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Light and Electron Microscopic Studies of the Pathogenesis of Vaccinia Virus Infection in Mouse Brain (41479)

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Abstract. Vaccinia virus replication in weanling mouse brains was examined by light and electron microscopy 2 to 5 days after inoculation, i.e., during the period of maximal viral infectivity. Replication sites were detected in meningeal cells, adventitial cells of meningeal arterioles, and small nonneuronal cells of the brain. No evidence was found for replication in neurons, although these cells were altered indirectly. The virus can be described most accurately as being leptomeningoencephalitic.

The natural history of poxvirus infection usually includes involvement of the respiratory tract, reticuloendothelial system, liver, and skin. Infection of the brain is rare, but is of interest because of the possibility that encephalitis following immunization with vaccinia virus may be a manifestation of infection. It is unlikely that it results from a toxic effect (1). Experimental inoculation of brain with vaccinia virus has revealed that some strains proliferate in nervous tissue. Uncertainties exist, however, as to the types of cells involved and the natural history of intracerebral infection. Several reports have appeared suggesting that the principal lesions of cerebral infections are a leptomeningitis and/or ependymitis (2–5). Most of these studies have been conducted in mice, and the approach has been principally through light microscopy, including the application of immunofluorescence. An exception to the usual reports has been a study in which a racoon poxvirus inoculated into mice was found to cause necrotic foci in dorsal spinal ganglia and severe demyelination with necrosis in the lumbar plexuses (6). These findings suggest a modification of neurons due to a direct viral infection. Where it has been considered, there is no evidence for viral replication in glial cells. Immunofluorescence microscopy is not sensitive enough to justify the conclusion that immunofluorescent-negative cells are not infected. Conversely, positive immunofluorescence does not guarantee intracellular viral replication. Electron microscopy permits direct observation of the characteristic morphology of poxviruses in both their replicating and mature forms. Accordingly, electron microscopy was brought to bear on the question of poxvirus neurovirulence and focused on the early period of maximal infectivity after the intracerebral inoculation of mice with vaccinia virus.

Materials and Methods. *Poxvirus.* Vaccinia virus, strain Levaditi-58 (7), is a virus capable of multiplying to a high level in mouse brain. It was assayed by dropping the virus on the chorioallantois of 11-day old chicken embryos, and after 2 days of incubation at 37° the results were scored as pock-producing units (ppu) per 0.05 ml of inoculum.

Mice. Three-week-old, NIH general purpose albino mice were employed. Virus was administered by injecting 0.02 ml of suspension into the right cerebral hemisphere of ether-anesthetized mice.

Viral replication in brain. Eighteen mice were inoculated with a selected virus dose and three mice were sacrificed each day thereafter, at which time the pooled brains were ground with RR Alundum and brought to a 20% suspension, by weight, in 0.85% NaCl solution. From the growth curve results, a suitable virus dose (100 ppu) was selected for further studies. Control mice, sham inoculated with NaCl solution, were run in parallel.

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Preliminary preparation of infected brain. For 6 days after intracerebral inoculation with 100 ppu of virus, each of two mice was anesthetized, and an infusion was begun through the left ventricle of the heart with 20 ml of cold (4°) imidazole–formalin solution (0.1 *M* imidazole–HCl, 10% formalin, pH 7.2). As the infusion was begun, the hepatic vein was cut. After infusion, the brain and spinal cord were dissected out and stored in imidazole–formalin solution at 4° for at least 24 hr. Control mice were processed in an identical manner.

Fixation and sectioning of brain. The cold, fixed brain was cut coronally into eight serial sections. Subsections for light microscopy were embedded in Paraplast and the caudal surface was sectioned to a thickness of 5 μm. The sections were stained with hematoxylin and eosin.

The specimens for electron microscopy were divided further by a medial cut, dividing them into right and left sides of the brain. Prior to postfixation, further subsections were made. The subsections were diced into pieces less than 1 mm in greatest diameter and were fixed in isoosmolal, buffered, 1.5% glutaraldehyde, pH 7.4, for 3 hr at 4° and then placed in a wash solution (isoosmolal sucrose solution with 0.02 *M* imidazole–HCl, pH 7.4) overnight at 4°. (The tissue used for electron microscopy had been kept at 4° until this point.) After a 1-hr exposure to 2% OsO_4 in sodium barbital buffer at pH 7.4, the specimens were dehydrated through an alcohol series, carried in propylene for two 15-min periods, held for 30 min in a 50/50 propylene–Maraglas mixture and embedded in Maraglas as described previously (1). Thin sections (about 500 nm) were cut with glass knives, and areas to be studied by electron microscopy were selected. Ultrathin sections (about 75 nm) were cut with diamond knives and placed on uncoated copper grids, stained with lead citrate, and studied by electron microscopy (1).

Results. *Viral replication in brain.* Vaccinia virus replication at three different doses is shown in Fig. 1. The virus replicates appreciably by 3 days in mouse brain. Accordingly, for electron microscopic studies the combination of a dose of 100 ppu and an examination period of 2–4 days postinoculation was selected as conditions under which viral replication would be expected to be extensive and yet tissue breakdown would be at a minimum.

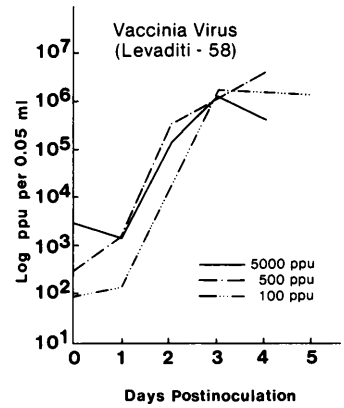

FIG. 1. Replication of vaccinia virus in mouse brain following intracerebral inoculation with three different doses of virus.

Signs of disease. At the 100 ppu dose, signs of disease began to appear at 3 days postinoculation. The mice appeared lethargic, hunched and trembling. Flaccid paralysis was noted, and body spasms occurred when the mice were twirled by their tails. By the fourth day, trembling continued and breathing became labored. The front legs no longer were used for locomotion. Animals surviving to the fifth day became totally immobilized, very sensitive to noise vibrations, and usually died in spasms. At this time, the brains were of a very soft consistency and covered with petechial hemorrhages. Some mice survived to Day 6, but none survived longer.

Light microscopy of infected brains. Histologically, the brain was essentially unremarkable during the first 2 days postinoculation (Fig. 2A). By the third and fourth days, many cortical areas showed nuclear shrinkage associated with early, occasionally marked, perinuclear clearing, especially in the neuronal layers (Fig. 2B). More extensive perinuclear clearing was present by the fifth day. A focal leptomeningitis and ependymitis, both of which included a polymorphonuclear

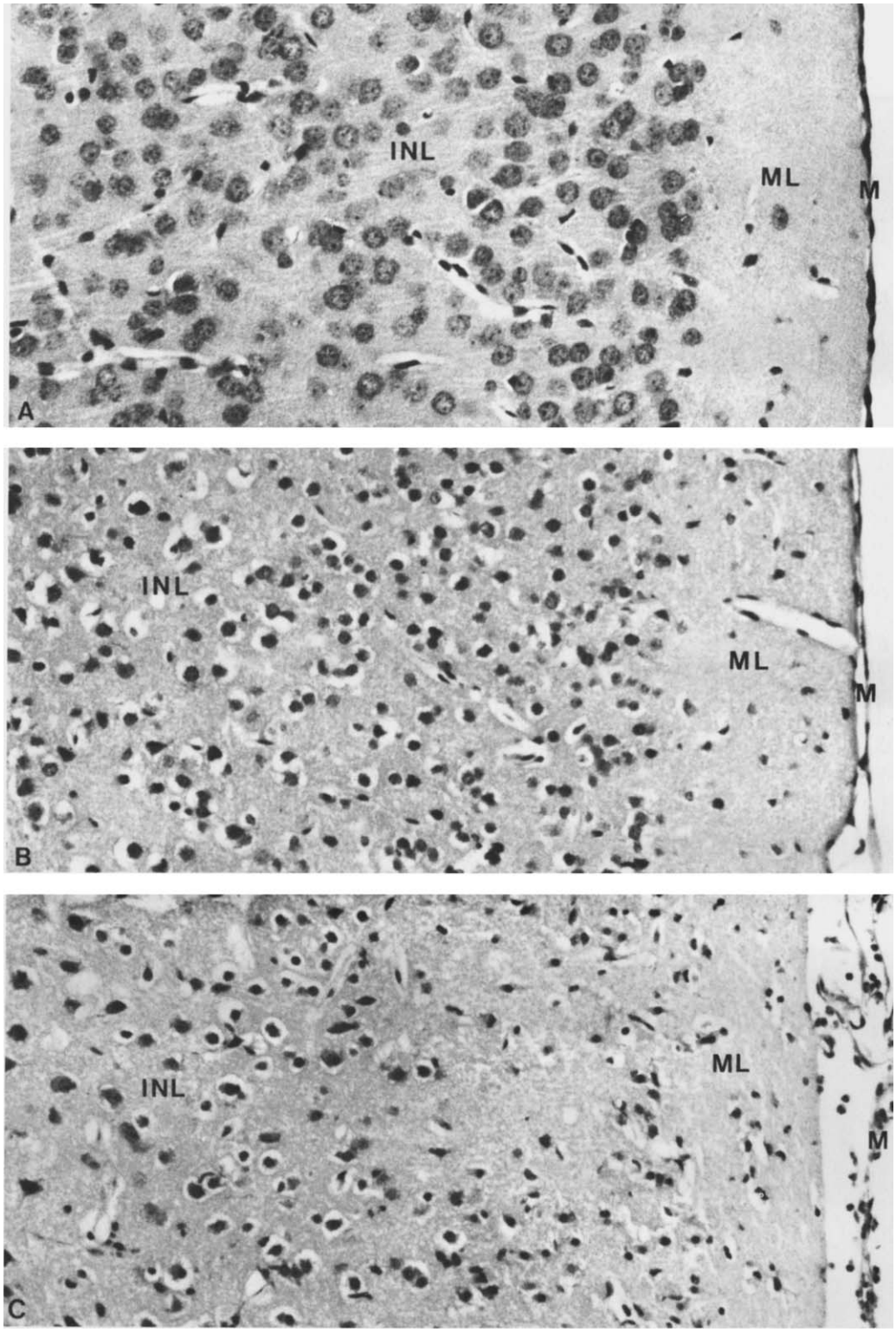

FIG. 2. Light microscopy of dorsal surface of parietal cortex of brain. (A) One day after virus inoculation. Meninges (M), molecular layer (ML), and inner neuronal layer (INL) appear normal. (B) Four days postinoculation. Nuclear shrinkage, nuclear hyperchromasia, and perinuclear clearing are evident in cells of the molecular and inner neuronal layers, especially cells that appear to be neurons. The neuropil has a spongy quality. Note that the meninges appear unremarkable. (C) Six days post-inoculation. Changes in molecular and inner neuronal layers persist. In addition, an inflammatory infiltrate, rich in neutrophils, involves the meninges. Hematoxylin and eosin, ×250.

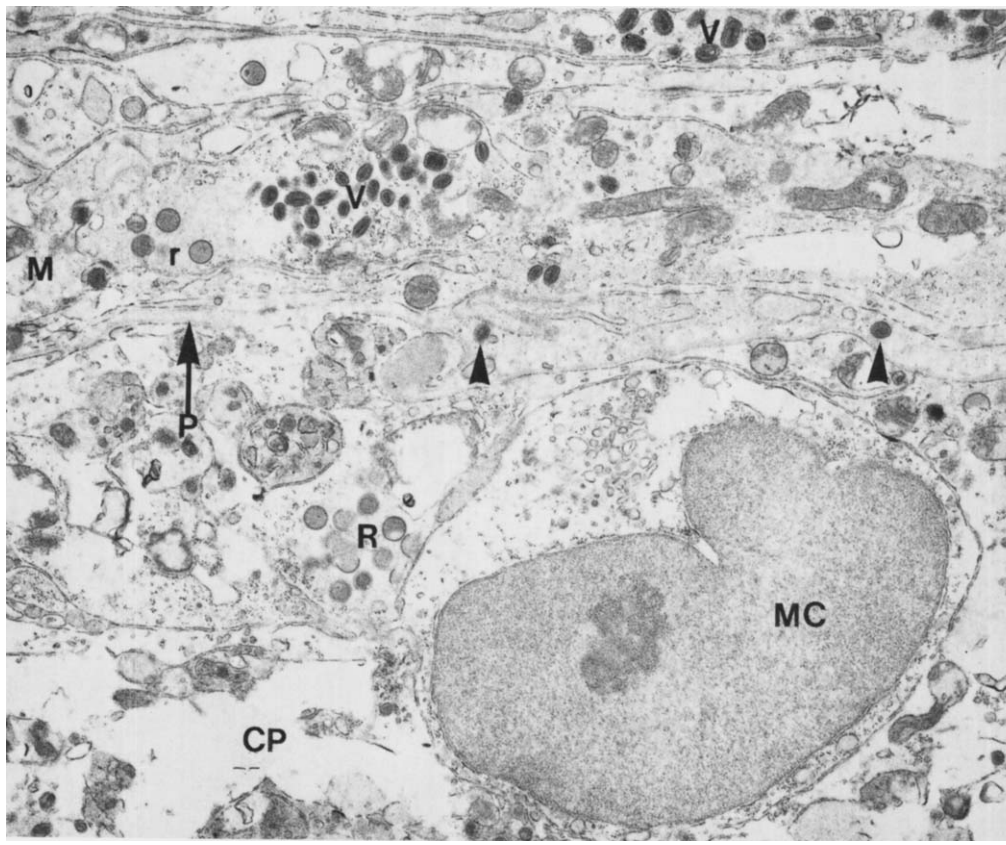

FIG. 3. Three days after virus inoculation. Meninges and submeningeal cortex. Above the pia (P), most meningeal cells (M) contain clusters of mature virus (V) and/or sites of viral replication (r). Arrowheads indicate individual mature viruses within the pia. Below the pia, the molecular layer neuropil contains swollen cytoplasmic processes (CP), one of which contains a site of viral replication (R). A mononuclear cell (MC) shows cytoplasmic swelling. Lead citrate, ×6600.

leukocyte infiltrate, developed between the fourth and sixth days. Generally, the focal leptomeningitis first appeared on the ventral surface and later (at 5–6 days) on the dorsal surface (Fig. 2C). Diffuse leptomeningitis and focal ependymitis found in terminal mice were occasionally associated with focal necrosis of the underlying brain.

Electron microscopy of infected brains. Brain sections from sham-inoculated control mice were well preserved and unremarkable in appearance.

In inoculated animals, virus was not readily demonstrable at 2 days. Mature virus and sites of viral replication were readily found at 3–4 days, at which time considerable degenerative alterations were noted in meningeal, neuronal, glial, ependymal, and vascular adventitial cells. In contrast, endothelial and smooth muscle cells of the cerebral vasculature were preserved. Viral structures were most easily found at 3 days, which corresponds with the peak of infectious virus titer (Fig. 1).

Between 3 and 4 days, both mature virus and sites of viral replication were clearly identified in many cells; meningeal cells were extensively involved (Figs. 3, 4) and adventitial cells of meningeal arterioles were also commonly involved (Fig. 4). In the cerebral cortex and cerebellum, numerous small, mononuclear cells of the neuropil of the molecular and neuronal layers contained sites of viral replication and mature virus (Fig. 5). The virus-containing cells frequently were adjacent to capillaries and neurons. These cells were interpreted as probably being glial cells.

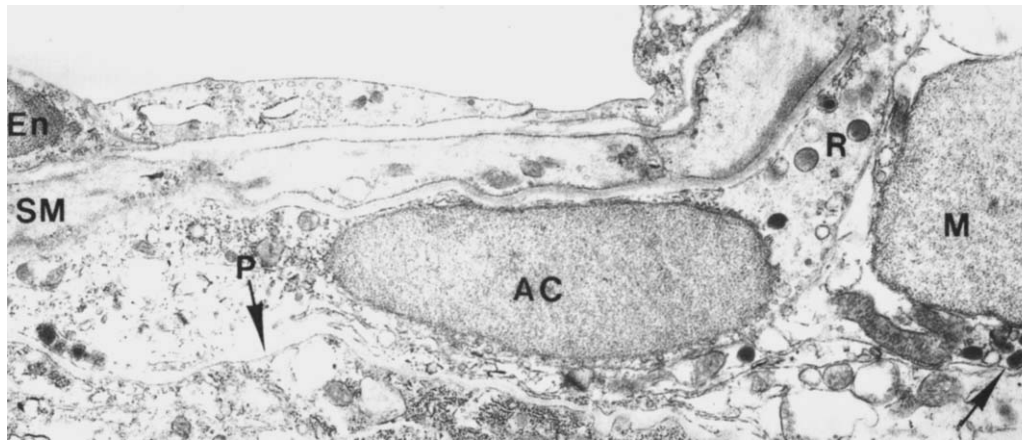

FIG. 4. Three days after virus inoculation. Section of meninges (above) and subjacent molecular layer. An arteriolar wall shows no virus in the capillary endothelial cells (En) or smooth muscle cells (SM). An adventitial cell (AC) contains a site of viral replication (R). Mature virus (arrow) can be seen in the cytoplasm of an adjacent meningeal cell (M). Pia (P). Lead citrate, ×6400.

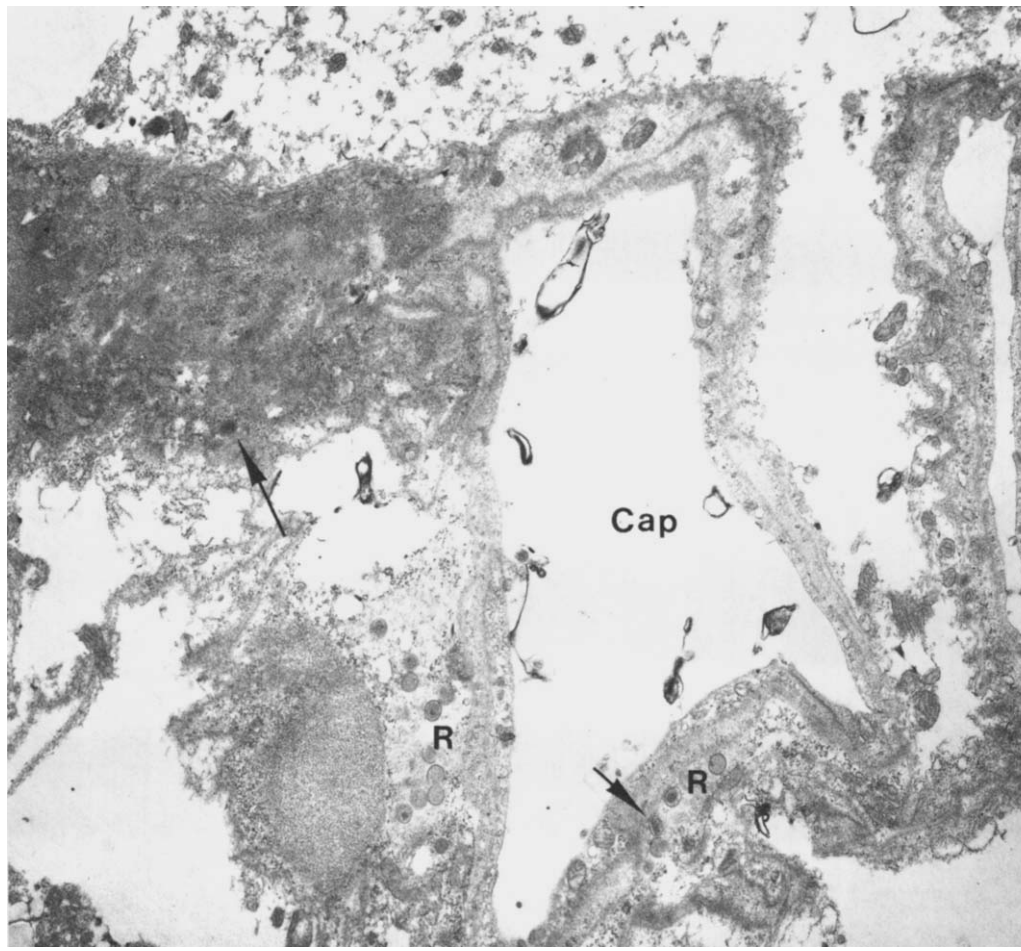

FIG. 5. Three days after virus inoculation. Cerebral cortex. A mature virus particle (short arrow) and sites of viral replication (R) are seen in two small paracapillary cells showing marked cytoplasmic swelling. A cell with dense cytoplasm contains a mature virus particle (long arrow). Capillary lumen (Cap). Lead citrate, ×6200.

To determine whether neurons contained viral material, a number of sections of cerebral cortex rich in neurons, as determined by light microscopy, were carefully selected and studied by electron microscopy. In no instance were viral structures found in neuronal cells, which frequently showed cytoplasmic clearing and other degenerative changes. In addition, viral structures never were identified in ependymal cells, endothelial cells, smooth muscle cells of blood vessels, or cells of the inflammatory infiltrates.

Discussion. Previous investigators have shown the presence of viral antigen in leptomeningeal cells of vaccinia virus-infected mouse brains, using immunofluorescence techniques (2, 4). The present study confirms that the meninges are a principal site of intracranial replication of vaccinia virus during the first 4 days after inoculation of brain and for the first time demonstrates unequivocally the presence of replicating viral structures in meningeal cells. In addition, virus replicates in the adventitial cells of blood vessels, principally meningeal arterioles, and in small nonneuronal cells of the cerebral cortex, presumably glial cells. Although ependymitis was observed, no virus was detected in ependymal cells, which frequently were focally denuded.

Blinzinger and co-workers, using electron microscopy, studied late stages of infection, i.e., 4–6 days postinoculation in adult mice (5). At that time, they found virus only in mononuclear phagocytes and arterial adventitial cells of the meninges. No evidence of neuronal, glial, or ependymal cell infection was found. The present study focuses on earlier stages of infection, in weanling mice, in contrast to Blinzinger's examination of the later stages of infection in adult mice. These differences in experimental design appear to account for the variation in findings. In fact, it is postulated from the results of these two studies that after initial infection of leptomeningeal, vascular adventitial, and glial cells of the brain, a secondary infection of late occurring inflammatory infiltrate may ensue.

In keeping with Blinzinger *et al.* (5), virus was not found within neurons, although in the present study marked alterations of these cells were noted by both light and electron microscopy. The neuronal changes are in parallel with a report on the indirect action of Newcastle disease virus on the neurons of mice (8). Since glial cells are thought to influence the transmission of nervous activity (9), consideration of this point may be appropriate in the present study. It has been shown that in primary cell cultures astrocytes release factors into the medium that promote the growth and prolong the survival of rat hippocampal neurons (10). In this regard, in the present study it was noted that small nonneuronal cells within the cerebral cortex frequently contained sites of viral replication. Although marked alterations of these cells, probably due to viral replication, precluded definitive structural identification, the cells presumably represent glial components. This presumption is supported by the absence of cellular inflammatory infiltrates, which might lead to the confusion of cell types within the brain, during the time period covered in this study.

The term "neurotropic" has been applied to numerous viruses replicating in the brain. It has been suggested that this is an unsuitable designation for poxviruses replicating in mouse brain since they do not replicate in neurons (11). Modern dictionaries, however, define "neurotropic" as designating agents having an affinity for nervous tissue (12, 13), thus making the term applicable to the aforementioned poxviruses. In the present study, viral replication (or infection) within neurons could not be detected, even though virus replicated in nonneuronal cells of the brain substance. The virus, however, effected an indirect neuronovirulence, as suggested by the observed paralysis of infected animals and the morphologic alterations in neurons. Care should be taken to recognize that some viruses are strictly "neuronotropic," replicating only within neurons. Viruses replicating in the brain also may be described as "encephalitic," causing inflammation of the brain, as distinct from its membranes (14). The virus examined in this study can be most specifically described as being leptomeningoencephalitic.

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