Evidence that Neurons Harbor Latent Herpes Simplex Virus

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The cell type(s) harboring latent herpes simplex virus in the spinal ganglia of mice was investigated. Taken together, results from immunofluorescence, electron microscopic, autoradiographic, and in situ nucleic acid hybridization methods suggest strongly that, in mice, latent virus is maintained in neurons.

Herpes simplex virus (HSV) is now known to induce latent infections in the peripheral and central nervous systems of experimental animals (10, 17, 20) and in the peripheral nervous system of humans (1, 2). The basis for this unique virus-tissue interaction is not understood, and a complete definition is of obvious importance to understanding the natural history of herpetic disease (5). To define further the phenomenon of latency, we have attempted to determine the cell type(s) involved in harboring latent virus. In this communication, we present evidence that, in murine sacrosciatic spinal ganglia, latent virus is maintained in neurons.

MATERIALS AND METHODS

Virus. The prototype HSV-1 strain (MacIntyre) used in these experiments was described earlier (4). Virus which had been serially passed 17 to 23 times in mouse brains was used for mouse inoculation, with a typical pool possessing a titer of 4×10^4 RK_{13} cell plaque-forming units (PFU) per ml.

Mice. Four-week-old outbred or inbred Swiss mice were used in all experiments; no differences in response were detected between them. Methods employed for infection of mice, dissection of tissues, and characterization of the clinical course of disease have all been presented earlier (4). In brief, infection was established by rubbing a drop of the virus inoculum on scarified and edematous foodpads of mice. Those mice that subsequently become paralyzed and later recovered were used as donors for latently infected spinal ganglia.

Maintenance of explanted, latently infected ganglia. Initially, attempts were made to employ ganglia which had been co-cultivated in vitro with monolayer cultures of RK_{13} cells, a method which results in the reactivation of virus from latently infected ganglia (17). We soon found that these ganglia were poorly preserved and failed to demonstrate virus-specific antigens when processed by immunofluorescence methods. In an attempt to provide a less artificial environment for these explants, the ganglia were enclosed in Millipore chambers (pore

size, 0.22 μm; Millipore Corp., Bedford, Mass.) and transplanted to the peritoneal cavity of uninfected mice. Viral antigens induced in ganglia maintained at this site were readily and reproducibly stained by immunofluorescence techniques.

Immunofluorescence techniques. Latently infected ganglia maintained in Millipore chambers were removed at various times, and frozen sections (6 μm) were cut on a cryostat. After fixation in acetone, the sections were stained with an antiviral antiserum by indirect immunofluorescence methods (4).

Electron microscopy. Latently infected ganglia to be examined by electron microscopy were also maintained in Millipore chambers. After an appropriate incubation time, ganglia were collected in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.3. They were postfixed in 1% osmic acid in the same buffer and further processed for microscopy (3).

Autoradiography. Uninfected or latently infected spinal ganglia were incubated in Leighton tubes for various times in medium containing 10 μCi of [³H]thymidine per ml (specific activity, 20 Ci/mmol). After appropriate labeling times, the ganglia were removed, and 6-μm frozen sections were cut and picked up on gelatinized glass slides (6). After fixation in cold methanol (4 h to overnight), the specimens were dried and then dipped in Kodak nuclear track emulsion (NTB-2) which had been diluted 1:1 with distilled water and brought to 43 C. Then the sections were drained, dried, and maintained for a 1-week exposure at –20 C. At that time, the sections were developed (Kodak D-19 developer), fixed, stained with buffered Giemsa, and examined microscopically.

In situ nucleic acid hybridization. For in situ nucleic acid hybridization experiments, herpesvirus deoxyribonucleic acid (DNA) was purified and used to prepare radioactive complementary ribonucleic acid (RNA), which was subsequently applied to sections of ganglia. To expand, herpesvirus DNA was extracted from synchronously infected RK_{13} cells 20 h after infection by use of the methods described by Saxton and Stevens (15). After extraction, viral DNA was purified by two CsCl density equilibrium centrifugations, with [³H]thymidine-labeled RK_{13} cell DNA used as a density marker. After the second centrifugation, the single peak of 260-nm absorbing material collected between densities 1.704 and 1.730 g/cm³

(well separated from the marker) was considered to be viral DNA. This DNA was used to prepare radioactive viral complementary RNA (cRNA), with [³H]-uridine triphosphate ([³H]UTP) used as the label (11). Based on the specific activity of the [³H]UTP, and the proportion of thymidine in HSV DNA, the specific activity of the cRNA was calculated to be 3.0×10^6 counts per min per μg. Viral specificity of the cRNA was established autoradiographically. Here, by the methods described below, it was shown to bind selectively in high concentration to isolated cells in sections prepared from sacrosciatic ganglia taken from mice infected with HSV in the ipsilateral footpad 4 days earlier. As shown previously (4), HSV is present at a high concentration in these ganglia. Ganglia from noninfected animals displayed only uniform background binding of this cRNA.

Latently infected ganglia to be used for in situ hybridization studies were incubated for 24, 48, or 63 h either in Leighton tubes in vitro or in Millipore chambers in vivo. After incubation, 6-μm frozen sections of the ganglia were cut, picked up on gelatinized slides, and processed by slight variations of methods described by others (6, 8). In our experiments, sections were fixed in chilled methanol-acetic acid (3:1) for 15 min, dipped into 95% ethanol, dried, and dipped twice in 0.4% agarose at 60 C. Then the agarose was allowed to "soft set" and the DNA was denatured by treatment with 0.07 N NaOH at 37 C (4 min). This was followed by rinses in 70 and 95% ethanol. After drying, the labeled viral cRNA (3.7×10^4 counts/min in 0.1 ml of 6 $\times$ standard saline citrate [SSC]) was added to each slide and the hybridization was carried out at 66 C for 22 h. After rinses in 6 $\times$ [SSC]) was added to each slide and the hybridization μg of pancreatic ribonuclease per ml in 2 $\times$ SSC. This was followed by two rinses in 6 $\times$ SSC, two rinses in 70% ethanol, and two rinses in 95% ethanol. Finally, the specimens were air-dried, filmed for autoradiography, exposed for 3 or 4 weeks, developed, stained, and examined as described in the preceding section.

RESULTS

Background. For an adequate orientation, it should be re-emphasized that the spinal ganglia used in all experiments were obtained from mice which had been previously infected in a rear footpad with HSV, had become paralyzed, and then had recovered from clinically apparent disease. In addition, as we discussed earlier (18), at the time of explantation, no virus or virus-specific products could be found in ganglia. However, infectious HSV is "reactivated" when these ganglia (but not associated sciatic nerves) are maintained either in organ culture or in Millipore chambers placed in vivo. These "reactivating ganglia" were used in all of the experiments which follow.

Anatomic experiments. The first argument which indicates that neurons harbor latent virus relates to the differing cell types in ganglia and nerves. Of the cell types present in the ganglia,

satellite cells surrounding neurons are generally accepted to be very similar, if not identical, to the nonmyelinating Schwann cells which are present in nerves as well as ganglia (13). Thus, the only cell types present in ganglia which are not also present in the sciatic nerves are neurons. Virus passes through the sciatic nerves to the ganglia after peripheral infection (4) and thus has an opportunity to establish a latent infection in the nerves. However, when sciatic nerves from mice with latent infections are maintained in vitro, no infectious virus can be induced (17). However, as is shown in Table 1, sciatic nerves from uninfected mice are capable of replicating virus when they are infected and maintained in vitro. In addition, ultrastructural evidence of viral replication was seen in a variety of cell types (including nonmyelinating Schwann cells) in such specimens. From these results, it can be concluded that the nerve from latently infected animals should have supported viral replication if virus had been there. Since the nerve, which contains all of the cell types present in ganglia except the neuronal soma, appears not to harbor latent virus, while latent virus is present in the ganglion, the neuronal soma appears to be the cell involved in harboring latent virus.

Immunofluorescence experiments. Immunofluorescence techniques were used to determine the cell type in which the first virus-specific antigens appeared when virus was being reactivated from latently infected ganglia. These ganglia were maintained in Millipore chambers in vivo and were processed for immunofluorescence 48 h after transplantation. A

TABLE 1. *Replication of HSV in murine sciatic nerves infected and maintained in vitro*[a]

| Time after infection (days) | Virus titer (PFU/ml) | | | |
| | Expt 1 | | Expt 2 | |
	Sample 1	Sample 2	Sample 1	Sample 2
0[b]	245	100	565	65
1	95	5	280	65
2	560	45	1,555	310
3	370	180	2,000	625
4	ND[c]	100	1,070	265

[a] For each sample, sciatic nerves from four mice were removed and infected for 0.5 h with 3×10^6 PFU of HSV contained in one or two drops of cell culture media (4). After extensive washing, the samples were incubated at 37 C on collagen-coated cover slips (21). Supernatant fluids from these cultures were assayed for virus at the time intervals noted.
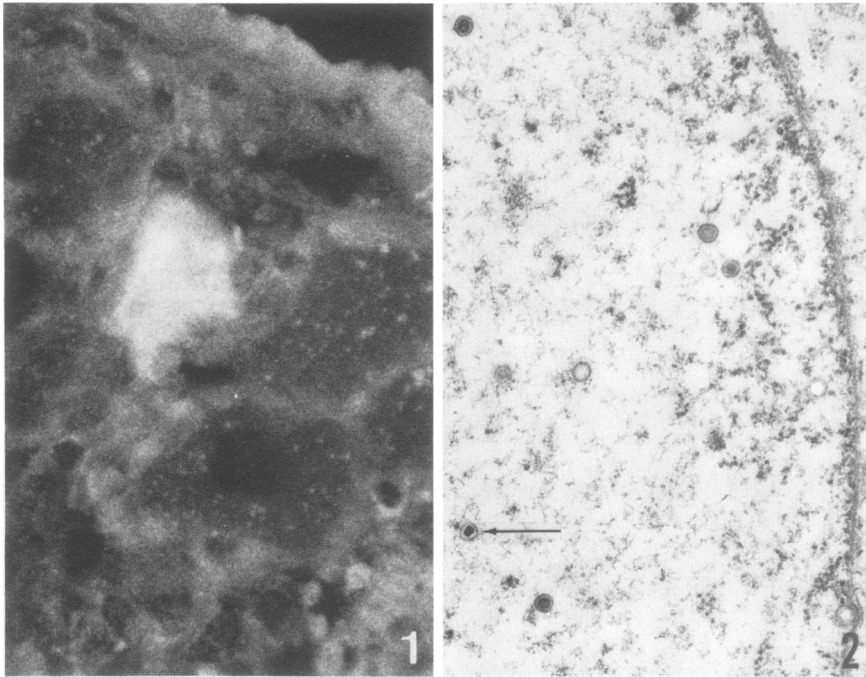
[b] Zero time = 1 h after wash.

[c] ND, Not done.

FIG. 1. *Light micrograph of a latently infected spinal ganglion processed after 48 h of incubation in a Millipore chamber maintained in vivo. The ganglion was sectioned on a cryostat and stained by the indirect immunofluorescence method for herpesvirus-specific antigens. One positively stained neuron surrounded by several unstained neurons and supporting cells can be seen. ×740.*

FIG. 2. *Electron micrograph of herpes simplex virus capsids and a nucleocapsid (arrow) in a neuronal nucleus from a latently infected spinal ganglion processed after 48 h of incubation in a Millipore chamber maintained in vivo. ×25,000.*

typical result (Fig. 1) demonstrates that antigens are induced initially in neurons, suggesting again that latent virus is associated with neurons.

Ultrastructural experiments. In an extension of the previous experiment, ultrastructural, rather than immunofluorescence, methods were employed. As in the immunofluorescence experiments, ganglia were maintained in Millipore chambers before they were processed for electron microscopy. Here, as there, the first virus-specific effects were seen in neurons. An example (Fig. 2) depicts a neuronal nucleus containing capsids and a nucleocapsid. At this time (48 h after transplantation), very few mature virions were seen in neuronal cytoplasm and no viral products of any type were seen in any other cell type. However, at later times, cells surrounding neurons became infected.

We attempted to extend these observations by examining ganglia allowed to reactivate in vitro in the presence of rabbit-anti-HSV antibody (2 times the concentration of neutralizing antibody present in latently infected mice). When such cells were examined early during reactivation, viral particles were restricted to the neuron.

Autoradiographic experiments. Although the previous experiments show that viral antigens and morphological particles first appear in neurons, they do not rule out the possibility that infectious (DNA-containing), subviral particles could be passing from an adjacent cell to the neuron. Therefore, by autoradiographic methods, the synthesis of viral DNA in reactivating ganglia was studied. Here, we looked for selective incorporation of radioactive thymidine into the nuclei of isolated neurons (which do not normally replicate DNA [13]). A portion of a ganglion maintained in vitro in the presence of 10 μCi of [^{3}H]thymidine per ml for 24 h is shown in Fig. 3. Very dense labeling of a neuronal nucleus can be seen, whereas label in supporting cells is not above background levels. In these sections, an occasional supporting cell did incorporate label, but only to a degree comparable to uninfected ganglia. No neurons were ever seen to be labeled when ganglia taken from nonlatently infected mice were processed identically.

In additional experiments, radioactive label was "chased" from the nucleus to the periphery of the neuron and to surrounding cells. Here, ganglia undergoing reactivation were labeled for 24 h and the medium was then replaced with one containing 100 × unlabeled thymidine for an additional 12 h. The result of one such experiment is shown in Fig. 4. Here, most of the label has moved peripherally in the neuron and into satellite cells surrounding it.

From the two experiments, it is clear that isolated neurons incorporating radioactive thymidine can be found in ganglia when herpes simplex virus is being "reactivated." In addition, the radioactive label can be "chased" peripherally from the neuronal nucleus to supporting cells.

To establish that the radioactive thymidine seen in these autoradiographic experiments was being incorporated into herpesvirus DNA, we employed in situ nucleic acid hybridization techniques. Latently infected ganglia were incubated for 24, 48, or 63 h in Leighton tubes in vitro or in Millipore chambers in vivo. After sectioning, the tissues were processed as described in Materials and Methods. The radioac-

tive cRNA label is restricted to a neuronal nucleus, thus demonstrating the presence of HSV DNA (Fig. 5). In these experiments, no amount of RNA above background was bound to sections prepared from uninfected ganglia.

DISCUSSION

Taken together, the indirect experiments presented here permit the tentative conclusion that latent herpes simplex virus is selectively harbored in the neurons of murine spinal ganglia. Although these data constitute strong support for this conclusion, we recognize that rigorous direct proof demands that cellular components of dorsal root ganglia be separated and that virus be shown to be limited to the neuronal fraction. However, despite the use of a variety of techniques, we have not been able to achieve such a separation. The other direct approach to the problem (which is currently being investigated) would employ in situ nucleic acid hybridization techniques on latently infected but nonreactivating ganglionic tissue taken directly from animals. Our preliminary experiments indicate that this method lacks sufficient sensitivity.
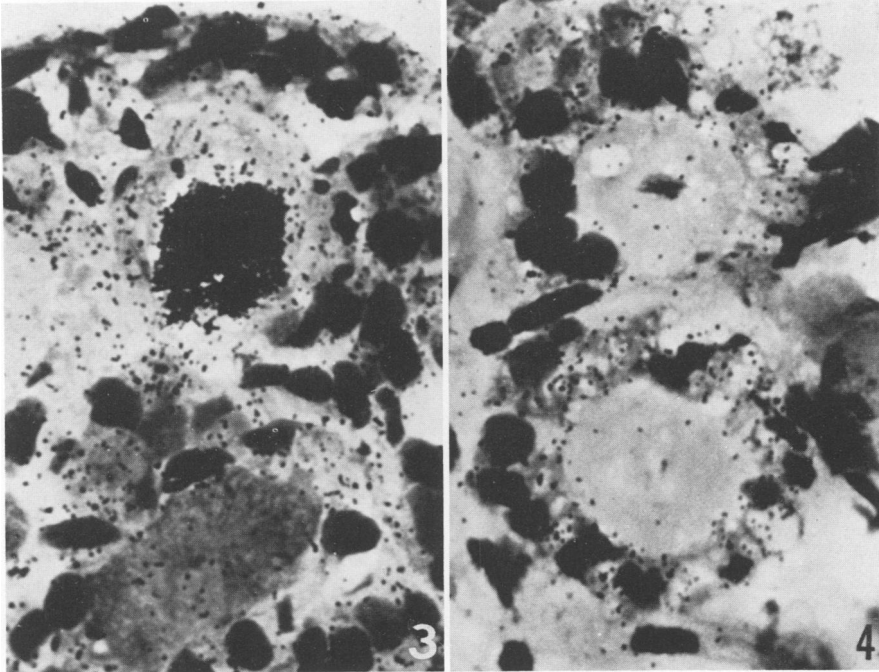

FIG. 3. *Autoradiograph of a latently infected spinal ganglion maintained in vitro for 24 h in the presence of* ^3H-thymidine. *The heavy concentration of silver grains over the nucleus of the upper neuron can be compared with a lower neuron which exhibits background labeling.* ×900.

FIG. 4. *Autoradiograph of a latently infected spinal ganglion which was maintained in vitro, "pulsed" with* ^3H-thymidine for 24 h, and "chased" with excess unlabeled thymidine for an additional 12 h. Silver grains can be seen at the periphery of two neurons and in the satellite cells surrounding them. ×900.
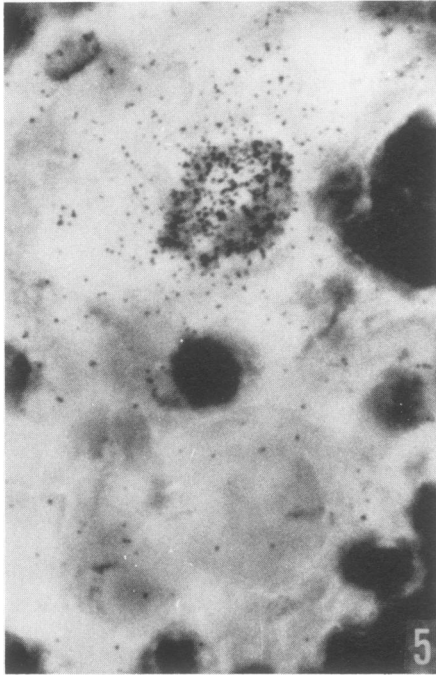
FIG. 5. *Autoradiograph of a latently infected spinal ganglion processed after 63 h of incubation in a Millipore chamber maintained in vivo. After sectioning, the specimen was subjected to in situ nucleic acid hybridization procedures involving radioactive herpes virus-specific complementary RNA. A concentration of silver grains denoting the presence of herpes simplex virus DNA is seen over a neuron. This can be compared with the unlabeled neuron below.* ×900.

The suggestion that neurons in sensory ganglia selectively harbor latent herpesvirus was first made by Goodpasture in 1929 (7). In subsequent reviews, Paine (12) and Kibrick and Gooding (9) also indicated that latent virus was probably associated with neurons. Finally, the suggestion recently received experimental support from the ultrastructural studies of Baringer and Swoveland (1). They also found that reactivating virus could first be seen in neurons.

Roizman (14), on the other hand, observed that factors which favor the multiplication of Schwann cells also appear to favor reactivation of latent HSV and suggested that supporting cells harbored latent virus. However, he also noted that nerve cell participation in recurrent infection is indicated by the distribution of vesicles (which correspond to areas of cutaneous innervation) and by the regular occurrence of eruptions after sectioning of the trigeminal root. In the same vein, Schwartz and Elizan (16) suggested that the astrocyte (or presumably a supporting cell in the peripheral nervous system) was the most likely site in which latent virus is harbored since, during acute infections, the neuron is permissive for viral replication while other cells exhibit abortive infections. Although we agree with the observations concerning the abortive infection of supporting cells (4; F. P. Knotts, M. L. Cook, and J. G. Stevens, J. Infect. Dis., in press), they appear not to play a role in latency; all of our data support the original hypothesis postulated by Goodpasture.

The finding that neurons harbor latent HSV fits nicely within the general hypothesis for pathogenesis of recurrent disease which we reviewed earlier (19). In brief, infection is postulated to progress centripetally from skin to sensory nerve to ganglion, where latent infections are established. Upon "reactivation," virus would travel centrifugally in nerves to the skin, where vesicles would be induced. We recently presented strong evidence that virus travels in axons (4). If, as our present data suggest (18), latent infection in ganglia is maintained by nonreplicating intracellular virus, then neuronal somas are the reservoirs most easily related to axonal movement. Alternative sites would necessitate passage of virus from neuron to surrounding cells and back during establishment of the latent state and subsequent reactivation.

Finally, since latent herpesvirus has now been described in the central nervous system (10), it is interesting to speculate concerning the cell type involved. The possibility that unique properties of the neuron might also offer a suitable environment for maintenance of latent virus in this location is of potential significance in the study of herpetic encephalitis and chronic, degenerative diseases of the central nervous system.

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