NOTES

Peripheral Infection in Culture of Rat Sensory Neurons by Herpes Simplex Virus

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The peripheral infection of neurons from dissociated rat sensory ganglia by herpes simplex virus was studied with a newly developed tissue culture system. The results indicated that the virus arrives at the neuronal soma by an axoplasmic route and that at least some of these neurons are productively infected since eventually all cells in the culture appeared to be destroyed by the virus. This system showed promise in elucidating the possible roles of various effector cells, antiviral antibody, or antiviral compounds in the mechanism of herpes simplex virus latency.

During the past decade, morphological and pathogenetic studies of herpes simplex virus (HSV)-induced encephalitis in experimental animals (5, 9), as well as the reactivation of HSV from explanted ganglia (2, 6, 12, 13, 14, 17) and the detection of latent HSV in human sensory ganglia (1, 2, 3, 7, 19), have established that the normal course of HSV infections of the sensory nervous system involves the migration of virus via axoplasmic flow in peripheral nerves to sensory ganglia. In acute infections, the virus moves from here either directly to the brain or through the spinal cord to the brain. Alternatively, the virus may establish a latent infection within the neurons of the sensory ganglia (5, 14). The precise conditions necessary for establishing this latent state have not been established. Various effector cells (8, 11) and antiviral compounds, including antibody (15, 16, 20), have been postulated.

In an effort to elucidate more precisely the mechanism of latency of HSV, we developed a tissue culture system which offered the possibility for peripheral HSV infection of sensory neurons. Such a system should afford greater control of experimental conditions than that inherent within *in vivo* studies. The experimental design of such a system based on a nerve tissue culture setup developed by R. Campenot (4) is reported herein together with some consequences of peripheral HSV infection.

The tissue culture plates for this system were prepared in the following way: 35-mm plates were coated with calfskin collagen (Calbiochem, La Jolla, Calif.). A scratched area made with an insect pin rake was placed in the bottom of the

dish (Fig. 1). A drop of Eagle 10/10 medium (Eagle medium supplemented with 10% heat-inactivated horse serum, 10% fetal calf serum, 3% embryo extract, and glucose [600 mg/100 ml] containing 0.6% methyl cellulose) was placed on the scratch. An 8-mm cloning cylinder (Bellco Glass, Inc., Vineland, N.J.) with one end coated with a ribbon of silicon high vacuum grease (Dow Corning, Midland, Mich.) was placed on

FIG. 1. Tissue culture system used for growing cultures of dissociated rat DRG cells. The basic culture chamber is a 35- by 10-mm tissue culture dish which has a scratched collagen-coated growing surface and contains a 8-mm cloning cylinder sealed to it with vacuum grease.

this drop of medium and secured to the bottom of the plate by gentle pressure. The inside of the cloning cylinder was filled with Eagle 10/10 medium and examined 12 to 24 h later to determine whether the hydrostatic head at this time indicated an effective vacuum grease seal.

Cultured dorsal root ganglia (DRG) cells were obtained by dissection of 17-day-old rat embryos from matings of Sprague-Dawley rats. The DRGs from 8 to 10 embryos were pooled, and after gentle teasing with a fine-tipped forcep, they were dissociated in 5 ml of 0.25% trypsin (1-250, ICN Nutritional Biochemicals, Cleveland, Ohio) in calcium- and magnesium-free Hanks balanced salt solution for 45 min at 37°C. The cells were then washed once, resuspended in 0.25 ml of Eagle (10/10) medium per embryo, and triturated until a cloudy suspension was obtained. Samples (0.05 to 0.1 ml) of resuspended cells (approximately 10,000 cells) were placed inside the cloning cylinder and incubated

overnight in a 5% CO₂ atmosphere at 37°C. The next day, fresh Eagle 10/10 medium was added to each cylinder and the area outside the cylinder. Within 48 h, there was almost confluent cell growth within the cylinder. At this time these cells were treated for 48 h with Eagle 10/10 medium containing 10 μ m of cytosine arabinoside (Sigma Chemicals, St. Louis, Mo.). The cells were then refed every 3 days with growth medium.

Initially, groups of neurons as well as satellite cells, Schwann cells, and fibroblasts attached to the culture dish. The cytosine arabinoside treatment seemed to preferentially eliminate most of the fibroblasts. After 2 weeks of growth in vitro, the cells within the cylinder appeared as shown in Fig. 2. The sensory neurons had many extensions of neuritic processes extending from each soma. Schwann cells were associated with these neuritic processes. Neurites tended to grow parallel to the scratches in the collagen and were

FIG. 2. Phase-contrast morphology of dissociated rat DRG cells maintained in culture for 2 weeks. The main portion of the photograph shows an area inside the cloning cylinder. Neuronal somas with prominent nuclei and nucleoli as well as neurites with attached Schwann cells are clearly visible. The inset shows a neurite with its attached Schwann cells emerging from underneath the cloning cylinder. The vacuum grease seal is visible at the left and a scratch is seen at the top. The large bar represents 30 μ m, and the smaller bar represents 60 μ m.

first observed to grow out under the cloning cylinder after 10 days of *in vitro* growth. Increased incubation enabled more neurites to emerge. An emerging neurite with its associated Schwann cells can be seen in Fig. 2 (inset).

At a time when several areas of a culture had emerging neurites (14 to 20 days), the area of the culture outside of the cloning cylinder was exposed to 10^5 plaque-forming units of HSV (MacIntyre strain). After 1 h of incubation at 37°C, the virus suspension was removed and replaced by growth media. At 8, 16, 24, 36, 48, and 72 h postinfection, areas inside and outside the cloning cylinder were washed in 0.01 M phosphate-buffered saline, pH 7.2, fixed in 95% ethanol, and then stained with fluorescein-labeled rabbit anti-HSV immunoglobulins (M.A. Bioproducts, Walkerville, Md.) to detect the presence of HSV-specific antigens. Duplicate cultures were utilized for each time period, and the experiment was performed several times with different DRG preparations.

At 8 and 16 h postinfection, the only cells which possessed definite HSV-specific antigens were the Schwann cells outside the cloning cylinder. Occasionally, one could see a hint of viral antigen in some neuronal somas located within the same scratch but on the other side of the seal from which the neurites seemed to emerge. By 24 h postinfection, isolated neuronal somas or small groups of neuronal somas displayed distinct granular fluorescence. An example is

shown in Fig. 3. These somas tended to be rounded rather than flat on the plate and were always in a scratched area that had neurites outside the chamber. The neurites and the associated Schwann cells inside the cloning cylinder between the seal and these fluorescing somas did not show HSV-specific antigens.

As the time postinfection increased to 36 or 48 h, HSV-specific fluorescence was detected in an increased number of neuronal somas as well as satellite cells and Schwann cells in the area within the cloning cylinder, which most likely was the origin of emerging neurites. During the latter stages of infection, all cells in culture contained HSV-specific antigens.

These observations regarding the sequential appearance of HSV specific-antigens in various areas of the culture system after virus inoculation outside the cloning cylinder suggest that the virus first infected neuritic extensions outside the cloning cylinders and then traveled within axocylinders to corresponding somas where a productive infection in at least some of the neurons occurred. Next, neighboring cells were infected, and finally all cells in all areas of the culture became involved. To our knowledge, this is the first report of an *in vitro* system which demonstrates the well-known phenomenon of axoplasmic transport of HSV that occurs *in vivo* (2, 5). The distances traveled by the virus and the times involved are consistent with *in vivo* HSV migration data (10).

FIG. 3. Immunofluorescent micrographs of HSV-peripherally infected cell culture. (A) A group of neuronal somas within the cloning cylinder of a mock-infected, control culture. No HSV-specific fluorescence is visible, although neuronal morphology is quite evident. (B) was taken outside the cloning cylinder at 16 h postinfection. Bright HSV-specific fluorescence can be seen in the Schwann cells attached to these emerging neurites. (C) A group of neuronal somas inside the cloning cylinder at 24 h postinfection. One soma clearly shows bright HSV-specific, granular fluorescence. These somas do not grow flat on the plate as their uninfected neighbors do. The magnification on each photograph is the same, with the bar representing 20 μ m.

These studies have allowed us to draw three conclusions concerning HSV infection of the cells in cultures. First, all cell types show evidence of viral antigens; therefore all cell types are susceptible to infection. Second, neuritic extensions seem not to display any signs of virus infection (i.e., retraction) until after their somas have viral antigens present. Thus, mere virus infection of the neurite itself does not cause retraction. Third, the outcome of this peripheral infection is not the latent infection observed for in vivo experimental HSV infections (6, 12, 14, 17) and for naturally occurring HSV infections of humans (2, 19). However, this system offers the possibility whereby one might study the mechanism of latency. Typical experiments under study are the addition of various effector cells (8, 11, 18) or compounds implicated in latency (15, 16, 18, 20) to areas of the culture system and observing their effect on the pathogenesis of the virus.

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