THE YALE JOURNAL OF BIOLOGY AND MEDICINE 60 (1987), 159–167

Latent Herpesvirus Infections of Neurons in Guinea Pigs and Humans

RICHARD B. TENSER, M.D.,[a,b] AND RICHARD W. HYMAN, Ph.D.[b]

[a]*Department of Medicine (Neurology) and* [b]*Department of Microbiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania*

Received May 21, 1986

Latent herpes simplex virus (HSV) infection of the trigeminal ganglion of guinea pigs and latent varicella-zoster virus (VZV) infection of the trigeminal ganglion of humans were studied by *in situ* nucleic acid hybridization. Guinea pig trigeminal ganglia were removed during the period of viral latency (four to five weeks after corneal inoculation of HSV), and human ganglia were removed at autopsy. Radiolabeled HSV and VZV DNAs were used to probe ganglion tissue sections for viral-specified RNA. Hybridization detected only over neurons was present in 46 percent of ganglia from 22 latently infected guinea pigs and from 33 percent of ganglia from 10 human subjects. These results support the conclusion that some viral transcription occurred during HSV and VZV latency.

INTRODUCTION

Evidence of virus infection of the nervous system is most simply concluded by the isolation of infectious virus from tissues of the central or peripheral nervous systems. With the advent of *in vitro* cell culture techniques, such isolations can be readily performed, for example, with poliomyelitis virus [1]. For some virus infections of the nervous systems, however, virus can be isolated only with difficulty, if at all. Examples include subacute sclerosing panencephalitis and progressive multifocal leukoencephalopathy. In these illnesses, evidence of viral infection may be concluded by the presence of virus-like particles in brain tissue [2,3] and by the presence of viral antigens in brain tissue, as detected by immunological means [4,5]. For other virus infections, even the detection of viral proteins is not possible. Viral infections of this last type include latent herpes simplex virus (HSV) and latent varicella-zoster virus (VZV) infections. Latent HSV and VZV infections of sensory ganglion neurons are probably the substrates of recurrent infections, including herpes labialis and genitalis (caused by HSV) and shingles (caused by VZV) [6]. The relationship between latent HSV infection and other diseases such as encephalitis is unclear, as is a possible pathogenic role for HSV in diverse illnesses of the nervous system, including multiple sclerosis and psychiatric illnesses.

Latent HSV infection of humans and experimental animals and latent VZV infection of humans are infections of neurons, most typically neurons of sensory ganglia. Infectious virus and viral antigens are not detected routinely in latently infected tissues, although HSV can be isolated with special cell culture procedures. It is possible that the failure to detect infectious HSV (and VZV) and viral antigens during latency reflects inadequate sensitivity of present-day assay methods; however, most investigators think that during latency HSV- (and VZV-) specified information is restricted and that HSV (and VZV) DNA is present in a noninfectious form [6]. The production of infectious virus in recurrent HSV and VZV infections is considered to be the result of a reactivation process in which viral DNA, RNA, proteins, and viral particles are synthesized via conventional virological mechanisms. Mechanisms underlying the initiation of viral reactivation from the latent state are unknown. The maintenance of a herpesvirus infection in a latent state implies viral or neuronal mechanisms of control. These mechanisms are presumably altered with reactivation.

To investigate further HSV and VZV latency, we employed the technique of *in situ* nucleic acid hybridization. By this procedure we utilized single-stranded and radiolabeled viral DNA to probe trigeminal ganglion tissues for HSV- and VZV-specified RNA. In investigations of latency we detected HSV RNA in trigeminal ganglion tissues of experimentally infected animals, and VZV RNA in human trigeminal ganglion tissues obtained at autopsy. Hybridization indicated the presence of at least partial viral transcription during latent HSV and VZV infections.

MATERIALS AND METHODS

Experimental Animals and Viruses

Strain 333 of HSV type 2 (HSV-2) was grown in primary rabbit kidney cells by standard methods. Virus harvested from these cultures was used for corneal inoculation of guinea pigs to induce latent infection of the trigeminal ganglia. Random-bred Hartley guinea pigs of both sexes weighing 200–250 g (Dutchland Laboratories, Denver, PA) were used. Under general anesthesia, bilateral corneal scarification was performed, and 5×10^4 plaque-forming units of virus were dropped on to each eye. Animals were observed for four to five weeks and sacrificed under general anesthesia. The trigeminal ganglia were removed and stored at $-70°C$. Cryostat sections (12 μ) on glass slides were fixed in ethanol at 4°C.

Human Tissues

Trigeminal ganglia were obtained at routine autopsy 6 to 12 hours after death. Two individuals had died of chronic neoplastic diseases; none had histories of recent VZV or HSV infections. Tissue sections (12 μ) were stored in ethanol at $-70°C$.

Preparation of Viral DNA Probes

The HSV probe used to examine guinea pig ganglia was prepared from purified HSV DNA. DNA was obtained from purified HSV virions as described previously [7]. Purified HSV DNA was radiolabeled *in vitro* by nick translation, by the method of Rigby et al. [8]. In the presence of DNA polymerase nicks in DNA were filled with ^3H-labeled nucleotides to produce radiolabeled DNA. Nick translated ^3H-labeled HSV DNA had a specific activity of approximately 2×10^7 cpm/μg of DNA; 20,000 cpm/μl. Strain 80-2 was used for the preparation of the VZV DNA probe. By using cloned VZV DNA fragments, a combination of recombinant VZV DNAs spanning the entire viral genome was prepared [9]. The VZV DNA probe was labeled *in vitro* by nick translation using [^{35}S] dATP. Probe VZV DNA contained approximately 10,000 cpm/μl.

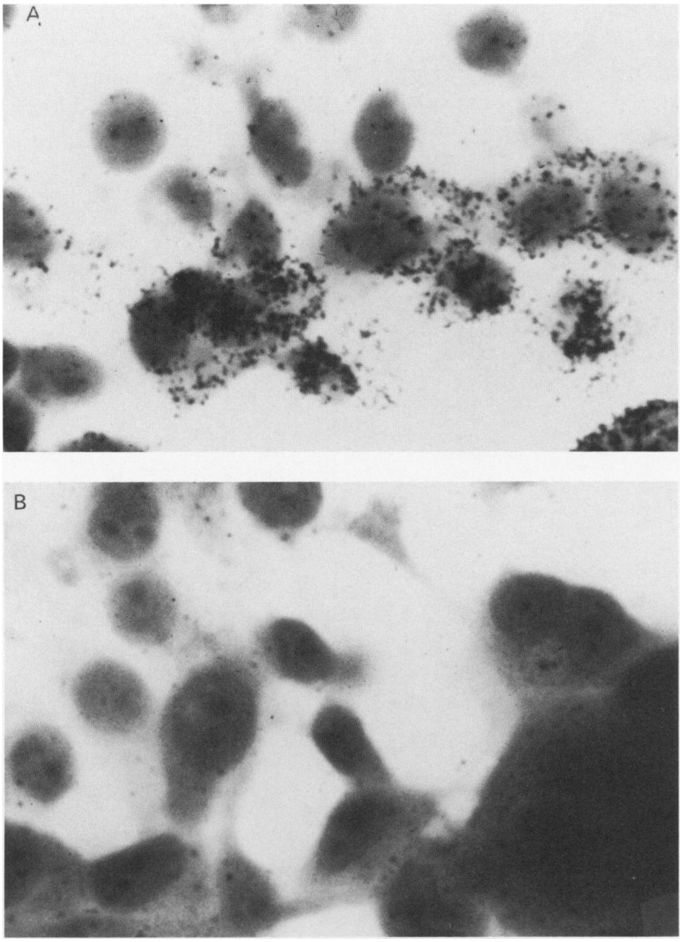

FIG. 1. Area of HSV cytopathology in Vero monolayer cells tested with ^3H-labeled HSV DNA probe, **A**, or with ^3H-labeled bacteriophage lambda DNA probe, **B.**

In Situ *Hybridization*

Although HSV DNA was labeled with ^3H and VZV DNA with ^{35}S, *in situ* hybridization reactions, carried out by the methods of Brahic and Haase [10], were similar. Probe DNA was denatured, and 10 μl of probe mixture containing 50 percent formamide and sodium chloride, 0.3 M sodium citrate, 0.3 M buffer (SSC buffer) was then overlaid on ganglion tissue sections (4 to 5 $\times$ 10^4 cpm of probe/slide). In some instances, ganglion target tissues were treated with DNase or RNase prior to hybridization. Hybridization was permitted to occur for 48 to 60 hours. Following hybridization, sections were washed extensively with SSC buffer and were then dehydrated in ethanol. Slides were covered with photographic emulsion (Kodak NTB-2) and exposed for four weeks (HSV) or three days (VZV). Following exposure in light-tight boxes, slides were developed and stained with Giemsa. Tissue sections were examined for the presence of silver grains, which indicated hybridization of probe DNA to viral nucleic acid present in the cells of the target tissue. Duplicate or triplicate sections of each ganglion tested were studied microscopically for evidence of silver grains. Sections on each slide were not sequential. Total numbers of neurons in

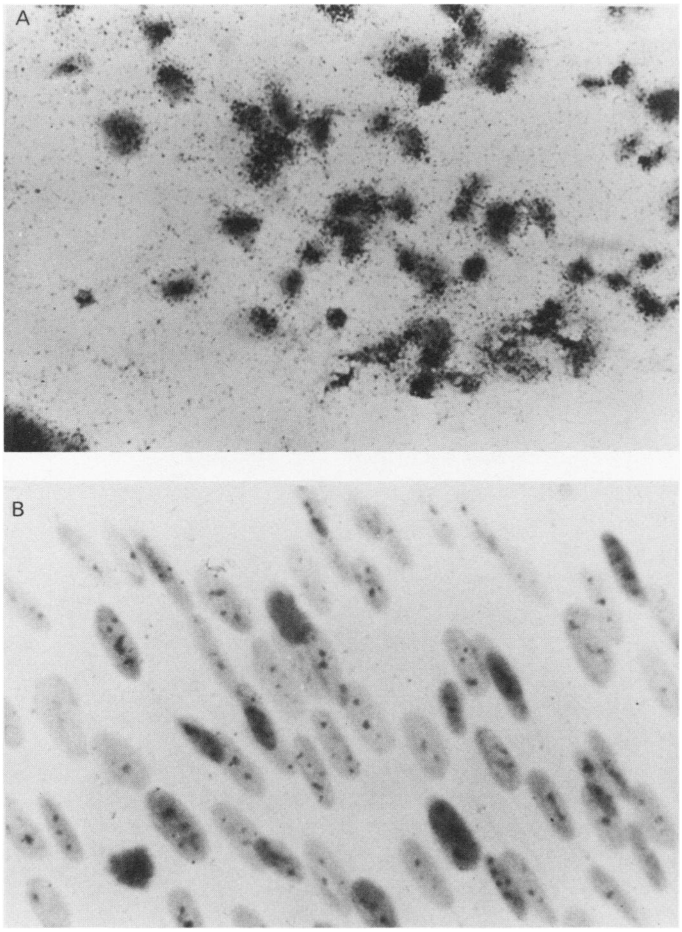

FIG. 2. Flow 5000 (human embryo) cells (Flow Laboratories) infected with VZV and tested with ^{35}S-labeled VZV DNA probe, **A**, or with ^{35}S-labeled bacteriophage lambda DNA probe, **B.**

sections, and proportions of neurons showing evidence of hybridization (hybridization-positive) were determined.

RESULTS

Hybridization Controls

Prior to use on tissue sections, viral DNA probes were tested on infected monolayer cells. To evaluate the HSV DNA probe, *in situ* hybridization was performed on HSV-infected Vero cells. Results were compared with similar hybridizations carried out with a bacteriophage lambda DNA probe control. As shown in Fig. 1A, the HSV probe hybridized to areas showing HSV cytopathic effects, whereas bacteriophage lambda DNA did not (Fig. 1B). Similarly, whereas the VZV DNA probe hybridized to human embryo cells infected with VZV (Fig. 2A), the bacteriophage lambda DNA probe did not (Fig. 2B). In studies in which the HSV DNA probe was used in hybridization experiments on cells infected with VZV, essentially no hybridization was noted (data not shown).

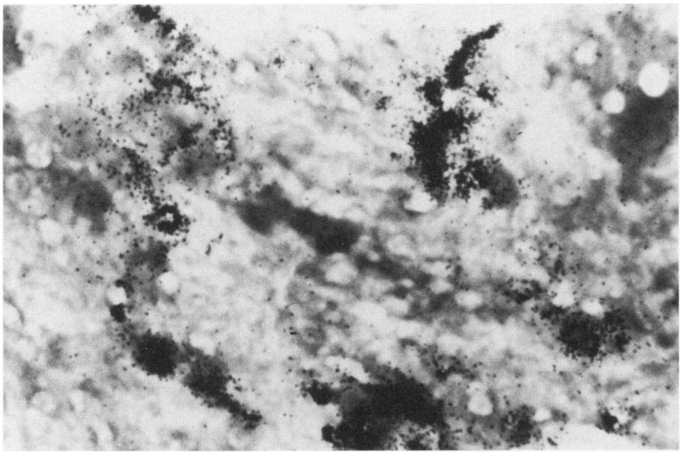

FIG. 3. Trigeminal ganglion tissue from a guinea pig sacrificed during the period of acute HSV ganglion infection (three days post-corneal inoculation of HSV) and tested with the HSV DNA probe.

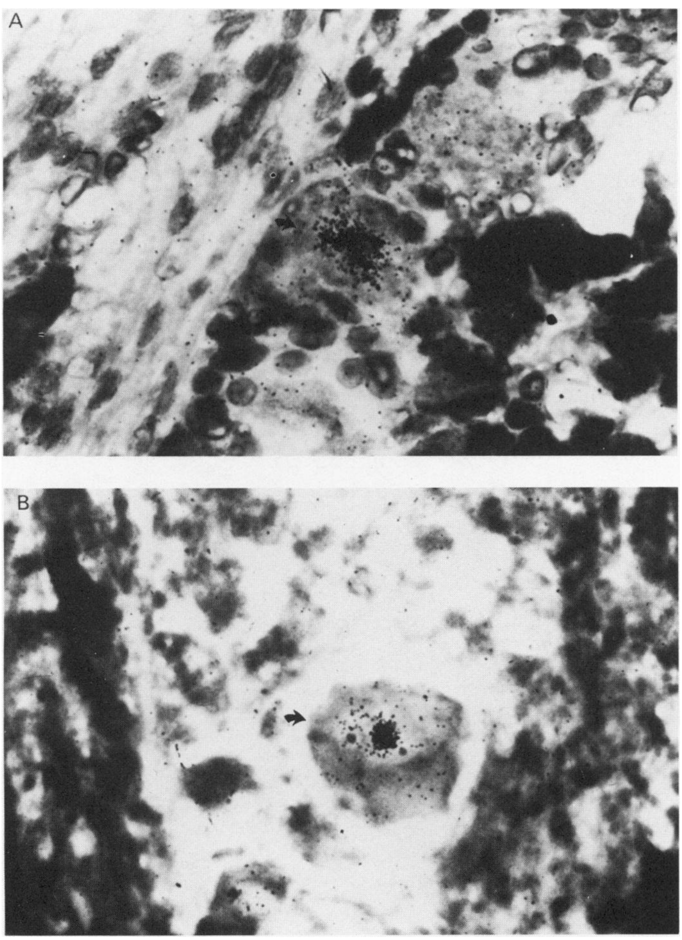

FIG. 4. Trigeminal ganglion tissue from a guinea pig sacrificed during the period of latent HSV infection (four weeks post-corneal inoculation of HSV) and tested with the HSV DNA probe, **A**, or with the same probe after treatment of the tissue with DNase, **B.** Arrows indicate neurons with evidence of hybridization.

TABLE 1
In Situ Hybridization of Radiolabeled HSV DNA Probe to Latently Infected Guinea Pig Trigeminal Ganglion Tissue

Probe Tested and Conditions	Trigeminal Ganglia with Hybridization/No. Tested (%)	Percentage of Cells (Neurons) with Hybridization
HSV DNA	20/43 (46)	0.3–5.0
Bacteriophage lambda DNA	0/43 (0)	0
HSV DNA with DNase pretreatment	18/39 (46)	0.3–1.0
HSV DNA with RNase pretreatment	3/38 (8)	0.4–0.6

Acute HSV Infection of Trigeminal Ganglion

An additional control was utilized in studies of experimental HSV infection of guinea pig trigeminal ganglion. Acute ganglion infection was evaluated in guinea pigs sacrificed three days after corneal inoculation; at this time infectious cell-free virus is present in ganglion tissue. As shown in Fig. 3, evidence of hybridization with the HSV probe was detected. Hybridization occurred over groups of cells and was present over neurons as well as non-neuronal cells. Hybridization was not apparent over ganglion tissue from uninfected animals (data not shown).

Latent HSV Infection of Guinea Pig Trigeminal Ganglia

Evidence of hybridization was seen over occasional neurons from latently infected guinea pigs when tested with the HSV DNA probe. As shown in Fig. 4A, clusters of grains were localized over the nuclei of cells. In all instances, hybridization was limited to neurons within ganglion tissue. When tissue sections were pretreated with DNase, there was little or no decrease of subsequent hybridization (Fig. 4B); however, after pretreatment with RNase, hybridization was markedly decreased or eliminated. Hybridization to ganglia from latently infected animals was not detected when the bacteriophage lambda probe was used. Hybridization results are summarized in Table

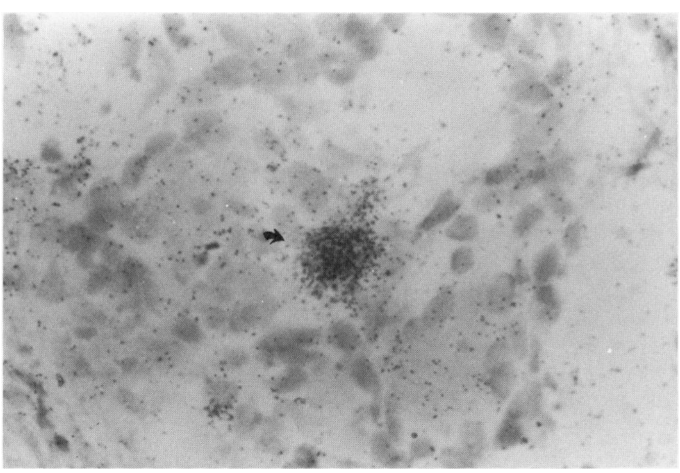

FIG. 5. Human trigeminal ganglion tissue tested with the VZV DNA probe. Arrow points to neuron with silver grains indicative of hybridization.

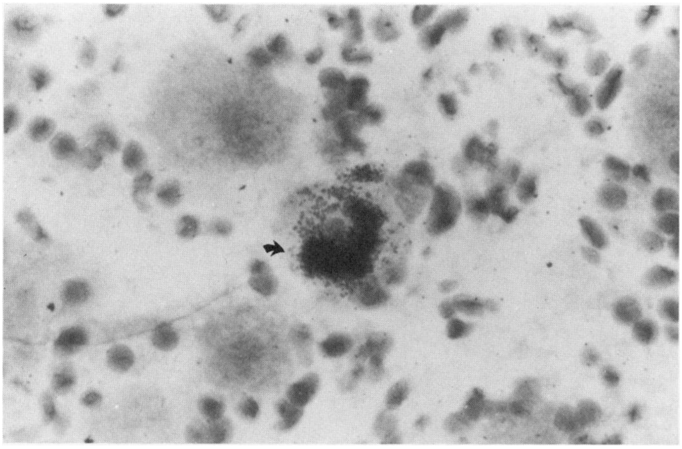

FIG. 6. Human trigeminal ganglion not tested with any radiolabeled DNA probe. Dense perinuclear grains *(arrow)* are the result of autofluorescent lipofuscin granules.

1. Of a total of 22 guinea pigs studied (43 trigeminal ganglia), hybridization was detected in 0.3 to 5 percent of neurons in 17 animals (77 percent). Two hundred and eighteen to four hundred and twenty neurons were examined for each ganglion.

Latent VZV Infection of Human Trigeminal Ganglia

Hybridization of the VZV DNA probe to human trigeminal ganglion tissue was also detected (Fig. 5). Although tissue preservation was not as good as in the guinea pig studies, clusters of grains were localized over neurons. Silver grains representing authentic hybridization within neurons were differentiated from the typically perinuclear neuronal grains due to autofluorescent lipofuscin (Fig. 6). As indicated in Table 2, 0.08 to 0.3 percent of neurons in three ganglia from nine individuals were hybridization-positive. For each ganglion, 448–607 neurons were examined. Evidence of hybridization was not detected when the bacteriophage lambda DNA probe was used.

DISCUSSION

The strength of *in situ* nucleic acid hybridization is the identification of viral nucleic acids in tissue sections in instances in which the nucleic acids are present in few cells, particularly where nucleic acid is present in a specific subpopulation of cells. Both of these conditions pertain to HSV and VZV latency of sensory ganglion neurons. Evidence has been provided that HSV and VZV latent infections of ganglia are infections of neurons, and latent virus was detected only in relatively few neurons. In our studies of HSV latency utilizing DNA-RNA *in situ* hybridization, 0.3 to 5 percent of neurons in the 46 percent of trigeminal ganglia that were positive showed

TABLE 2
In Situ Hybridization of Radiolabeled VZV DNA Probe to Human Trigeminal Ganglion Tissue

Probe Tested	Trigeminal Ganglia with Hybridization/No. Tested (%)	Percentage of Cells (Neurons) with Hybridization
VZV DNA	3/9 (33)	0.08–0.3
Bacteriophage lambda DNA	0/9 (0)	0

hybridization. In studies of VZV in human trigeminal ganglia by DNA-RNA hybridization, only 0.08–0.3 percent of neurons in the 33 percent of ganglia which were positive showed hybridization.

When *in situ* hybridization is performed on human tissues obtained at autopsy, the potential for virus reactivation between the time of death and removal of tissues needs to be considered. Partial reactivation might have occurred in our VZV study and in studies of HSV performed by Galloway and colleagues [11]. However, reports of similar hybridization results for HSV in experimental animal tissues [7,12] support the validity of the human studies.

It has recently been reported that cellular and HSV DNAs share homology [13–15]. For example, shared homology has been reported between human DNA and the *Bam*HI-S/P fragment of HSV DNA [14], and hybridization has been reported between a small DNA segment from the distal portion of the TR_L/IR_L regions of HSV DNA and human 28S ribosomal RNA and DNA [15]. Such homology presents potential difficulty in the interpretation of hybridization results. Several considerations, however, may limit the problem. First, if the region of the viral DNA with shared homology is known, hybridization with recombinant DNA fragments encompassing this region can be used as a control for hybridization with other viral DNA fragments. Studies with various animal species may show differing areas of "nonspecific" labeling by other DNA fragments. The lack of labeled cells in control tissue sections minimizes the overall problem. For RNA blot hybridization, a small degree of shared homology may result in spurious bands, because hybridization with RNA of all cells in the tissue samples is summed. For *in situ* hybridization, however, individual cells are examined, and it is unlikely that minimal shared homology will result in clusters of grains over the cells. If the presence of reactivated virus can be excluded as the reason for hybridization, the labeling of few neurons in latently infected ganglia with radiolabeled viral DNA would be open to two interpretations; both are of interest. More likely it would indicate the presence of viral-specified RNAs; less likely, it would indicate the presence of cellular RNA that has specifically accumulated in latently infected cells.

In studies of viral latency utilizing *in situ* hybridization, an important concern is whether the probe is detecting reactivating or reactivated virus, as mentioned in the preceding paragraphs. It is quite apparent that the detection of viral nucleic acid may be improved by reactivation. For example, improved labeling of sensory ganglion neurons after a brief period of *in vitro* cultivation was demonstrated by Stevens and others in a DNA-DNA hybridization study [16]. In the present hybridization study and in the studies mentioned above, cultivation was not performed. In our studies of ganglia from latently infected animals, cell-free virus was not detected [unpublished observation]. Therefore, within the limits of the sensitivity of the methods used to detect infectious virus, it can be concluded that "complete" reactivation had not occurred. That "incomplete" reactivation occurred, whereby limited viral RNAs were expressed during latency, is possible. This hypothesis requires some discussion of the definitions of latency. "Incomplete" reactivation may be common, or it may occur occasionally in cells in which viral RNA is not expressed. "Incomplete" reactivation may be an important process because it implies a level of cell and/or viral control on the reactivation process. The determination of such limited expression of viral RNA and protein is, in fact, a major objective of future studies.

ACKNOWLEDGEMENTS

Supported by Javits Neuroscience Investigator Award NA 20684 (RBT) and grant CA 16498 (RWH). We acknowledge the secretarial assistance of K. Rank and the editorial assistance of M. Clement.

REFERENCES

1. Melnick JL: Isolation of virus and the development of neutralizing and complement-fixing antibodies in poliomyelitic patients. Ann NY Acad Sci 61:1005–1010, 1955
2. Tellez-Nagel I, Harter DH: Subacute sclerosing leukoencephalitis: ultrastructure of intranuclear and intracytoplasmic inclusions. Science 154:899–901, 1965
3. Tenser RB, Sommerville KW, Mummaw JG, Frisque RJ: Isolation of JC virus capsomer-like structures from progressive multifocal leukoencephalopathy brain. J Neurol Sci 72:243–254, 1986
4. Hall WW, Choppin PW: Measles-virus proteins in the brain tissue of patients with subacute sclerosing panencephalitis. New Eng J Med 304:1152–1155, 1981
5. Weiner LP, Narayan O, Penney JB Jr, Herndon RM, Feringa ER, Tourtellotte WW, Johnson RT: Papovavirus of JC type in progressive multifocal leukoencephalopathy. Arch Neurol 29:1–3, 1973
6. Tenser RB: Herpes simplex and herpes zoster. Neurol Clin 2:215–240, 1984
7. Tenser RB, Dawson M, Ressel SJ, Dunstan ME: Detection of herpes simplex virus mRNA in latently infected trigeminal ganglion neurons by *in situ* hybridization. Ann Neurol 11:285–291, 1982
8. Rigby PWJ, Dieckmann M, Rhodes C, Berg P: Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. J Mol Biol 113:237–251, 1977
9. Hyman RB, Ecker JR, Tenser RB: Varicella-zoster virus RNA in human trigeminal ganglia. Lancet ii:814–816, 1983
10. Brahic M, Haase AT: Detection of viral sequences of low reiteration frequency by *in situ* hybridization. Proc Natl Acad Sci USA 75:6125–6129, 1978
11. Galloway DA, Fenoglio C, Shevchuk M, McDougall JK: Detection of herpes simplex RNA in human sensory ganglia. Virology 95:265–268, 1979
12. Stroop WG, Rock DL, Fraser NW: Localization of herpes simplex virus in the trigeminal and olfactory systems of the mouse central nervous system during acute and latent infections by *in situ* hybridization. Lab Invest 51:27–38, 1985
13. Maitland N, Kinross J, Busutti A, Ludgate S, Smart G, Jones K: The detection of DNA tumour virus-specific RNA sequences in abnormal human cervical biopsies by *in situ* hybridization. J Gen Virol 55:123–137, 1981
14. Jones TR, Hyman RW: Specious hybridization between herpes simplex virus DNA and human cellular DNA. Virology 131:555–560, 1983
15. Jones TR, Parks CL, Spector DJ, Hyman RW: Hybridization of herpes simplex virus DNA and human ribosomal DNA and RNA. Virology 144:384–397, 1985
16. Cook ML, Bastone VB, Stevens JG: Evidence that neurons harbor latent herpes simplex virus. Infect Immun 9:946–951, 1974