Demyelination and Cytopathic Effects in Cultures of Mammalian Dorsal Root Ganglia Infected with Encephalomyocarditis Virus

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Replication of encephalomyocarditis virus and its cytopathic effects were studied in myelinated cultures of dorsal root ganglia obtained from newborn mice. Six hours after infection virus progeny was detected in the culture. At 24 h the virus titer reached $2 \times 10^4$ PFU per culture and remained at this level until 48 h. The first cytopathic alterations began at 24 h and consisted of rounding of Schwann and satellites cells and their detachment from neurons. Later, bead-like swellings of the myelin appeared along the axons followed by splitting and degeneration of lamellae. The cytopathic effect in the neurons started 29 h after infection, reaching complete neuronolysis at 48 h. Virus particles, scattered or arranged in crystal-like aggregates, were first seen in the cytoplasm of glial cells and then in neurons and axons.

The encephalomyocarditis virus (EMC) is a highly neurotropic RNA virus which causes myelitis or encephalitis in small animals. It is known to cause damage to various organs, such as myocardium, endocardium, aorta, kidney, pancreas, and liver (4–6, 8). EMC virus enters the brain before multiplication in the different organs, grows rapidly there, and causes paralysis or death to the infected animals (8). Since neural tissue is the target organ of such infection, it would be of interest to study the relationship between the virus and this tissue.

Virus-host relationship in cultures of neural origin has been previously described. Most of the work done in this respect dealt with enveloped viruses such as herpes (9, 10, 12), rabies (13), measles (17), subacute sclerosing panencephalitis virus (16, 18), and visna (3), but there is no information of the neuropathic effects in vitro of enteroviruses (naked, RNA). The present investigation was undertaken to study the cytopathogenesis of EMC virus in long-term cultures of myelinated dorsal root ganglia (DRG) from newborn mice.

MATERIALS AND METHODS

Virus. The virus was grown in a suspension of BHK-21 cells. It was used as supernatant fluid cleared of cells and cell debris by centrifugation and stored at −70 C until used.

Tissue culture. DRG were obtained from newborn white mice (Ness-Ziona strain) and cultivated in the double cover slip Maximow assembly (14). The ganglia were explanted on either glass or silicon rubber membrane (19). The nutrient medium consisted of 38% modified Simm balanced salt solution (BSS), 10% Eagle basal medium, 27% fresh human serum, and 20% fresh chicken embryonic extract. The medium was buffered with 5% of a 0.15 M stock solution of TES (2-aminoethane sulfonic acid) buffer and supplemented with glucose (60 μg/ml) and gentamicin (16 μg/ml).

Virus infection. At 1 month in vitro when cultures were abundantly myelinated, they were infected with 0.05 ml of virus suspension containing about $4 \times 10^4$ PFU of EMC virus. After a 1-h absorption at 34.5 C, the inoculum was removed by washing each culture in Simm BSS and replaced by 0.05 ml of normal nutrient medium. At various times after infection the culture medium from duplicate cultures was taken and separately assayed for the presence and titer of virus. For determination of cell-associated virus, the entire cell mass was removed from the cover glass and placed in 1 ml of Eagle medium. The cells were frozen and thawed to obtain cell disruption. The virus titers were determined by a plaque assay on BHK-21 cell monolayers.

Light and electron microscopy. Infected cultures were observed under bright-field and phase-contrast microscopy to follow the sequential order of morphological alterations.

For electron microscopy ganglia cultivated on silicon rubber were fixed as whole mounts in 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, and dehydrated. Embedding was carried out as previously described (20). Sections were cut with a OMU-2 Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and observed in a JEM 100B electron microscope.

RESULTS

Viral assay. First evidence of the presence of cell-associated virus was found 6 h after infec-
tion with $10^3$ virus, whereas free virus in the medium was detectable only after 8 h. At about 24 h postinfection (PI) the titer of the virus reached a maximum of $2 \times 10^4$ PFU per culture, remained at this level for an additional 24 h, and only then began to decline. A growth curve of EMC virus in DRG cultures is presented in Fig. 1.

**Light microscopy.** Neurons, satellite, and Schwann cells in culture showed typical morphological characteristics. Myelin appeared between the second and third week in vitro (2).

The first alterations in cells infected by EMC virus were detected 24 h PI. They were characterized first by a marked increase in number and size of vacuoles in the cytoplasm of macrophages and fibroblasts. In the following hours, Schwann cells and satellites appeared rounded up and looked as if detaching from the nerve cells. This was accompanied by changes in myelin sheath in the form of head-like swellings along the axons. Degeneration of myelin proceeded, showing fragmentation and breakdown into small refringent droplets (Fig. 2B). Its almost complete loss was reached at 48 hours PI. The chronological sequence of cytopathic changes consisted of appearance of refractile granules and small vacuoles in the cytoplasm of neurons and nonmyelinated axons. This was followed by coarser granulations and increase in the size of vacuoles accompanied by dislocation of cellular organelles to an eccentric position (Fig. 3).

Control experiments showed that cytopathic effects (CPE) and myelin deterioration were due to virus multiplication and not to the presence of cell debris or toxic effects of inactive dead virus.

**Electron microscopy.** Specimens for electron microscopy were selected on the basis of observations in light microscopy according to degenerative disorders in the myelin and neurons. Myelin lesions consisted of marked dilatations with intralaminar disjunctions and splitting of lamellae (Fig. 4B). Axons appeared swollen, and their microtubuli and mitochondria were disorganized. Viruses were first seen in the cytoplasm of Schwann cells 29 hours PI (Fig. 5). The latter became vacuolated, and the nucleus was lobulated (Fig. 6). The first signs of CPE in the neuron somas became evident at 29 h. Vacuoles appeared throughout the cytoplasm; some of these vacuoles may be derived from swollen mitochondria which can still be recognized by remnants of cristae. There were indentations of the nuclear membrane. At this stage virus particles in crystal arrangements were seen in the cytoplasm (Fig. 7). These lesions became more accentuated at a more advanced cycle of infection (Fig. 8).

**DISCUSSION**

The data presented demonstrate the replication of EMC virus in myelinated cultures of DRG of newborn mice. Virus could be detected after 6 h in the cells and after 8 h in the supernatant. The maximal infectious titers ranged between $10^4$ to $2.10^4$ PFU per culture.

Explants of cultured DRG contain, besides the neuronal populations, other cells such as fibroblasts and macrophages derived from connective tissue. Therefore, in the analysis of virus replication one has to consider the mixed population of cells as a whole and not neurons and glial cells only. Nevertheless, observations at the ultrastructural level revealed the presence of viral particles in the cytoplasm of glial cells and in nerve somas. The similarity in size and density of scattered viruses and ribosomes made it difficult to distinguish between the two, thus no such distinction will be attempted here. The determination of the presence of viruses was based on the arrangement of particles into aggregates or crystals.

The CPE observed in our model system resemble the patterns described in a variety of non-neural systems infected with EMC virus in vitro and in vivo (7, 11). Similar CPE were described in neural systems infected with different viruses (3, 12, 13, 17, 18). In our case, however, besides the changes in the cellular architecture and the presence of crystalline...
Fig. 2. Myelinated axons in DRG cultures. (A) Fragment of normal myelinated axon. R indicates node of Ranvier. ×1,600. (B) Forty-eight hours after infection with EMC. Arrows point to swelling and destruction of myelin. ×1,440.
FIG. 2B
Fig. 3. DRG culture 24 to 48 h PI, from earliest CPE until complete neuronolysis. ×600. (A) Early alteration in neuron: note swelling of satellite cell (S) and retraction of myelin sheath at node of Ranvier (R). (B) advanced morphological changes in neurons: fine cytoplasmic granulations. Swelling and detachment of satellite cells (S). (C) Coarser granulations and large vacuoles (V) within cytoplasm of a neuron and in axon (A). (D) Neuronolysis and advanced state of myelin degeneration; remnants of fragmented myelin and complete loss are indicated by arrows.
FIG. 4. Fragments of myelinated axons fixed and processed under the same conditions. (A) Normal myelinated axon: note the integrity of microtubules and myelin lamellae. ×34,000. (B) Myelinated axon 48 h PI: advanced stage of damage to cytoplasmic structures; splitting and disruption of lamellae. ×48,000.
Fig. 5. DRG culture 29 h PI. Portion of a Schwann cell. Arrow indicates EMC virus tightly packed within the crystal aggregate. ×54,600. Inset: higher magnification of the same crystal aggregate. ×150,400.
FIG. 6. DRG culture 48 h PI. Crystal array of EMC virus particles (arrow), large vacuoles within cytoplasm of Schwann cell. Note lobulated nucleus. ×20,100.

FIG. 7. DRG culture 48 h PI. Initial stages of nerve cell damage. Arrow: aggregates of virus particles. ×45,800. Inset: higher magnification of the crystal array. ×136,400.
viral aggregates in the cytoplasm of DRG cells infected with EMC, we observed marked alteration of myelin consisting of splitting, fragmentation, breakdown, and complete loss of myelin. Similar patterns of demyelination were only described in other pathological in vitro conditions caused by nonviral agents (15, 21).

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LITERATURE CITED