Application of Freeze-Etching Method to the Study of Reovirus-Infected LLC-MK₂ Cells

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A continuous cell line of rhesus monkey kidney cells, LLC-MK₂, was infected with reovirus type 1 (Lang). The cells were freeze-etched as a method for observing the structural details of the reovirus-induced cytoplasmic inclusion. Information on maturation may be obtained by preparing infected cells for electron microscopy by freeze-etching.

Increasing use has recently been made of freeze-etching preparative techniques (1, 3) for the electron microscopic analysis of cell structure. This method is inherently free from the chemical and dehydration artifacts of other preparative techniques and should be expected to add additional worthwhile information to our understanding of the evolving structural changes occurring in virus-infected cells. Consequently, we report here the use of freeze-etching as a method for observing the structural details of the reovirus-induced cytoplasmic inclusion in LLC-MK₂ cells.

MATERIALS AND METHODS

LLC-MK₂ cells, a continuous cell line of rhesus monkey kidney cells, were grown in monolayer culture in Eagle's minimum essential medium (MEM) with 2% inactivated fetal calf serum. These cultures, in Blake bottles containing from 1.5 × 10⁶ to 2.0 × 10⁶ cells, were washed three times in MEM and infected at a multiplicity of approximately 3 with reovirus type 1 (Lang). Cultures of the same age on cover slips were similarly infected. Control cultures in Blake bottles and on cover slips were inoculated with a volume of MEM equivalent to the virus inoculum.

The reovirus inoculum was allowed to absorb for 1 hr at 37 C, and then the medium was replaced in both infected and control cultures.

Thirty-six hr later the Blake bottle cultures, containing infected and control cells, were harvested by scraping with a rubber policeman. Each cell suspension was divided into two samples, and these were collected as a centrifugal deposit and processed for electron microscopy.

For thin-sectioning examination, the cells in one sample were fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, and, after dehydration in an ethanol series, embedded in a mixture of Epon and Araldite. Staining was achieved by including uranyl acetate in the 70% ethanol during dehydration and by treatment of the sections with lead citrate.

The cells in another sample were slowly infiltrated with 40% glycerol and then rapidly frozen with liquid Freon in preparation for freeze-etching. This procedure was performed by using Balzers system (2), with an etching time of 60 sec at a temperature of −100 C and a knife temperature of less than −150 C. The fractured specimen was shadowed with platinum-carbon and the replica freed from the specimen by floating on Javel water.

Specimens for electron microscopy were examined with a Philips EM300 with Kodak fine-grain positive film.

Cover slip cultures, both infected and control, were fixed for 30 min in methanol-acetone-phosphate-buffered saline (5:3:2) and stained with 0.007% coriphosphene orange in McIlvaine's buffer (pH 5.8). This stain reacts with nucleic acids in the same manner as the more popular acridine orange. Microscopic observations were made with a Zeiss Ultraphot II by using an HBO ultraviolet light source.

RESULTS

Figure 1 contains photomicrographs of cells in the coriphosphene orange-stained infected monolayer culture (Fig. 1A) and of the uranyl acetate-stained thin section preparation of similar cells (Fig. 1B). The virus inclusion is usually clearly demarcated in the perinuclear region of the cytoplasm and stains light green, as is typical of double-stranded nucleic acids. In addition, virions within the inclusion are marshalled in pseudo-crystalline arrays.

Figure 2 depicts a replica of a single infected LLC-MK₂ cell prepared for electron microscopy by the freeze-etching technique. It can be observed that the plane of cleavage has passed through both nucleus and cytoplasmic inclusion. The inclusion, which in this cell surrounds the
FIG. 1. (A) Photomicrograph of reovirus type 1 (Lang)-infected LLC-MK² cells stained with coriphosphene orange and observed by ultraviolet light microscopy. X 720. (B) Electron photomicrograph of a thin-sectioned LLC-MK² cell infected with reovirus type 1 (Lang) and stained with uranyl acetate (magnification 18,150X).
Fig. 2. Electron photomicrograph of a replica from a freeze-etched specimen of reovirus type I (Lang)-infected LLC-MK2 cells. × 12,100. Insert is an enlargement of the indicated area. × 104,000.
nucleus, appears set apart from it by an area of finely granular cytoplasm. The body of the inclusion is seen to contain a large number of spherical particles. The insert of Fig. 2 shows an enlargement of one of the areas containing these particles, and they are comparable in size to mature reovirus virions.

Preparations of control cells observed by these techniques revealed only typical, normal LCC-MK₂ cell morphology.

**DISCUSSION**

Although the assembly of reovirus in a cytoplasmic inclusion has been observed previously by the technique of thin-section (4), additional information on maturation may be obtained by preparing infected cells for electron microscopy by freeze-etching. It is apparent that the resolution of the method is sufficient so that useful data dealing with the organization of the inclusion may be obtained, free from drying and fixation artifacts associated with other techniques, and should not be overlooked by virologists interested in ultrastructure. Additional data dealing with the fine structure of the inclusion and the virus particles contained therein are being prepared for a subsequent publication.

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**LITERATURE CITED**

1. Bachi, T., W. Gerhard, J. Lindenmann, and K. Mühlethaler. 1969. Morphogenesis of influenza A virus in Ehrlich ascites tumor cells as revealed by thin-sectioning and freeze-etching. J. Virology 4:769–776.
2. Moor, H., and K. Mühlethaler. 1963. Fine structure in frozen etched yeast cells. J. Cell Biol. 17:609–628.
3. Steere, R. L. 1957. Electron microscopy of structural detail in frozen biological specimens. J. Biophys. Cytol. 3:45–57.
4. Tournier, P., and M. Plissier. 1963. Le développement intracellulaire du reovirus. La Presse Med. 68:683–688.