Age-Related Neurocytrotropism of Mouse Cytomegalovirus in Explanted Trigeminal Ganglions

Gustave L. Davis, MD, Konrad W. Krawczyk, BA, and Mary M. Hawrisiak, ScM

Human cytomegalovirus (CMV) causes severe congenital neurologic disease; adult neural infection is associated with transient cranial nerve palsies. In our experimental model, mouse CMV (MCMV) infects neurons and Schwann and satellite cells of cranial nerve ganglions. To study the fate of MCMV in nerve tissue, we explanted trigeminal ganglions from newborn, suckling, and weanling mice, 3 to 36 days after intracranial inoculation. Negative explants were co-cultivated with mouse embryo tissue culture (METC) to test for latency. MCMV, identified by electron microscopy, replicated in fibroblasts, Schwann cells, and/or satellite cells and neurons of trigeminal explants from newborn and suckling, but not weanling, mice. No latent virus was detected by our methods. The age differences in viral replication may be due to the age-dependent intrinsic cellular mechanisms and host inflammatory and immunologic response. Though neurons are infected, they remain relatively resistant to CMV replication. (Am J Pathol 97:261-276, 1979)

The effects of congenital cytomegalovirus (CMV) infection on the nervous system vary from severely destructive cytomegalic inclusion disease with attendant microcephaly and death to less severe congenital or neonatal infection associated with infantile spasms, mental retardation, speech and learning disorders, and varying degrees of hearing loss.1-6

The pattern of adult nervous system CMV infection is entirely different, manifested as transient cranial nerve palsies and/or ascending myelitis in immunologically intact individuals.7-10 Acute hearing loss in adults has not been documented with CMV infection,11 though a recent case report suggests that the development of tinnitus and vertigo and worsening of existing hearing loss in an adult was due to activation of latent prior congenital CMV infection.12 More common are non-neural manifestations of infection in immunologically compromised adults. The vast majority of

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Supported by the Deafness Research Foundation, the Biochemical Research Support Funds of The Jewish Hospital of St. Louis, and a Senior International Fellowship (F06 TW00132) of the Fogarty International Center of the National Institutes of Health.

Submitted in part to fulfill the requirements for Honors in Biology at Washington University, St. Louis, Missouri, March 21, 1977, by Mr. Krawczyk, who is a student at the Chicago Medical School.


Accepted for publication June 12, 1979.

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healthy adults have antibody to CMV, evidence of prior asymptomatic infection. Although CMV shares with other herpesviruses characteristics of cytolysis and latency, CMV has not been recovered from cranial or sensory dorsal root ganglia, as have herpesvirus hominus and varicella zoster.\textsuperscript{15,16}

The few cases of cytomegalic inclusion disease in which the inner ears have been examined histopathologically show infection of the cochlea, an endolabyrinthitis.\textsuperscript{6,17-19} However, the spectrum of congenital CMV central nervous system infection, including brain, brain stem, and cranial nerves, indicates a much more global pattern of disease.\textsuperscript{20} It is not yet clear whether the inner ear viral infection is the major mechanism for the hearing loss or merely represents dissemination of the virus.

In our murine model of CMV infection of the inner ear, a perilabyrinthitis following intracranial inoculation of mouse CMV, we observed CMV-infected cells in trigeminal (Figure 1), glosso-pharyngeal, and spiral ganglia \textit{in vivo}. Newborn mice were extremely susceptible to infection, with a high rate of morbidity and mortality; older suckling and weanling mice were less susceptible and survived without evidence of clinical infection. Thus, experimental murine models with mouse CMV parallel the human experience with variation in both host susceptibility to the virus and the apparent ability of neural tissues to support viral replication.

We studied explanted trigeminal ganglia from intracranially inoculated mice to correlate viral recovery with host age and to identify which ganglion cells are infected.

\textbf{Materials and Methods}

The mouse colony free of mouse cytomegalovirus (MCMV) was developed from mice originally obtained by the late Dr. Margaret G. Smith. MCMV has never been histologically identified in these animals, nor has culture of various tissues from the mice yielded a cytopathic effect typical of MCMV in mouse embryo tissue culture. We have not undertaken specific serologic tests for MCMV and have not tested for other specific pathogens or viruses.

The MCMV was originally obtained from Dr. Roger Smith (University of Cincinnati) and Dr. Donald Henson (National Institutes of Health). It has been maintained by animal passage. Ten percent (weight by volume) aliquots of infected salivary gland supernatant have also been maintained at -70°C. The infectivity of the salivary gland extracts was proven by observation of the cytopathic effect in inoculated mouse embryo tissue cultures. Viral inoculums were quantitated by determining the dose of virus necessary to infect half of the tissue cultures inoculated (TCID\textsubscript{50}). Mice received intracranial inoculations of 0.02-ml dilutions of the viral stock solution, which varied between $10^{-4.5}$ and $10^{-5.5}$ TCID\textsubscript{50} per milliliter. The several newborn mice which received $10^{-4.5}$ TCID\textsubscript{50}/ml inoculums did not live beyond nine days after inoculation, thus necessitating a lower viral dose ($10^{-5.5}$/ TCID\textsubscript{50}/ml).

Newborn mice were inoculated 1 day after birth and were sacrificed every subsequent third day up to 21 days after birth. Suckling mice were inoculated on the sixth day after
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birth and sacrificed each subsequent third day for 30 days. Weanling mice were inoculated 21 days after birth and sacrificed each subsequent third day for 38 days.

Controls consisted of uninoculated mice sacrificed every third day after birth through 57 days and 2 21-day-old mice receiving inoculations of virus inactivated by heat at 56 C for 30 minutes. One of these latter animals was sacrificed 3 days after inoculation, the other 44 days after inoculation.

The mice were killed by decapitation. The skin of the head was removed and, with the aid of a dissecting microscope, the skull was hemisected in the midsagittal plane. The trigeminal ganglia were exposed by the removal of the cerebrum and cerebellum. One excised ganglion was washed in Hanks' balanced salt solution containing penicillin (200 U/ml) and streptomycin (100 µg/ml) and then minced in Liebowitz' L-15 medium with 20% fetal calf serum, penicillin, and streptomycin and pipetted into tissue culture flasks. The minced ganglion explants were incubated at 37 C in ambient air and observed for the presence of cytopathic effect each subsequent third day.

The contralateral ganglion was removed and fixed in 2% glutaraldehyde, washed in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Araldite 502. Thick sections were cut and stained with 1% toluidine blue and were examined with a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 201C electron microscope.

Tissue cultures were maintained with 1-ml aliquots of the L-15 medium with fetal calf serum and antibiotics. Each seventh day after explantation, up to 21 days, 0.1 milliliter of the supernatant fluid was drawn off and absorbed onto mouse embryo tissue culture (METC) for 1 hour. The inoculated METC was then supplemented with minimal essential medium with 3% fetal calf serum and antibiotics and incubated at 37 C in the ambient air.

At the first sign of a cytopathic effect—cytolysis and/or inclusions or enlarged cells—the explant culture was fixed in 2% glutaraldehyde and washed in 0.1 M phosphate buffer for two hours. The cells were dehydrated in graded alcohols and embedded in Araldite 502 within the plastic tissue culture flasks. The flasks were broken, and the fragments of Araldite containing the embedded cells were peeled from the adherent plastic flask pieces. The Araldite fragments were trimmed and then mounted at the ends of Araldite blanks. Thick and ultrathin sections were then cut and stained appropriately, as above.

If no cytopathic effect was seen in the ganglion explant cultures after 21 days of incubation, or in the three supernatant fluid cultures, the explants were trypsinized, and the dispersed cells were co-cultivated with mouse embryo tissue culture for up to an additional 21 days with the use of the minimal essential medium regimen. These co-cultivated tissues were fixed for electron-microscopic examination as above at the first appearance of a cytopathic effect or, in its absence, at the end of 21 days.

Results

Survival Data (Table 1)

Of 75 infected newborn mice, only 18 (24%) survived long enough to be examined, and none lived past 21 days postpartum. Many of the non-survivors were sick and were eaten by the mothers. Seventeen of the 18 suckling mice survived (94%), and all of the 13 weanling mice and control mice lived until they were killed. The suckling, weanling, and control mice showed no outward sign of illness.

Tissue Culture

Newborn Mice (Table 2)

The cytopathic effect characteristic of MCMV occurred consistently in cells growing from infected newborn mouse trigeminal ganglia ex-
<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Day after birth inoculated</th>
<th>Number of cultured ganglions examined per number of mice killed on day after inoculation</th>
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<td>Experimental mice</td>
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Inoculation titer = 0.02 ml; TCID₅₀ = 10⁵.5/ml.

* Differences between "number of mice injected" and "number of mice" are those that died or were eaten before they were examined.
† Inoculum had been previously heated to 56 C for 30 minutes.
Table 2—Mice Inoculated at Birth (Newborn)

<table>
<thead>
<tr>
<th>Day after inoculation killed</th>
<th>Viral dilution</th>
<th>CPE in explant tissue</th>
<th>CPE due to virus in supernatant fluid</th>
<th>CPE in co-cultivated trypsinized cultures</th>
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CPE = cytopathic effect; EM = electron microscopy.
planted 3, 6, 9, and 12 days after inoculation. Three of these explants and 4 subsequent cultures were contaminated and were not adequate for study. The supernatant fluids from the positive explants, but not the negative ones, produced a cytopathic effect after inoculation onto METC. The viral nature of this cytopathic effect was confirmed by electron-microscopic identification of virions. No virions were found in the 3 negative explants, co-cultivated ganglions, or any of the contralateral ganglions. Cocultivation of negative explants on METC for up to an additional 21 days failed to show evidence of viral replication.

Suckling Mice (Table 3)

The virus was recovered from the trigeminal ganglion explants from the 6-day-old suckling mice up to 21 days after inoculation. This virus recovery was less consistent than with the newborn mice, and the delayed appearance of the cytopathic effect in cultures of supernatant fluid suggested the presence of lower concentrations of the virus, but titrations were not carried out. The presence of the virus in the positive explants was again confirmed by the cytopathic effect in inoculated METC and by electron-microscopic identification of virions. No virions were seen in negative cultures or contralateral ganglions.

Weanling and Control Mice

No virus was recovered from these cultures, and virions were not seen in any of the material examined with the electron microscope.

Microscopy

Cellular growth from explanted ganglions was quite variable. Within 2 to 3 days after explantation, a peripheral monolayer was present. Due to the immaturity of cells, it was extremely difficult to identify the cell types in explants from newborn animals. By 6 days of age, however, large granular neurons were distinguishable from smaller, spindle-shaped cells. Neurons were clearly identified by their large size, central nucleus with distinct nucleolus, and relatively clear nucleoplasm. Neuronal cytoplasm contained elongated mitochondria, Nissl bodies and microtubules, and dense granules that became more frequently seen as the cultures aged. Axonal and dendritic extensions of cell bodies were found in different planes of the sections. Differentiation among cells was easier in explants from older mice and as the cultures aged in vitro (Figure 2).

There was extensive necrosis in trigeminal ganglions removed from infected newborn mice 3 days after inoculation and maintained as explant
Table 3—Mice Inoculated at Birth (Sucklings)

<table>
<thead>
<tr>
<th>Day after inoculation killed</th>
<th>Viral dilution</th>
<th>CPE in explant tissue</th>
<th>CPE with supernatant fluid</th>
<th>CPE in co-cultivated negative trypsinized cultures</th>
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CPE = cytopathic effect; EM = electron microscopy.
culture for 1 week. Both naked and enveloped herpes-type nucleocapsids were present in the lysed nuclei and in extracellular fluids among cell debris and collagen fibrils (Figure 3). Intracellular crystalline arrays of nucleocapsids, an unusual occurrence with CMV but characteristic of herpes simplex virus, were present in infected cells. However, the extensive viral cytopathic effect obscured the identity of these infected cells.

We were able to differentiate among cell types infected in 1-week explants obtained from newborn mice 6 days after inoculation and from infected sucklings. Neurons were infected, but virions were only observed in the nuclei (Figure 4). Though many infected lysed unidentifiable cells were present in older cultures, we could not identify intermediate stages of viral replication in neurons, such as budding and envelopment from the inner nuclear membrane and formation of cytoplasm in dense bodies incorporating virions. No virions were seen in neuronal cytoplasm or axons. Infection of remaining smaller spindle cells was common. Differentiation among these non-neuronal ganglion cells infected often required identification of intracytoplasmic large myelin figures (Schwann cells) or juxtaposition to neuronal cell bodies (satellite cells) (Figure 5).

Contralateral nonexplanted ganglions and those ganglions that yielded no tissue culture evidence of viral replication did not contain virions.

Discussion

This study presents additional evidence for an age-related neurocytropism of mouse cytomegalovirus. The intracranial inoculum of MCMV fatal to newborn mice within 8 days of infection causes a meningoencephalitis and cranial neuronitis similar to that reported by Luca and Margolis and Kilham. A reduction in viral titer to permit survival of 75% of the infected newborn mice results in a chronic persistent trigeminal nerve infection in both newborn and suckling mice without morphologic evidence of MCMV. Viral replication is age-restricted, since no virus is found in ganglions from newborn and suckling mice 21 days after inoculation or from inoculated weanling mice.

Infected newborn mice do not live long enough to permit age-matched comparisons with infected weanlings (Table 1). There is, however, overlap between infected suckling and weanling mice at 24, 27, and 30 days of age. The absence of MCMV in ganglions from suckling mice more than 27 days of age indicates that MCMV is either repressed or eliminated as the animal develops. Such restriction of viral replication may prevent the virus from reaching the trigeminal ganglions or yield insufficient amounts for successful replication in the weanling ganglions. The failure of Mar-
golis and Kilham to demonstrate age-related differences in central nervous system infection with MCMV may be explained by their use of comparatively high titers of virus necessary to yield morphologic evidence of encephalitis in their relatively short-term experiments.

Schneider, Willson, and their co-workers could find no differences in the rate of MCMV infection of cultures of fetal mouse spinal cord and dorsal root ganglions of different in vitro ages. They noted that the pattern of intracellular virus formation varied with the age of the culture; but due to extensive cytolysis and distortion of morphologic characteristics, they were unable to determine the infected host cell type—neuron, satellite cell, or fibroblast. Cellular mitotic activity is not a factor in this age dependency of viral replication as Schwartz et al suggest, since MCMV can infect postmitotic cerebral, cerebellar, and cranial-nerve neurons. Herndon, Rooss, and co-workers explained the greater susceptibility of immature neurons to measles infection as a failure of maturing host neurons to produce sufficient amounts of ribonucleic acid to support neurotropic measles virus replication.

In addition to the as yet undefined intrinsic host cell factors, both cellular and humoral immune mechanisms affect viral replication and latency. Thymectomized or "nude" mice and those treated with antilymphocyte serum or prednisone have widespread dissemination of relatively mild CMV infection as well as activation of otherwise latent infections. An age-related protective effect of macrophages is also postulated by Selgrade and Osborne. Genetic variation in susceptibility to MCMV infection among inbred strains of mice may be extremely great. Rajcani et al demonstrated that the recovery of latent herpes simplex virus from explanted trigeminal ganglions was markedly decreased by the application of immune serum to the culture system.

Although we found MCMV virions in intact neuronal nuclei, a morphologic maturation sequence of the envelopment of nucleocapsids, the formation of cytoplasmic bodies, and cytolysis was not seen. This may be due to the loss of cellular characteristics of the neuron with viral replication or to the lack of such a sequence. In addition to the cell's age-related ability to support a viral replication and extrinsic immunologic factors, anatomic considerations may be important in determining which cells are infected.

Schneider and Willson and their associates infected spinal cord and dorsal root ganglion cultures with mouse CMV and reported that satellite-cell infection invariably preceded neuron infection and suggested a "resistance of neurons in the dorsal root ganglion to infection."

In our pre-
vious in vitro studies of the effect of mouse CMV on the developing ear, Schwann and satellite-cell infections were much more prominent than neuronal involvement. Both Schneider's group, working with MCMV, and Rajcani and Conen, working with herpes simplex virus, found that the supporting cells, satellite and Schwann cells, are infected before neurons. The amount of infected virus available to the neuron in vivo may therefore be restricted. Unlike herpes simplex virus, axonal spread from peripheral site of inoculation to the sensory ganglion has not been demonstrated with cytomegalovirus, and in vivo neuronal infection may be restricted by the spread of the virus among surrounding cells.

If neuronal infection is restricted, can CMV cause a latent infection similar to that demonstrated with herpes simplex or varicella-zoster in nerves or CMV in non-neural tissues? Clinical and experimental studies with other related herpesviruses suggest that it can, and that our inability to demonstrate such latency in our infected weanling mice could be due to the insensitivity of our cellular substrate. The biochemical demonstration of viral nucleic acids and the immunologic identification of viral antigen in the absence of viral replication in target cells may be better proofs of latency.

Although we have histologically shown HCMV in the human labyrinth, it has not been isolated from, or shown in, cranial nerves, as have the other herpesviruses. Stagno et al, using an anticomplementary fluorescent antibody technique, suggested that HCMV antigen is present in the organ of Corti and spiral ganglion neurons in the absence of virions in those tissues in children with CMV endolabyrinthitis.

Our experimental model indicates that MCMV can infect cranial-nerve ganglion cells, spreading by cell–cell contact from perineural fibroblasts to Schwann cells, satellite cells, and, ultimately, the neuron. Ganglion cells from newborn and suckling mice support viral replication yielding a persistent but limited infection. Although we have confirmed an age-related neurocystotropism of MCMV, the latent potential of MCMV in the neuron remains unknown.

References
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40. Baringer JR, Swoveland P: Persistent herpes simplex virus infection in rabbit trigeminal ganglia. Lab Invest 30:230-240, 1974


Acknowledgments

We wish to thank William Kraft and Robert Henry for their valuable technical assistance in carrying out this work.
**Figure 1**—Intranuclear inclusions (arrows) are present in cells of the mouse trigeminal ganglion following intracranial inoculation with MCMV. Adjacent, large ganglion cells are apparently uninfected. (H&E, enlarged from ×1000)

**Figure 2**—Monolayer of cells growing from a newborn trigeminal ganglion explanted 18 days after inoculation contains large neurons (N), smaller Schwann-satellite cells (S), and neuronal axons (A). (Lead citrate and uranyl acetate, ×3000)
Figure 3—This lysed infected cell from a newborn trigeminal ganglion removed 3 days after infection contains a crystalline array of herpes-type nucleocapsids. Note the budding of virions into the perinuclear cistern (arrow). (Lead citrate and uranyl acetate, x13,325)

Figure 4—An intact neuron from a newborn ganglion removed 9 days after inoculation contains scattered intranuclear nucleocapsids (inset) without intranuclear inclusion formation. (Lead citrate and uranyl acetate, x4500; inset, x18,600).
Figure 5 — An infected suckling ganglion removed 15 days after inoculation contains a neuron and 2 adjacent satellite cells. The lower satellite cell contains intranuclear nucleocapsids (inset) that form an intranuclear inclusion with aggregates of chromatin. (Lead citrate and uranyl acetate, ×3560; inset, ×17,000).