Ultrastructure of Junín Virus in Mouse Whole Brain and Mouse Brain Tissue Cultures

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Comparative ultrastructural studies were performed on the development of Junín virus in mouse brain and in cerebellum explants and brain monolayers of the same animal. In mouse brain, neurons and astrocytes released virus particles by a budding mechanism identical to that previously described for this virus. In the neurons, the viral multiplication took place in the perikarion as well as in the cytoplasmic processes, including areas near synapses. Viral particles were observed emerging from pericapillary neurons and astrocytes. In the explants, the budding also occurred in neurons and astrocytes. In the monolayers, however, the virus originated in astrocytes and cells of fibroblastic appearance, which were the two cell types that developed in this substrate. These results indicate that the characteristics of the development of Junín virus in mouse brain are faithfully reproduced in cerebellum explants from the same animal, thus allowing some extrapolation of data from one system to the other. The explant proved to be a better model than the monolayer, not only because it reproduced the structural complexity of nervous tissue better, but also because it contains neurons and astrocytes, i.e., the two cell types that release the virus in the in vivo system.

Once the morphological identification of Junín virus, the agent of Argentinian hemorrhagic fever, was achieved (11, 12, 14), we decided to attempt its growth in some more complex cell substrates. The main object was to correlate the sequential stages of viral multiplication in a living animal with those achieved with culture systems. Since the Junín virus is encephalitogenic when inoculated into newborn mice, mouse brain was utilized as an in vivo model, and cerebellum explant and brain monolayers of the same animal were chosen as in vitro models.

The virological studies were preceded by light and electron microscopic observations to determine the nature of cell types developed in each of those systems.

MATERIALS AND METHODS

Virus. The Gar strain of Junín virus (11, 12) was used. The absence of contaminants from this strain, including the agent of lymphocytic choriomeningitis, has been well documented (12).

Mice. Litters of 1- to 2-day-old albino mice were infected by intracerebral route with 0.02 ml of mouse brain suspension containing 10^4 Junín virus ($10^{5.7}$ mean lethal dose [LD_{50}]/ml). The animals were sacrificed 6 to 15 days after inoculation and brains were dissected for electron microscopic study.

Explants. Mouse cerebellum explants were carried

out according to Maximow's double cover slip technique in chicken plasma clot (17, 18).

An extract of chicken embryo was added to the growth medium (19) to induce coagulation. The growth medium was opportunely replaced by minimal essential medium with 2% inactivated fetal bovine serum.

Two types of explants were performed. The first was cerebellum of 24-h-old healthy mice inoculated with the virus after an 8- to 12-day growth in the medium. The infection was carried out with 0.1 ml of mouse brain suspension containing $10^{5.7}$ LD_{50} of Junín virus per ml; the second was cerebellum obtained from mice that had been inoculated intracerebrally within the first 24 h of life with 0.02 ml of mouse brain suspension containing $10^{5.7}$ LD_{50} of Junín virus per ml which were explanted afterwards at different intervals.

Titration of the explants was not attempted.

Monolayers. Cells from suckling mouse brain were dispersed by routine trypsinization techniques and seeded at a concentration of 10^6 /ml in the medium recommended by Illavia and Webb (7). Monolayers were obtained within 6 to 10 days in bottles and Leighton tubes.

Two types of monolayers were performed. The first was from the brains of 24- to 48-h-old healthy mice which, once its development was complete, was inoculated with Junín virus at a multiplicity of infection of 0.1 to 0.01 (i.e., 0.1 to 0.01 mouse LD_{50} per cell); after infection, the growth medium was replaced by minimal essential medium with 2% inactivated fetal bovine serum. The second was from the brains of mice inoculated intracerebrally within the first 24 h of life

with 0.02 ml of mouse brain suspension, containing $10^{5.7}$ LD₅₀ of Junín virus per ml and collected 3 to 6 days after infection.

Titration. Titration was carried out by intracerebral inoculation in suckling mice. The Reed and Muench method was used.

Controls. Brains of mice inoculated with brain suspensions from healthy mice, and mouse brain monolayers and cerebellar explants inoculated with supernatants fluids from uninfected mouse brain tissue cultures, were used as controls.

Stains for light microscopy. All types of cell cultures were stained with Giemsa and Holmes techniques. The monolayers were also stained with the following method: (i) thorough washing of bottle or Leighton tube with distilled water; (ii) fixation in 4% paraformaldehyde for one or preferably several days; (iii) thorough washing in distilled water; (iv) treatment with a solution of saturated uranyl acetate in 50% ethanol for 1 h at 37°C; (v) thorough washing in distilled water; (vi) impregnation in freshly prepared Wilder's silver bath (to which 20 drops of pyridine were added for every 10 ml of solution) for 15 min at room temperature; (vii) development in the reducing solution for 5 min, with special care not to shake the slide or the solution.

The reducing solution is prepared by dissolving 2.5 ml of 10% gelatin in 95.5 ml of distilled water; once the gelatin has been completely dissolved by thorough shaking, 2.0 ml of pure formalin are added. At this point, the slide is examined under the microscope. If the stain is too pale, which is often the case, steps (v) to (vii) should be repeated as many times as needed until a total visualization of the astrocytes and their gliofibrils is obtained. Overstained slides can be decolorized as needed with 2% iron alum (prepared by pulverizing the salt in mortar, dissolving it in distilled water, and centrifuging the solution at 800 g for 10 min to eliminate undissolved particles) and steps (v) to (vii) can be repeated until a satisfactory result is obtained. Slides can be toned with gold chloride as follows: (i) impregnation in 0.2% gold chloride for 2 min; (ii) washing in distilled water; (iii) fixation in 5% sodium thiosulfate for 2 min; (iv) washing in tap water, dehydration, and mounting.

Electron microscopy. Minced mouse brain and mouse cerebellum explants were fixed in 4% paraformaldehyde in Millonig buffer for 60 min. Mouse brain cell monolayers were scraped and centrifuged at $800 \times g$ for 10 min, and the pellets were fixed in 1% paraformaldehyde in Millonig buffer for 20 min. Thereafter, all materials were equally treated as follows: they were postfixed in 1% osmium tetroxide for 90 min; washed several times in 50% ethanol; treated with saturated uranyl acetate in 50% ethanol for 2 h; dehydrated in graded ethanol; treated with acetone; and embedded in Vestopal.

Sections were cut with glass knives, sequentially stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop I electron microscope.

RESULTS

Mice. The infective titer of mouse brain

studied by electron microscopy was $10^{7.7}$ LD₅₀/ml.

The ultrathin sections did not show any morphological differences between the brain cells of the inoculated animals and the controls. It was found that neurons and astrocytes were able to release the virus (Fig. 1 and 2). This was done through a budding mechanism similar to the one described in other substrates (11, 12, 14). In the budding zone, a thickening of the cytoplasm appeared against which dense, ribosome-like granules were attached. As the particle emerged, the cell membrane formed an envelope around them. A distinct pedicle joining the virus to the budding zone was frequently seen. Finally, the pedicle disappeared. In the neurons, the viral buds were seen in the perikarion as well as in the cell processes, including the synaptic zone (Fig. 1). Occasionally, particles arising from pericapillary neurons and astrocytes were observed (Fig. 3 and 4). At this time (10 days after infection), Junín virus development was not seen in microglial cells from the living animal. However, more research in this area is required.

The number of particles was rather small and zonal variations were prominent. In some cases, there were more virions in the peripheral zones of the brain than in the central parts; this is perhaps the result of diffusion through cerebrospinal fluid pathways along intercellular spaces.

Explants. The growth of the explants from cerebellum of 24-h-old healthy mice was, in general, satisfactory. The cells began to grow approximately 2 days after the explantation. Parallel rows of spindle cells were seen irradiating from a central nucleus; these progressively increased in size and acquired a stellate appearance in the peripheral portions. The explants grew well in a period of 8 to 10 days. Infection with Junín virus was carried out in well-adapted cells showing no degenerative signs.

Electron microscopic study revealed an elaborate development of neural tissue. Neurons, neuroglia, ependymal cells, and myelin were all present, as previously described by Ross et al. (22). In some cases, neurons were small and had a rather poor development of the granular endoplasmic reticulum. The neuronal nature was confirmed by the indentation of the nucleus, the large and well-developed Golgi apparatus, the presence of subsurface cisternae, (21) and the occasional findings of Nissl's substance in the form of onion-like corpuscles (16).

The usual interval for visualization of viral particles was in the neighborhood of 2 weeks after inoculation (Fig. 5). Neurons and astrocytes were both able to release the virus (Fig.

FIG. 1. Mouse brain 10 days after intracerebral inoculation. Two virus particles budding from the plasma membrane of a dendritic thorn (arrow). A synapse is seen at the bottom. $\times 60,000$.

FIG. 2. Mouse brain 10 days after intracerebral infection. Virus particle with a pedicle budding from the plasma membrane of an astrocytic process. $\times 60,000$.

FIG. 3. Mouse brain 8 days after intracerebral inoculation. A Junín virus particle is seen between a neuron and a capillary basal lamina. Capillary lumen (L). $\times 40,000$.

FIG. 4. Mouse brain 8 days after intracerebral infection. Junín virus particle budding from a pericapillary astrocytic process (arrow). Note the capillary basal lamina and the endothelial cell. $\times 40,000$.

FIG. 5. Mouse cerebellum explant after 27 days of growth and 13 days of Junín virus infection. Virus particles budding from several cytoplasmic processes. $\times 40,000$.

FIG. 6. Mouse cerebellum explant after 27 days of growth and 13 days of Junín virus infection. Three virus particles (arrows) near a cytoplasmic process (probably a dendrite) of a neuron. $\times 30,000$.

6 and 7) and to reproduce the images that were present in the mouse brain. Viral buds were seen up to the boundary between the explant and the nutrient clot (Fig. 7). Mature viral particles were found even inside the clot.

In the explants from previously inoculated

suckling mice, the results were variable. An outstanding fact was that the older the animal, the lower the probability of obtaining a good explant. The viability was maintained for only a few days, followed by a phase of degeneration and necrosis. So far, our most satisfactory

results have been obtained by inoculating the animals within the first 24 h of life and initiating the explants within the next 3 days. However, even under the best circumstances the viral development was poor, perhaps inferior to that obtained with the first type of explant (Fig. 8).

Monolayers. The monolayers resulted partly from cell multiplication and partly from growth of microexplants. From the latter, one could see small polygonal cells enlarging progressively towards the periphery and acquiring a spindle or stellate configuration with numerous complex prolongations. Giant cell forms were com-

FIG. 7. Mouse cerebellum explant after 27 days of growth and 13 days of Junín virus inoculation. Peripheral astrocytes in contact with the nutrient clot. Budding virus particle from the plasma membrane of an astrocyte (arrow) and several mature virus particles can be seen. Nutrient clot (C). $\times 30,000$.

FIG. 8. Mouse cerebellum explant 16 days after intracerebral inoculation. The cluster of virus particles present in the intercellular space is unusual in this type of explant. $\times 40,000$.

mon. The silver staining of straight, parallel, cytoplasmic fibrils demonstrated its astrocytic nature (Fig. 9), confirming previous studies of Boulton et al. in mice (1). The presence of astrocytes has been repeatedly shown in monolayers derived from brain tissue of different animals (2, 9, 23) with the additional development of nonclassified cells (9) and of cells of doubtful interpretation (2). By electron microscopy, the astrocytes showed a greater number of vacuoles and more rounded and swollen mitochondriae than in the explants. In addition to the astrocytes, which were the predominant elements, there were smaller cells with central nuclei formed by condensed chromatin and long, thin cytoplasmic processes packed with ribosomes (Fig. 10), reproducing the morphological characteristics of microglia cells (25).

By light microscopy, the infected monolayers did not differ from the controls, supporting without obvious alterations the viral multiplication, the same as described in Kyasanur Forest (6, 8), Langat (1, 6-8), and West Nile (8) viruses.

The maximal infective titers of the supernatants of brain monolayers infected after their development ranged between $10^{5.5}$ and $10^{6.5}$ LD₅₀/ml. These titers were observed between days 7 and 15 after the inoculation. On the other hand, in monolayers from mice inoculated by intracerebral route, the infective titers were lower ($10^{4.5}$ LD₅₀/ml), but the viral multiplication was detected at least up to day 35 of observation.

Both of the two main cell types present in the monolayers, i.e., astrocytes and microglia (fibroblasts), were able to release the virus through the budding mechanism already noted (Fig. 11 and 12). The number of particles was rather small and variable from one culture to another. In the interstitial spaces between the cells, where the thin surface folds were abundant, there was a greater chance to find virus particles than in the free, smooth surface of the cells.

The mature particle present in each of these systems (whole brain and tissue cultures) was composed of an inner core surrounded by a unit membrane (envelope). The core exhibited a clear zone with granules and a dense peripheral zone. The inner leaflet of unit membrane was masked by this dense peripheral zone, whereas the middle and outer leaflets were clearly visible (Fig. 13a). In the very thin sections, the outer surface of the particle appeared rather smooth (Fig. 13a), without the spikes present in the thicker sections (Fig. 13b).

DISCUSSION

From these results, it appears that the development of Junín virus in the mouse brain is faithfully reproduced in the cerebellum explants of the same animal. This extrapolation of data from an in vitro system to an in vivo system has been repeatedly referred to in the literature (3, 5, 9, 13, 20). Cerebellum explant has proved to be a better model than brain

FIG. 9. Mouse brain monolayer after 30 days of growth in Leighton tube. Giant astrocytes with cytoplasmic fibrils. (Silver stain) $\times 500$.

FIG. 10. Mouse brain monolayer after 29 days of growth and 7 days of *Junfn* virus infection. Microglia (or fibroblastic) cell. $\times 12,000$.

FIG. 11. Mouse brain monolayer after 29 days of growth and 15 days of *Junfn* virus infection. Several virus particles can be seen budding from the plasma membrane of an astrocyte. $\times 40,000$.

FIG. 12. Mouse brain monolayer after 29 days of growth and 15 days of Junín virus infection. Virus particles budding from the plasma membrane of a fibroblastic (or microglia) cell. Some astrocytic processes are also evident. $\times 40,000$.

FIG. 13a. Mature Junín virus particle in extracellular space near a plasma membrane. Notice the granules and the peripheral dense zone of the core surrounded by an envelope. The structural similarity between the virus envelope and the trilaminar plasma membrane is clearly seen. Mouse cerebellum explant. $\times 200,000$. (b) Junín virus particle budding from an astrocyte. In this rather thick section surface projections (spikes) are evident. Mouse cerebellum explant. $\times 200,000$.

monolayers because it not only imitates the structural complexities of the nervous tissue, but it also contains neurons and astrocytes, which are precisely the two cell types that release the virus both in the explant and in the living animal. Furthermore, the sequential stages of viral budding observed in the cells of mouse brain were repeated in the cells of cerebellum explant.

Explants of healthy animals (24 to 48 h old) infected after the cellular growth were superior to explants of animals inoculated intracerebrally. The reason of this was that in the latter, the explants were initiated 3 to 8 days after inoculation, a term rather excessive to obtain a good explant. This period of 3 to 8 days was necessary to reach the maximal infective titer in mouse brain (10).

Mouse brain monolayers did not provide additional information concerning viral development. The infective titers of supernatants were slightly lower than those obtained in mouse fibroblasts monolayers, which ranged from $10^{5.8}$ to $10^{7.7}$ LD₅₀/ml (11), and those obtained in rabbit kidney and hamster kidney monolayers, which ranged from $10^{6.9}$ to $10^{7.2}$ LD₅₀/ml (12), and no increase was detected in the number of viral particles.

In the cytoplasm of infected cells we did not

find the masses of dense granules described in other systems and interpreted as inclusion bodies (14). The budding was the only stage of viral morphogenesis present in the living animal and in the cell cultures.

Numerous particles joined to its budding locus by a pedicle were noticed. This finding suggests that the separation of the particle from the cell is a rather slow process.

The comparison of these in vivo and in vitro systems revealed that Junín virus reached higher development in living animal cells than in tissue culture cells as shown by the higher infective titers and the increased amount of virus particles. It is possible that the greater reaction of living animals is a consequence of an immunological response. In this respect, it was reported that mice infected intracerebrally with Junín virus were significantly protected by thymectomy (24) or by immunosuppressive measures (4, 15).

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