk of latent viral DNA did not correspond with the highest prevalence of LAT+ neurons. Viral DNA was most abundant in spinal ganglia directly innervating the inoculation site and the amount recovered, which was similar to that found previously in human trigeminal ganglia, suggested that LAT+ neurons each contain hundreds of copies of HSV DNA. In stark contrast, although LAT+ neurons were most abundant in neighbouring ganglia, viral DNA was scarce (approx. 20 copies/LAT+ cell). These data indicate that amplification of HSV DNA sequences is greatest in ganglia previously shown to be associated with viral antigen expression during the productive phase of primary infection.

Herpes simplex virus (HSV) is characterized by its ability to persist in a latent form in sensory ganglia of the peripheral nervous system (PNS) after a brief phase of productive infection (Stevens & Cook, 1971). Latently infected ganglia taken from humans and experimental animals contain a strikingly large amount of viral DNA (Rock & Fraser, 1983; Efstathiou et al., 1986) that is widely assumed to reside in neurons, first because these cells have been convincingly shown to be the site of HSV reactivation (McLennan & Darby, 1980) and second because virus encoded RNA molecules [latency-associated transcripts (LATs)] accumulate in nuclei of neurons (Stevens et al., 1987). Typically, approximately 1% of ganglionic neurons appear to be latently infected, as judged by counting either infectious centres after cocultivating dissociated ganglia with susceptible cells (Walz et al., 1976; Nicholls & Blyth, 1989) or LAT+ cells following in situ hybridization (ISH) (e.g. Tenser et al., 1989). On this basis, latently infected neurons have been calculated each to contain many copies of the virus genome (Efstathiou et al., 1986), suggesting that HSV DNA is amplified during the establishment phase. However, substantial amplification of viral DNA is apparently not essential for establishment of latency because a replication-defective mutant virus can persist in the PNS of experimentally infected animals (Clements & Stow, 1989; Coen et al., 1989; Leib et al., 1989; Leist et al., 1989; Dobson et al., 1990; Kosz-Vnenchak et al., 1990; Steiner et al., 1990); under these conditions the amount of HSV DNA recovered from latently infected ganglia is reduced (Efstathiou et al., 1989; Tenser et al., 1989; Friedrich et al., 1990; Katz et al., 1990; Valyi-Nagy et al., 1991). Therefore, it is likely that either the number of cells harbouring virus genomes has been underestimated or HSV DNA is not distributed evenly amongst infected cells during latency.

We have investigated these issues using an experimental system that makes novel use of the segmental sensory innervation of flank skin. In mice the thoracic and lumbar regions of the PNS are divided into 13 (T1 to T13) and five (L1 to L5) structural segments respectively, each innervating corresponding segments of skin (dermatomes). On the trunk adjacent dermatomes overlap by approximately 50%. We have shown recently that the distribution of acute and latent infection in the PNS can be mapped with precision after introduction of HSV-1 into the midflank skin of C57BL10 mice (Speck & Simmons, 1991). Productive infection, as judged by virus recovery and the presence of viral antigens and mRNA, was restricted to ganglia innervating the site of inoculation (around T9). In contrast, latent infection, assessed by the presence of LAT+ neurons and reactivation in vitro, was much more widespread.

Using the same system we compared the amount of HSV DNA that persists in each spinal segment (T8 to L1) with the number of LAT+ neurons present at the corresponding level, and showed that, after recovery from acute infection with a wild-type strain of HSV-1,
viral DNA sequences are not distributed evenly among latently infected cells. Some LAT+ neurons contain very few copies of the virus genome and cells of this type (in which HSV DNA is scarce) are alone sufficient to account for reactivation of infection after explantation of ganglia. In contrast, the bulk of the viral DNA recovered from latently infected tissue in this system appears, judged by its location, to be a residuum of abortive infection and is likely to represent the 'endless' form of latent DNA detected by Rock & Fraser (1983) and Efstathiou et al. (1986). The biological relevance of this DNA remains to be established.

To limit the spread of productive infection and maximize survival, resistant C57BL10 mice (Specific Pathogen-Free facility, Animal Resources Centre, Perth, Western Australia) were used. Adult animals were infected (day 0) with $3 \times 10^4$ p.f.u. HSV-1 strain SC16 (Hill et al., 1975) by scarification of the left flank (T9) as described previously (Simmons & Nash, 1984; Speck & Simmons, 1991). Ganglia spanning T8 to L1 were removed from latently infected mice 23 days post-infection and tested for the presence of either HSV DNA (62 animals) or LAT+ neurons (25 animals). Segmentally pooled ganglionic DNA samples were digested with BamHI and analysed by Southern blot hybridization using a 32p-labelled cloned probe (pBAZ-1) from the thymidine kinase region of HSV-1 (strain F). Filters were washed at a final stringency of $T_m - 15$ °C and subjected to phosphor image analysis (PhosphorImager 400, Molecular Dynamics). Phosphor image analysis provided an alternative to conventional autoradiography by using a photostimulable storage phosphor screen instead of X-ray film. This system is more sensitive (15-fold higher for 32P) and offers a much greater linear dynamic range than X-ray film (Johnston et al., 1990). Images were stored digitally and accurate quantification of bands, in relation to reconstructions, was carried out using ImageQuant software (V3.0, Molecular Dynamics) (Fig. 1 and Table 1). Virus sequences were detected readily on a 17 h phosphor image in ganglionic samples from T8 and T9, and were less abundant at T10 and T11 (Fig. 1b). A further analysis of samples between T10 and L1 showed (on a 48 h image) a small but quantifiable amount of DNA at T12. In some experiments extremely faint signals (approx. 0.001 copies/cell) were noted in T13 and L1 samples, but we have been unable to quantify this material precisely.

To assess accurately the relative number of LAT+ primary sensory neurons at each spinal level, an exhaustive examination of ganglia (25 from each segment) was undertaken by ISH. Segmentally pooled ganglia were fixed in periodate-lysine-parafomaldehyde (McLean & Nakane, 1974) immediately after removal and paraffin-embedded, and 5μm sections were collected onto 3-aminopropyltriethoxysilane-coated slides. The ISH protocol used to detect LATs was a modification of that described previously (Speck & Simmons, 1991). Digoxigenin-labelled strand-specific riboprobes generated from plasmid pBS-0, comprising a

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**Table 1. Relationship between HSV DNA and LAT+ neurons in spinal segments T8 to L1**

<table>
<thead>
<tr>
<th>Spinal segment</th>
<th>HSV genome equivalents/cell</th>
<th>LAT+ profiles/section ± S.E.M. (%)</th>
<th>HSV genome equivalents/LAT+ neuron†</th>
</tr>
</thead>
<tbody>
<tr>
<td>T8</td>
<td>0.056</td>
<td>0.25 ± 0.04 (0.20)</td>
<td>280</td>
</tr>
<tr>
<td>T9</td>
<td>0.048</td>
<td>0.3 ± 0.06 (0.23)</td>
<td>209</td>
</tr>
<tr>
<td>T10</td>
<td>0.016</td>
<td>1.19 ± 0.21 (0.93)</td>
<td>17</td>
</tr>
<tr>
<td>T11</td>
<td>0.014</td>
<td>0.75 ± 0.11 (0.58)</td>
<td>24</td>
</tr>
<tr>
<td>T12</td>
<td>0.006</td>
<td>0.44 ± 0.05 (0.34)</td>
<td>18</td>
</tr>
<tr>
<td>T13</td>
<td>0.001</td>
<td>0.17 ± 0.04 (0.13)</td>
<td>8</td>
</tr>
<tr>
<td>L1</td>
<td>~0.001</td>
<td>0.08 ± 0.02 (0.06)</td>
<td>17</td>
</tr>
</tbody>
</table>

* The percentage of LAT+ neuronal profiles/ganglionic section was calculated using an estimate of total neuronal profiles/ganglionic section of 128.4 ± 3.3 (± S.E.M.). This estimate was derived from pooled ganglia from C57BL10 mice (D. Tscharke & A. Simmons, unpublished results).
† The number of genomic equivalents/LAT+ neuron was calculated by assuming that 10% of ganglionic cells are neurons (see text).
2.6 kb BamHI-SalI fragment of HSV-1 (KOS) spanning map units 0.79 to 0.807 cloned into Bluescribe M13- (Stratagene Cloning Systems) were used. Tissue sections were hybridized overnight at $T_m - 25 \, ^\circ C$ and washed to a maximum stringency of $T_m - 13 \, ^\circ C$. Bound probe was detected using alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer Mannheim). Multiple slides (each containing sections through approx. 10 ganglia) were used to determine mean LAT+ neuronal profiles/ganglion using a 1 mm graticule (Leitz 519-950) to assist counting when necessary. By assessing several hundred ganglionic sections at each level statistically defensible accuracy was assured. Absolute LAT+ neuron numbers were not calculated, first because there is dispute regarding the correction factors required to generate this information (Smolen et al., 1983) and second because comparative data were sufficient in this context. In stark contrast to HSV DNA, LAT+ neurons (Fig. 2) were readily detected in all spinal segments between T8 and L1, and there was no apparent difference in the intensity of the LAT signal between segments. When the number of copies of viral DNA detected at each level was related to the proportion of neurons containing LATs, two distinct patterns emerged. In ganglia that had little or no direct neural connection with the inoculation site (T10 to L1) there were approximately 20 HSV genome equivalents/LAT+ neuron, based on the standard assumption that approximately 10% of ganglion cells are neurons (Walz et al., 1976). In contrast, at T8 and T9, the abundance of viral DNA was at least 10-fold that at T10 to L1 in relation to each LAT+ cell (Table 1), and up to 50-fold greater in absolute terms.

The possibility of differences in numbers of neurons between spinal levels influencing our estimates of viral DNA copy number/LAT+ neuron (Table 1) is considered unlikely because studies on mammalian ganglia (Hatai, 1902; Holmes & Davenport, 1940) have shown that they vary by less than twofold over a wide range of ganglionic levels.

HSV DNA extracted from latently infected tissue has been quantified and structurally characterized previously (Rock & Fraser, 1983; Efstatthiou et al., 1986). The molecules lack free ends, compatible with the presence of circular or concatemeric structures, and they can be readily separated from chromosomal DNA, indicating that at least the bulk of the virus genomes are not integrated into the genetic material of the host (Mellerick & Fraser, 1987). The amount of latent viral DNA recovered from the most abundantly infected C57BL10 mouse ganglia (e.g. 0.056 copies/cell at T8) is similar to that recovered from human trigeminal ganglia, but several-fold lower than that reported previously for BALB/c mice (Efstatthiou et al., 1986), which we attribute to the restrictive infection experienced by black mice after flank inoculation (Simmons & LaVista, 1989). Nevertheless, in T8 and T9 ganglia, as in other systems, LAT+ neurons would appear to contain hundreds of HSV DNA molecules. This calculation assumes that the population of cells harbouring HSV DNA is homogeneous, but the data presented here strongly suggest that this is not the case.

We have shown that many LAT+ neurons, such as those between T10 and L1 in the system described here, contain only a small number of HSV genomes, despite the presence of much higher levels of DNA in ganglia innervating the inoculation site. There are three potential explanations of these data. First, LAT+ neurons at T8 and T9 may contain more viral DNA than those at T10 to L1, perhaps as a consequence of virus gene expression during the establishment phase (Speck & Simmons, 1991). In this context, circular and concatemeric DNA molecules are both thought to exist at different stages of the replicative cycle of HSV (Roizman & Sears, 1990). Second, LAT+ neurons detected at T8 and T9 may represent a mixed population of cells, each of which contains either a high (200+) or low (approx. 20) viral DNA copy number. Finally, the bulk of the DNA recovered from the PNS of latently infected hosts (found at T8 and T9 in the system described here) may not reside in LAT+ neurons. Others have reported that LATs accumulate to wild-type levels under conditions that preclude amplification of the virus genome (Tenser et al., 1989; Valyi-Nagy et al., 1991) and in our experiments the abundance of virus transcripts in the nuclei of neurons appeared to be similar at all levels tested, despite the potential difference in the number of DNA templates present. Direct comparison of the levels of viral DNA and RNA recovered from each spinal segment in this model should further characterize this...
apparent dissociation between transcriptional activity and DNA copy number.

Using the standard technique of explant culture (Stevens & Cook, 1971) and the same experimental model, we have shown previously that latent HSV can be reactivated readily from all segments between T6 and L1 despite a much more restricted acute infection (Speck & Simmons, 1991), suggesting that LAT+ neurons containing very few virus genomes are an important reservoir of infection. The paucity of HSV DNA in these cells is compatible with the lack of detectable virus gene expression in ganglia distant from those innervating the inoculation site during the acute phase of infection (Speck & Simmons, 1991) and the ability of replication-competent mutant viruses to establish latency (e.g. Leib et al., 1989). Limitations imposed by the sensitivity of ISH mean that our measurement of the proportion of neurons containing LATs (e.g. 0.34% at T12) must be regarded as the lower limit of the true figure and therefore the actual number of HSV DNA molecules maintained in LAT+ cells may be even lower than what we have calculated. However, it seems likely that limited amplification of HSV DNA sequences occurs between T10 and L1, perhaps, as suggested by Sears & Roizman (1990), by the interaction of cellular enzymes with the virus genome.

The model system described here makes novel use of the segmental innervation of the thorax and abdomen. Spread of virus (via the ‘back-door’ route) to parts of the PNS adjacent to those innervating the inoculation site is well documented (Tullo et al., 1982). Although the factors that determine whether a neuron becomes productively or latently infected with HSV are poorly understood, careful manipulation of mouse strain, virus dose and site of inoculation enabled us to select only a single outcome, latency, following spread of virus within the PNS. The system allows the establishment and maintenance of latency to be characterized without the potential complications introduced by concurrent or resolved productive infection.

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References


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