Synchronous appearance of antigen-positive and latently infected neurons in spinal ganglia of mice infected with a virulent strain of herpes simplex virus

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Studies with replication-defective mutants of herpes simplex virus (HSV) have defined the minimum requirements for establishment of latency, but their behaviour may not reflect the course of events following infection by wild-type HSV, in which ability to express viral genes has not been precluded by a genetic lesion. To address this issue we devised a strategy for studying establishment of latency by a virulent strain of HSV, based on the distinctive molecular characteristics of latently infected neurons. By combining in situ hybridization for detection of latency-associated transcripts with immunohistochemical analysis of viral proteins we demonstrate here that antigen-positive and latently infected neurons appear synchronously in spinal ganglia during the earliest stages of acute ganglionic infection. This is consistent with early divergence of the molecular pathways leading to productive and latent infection, supporting and extending the results obtained with viral mutants.

During primary infection, herpes simplex virus (HSV) invades the peripheral nervous system (PNS) where it is sequestered in neurons in a non-replicating latent state for the life of the host (Stevens, 1989). Latent infection conveys an enormous survival advantage to the virus, yet the molecular events that determine whether a neuron becomes productively or latently infected are poorly understood. Establishment of latency is concurrent with the productive phase of primary HSV infection in sensory ganglia (Stevens, 1989), which complicates the approach to a key question regarding latent infection, namely whether viral genes are expressed during the establishment phase. Replication-defective mutant viruses have been studied intensively in attempts to overcome this problem.

Three major classes of HSV genes are recognized: immediate early, early and late, based on the tightly regulated sequence in which they are expressed during productive infection (Roizman & Sears, 1990). All classes include genes that are required for virus replication in cell culture, and mutation or deletion of these essential genes is a strategy frequently used to interrupt the infectious cycle at a defined stage. Numerous replication-defective viruses (Clements & Stow, 1989; Coen et al., 1989; Dobson et al., 1990; Katz et al., 1990; Kosz-Vnenchak, 1990; Leib et al., 1989; Leist et al., 1989; Steiner et al., 1990; Tenser et al., 1989) have been shown to establish latency as defined by the presence of latency-associated transcripts (LATs) in situ, or by that of rescuable DNA, or by their ability to be reactivated in cultures of explanted tissue. Of particular relevance are those with lesions in Vmw65 (VP16) (Steiner et al., 1990) or in immediate early genes (Clements & Stow, 1989; Dobson et al., 1990; Katz et al., 1990; Leib et al., 1989), because these suggest that initiation of the molecular pathways associated with productive infection is not essential for persistence of the HSV genome. However, studying the pathogenesis of herpes simplex by means of mutated viruses creates several interpretative difficulties. First, these experiments do not show unequivocally that wild-type viruses, which are not compromised in their ability to express immediate early genes, are also able to follow a pathway to latency which diverges from that of productive infection at such an early stage. Second, the defect may be manifested at other steps in the experimental system employed, for example in replication at the site of inoculation or in cells used in reactivation studies. Hence, although studies of mutants may delineate the minimum requirements for persistence of the genome, they might not reflect the natural course of events following infection by wild-type viruses.

In addressing this issue, we recently showed that, after introducing wild-type HSV-1 into the flank of mice, viral antigen expression during the acute phase of infection is restricted to fewer spinal segments of the PNS than subsequent latency, suggesting that antigen-positive cells are not essential precursors of latently infected neurons.
molecular characteristics of latently infected neurons. None of the viral proteins associated with lytic infection are expressed during latency but neurons harbouring HSV contain abundant nuclear LATs, of unknown function, derived from a limited region of the viral genome (Stevens et al., 1987). For the purpose of this study latently infected neurons were defined as those cells containing LATs in the absence of detectable viral proteins (Ag-, LAT+). We reasoned that if there is no precursor relationship between Ag+ and Ag-LAT+ cells then, given sufficient sensitivity of the in situ hybridization (ISH) procedure, both would be detectable in the PNS in the earliest stages of ganglionic infection. Using a dual labelling procedure for simultaneous detection of viral antigens and LATs we report here synchronous appearance of Ag+ and Ag-LAT+ neurons in spinal ganglia 3 days after inoculation of mouse flanks. This is consistent with early divergence of the molecular pathways leading to productive and latent infection, supporting and extending our previous observations and data obtained with viral mutants.

Female BALB/c mice (obtained from the Specific Pathogen Free Facilities, Gilles Plains Animal Resource Centre, Adelaide, South Australia) were infected at greater than 8 weeks of age with a well characterized strain of HSV-1, designated SC16 (Hill et al., 1975). The master stock of this virus has not been passaged extensively in vitro and has been shown to be virulent in mice by various routes of inoculation (Harbour et al., 1981; Simmons & Nash, 1984). Virus was grown and titrated in Vero cells, stored at −70 °C until required and mice were infected on the skin of the left flank (day 0) as previously described (Simmons & Nash, 1984; Speck & Simmons, 1991). Briefly, after depilation with Nair (Carter Wallace, French’s Forest, New South Wales, Australia), a small patch of skin defined by the sphen tip and corresponding to the tenth thoracic dermatome was scarified with a 27-gauge needle through a 10 μl drop of virus suspension containing 3 × 10^4 p.f.u. of SC16. On days 2 to 5 and on day 45 after infection, the left sensory dorsal root ganglia (T9 to T13) were removed from groups of 10 mice, fixed immediately in periodate-lysine-paraformaldehyde (McLean & Nakane, 1974) for 60 min and transferred to 50% ethanol. Daily samples were pooled, paraffin-embedded and 5 μm sections were collected onto glutaraldehyde-activated 3-aminopropyltriethoxysilane-coated slides. Viral antigens were detected using rabbit antiserum to HSV-infected cells, and binding of primary antiserum was detected using swine anti-rabbit Ig, followed by rabbit peroxidase anti-peroxidase conjugate (all from Dakopatts). Reactions were allowed to proceed for 30 min at 37 °C with a 10 min wash in 50 mM-Tris-HCl pH 7.5 between each step and bound antibody was detected using 3,3′-diaminobenzidine (0.5 mg/ml, containing 0.1% H2O2). Using this method we have previously shown that detection of viral proteins is of similar sensitivity to detection of their mRNAs by ISH (Speck & Simmons, 1991). For dual staining, tissues were transferred after peroxidase staining to non-ionic detergent (0.08% Triton X-100 for 10 min at room temperature) to facilitate probe access (Gowans et al., 1989). Diaminobenzidine, the substrate for the immunoperoxidase reaction, is insoluble in water and ethanol and is resistant to the ISH procedure (Gendelman et al., 1985; Gowans et al., 1989). The subsequent ISH procedure using 125I-labelled riboprobes opposite in sense to LATs has been described previously (Speck & Simmons, 1991). Briefly, riboprobes were generated from plasmid pBSO (a gift from S. Efstathiou, Cambridge University, U.K.), which consists of a 2557 bp BamHI-SalI fragment of HSV-1 strain KOS spanning map units 0.79 to 0.807, cloned into Bluescribe M13− (Stratagene Cloning Systems). Templates were linearized prior to transcription and probes were labelled to a specific activity of 5 × 10^6 d.p.m./μg using [125I]CTP (NEN) under reaction conditions generating an average size of around 1.5 kb. Tissue sections were hybridized overnight at Tm −25 °C with 1.6 × 10^2 pg of riboprobe/section and unbound probe was removed by three sequential 2 h washes of increasing stringency, up to Tm −10 °C. Bound probe was detected autoradiographically using LM-1 emulsion (Amersham) using exposure times of 2 to 3 days after which sections were lightly counterstained with rapid haematoxylin. For estimation of signal intensity, grains in photographic emulsion were counted under oil (×1200). Grain counts were used to calculate the number of target sequences present using the method of Gowans et al. (1989). The mean grain count over sensory neurons (nucleus and cytoplasm) in uninfected tissue was 30 and cells with >100 grains were considered unequivocally LAT+, placing the theoretical lower limit of LAT detection at 130 copies/cell.

A large number of randomly selected ganglionic sections (ranging from 180 on day 2 down to 47 on day 5) was used for enumeration of total cell profiles with the characteristic appearance of primary sensory neurons. In addition Ag+ LAT−, Ag+ LAT+ and Ag− LAT+ neuronal profiles were enumerated separately (Table 1). On day 2 neither viral antigens nor LATs were detected. On day 3, Ag+LAT− and Ag−LAT+ neurons (Fig. 1a, b) were observed, although Ag and LAT signals were not mutually exclusive. Approximately 11% of Ag+ cells also contained LATs (Fig. 1c and Table 1). The presence of Ag+ LAT+ neurons demonstrates that the probe used for ISH was able to penetrate immunohistochemically stained cells and is consistent with the observation that
productively infected cultured cells express LATs at high m.o.i. (Spivack & Fraser, 1988a). All three types of infected cell (Ag+LAT+, Ag+LAT− and Ag−LAT+) increased in number during the course of acute infection.

The intensity of LAT-specific autoradiographic signals on days 3 to 5 (typified in Fig. 1b) was at least an order of magnitude less than that typically observed in latently infected neurons 45 days after infection (Fig. 2). We estimate that on days 3 to 5 Ag−LAT+ neurons each contain up to $10^3$ viral transcripts, in contrast to approximately $10^4$ copies of LAT commonly detected on day 45.

This study relied on a series of snapshots of events taken at daily intervals, because it is not possible to follow individual neurons through a time course. The apparently synchronous appearance of Ag+ and latently infected cells between 48 and 72 h after infection suggests that the molecular pathways leading to latency and productive infection may diverge at an early stage, even when HSV gene expression has not been precluded by a lesion in the viral genome. However, on the basis of daily sampling, the possibility cannot be excluded that Ag−LAT+ cells were generated by a transient burst of gene expression that went undetected because it fell, in all animals tested, between days 2 and 3.

There are three possible reasons for the observed increase in the number of Ag−LAT+ neurons between the onset and peak of acute infection. First, this increase parallels the rising number of Ag+ cells and may reflect the generation, by viral spread, of new sites of latency. Second, temporal accumulation of LATs (Spivack & Fraser, 1988b) may render latently infected neurons, present from the outset, more readily detectable as time progresses. Finally, although it now seems certain based on studies with mutant and virulent strains of HSV that production of viral polypeptides is not essential for establishment of latency, it does not follow that expression of viral genes necessarily precludes subsequent entry into a latent form of infection. Indeed the presence of Ag−LAT+ neurons during the establishment phase supports the possibility of an additional pathway leading to persistence of viral genomes. In this respect

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<th>Time after infection (days)</th>
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**Fig. 1.** Analysis of the establishment of HSV latency by combined immunohistochemical detection of viral antigen and ISH for LATs. (a) Photomicrograph showing several Ag+ neurons (brown areas) and a single Ag−LAT+ neuron (dense collection of grains) in a spinal ganglion 3 days after inoculation of flank skin. Bar marker represents 10 μm. (b) Photomicrograph showing different outcomes of infection in adjacent neurons. The collection of silver grains (approx. 350) over the LAT+ neuron (arrow) represents approximately $10^3$ copies of LAT. The number of grains over surrounding cells (20 to 30/cell) typifies the level of background associated with uninfected material. Bar marker represents 5 μm. (c) High power photomicrograph of an Ag+LAT+ neuron. Bar marker represents 5 μm.
the strikingly large amount of viral DNA that can be recovered from latently infected tissue merits discussion. Neither our observations with virulent HSV-1, nor those showing that replication-defective mutants can establish latency, provide a straightforward explanation for the presence of several hundred copies of the viral genome for each latently infected neuron (Rock & Fraser, 1983; Efstathiou et al., 1986). Whether HSV genomes are amplified by cellular factors, as suggested (Sears & Roizman, 1990), or by viral mechanisms (or both) remains to be shown. Further dissection of viral gene activity during the establishment phase using combined ISH and immunohistochemistry, as described here, may provide an initial approach to these questions.

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References


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