Neurons containing latency-associated transcripts are numerous and widespread in dorsal root ganglia following footpad inoculation of mice with herpes simplex virus type 1 mutant in1814

M. S. Ecob-Prince,1* C. M. Preston,2 F. J. Rixon,2 K. Hassan1 and P. G. E. Kennedy1

1Department of Neurology, University of Glasgow, Institute of Neurological Sciences, Southern General Hospital, Glasgow G51 4TF and 2MRC Virology Unit, Church Street, Glasgow G11 5JR, U.K.

The herpes simplex virus type 1 (HSV-1) mutant in1814 lacks the ability to trans-activate immediate early gene transcription and enter lytic replication but it can establish and reactivate from latency. We therefore investigated the number of neurons that expressed latency-associated transcripts (LATs) in animals latently infected with in1814, the rescued revertant (1814R), or wild-type (wt) HSV-1. The percentage of LAT+ neurons increased with increasing doses of each of the viruses. After inoculation of equal amounts of infectious virus many more LAT+ neurons were observed in animals infected with in1814 than with 1814R or wt HSV-1. Whereas the LAT+ neurons in animals infected with 1814R or wt HSV-1 were largely confined to lumbar dorsal root ganglia (DRG) L4/L5/L6 (those which innervate the lower leg), in animals infected with in1814 they were also present in DRG not directly involved with such innervation (thoracic 12 and 13, L1, L2 and L3). We concluded that the large number of LAT+ neurons observed with in1814 was related to the high particle numbers in the inoculum and that spread of virus was related to limited replication as well as to the low neurovirulence of in1814. This spread was not unique to in1814 but when it occurred with more virulent viruses such as 1814R or wt HSV-1, it resulted in the death of the host.

Introduction

When herpes simplex virus (HSV) is inoculated intradermally into a mouse it establishes a latent infection in the sensory neurons of the ganglia that innervate the dermatome (Stevens & Cook, 1971). In the case of the footpad this is mainly the dorsal root ganglia (GRG) of lumbar segment 5 (L5), with some contribution also from L4 and L6 (Greene, 1959). Although latent viral DNA can be found in the relevant ganglia (Efstathiou et al., 1986; Katz et al., 1990), it cannot be detected on an individual cell basis by in situ hybridization for reasons that are not yet understood (Stevens, 1989). However, there is limited transcription of the HSV genome during latency and latency-associated transcripts (LATs) accumulate in the nucleus of neurons in mice (Deatly et al., 1987; Spivack & Fraser, 1987; Stevens et al., 1987), rabbits (Rock et al., 1987; Wagner et al., 1988) and humans (Croen et al., 1987; Gordon et al., 1988; Wechsler et al., 1988) where they can be detected by in situ hybridization. The major LAT is a 2.0 kb RNA which may be an intron spliced from an 8.3 kb transcript (Farrell et al., 1991) or an alternative transcription unit using the same promoter as the 8.3 kb mRNA (Spivack et al., 1991). The function of LATs remains unclear but viruses from which the LAT gene has been deleted can establish, maintain and reactivate from latent infections (Javier et al., 1988; Ho & Mocarski, 1989; Steiner et al., 1989; Izumi et al., 1989; Block et al., 1990), although reactivation in some cases is delayed or less efficient (Leib et al., 1989a; Hill et al., 1990; Trousdale et al., 1991). This reduced reactivation may, however, be the result of fewer latently infected cells in the DRG from which reactivation can take place, supporting the idea that LATs have a role in the efficient establishment of latent infections (Sawtell & Thompson, 1992). Nevertheless, the presence of LATs in a cell is thought to be a marker of latent virus infection.

The percentage of neurons in which a latent infection becomes established is very low. Detection of viral nucleic acid suggests that between 1% and 5% of the neurons are involved (Galloway et al., 1979; Stevens, 1989; Speck & Simmons, 1991; Rock et al., 1992). However, when DRG were serially sectioned to quantify LAT+ cells, only 24 (0.65%) were found in 3500 neurons of the L5 ganglion (Tenser et al., 1989). We therefore investigated whether a mutant of HSV, which was less able than wild-type (wt) HSV to enter a lytic replication cycle, would establish larger numbers of LAT+ neurons.

The mutant in1814 (Ace et al., 1989) has a 12 bp
insertion in the gene for Vmw65 (VP16, x-TIF) which disrupts the trans-activation function but not the assembly function of the protein. Vmw65 is present in the virus tegument and trans-activates the immediate early (IE) genes which in turn control the early and late genes in a cascade leading to lytic replication (Goding & O'Hare, 1989). Without functional Vmw65 the expression of IE genes is reduced and lytic replication usually fails (Harris & Preston, 1991). After corneal inoculation of mice, in1814 establishes latent infections in the trigeminal ganglia from which it reactivates on explantation (Steiner et al., 1990). We therefore investigated the number of LAT+ neurons in DRG of mice latently infected with in1814, the wt HSV-1 from which it was derived or the rescued revertant (1814R) of the mutant (Ace et al., 1989). It was found that after inoculation of equal p.f.u. in1814 established a greater number of LAT+ neurons in a more widespread number of DRG than did the rescued or wt viruses. The large number of LAT+ neurons were probably the result of the large number of particles present in the inoculum together with limited replication (at least in the DRG) of in1814. This spread was not unique to in1814 but the non-neurovirulent phenotype of the virus meant that the mice survived whereas a similar spread with the virulent 1814R or wt HSV-1 viruses could result in the death of the host.

Methods

Virus stocks. All virus stocks were produced and assayed by titration in BHK cells (clone 13) grown in Eagle's medium with 10% newborn calf serum, 10% tryptose phosphate broth and antibiotics. The wt HSV-1 virus used in these studies was Glasgow strain 17+ (Brown et al., 1973). The insertion mutant of HSV-1, in1814, and its rescued revertant, 1814R, have been described previously (Ace et al., 1989). The mutant in1851 was derived from in1814 and, in addition to lacking a functional Vmw65 protein, has the Escherichia coli β-galactosidase gene controlled by the simian virus 40 early promoter inserted into the thymidine kinase (TK) gene, disrupting its function. Virus particle concentrations were determined by comparison of virus stocks with bead preparations of known concentrations in the electron microscope. The viral stocks were as follows: HSV-I strain 17+, 7 × 10^9 (1.03 × 10^11); 1814R, 5 × 10^9 (1.0 × 10^11); in1814, 3 × 10^8 (4.4 × 10^10); in1851, 4 × 10^7 (8.7 × 10^9). The first value refers to p.f.u./ml; the second in parentheses, particles/ml.

Inoculation of mice. Virus stocks were diluted in PBS containing 10% newborn calf serum and 0.025 ml was inoculated subcutaneously into the right rear footpad of 3 to 4 week-old male BALB/c mice. Mice were handled in accordance with the Animals (Scientific Procedures) Act 1986.

Dissection of DRG. Ten to 12 weeks after the inoculation, mice were killed and DRG immediately dissected out and fixed for 1 h in 4% paraformaldehyde at 4°C. Using the lowest rib to locate thoracic 13 (T13) DRG, the DRG of T12 to L6 were removed. The fixed DRG were then washed in buffer, dehydrated and embedded together in a paraffin block in a pattern such that individual DRG could each be identified (Fig. 1). DRG from the left side were also excised in some mice. Serial 5 µm sections were cut and three sections were put onto
Fig. 2. Structure of the prototype HSV-1 genome including the long terminal repeat (TR), long unique (U), long internal repeat (IR), short internal repeat (IR), short unique (U) and short terminal repeat (TR) regions. The BamHI B restriction fragment is enlarged to show the relative positions of the immediate early 1 (IE1) gene and the major 2 kb LAT. The 2563 kb riboprobe used in this study recognizes the LATs and, in the opposite direction of transcription, also recognizes IE1 mRNA.

Each baked glass slide which had been coated with aminopropyltriethoxysilane (Maddox & Jenkins, 1987) for use in in situ hybridization was then baked for 1 h at 37 °C, acetic anhydride (0.25%) in 0.1 M-triethanolamine for 10 min at room temperature) and dehydrated again before adding the probe. The probe was diluted 10-fold in stock hybridization mix (30% formamide, 10% dextran sulphate, 20 mM-Tris-HCl pH 8.0, 0.3 M-NaCl, 5 mM-EDTA, 10 mM-sodium phosphate buffer pH 8.0, 0.5 mg/ml yeast tRNA and 1 x Denhardt's solution) and heated at 80 °C for 2 min before quenching on ice. Hybridization was at 50 °C for 18 to 21 h. Slides were then washed at 65 °C in 50% formamide in 2 x SSC (0.3 M-NaCl, 0.03 M-trisodium citrate) with 0.1 M-DTT for 20 min, digested with RNase A (20 µg/ml in 0.5 M-NaCl with 10 mM-Tris-HCl pH 7.5 and 5 mM-EDTA) for 30 min at 37 °C, washed in 50% formamide and then washed in decreasing concentrations of SSC before being dehydrated in alcohols containing 0.3 M-ammonium acetate. Dried slides were coated with 11% P.K5 nuclear emulsion and exposed for 6 days at 4 °C before being developed in D19 and fixed in Hypam. Slides were then counterstained with haematoxylin, dehydrated and mounted.

In each in situ hybridization series, coverslips of BHK cells infected for 24 h with wt HSV-1 were included as a positive control and as a control for any experimental variation in the hybridization signals. Sections of mouse DRG which were lytically infected with HSV-1 also acted as positive controls, whereas sections of uninfected DRG with the LAT probe, or infected DRG with the probe transcribed in the opposite sense to LAT, were negative controls. The signal could be removed by prior treatment with RNase. Because the DRG of one side of each animal were all contained in the same block, the level of signal could be compared directly between different DRG as they were all subject to the same variation in section or emulsion thickness, etc. Hybridization backgrounds were optimized to reduce the background grains to the lowest level possible, which was 0 to 10 grains (average of three) per neuronal nucleus.

Preparation of probe. HSV-1 BamHI B was cloned from wt HSV-1 genomic DNA into BamHI-digested pAT153 to give pGX48 (Perry et al., 1986). This was digested with SalI and BamHI generating a 2563 bp fragment which was purified and inserted into a SalI/BamHI-digested pT7T3 19U vector (Pharmacia) to generate pGFM1. This fragment contains the sequence from nucleotides 120902 to 123459 of the HSV-1 genome (Perry & McGeoch, 1988). Their location with respect to the IE1 and LAT genes are shown in Fig. 2. This vector allows selective in vitro transcription of either strand of a cloned DNA fragment using the T7 or T3 promoters flanking the multioligonucleotide site. The pGFM1 was linearized and the RNA transcribed in the presence of [35S]UTP using the appropriate restriction enzyme and polymerase: SalI digestion and T3 polymerase were used to produce a probe for the LATs and BamHI digestion and T7 polymerase were used to transcribe a control (with respect to length and G + C content) probe which was specific for the IE1 mRNA. The non-incorporated nucleotide was removed using Bio-Rad Bio-Gel (100 to 200 mesh) and the probe diluted in 10 mM-DTT to give 1 ng RNA/kb/µl. It was stored at −20 °C and used within 8 weeks of preparation.

Counts and estimates of LAT* neurons. Initially all the LAT* nuclei and all the neuronal nuclei (with or without LATs) were counted in all the serial sections through a DRG. About two-thirds of the neuronal nuclei are split between two consecutive sections and thus summations of total nuclei in all sections of a DRG were corrected by dividing by a factor of 1.66. Such corrected values for the total numbers of LAT* neurons or total numbers of neurons per DRG are presented in Table 1. The percentage of LAT* neurons present in a particular DRG was then estimated by dividing the total number of LAT* nuclei by the total number of neuronal nuclei counted. The data in Table 1 also emphasize that DRG at different levels of the spinal cord contain different numbers of neurons and that the larger a DRG, the more sections are needed to cut through it. It is therefore misleading to express LAT* cells/section or to use an ‘average’ number of neurons of DRG, particularly in the lumbar region. There is also considerable variation in neuronal numbers between the L5 DRG of different animals.

A sampling method was then devised which would give a reproducible value for the percentage of LAT* cells which was within a percentage point of the total counts on serial sections. This was achieved by counting the number of LAT* nuclei and the number of total neuronal nuclei in each of at least 10 sections involving at least

<table>
<thead>
<tr>
<th>Level of DRG</th>
<th>No. neurons per DRG</th>
<th>No. LAT* per DRG</th>
<th>LAT* cells (%)</th>
<th>Total no. sections</th>
<th>LAT* cells per section*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4</td>
<td>4327</td>
<td>260</td>
<td>6</td>
<td>51</td>
<td>5.1</td>
</tr>
<tr>
<td>L5</td>
<td>3304</td>
<td>429</td>
<td>13</td>
<td>34</td>
<td>7.9</td>
</tr>
<tr>
<td>L5</td>
<td>4657</td>
<td>605</td>
<td>13</td>
<td>62</td>
<td>9.8</td>
</tr>
<tr>
<td>L6</td>
<td>2550</td>
<td>76</td>
<td>3</td>
<td>76</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Calculated from data in the table.
Table 2. Clinical summary of mice following footpad inoculation

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titre (particle number)/ml</th>
<th>Proportion showing clinical signs</th>
<th>Death rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt HSV-1</td>
<td>$10^6 (1.5 \times 10^7)$</td>
<td>100% (5/5)</td>
<td>100% (5/5)</td>
</tr>
<tr>
<td></td>
<td>$10^5 (1.5 \times 10^6)$</td>
<td>47% (14/30)</td>
<td>40% (12/30)</td>
</tr>
<tr>
<td></td>
<td>$10^4 (1.5 \times 10^5)$</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td>1814R</td>
<td>$10^6 (2 \times 10^7)$</td>
<td>60% (22/37)</td>
<td>27% (10/37)</td>
</tr>
<tr>
<td></td>
<td>$10^5 (2 \times 10^6)$</td>
<td>31% (12/39)</td>
<td>10% (4/39)</td>
</tr>
<tr>
<td></td>
<td>$10^4 (2 \times 10^5)$</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td></td>
<td>$10^3 (2 \times 10^4)$</td>
<td>0% (0/7)</td>
<td>0% (0/7)</td>
</tr>
<tr>
<td>in1814</td>
<td>$10^6 (1.5 \times 10^7)$</td>
<td>70% (14/20)</td>
<td>10% (1/20)</td>
</tr>
<tr>
<td></td>
<td>$10^5 (1.5 \times 10^6)$</td>
<td>52% (22/42)</td>
<td>17% (7/42)</td>
</tr>
<tr>
<td></td>
<td>$10^4 (1.5 \times 10^5)$</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td></td>
<td>$10^3 (1.5 \times 10^4)$</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td>in1851</td>
<td>$10^6 (2.2 \times 10^9)$</td>
<td>60% (22/37)</td>
<td>27% (10/37)</td>
</tr>
<tr>
<td></td>
<td>$10^5 (1.5 \times 10^8)$</td>
<td>52% (22/42)</td>
<td>17% (7/42)</td>
</tr>
<tr>
<td></td>
<td>$10^4 (1.5 \times 10^7)$</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td></td>
<td>$10^3 (1.5 \times 10^6)$</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
</tr>
</tbody>
</table>

1000 neurons, taken through the central third of each DRG, then expressing the LAT+ nuclei as a percentage of total neuronal nuclei. Data in Tables 3 and 4 were derived by this method. Counts of LAT+ neurons were performed at 200-fold magnification.

Immunocytochemistry and plaque assays of lytically infected DRG.

For immunocytochemistry, DRG were excised, fixed in 4% paraformaldehyde for 1 h at 4 °C, dehydrated and embedded in paraffin. Sections were stained with a rabbit polyclonal antibody to HSV (Dakopatts) and visualized by a peroxidase ABC reaction (Vectastain). For detection of cell-free virus, DRG were homogenized individually in 600 μl of PBS with 10% serum, frozen and thawed three times, sonicated to release any replicating virus, and cell debris was removed by centrifugation. Cell-free 100 μl samples were assayed on BHK cells in the presence of hexamethylene bisacetamide which enhances the plaquing efficiency of in1814 and viruses derived from it (McFarlane et al., 1992), and the number of plaques was counted on duplicate plates.

Results

Clinical effect of viruses on animals

Inoculation of virus into the rear footpad caused clinical symptoms in some mice 5 to 10 days post-infection (p.i.) which consisted of either ipsilateral foot-drop, ipsilateral hindlimb paralysis or bilateral hindlimb paralysis (Table 2). Animals with severe symptoms were sacrificed and recorded as a death. Deaths recorded after inoculation of in1814 were all a consequence of humane actions, but in addition to those put down, a few mice inoculated with wt HSV-1 or 1814R died as a direct consequence of virus infection. Clinical symptoms in other mice were usually resolved within 48 h of onset. If the effects of the three viruses are compared on the basis of equal infectivity both in1814 and 1814R caused less severe clinical symptoms than did wt HSV-1 but only in1814 killed no mice directly. However, on the basis of particle numbers, the effects of in1814 were far less than those of either wild-type or rescued viruses. Thus, although in1814 is apparently neuroinvasive after footpad inoculation of mice, causing extensive clinical signs, it is much less neurovirulent than wild-type or rescued virus.

LAT grain density in serially sectioned DRG

LATs were found in the nuclei of neurons (identified on the basis of morphology, size and location in the DRG) which were distributed evenly throughout the DRG. Rarely, grains were found over small nuclei which may have been non-neuronal cells but which could not be identified unequivocally as such on the grounds of morphology alone, despite the thinness of the sections used. The different density of grains over neuronal nuclei was classified into three groups, illustrated in Fig. 3, as light (25 to 50 grains), medium (between 50 and 120 grains) or dense (those with so many grains as to be uncountable). Observation of serial sections such as those in Fig. 4 showed that most of this variation represented sections through the same nucleus. When sectioning split a nucleus, both sections appeared heavily stained as in neuron 1 in Fig. 4(a, b). If one section contained only a segment of the nucleus, it appeared as light or medium depending on how large a piece of the nucleus was involved. For example, neuron 2 in Fig. 4 is negative in (a), medium in (b) and heavy in (c). Neuron 3 is light in (a), medium in (b) and heavy in (c). However, in some cases where the section clearly cut through a substantial part of the nucleus, there was still only a light density of grains which, in serial sections, did not evolve into a denser pattern. Examples of this can be seen in neuron 4 in Fig. 4(b and c) and in the bottom neuron marked L in Fig. 3. These observations indicate that the level of the LAT signal is actually lower in some neurons than in others. Neurons with a low intensity LAT signal were found at all levels of the DRG and were not restricted to, or characteristic of, any particular virus strain.

LAT+ neurons in DRG of latently infected mice

The number of LAT+ cells was estimated in DRG from mice latently infected with wt HSV-1, 1814R or in1814 and the results, giving the average percentage of
LAT-positive neurons for DRG at each segmental level, are presented in Table 3. For each virus, the percentage of LAT+ neurons increased with increasing doses of virus inoculum. In animals latently infected with wt HSV-1, LAT+ nuclei were confined to the L4/L5/L6 DRG levels (which innervate the lower leg) and involved a maximum of about 3% (range 1.3% to 4.7%) of the neurons in L5 after inoculation with 10^5 p.f.u. Animals inoculated with higher doses of virus were all killed. In mice latently infected with 10^6 p.f.u. of in1814R, maximum levels of about 1.3% (range 0.1% to 2.3%) LAT+ nuclei were found in L5. However, in animals surviving an input of 10^6 p.f.u. 1814R, there was a higher percentage of LAT+ neurons in L5 (10.9%; range 3.4% to 22.0%) and lower numbers of LAT+ neurons were also present in DRG T12 to L3 (which do not directly innervate the lower leg) although the highest levels were still in the L4/L5/L6 region. In mice latently infected with in1814, the highest percentages of LAT+ neurons were in L5 DRG and increased with increasing doses of virus to a maximum of 19.8% (range 13.7% to 32.5%) at 10^6 p.f.u., with LAT+ neurons often being present in DRG T12 to L3 as well as in L4 to L6. Thus, at an equal infectivity, wt HSV-1 established the lowest number of LAT+ neurons and these were confined to DRG in the L4/L5/L6 region. In contrast, infection with in1814 resulted in the establishment of many more LAT+ neurons and these were in

Fig. 3. Section of a DRG, 10 weeks after infection with 10⁴ p.f.u. of in1814, which has been probed for the presence of LATs by in situ hybridization. The range of signal intensity has been classified into three groups: light (L), medium (M) and heavy (H). Note that in the neuron marked L, the nucleus is clearly visible suggesting that the low level of signal here is not due to the section containing only a thin segment of a nucleus. Bar marker represents 50 µm.

Fig. 4. Serial sections of a DRG, 10 weeks after infection with 10⁴ p.f.u. of in1814, which have been probed for the presence of LATs by in situ hybridization. The positions of four selected neurons are marked by arrows. Bar marker represents 50 µm.
Table 3. Average percentage of LAT⁺ neurons in different levels of thoracic or lumbar DRG after different doses of virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>P.f.u.*</th>
<th>No. of animals</th>
<th>Average of LAT⁺ neurons (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T12</td>
</tr>
<tr>
<td>wt HSV-1</td>
<td>10³</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1814R</td>
<td>10³</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁶</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>in1814</td>
<td>10³</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>3</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>7</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>10⁶</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>in1851</td>
<td>10³</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

* P.f.u. injected into the rear footpad of 3- to 4-week-old BALB/c mice.

Table 4. Percentage of LAT⁺ neurons in DRG of the right (ipsilateral to the inoculum) and left (contralateral) side of individual latently infected animals

<table>
<thead>
<tr>
<th>Virus</th>
<th>P.f.u.</th>
<th>Side</th>
<th>LAT⁺ neurons in each DRG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T12</td>
</tr>
<tr>
<td>wt HSV-1</td>
<td>10³</td>
<td>Right</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0</td>
</tr>
<tr>
<td>1814R</td>
<td>10³</td>
<td>Right</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0</td>
</tr>
<tr>
<td>in1814</td>
<td>10³</td>
<td>Right</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0.4</td>
</tr>
<tr>
<td>1814R</td>
<td>10⁴</td>
<td>Right</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>Right</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>10⁶</td>
<td>Right</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0</td>
</tr>
<tr>
<td>in1814</td>
<td>10³</td>
<td>Right</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>Right</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁵</td>
<td>Right</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* ND, Not determined.

all DRG including T12 to L3. Animals latently infected with 1814R had fewer LAT⁺ neurons than in animals infected with in1814 but these were also found in T12 to L3 in animals surviving the high-dose infection.

The high number of LAT⁺ neurons in animals infected with in1814 could be due to particles unable to form a plaque on BHK cells nevertheless establishing LAT⁺ cells. When the number of LAT⁺ neurons in animals latently infected with in1814 and 1814R were compared on the basis of equal particle numbers (e.g. 10⁵ p.f.u. 1814R compared with 10³ p.f.u. in1814, and 10⁶ p.f.u. 1814R compared with 10⁴ p.f.u. in1814), the two viruses were found to have established similar levels of LAT⁺ neurons. A widespread distribution of LAT⁺ neurons was also observed in animals latently infected with either virus at the higher doses. The presence of large numbers of particles in the inoculum might, therefore, be involved in the establishment of such widespread LAT⁺ neurons.

LAT⁺ neurons in DRG contralateral to the inoculation side

We also investigated whether virus could spread to the contralateral, uninoculated, side of mice which had been inoculated with 10⁵ p.f.u. of all three viruses or with 10⁶ p.f.u. of 1814R or in1814 (Table 4). The percentage of LAT⁺ neurons in the contralateral DRG was variable although more common after inoculation of in1814, but
they were not found in all mice investigated. There did not appear to be any specific correlation between the percentage of LAT\(^+\) neurons in the ipsilateral and contralateral sides at a particular level of DRG.

The spread of in1814 to levels of DRG other than those directly innervating the footpad was unexpected since replication had not been detected in the eye or trigeminal ganglion after ocular inoculation with the virus (Steiner et al., 1990). To determine whether replication occurred in DRG, we used two approaches. First, we examined the distribution of LAT\(^+\) neurons after inoculation of a virus derived from in1814, which had a similar particle:p.f.u. ratio, but which also lacked a functional TK gene (necessary for replication of HSV in neurons). Second, we investigated directly whether there was virus replication in the DRG during the establishment of latency after footpad inoculation.

**LAT\(^+\) neurons in DRG of mice latently infected with TK virus**

The mutant in1851 has an insertion in the TK gene and a particle:p.f.u. ratio (2175:1) which is slightly higher than that of in1814 (1470:1). We could thus determine whether high particle numbers in the absence of the ability to replicate in neurons resulted in high numbers and widespread distribution of LAT\(^+\) neurons along the spinal ganglia.

Ten animals were inoculated in the rear footpad as usual. None showed any clinical signs at any time (Table 2). Six weeks later the mice were killed and the DRG of four investigated for the presence of LAT\(^+\) cells in different levels of DRG (Table 3). LAT\(^+\) neurons were found in DRG outside the region of L4/L5/L6 in only one of the four mice, and the average percentage of LAT\(^+\) neurons in L5 was lower after 10\(^8\) p.f.u. of in1851 (2.2 x 10\(^9\) particles) than after 10\(^9\) p.f.u. of in1814 (1.5 x 10\(^8\) particles). This observation suggests that the spread of virus to establish LAT\(^+\) neurons in DRG of different levels in animals infected with in1814 was not solely attributable to the high numbers of particles present in the inoculum, but may have involved virus replication in the DRG.

**Virus replication in DRG L5**

L5 DRG were excised from animals 4 days after footpad inoculation with 10\(^8\) p.f.u. of either in1814 or wt HSV-1 and were processed for immunocytochemistry or were homogenized individually and assayed in BHK cells to detect cell-free virus. In each of three animals infected with in1814 we found evidence of virus antigen in about 10 foci per section of usually single cells. In L5 DRG infected with wt HSV-1, virus antigen was found in more than 35 foci per section, most of which were single cells but with a few foci containing small groups of cells.

Cell-free virus was detected in L5 DRG from each of three animals 4 days after inoculation with 10\(^8\) p.f.u. of in1814 or wt HSV-1. Levels of 12, 81 and 138 p.f.u. per DRG were found for in1814-infected mice and there were 15, 204 or 1032 p.f.u. per DRG for wt HSV-1. Thus in1814 did express virus antigens and produce infectious virus in mouse DRG following footpad inoculation.

**Discussion**

We have shown that after inoculation via the footpad, in1814 establishes many more LAT\(^+\) neurons than are found after infection with equal amounts of infectious wt HSV-1 or 1814R. After inoculation of 10\(^8\) p.f.u. maximum levels of LAT\(^+\) neurons in L5 were about 3% for wt HSV-1, about 1:3% for 1814R and about 9:3% for in1814. In animals surviving inoculation with 10\(^8\) p.f.u. of 1814R, maximum levels of LAT\(^+\) neurons in L5 rose to 10-9% but in animals infected with in1814, the rise was even higher, to an average of 19-8% of the neurons in L5. Since L5 DRG contain about 4000 neurons, based on the figures for the average number of LAT\(^+\) neurons presented in Table 3, as many as 792 neurons were latently infected after inoculation of 10\(^6\) p.f.u. of in1814, compared to a maximum of about 436 in mice with 10\(^8\) p.f.u. of 1814R or about 120 LAT\(^+\) neurons in mice with 10\(^8\) p.f.u. of wt HSV-1.

It was surprising that a virus potentially unable to replicate could establish larger numbers of LAT\(^+\) neurons in animals than mutant or wild-type viruses used at a similar input level of infectious virus. However, when the percentages were compared on a particle basis, the viruses all produced similar levels of LAT\(^+\) neurons in the L5 DRG. Since replication is not necessary to establish latent infection (Coen et al., 1989; Tenser et al., 1989; Leib et al., 1989b; Valyi-Nagy et al., 1991) many of the particles that are unable to form a plaque on BHK cells may be able to establish latent infections. However, the mutant in 1851, which also had a high particle:p.f.u. ratio but which should have been unable to replicate in neurons, established fewer LAT\(^+\) neurons than in1814, suggesting that increased particle number does contribute to, but may not entirely account for, the increased number of LAT\(^+\) cells.

The second striking characteristic of DRG latently infected with in1814 was that, at doses about 10\(^4\) p.f.u. LAT\(^+\) neurons were found in DRG both within and outside the region of innervation of the lower leg on the ipsilateral and, to a limited extent, also on the contralateral side. In animals latently infected with wt HSV-1 or 1814R, LAT\(^+\) neurons were generally confined to the L4/L5/L6 DRG of the ipsilateral side. This distribution
infection could result in involvement of the central nervous system, but this produced clinical signs unlike those found in the present study. In fact, the clinical signs described here most closely resemble their description of mice after direct inoculation of the sciatic nerve, and probably suggest that if haematogenous spread did occur, it was not significant.

The second possibility is that spread of input virus was by trans-synaptic transmission, shown to occur when HSV strain SC16 was inoculated directly into peripheral nerves (Ugolini et al., 1989). In the current study the spread of in1814 presumably also involved such trans-synaptic transmission and several routes are available. Following footpad inoculation, virus particles could enter sensory axons and be taken directly via the DRG into the spinal cord where they could be transmitted to interneurons and thereby travel vertically in the cord to other levels of DRG. Alternatively, virus particles could enter primary afferent fibres which go directly to the cord and travel several segments before synapsing on ascending or reflex arc pathways, which again involve DRG.

The third possibility is that in1814 underwent replication in the DRG and that spread was via progeny particles. We detected limited replication in the DRG and the resultant sensory loss probably accounts for the transient paralysis from which the mice recovered within 48 h. The severe paralysis in some animals suggests that neurons, perhaps anterior horn cells, had been killed either as a direct result of virus replication or indirectly via a possibly limited inflammatory response to virus replication.

We therefore conclude that the high particle number in in1814 results in the establishment of a large number of LAT+ neurones, and that by limited replication and intra-axonal transport, and or trans-synaptic transmission of input particles and progeny virus, LAT+ neurones become established outside the DRG directly innervating the lower leg. The non-neurovirulent phenotype of in1814 (Ace et al., 1989; Steiner et al., 1990; results presented in this paper) means that such spread does not result in the death of the host. Although a similar spread almost certainly occurs in mice infected with wt HSV-1 or 1814R, the neurovirulent phenotype of these viruses results in death of the infected neurons, often leading to death of the animal. Since we were able to investigate the distribution of LAT+ neurones in only those animals which survive infection, we were probably looking at a selected subset of animals in which extensive spread did not occur after infection with wt HSV-1 or 1814R. In contrast, the non-neurovirulent phenotype of in1814 results in greater survival of animals despite extensive spread of the virus which can be determined by the expression of LATs. Even in animals which survive inoculation with wild-type virus, there could be spread of virus which we cannot detect because it kills the cells concerned and therefore fails to establish a latent infection which we can detect by the presence of LATs.

The significance of lower levels of LATs in a small proportion of the total population of LAT+ neuron is unclear. HSV is not thought to reactivate spontaneously in BALB/c mice, so they are unlikely to represent cells in which reactivation from latency has occurred. They were found at all levels of DRG and were present after infection with either of the three viruses. They may represent a natural variation in LAT gene expression but whether this variation occurs with time or is a characteristic of particular neurons is not clear from our results.

When in1814 was inoculated onto mouse cornea, lytic replication was undetectable (Steiner et al., 1990) although subsequently a low level of viral transcription and DNA synthesis was found (Valyi-Nagi et al., 1991). Accumulation of LATs was also greater in trigeminal ganglia infected with wt HSV-1 than with in1814. In contrast, not only did we find limited replication of in1814 in the DRG but, on the basis of equal infectivity in the inoculum, we found larger numbers of LAT+ neurones in the DRG infected with in1814 than after infection with wt HSV-1. We would therefore agree with others that the site of inoculation or the type of neuron infected may be an important influence on the outcome of infection by the same viruses in similar animals (Sawtell & Thompson, 1992; Rodahl & Stevens, 1992).

Speck & Simmons (1991) also investigated the distribution of LAT+ neurones in thoracic and lumbar ganglia following inoculation of HSV-1 strain SC16 onto mouse flanks. They found that LAT+ neurones were not only in thoracic DRG T9, T10 and T11 (those innervating the site of inoculation) but also from T6 to L1. Their results and ours suggest that LAT+ neurones can be found in DRG outside the immediate levels that innervate the site of the inoculum. Whereas we used a non-neurovirulent virus (in1814) in a susceptible mouse strain (BALB/c), Speck & Simmons (1991) used a virulent strain of virus (SC16) in a resistant mouse strain (C57BL/6). In either combination, the ability of the virus to kill the host is reduced and widespread distribution of LAT+ neurones can be demonstrated in surviving animals. However, not all non-neurovirulent virus strains, for example the TK- in 1851 described in this report, appear
able to establish LAT+ neurons in such widespread DRG, suggesting that LAT gene expression may depend on the particular virus–host cell interaction.

In contrast to our results, however, Simmons et al. (1992) showed that the percentage of LAT+ neurons was least in the DRGs that innervated the site of inoculation, where virus replication and DNA levels had been shown to be greatest. We found that the highest levels of LAT+ neurons were generally in the L4/L5/L6 DRG which innervate the footpad. This apparent discrepancy may be a consequence of another difference between the two models of latency. When the flank is inoculated in the T9 dermatome, virus travels down sensory axons to T9 (with some spread to T8 and T10 DRG) where it replicates and possibly establishes latency. Virus then re-emerges 5 days later to for a zosteriform rash over the lateral sides of the mouse after footpad inoculation. This could be that virus from the zosteriform rash causes a depletion in the neurons of T9 DRG which harbour latent virus, whereas neurons in adjacent dermatomes are less affected. In the footpad model, virus is inoculated intradermally causing an initial redness of the footpad but with no evidence of lesions in the periphery, suggesting that subsequent reactivation in the DRG did not occur.

In conclusion, when compared to inocula of equal numbers of p.f.u. of wt HSV-1 or 1814R, in1814 establishes a greater number of LAT+ neurons in more widespread DRG on both the inoculated and contralateral sides of the mouse after footpad inoculation. The large number of LAT+ neurons are probably a reflection of the high particle numbers present in the inoculum and their spread is enhanced by limited replication. This spread is not unique to in1814, but the non-neurovirulent phenotype of the virus means that the mice survive whereas a similar spread with more virulent viruses (wt HSV-1 and 1814R) may result in death.

We would like to thank Professor John Subak-Sharpe for his continued interest and support, and Mr Jim Aitken for counting the particle numbers in the electron microscope. This work was supported by grants to M.S.E-P. and P.G.E.K. from The Wellcome Trust, The BUPA Medical Foundation and Bayer UK.

References


Leib, D. A., Coen, D. M., Bogard, C. L., Chicks, K. A., Yager,


(Received 11 September 1992; Accepted 25 January 1993)