Morphological and Cytochemical Studies on Lymphocytic Choriomeningitis Virus

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Lymphocytic choriomeningitis (LCM) virus was observed by electron microscopy in thin sections of infected tissue cultures. The particles were pleomorphic and varied greatly in size. The smaller particles (50 to 200 nm) appeared to be spherical, whereas the largest (over 200 nm) were often cup-shaped. All particles contained one to eight or more electron-dense granules which were removed by ribonuclease. The particles were formed by budding from the plasma membrane and appeared to have spikes. The morphological evidence suggests that LCM should be considered as belonging to the presently unclassified group of lipoprotein-enveloped ribonucleic acid viruses.

In examining the background "noise" of viral flora of mice, one of us 10 years ago spent many fruitless hours searching for the virions of lymphocytic choriomeningitis (LCM) virus with the electron microscope. The cells of ascites tumors of mice and guinea pigs infected with LCM were carefully examined. No structures were found in the infected cell lines that were not also present in the uninfected controls. Yet, the infected cell lines when tested in susceptible mice contained what appeared to be a high titer of virus.

Recently, tissue culture systems for propagation of LCM virus have become available, as well as a fluorescent antibody test for evaluation of the number of cells infected (17). With these new techniques available, it was decided to reinvestigate the possibility of observing the LCM virion, using thin sections of infected tissue culture cells.

**Materials and Methods**

**Virus.** Three strains of LCM virus were used. The majority of studies were carried out with the Fo-2 strain of virus, recovered from a contaminated hamster tumor (8). Confirmatory studies were performed with the CA 1371 strain (a mouse brain-passaged line originally obtained from C. Armstrong) and with virus in tissues of congenitally infected mice of the colony initiated by Haas in 1940 (6).

**Tissue cultures.** Primary cultures of African green monkey kidney (AGMK) cells were maintained in Eagle’s minimal essential medium (MEM) with 2% unheated fetal calf serum. The 3T3 line of mouse cells (16), obtained from M. N. Oxman, was grown and maintained in MEM with 10% unheated fetal calf serum. All media contained penicillin (250 μg/ml) and streptomycin (250 μg/ml).

The cells were maintained at 36 C in 32-oz (946 ml) flasks or 50-mm plastic petri dishes; the latter were kept in a humidified CO₂ incubator. Cultures were infected with freshly prepared, clarified 10% extracts of infected or control mouse tissue; the virus inocula generally contained about 10⁶ mouse ID₅₀ per 0.1 ml. Flask cultures received 1.0 ml, and dish cultures 0.1 ml. Culture fluids were changed 24 hr later. AGMK cells were generally inoculated after the cell sheet was confluent and harvested 7 days later. The 3T3 cell cultures were inoculated 24 hr after planting, and were generally harvested 3 days later.

**Fluorescent antibody tests.** In many experiments, replicate cultures were grown on glass cover slips and fixed for fluorescent antibody (FA) staining to evaluate the proportion of cells infected at time of harvest for electron microscopic examination. The direct FA procedure was used as previously described (17).

**Electron microscopy.** Control and infected tissue culture cells were scraped off the culture vessel and centrifuged; the cell pellets were fixed in one of three ways. Those pellets destined for Epon-araldite embedding (9) were fixed either in 3% glutaraldehyde followed by chrome osmium (3) or in chrome osmium followed by 10% Formalin containing 0.5% uranyl acetate. Epon-araldite-embedded sections were stained with a saturated solution of uranyl acetate followed by lead citrate (13). Cell pellets destined for glycol methacrylate (GMA) embedding were fixed in 2% glutaraldehyde. Sections from this material were treated with 0.1% ribonuclease (Worthington Biochemical Corp., N.J.), or 0.1% deoxyribonuclease (Worthington Biochemical Corp.), or 0.025% Pronase (Calbiochem, Los Angeles, Calif.). The sections were then stained with 0.5% aqueous uranyl acetate at pH 4.9 followed by lead citrate. Electron micrographs were taken with a Siemens Elmiskop I fitted with an anticontamination device and an automatic shutter. A 50-μm objective aperture and 80-kv accelerating voltage were used.
RESULTS

Morphological studies. No differences between control and infected cultures could be identified in the first material examined, but it was found that only 20% of the cells were positive in the FA test. In subsequent attempts in which 50% or more of the cells exhibited fluorescence, a new type of particle was identified in association with approximately 5% of the cells examined. For some reason, particles were more readily found in material first fixed in glutaraldehyde than in material first fixed in chrome osmium. These particles were extracellular and varied greatly in size, from 50 to over 300 nm in diameter. The smaller- and medium-sized particles appeared to be spherical; the larger ones were apparently cup-shaped (Fig. 1). Occasionally, large numbers of particles were observed grouped together, apparently trapped in cell debris (Fig. 2). Other particles were present in the narrow spaces between cells (Fig. 3). The surface of the particles could, in some instances, be seen to be covered with thin spikes (Fig. 1 and 4). The covering envelope was sometimes resolved as a unit membrane (Fig. 2). Budding from the plasma membrane was observed, the plasma membrane at the site of the bud possessing spikes (Fig. 5). Occasional larger particles contained a membrane-bound vacuole (Fig. 6), and the large majority of particles of all sizes contained one or more spherical electron-dense granules 20 to 30 nm in diameter (Fig. 1–5); as many as eight of these granules were observed in a single particle on cross section. Comparable particles were never observed in uninoculated AGMK cultures or in cultures inoculated with control mouse tissues.

Fluid from a particle-positive AGMK culture was tested for the presence of contaminating murine viruses, and was found negative for Sendai, pneumonia virus of mice, Reo 3, K virus, polyoma, Théler’s virus, mouse adenovirus, minute virus of mice, and lactic dehydrogenase virus. Also, the seed stocks of the FO-2 and CA 1371 viruses were free of these agents.

Similar results were obtained with 3T3 cells; particles were not found in control cultures but were readily identified in infected cultures. They had the same morphology and variation in size (Fig. 6 and 7), and were occasionally observed in the process of budding (Fig. 8). Particles were numerous on the third day after inoculation, at which time over 90% of cells showed immuno-fluorescent-stainable antigen, and the titer of virus in the culture fluid was high (≥ 10⁵ mouse ID₅₀ per ml). On the seventh day, when the percentage of FA-positive cells and titer of virus had decreased markedly, the particles were difficult to find.

Tissues of mouse fetuses congenitally infected with LCM were also examined. No particles could be found in fetal liver, pancreas, or uterus, but a small number were found in the kidney. These particles possessed the same morphology as those found in infected tissue cultures (Fig. 9).

Cytochemical studies. Ultrathin sections of LCM-infected AGMK and 3T3 cells embedded in water-miscible GMA were subjected to digestion with 0.1% ribonuclease, 0.1% deoxyribonuclease, 0.025% Pronase, 0.025% Pronase followed by 0.1% ribonuclease, or 0.025% pronase followed by 0.1% deoxyribonuclease. Simultaneously in each experiment, consecutive sections were incubated in the corresponding diluent for the enzyme employed to provide controls for nonenzymatic extraction.

Incubation in 0.1% ribonuclease for 20 to 25 min resulted in the disappearance of the densely staining ribosomes from the cytoplasm (Fig. 10); the rest of the cytoplasm became homogeneous and moderately dense. Slightly longer digestions (30 to 40 min) brought about the removal of the fibrillar and granular components of the nucleolus. No effect was apparent on the other components of the nucleus or cytoplasm even with much longer digestions (2 hr). The dense staining granules contained within the LCM virions were unaffected during the shorter incubation times. However, when sections were exposed for 1 hr or longer to 0.1% ribonuclease, electron-lucent areas appeared in these particles in spaces previously occupied by the dense granules (Fig. 11a, b, c). The clear areas corresponded in number and distribution to those areas occupied by the dense granules within the virions observed in control sections (Fig. 12a–c). In sections incubated for 2 hr in 0.1% ribonuclease, no granule-containing particles could be found, although five 200-mesh grids with three enzyme-treated thin sections per grid were carefully examined at high magnification in the electron microscope. This result was reproducible for particles found in both infected AGMK cell cultures and in mice 3T3 cell cultures.

Exposure of thin sections to 0.1% deoxyribonuclease and to 0.025% Pronase followed by 0.1% deoxyribonuclease failed to have any effect on the ultrastructural appearance of the LCM virion (Fig. 13 and 14). The activity of the deoxyribonuclease was manifest in the striking loss of density of the nuclear chromatin (Fig. 15).

Cells in sections incubated in 0.025% Pronase alone exhibited an overall reduction in stain intensity (Fig. 16). As described previously for other proteases (15), the matrices of the mitochondria, the agranular component of the nucleo-
FIG. 1. A group of particles near the border of an AGMK cell (lower left). They vary in size from 70 to over 300 nm; the larger particles are apparently cup-shaped (arrows). Spike-like processes are evident on the surface of some particles, and many contain electron-dense granules averaging 22 nm in diameter. (All figures are of electron micrographs, and the horizontal line on each figure represents 0.1 μm.) Approximately × 70,000.
lus, and the contents of the cisternae of the ergastoplasm were the organelles most obviously affected. Although the LCM virus particles showed somewhat less contrast, the staining of their dark granules was unaffected (Fig. 17a and b). In fact, the granules often appeared more dense in contrast to the reduced intensity of the background. Preincubation of sections in Pronase before exposure

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**Fig. 2.** A group of particles apparently caught in cell debris in an AGMK tissue culture. A unit membrane may be seen on one particle (arrow). Approximately × 70,000.

**Fig. 3.** Several particles present in the interstices between two adjacent cells in an AGMK tissue culture. Approximately × 105,000.

**Fig. 4.** Several particles near the border of a cell in an AGMK tissue culture. One possesses clearly defined spikes on its surface (arrow). Approximately × 105,000.
FIG. 5. Several extracellular particles are present in an AGMK tissue culture. In addition, several buds with surface spikes are evident (arrows). Approximately × 100,000.
to 0.1% ribonuclease had no appreciable effect on the rapidity of the specific removal of the dense granules from the LCM virions (Fig. 18a and b).

DISCUSSION

To our knowledge, there has been no previous report of the electron microscopic observation of LCM virus. On the face of it, this is rather surprising, since the virus has been known and worked on for over 30 years. There appears to be an explanation for this, however. With approximately 50% of the cells of an infected culture fluorescing, virions were found associated with only approximately 5% of the cells. On this basis, if only 5 or 10% of the cells fluoresced, the chances of finding particles would be small. Also, the likelihood of identification if seen would be negligible. In infected mice, the previous source of tissue for electron microscopic studies, the proportion of cells infected is generally relatively small (17).

The fact that the particles are primarily extracellular in position and the fluorescence is cytoplasmic suggests that the staining is not of com-

FIG. 6. Particles in an extracellular position near the border of a cell in a mouse fibroblast culture (3T3) infected with LCM. One particle contains a vacuole (arrow). Approximately × 70,000.

FIG. 7. Extracellular particles in a 3T3 culture infected with LCM. In size range and morphology, the particles in this figure and in figure 6 are identical to those in the other figures. Approximately × 70,000.

FIG. 8. Particles in a 3T3 culture infected with LCM. One particle is budding (arrow), and spikes are evident on the surface of the other. Approximately × 70,000.

FIG. 9. Two particles are present between kidney cells of a mouse fetus congenitally infected with LCM. The particles are identical to those in the other figures. Approximately × 70,000.
FIG. 10. A thin section of an LCM-infected African green monkey kidney cell dehydrated and embedded in water-miscible GMA and floated on 0.1% ribonuclease at pH 6.6 and 37 C for 20 min. The dense staining ribonucleoprotein particles have been removed from the cytoplasm. The cytoplasm has become moderately electron-dense; the mitochondrial cristae, membranes of the nuclear envelope, and endoplasm reticulum appear in negative contrast due to the absence of OsO₄ in the fixation procedure. Approximately × 24,500.
FIG. 11. (a-c). LCM virus particles are seen lying in the extracellular space in sections incubated with 0.1% ribonuclease for (a) 30 min, (b) 60 min, and (c) 2 hr, respectively. Electron-dense granules are readily apparent in the virion pictured in 11(a), although the ribosomes were completely removed from the cytoplasm. In Fig. 11(b), the virion contains one dense granule; several clear areas may be seen in which other granules were presumed present before incubation (arrows). Particle (11c) after 2 hr of exposure to ribonuclease contains no granules; granules could not be found in any of the particles contained in any of the sections treated for 2 hr with ribonuclease.

FIG. 12. (a-c). These micrographs show LCM virions in sections incubated in distilled water (pH 6.6) at 37°C for 60 min, 90 min, and 2 hr, respectively. The granules in the virions and the ribosomes in the cytoplasm are unaffected. Figures 11 and 12, approximately × 70,000.
Fig. 13 and 14. LCM particles in GMA-embedded thin sections exposed to 0.1% deoxyribonuclease for 1 hr and 2 hr at 37 C, respectively. Deoxyribonuclease hydrolysis had no effect on the staining of the intravirion granules. The particles in Fig. 13 which apparently lack dense granules are not a result of the digestion procedure, but are commonly found in ultrathin sections. This result is presumed to be produced by the plane of section which may exclude the part of the virion containing the granules. Approximately × 70,500.
FIG. 15. Low-power micrograph of cells incubated with 0.025C Pronase for 15 min followed by 0.1C deoxyribonuclease for 60 min. The deoxyribonuclease has completely removed the staining of the normally dense nuclear chromatin proximal to the nuclear envelope. Approximately × 24,500.
FIG. 16. Part of a GMA-embedded AGMK cell that has been hydrolyzed for 60 min with 0.025% Pronase at 37 C. An overall reduction in the density of the nuclear sap and cytoplasm has occurred. The formerly dense matrices of the mitochondria (M) are the structures most prominently affected. The ribosomes remain densely stained. Approximately × 24,500.
FIG. 17. (a and b). Particles following digestion with 0.025% Pronase at 37 C for 1 hr and 2 hr, respectively. The dense granules are unaltered; in Fig. 17b (arrow), they appear more dense in contrast to the reduced electron opacity of the background following protease hydrolysis. Approximately \( \times 70,500 \).

FIG. 18. (a and b). LCM particles (arrows) are seen in the extracellular space in sections treated with 0.025% Pronase for 1 hr followed by 0.1% ribonuclease for 30 min. Their granules are intact and densely stained, although all the ribosomal staining was removed after only 5 to 10 min of exposure to ribonuclease. Approximately \( \times 70,500 \).
pleted virions, but may be the soluble antigen. In any case, however, there is a correlation between the percentage of fluorescent cells and the ease with which particles can be found.

The pleomorphism and wide variation in size of the virions were not expected. Evidence from filtration and centrifugation experiments indicated a particle size of approximately 40 to 60 nm (11, 12, and 14). In this study, many particles were observed that were in the 60-nm-size range, but the majority were considerably larger. This does not represent a real discrepancy with the earlier estimates of size, since the biophysical procedures would have measured only the smallest particles. Also, it is conceivable that in the biophysical separations only the particles 60 nm or less in diameter retained their infectivity, whereas the larger particles might have been damaged.

All of the evidence at hand suggests that this virus replicates in the cytoplasm and buds from the plasma membrane, utilizing the modified plasma membrane as an outer envelope.

In a preliminary report (2), it was pointed out that the larger particles bear a superficial resemblance to mycoplasma. This is true, but the two are distinguishable from one another on the basis of the larger size of mycoplasma, the absence of deoxyribonucleic acid strands in the LCM virions, and the presence of spikes on their surface. Mycoplasma particles possess relatively smooth surfaces. Attempts to cultivate mycoplasma from the inocula and infected cultures were consistently negative.

In this study, the chemical nature of the LCM virion was investigated with respect to its staining properties and structural appearance in thin sections subjected to a number of specific enzymatic digestions. The results indicated good specificity of enzymatic activity with regard to target components within the sections. For example, ribonuclease digestion resulted only in the loss of ribosomal staining or the ribonucleic acid-containing structures in the nucleus. Incubation with Pronase or deoxyribonuclease had no effect on these structures. The reliability and reproducibility of this technique have been well-documented (1, 4, 5, 7, 15).

The dense granules contained within the LCM virus were susceptible to hydrolysis by ribonuclease but not by deoxyribonuclease. This phenomenon indicates that their strong affinity for lead staining is due to the presence of ribonucleic acid (RNA). However, the granules were more resistant to ribonuclease action than the cytoplasmic ribosomes or nucleolar structures. Exposure to ribonuclease, two to three times longer than that required for ribosomal degradation, was needed to completely disrupt their structural integrity. Several possibilities may be suggested to explain this phenomenon. First, these dense structures lie within the small membrane-bound virus particle and may not be directly exposed to the enzyme during flotation of the section. Second, the granules may contain RNA in conjunction with a protein which may impede the rapidity or efficacy of enzymatic hydrolysis. Third, these dense bodies may contain RNA structurally distinct from that in the ribosomes, requiring longer hydrolysis to eliminate its affinity for the electron-dense lead stain.

The first explanation is unlikely, since in all the sections the granules in LCM virions were susceptible to degradation by exposure to ribonuclease alone. In an attempt to distinguish between the two other possibilities, sections were preincubated with Pronase before hydrolysis with ribonuclease or deoxyribonuclease. This procedure had no significant effect in reducing the length of the incubation time required to remove the dense staining granules. This outcome suggests that the RNA in these granules is not intimately associated with protein. Ribosomal staining in sections pretreated with Pronase was lost more rapidly upon incubation with ribonuclease (5 to 10 min). These results infer that the dense granules within the LCM virion are not simply ribosomes which happen to be incorporated into the body of the virus indiscriminately during the budding process. Therefore, the most plausible explanation is that a physicochemical difference exists between the RNA in the LCM granules and that in the ribosome, which is reflected in the increased resistance of the former to ribonuclease under these experimental conditions. It is tempting to assume that these granules represent the viral genetic material; if this is the case, the presence of multiple granules would suggest that many of the virions may carry more than one copy of the genome.

The morphological and cytochemical data presented here, together with the previously known properties of the virus, such as its chloroform sensitivity, elaboration of soluble antigen, and resistance to IUDR (10), indicate that LCM virus falls into the presently unclassified category of lipoprotein-enveloped RNA viruses such as the avian and murine leukoses, mouse hepatitis, and other viruses. Studies using the negative staining procedure are in progress to attempt to clarify the structure of the internal components and the surface structure of the particle.

**Literature Cited**


