Electron Microscopy of Monkey Kidney Cell Cultures Infected with Rubella Virus

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Two rubella virus strains isolated in this laboratory were investigated in terms of their growth in LLC-MK₂ cell cultures and their effect on cell morphology. Rubella virus grew readily in LLC-MK₂ cells, but cytopathic effects of the virus were not observed in infected cultures. Such infected cultures can be subcultured indefinitely and continue to shed virus. Examination of rubella-infected cell cultures by electron microscopy showed the presence of annulate lamellae in the cytoplasm of 15% of the cells. No changes were evident in the nuclei. These membranous inclusions varied in complexity from parallel arrays of annulate lamellae to large lamellar structures of complex morphology. An occasional cell contained a crystal lattice structure in association with the lamellae. Larger inclusions, consisting of disorganized arrays of "unit" membranes, were also found. Uninfected cells were devoid of annulate lamellae, crystals, and complex membranous inclusions. No viruslike particles were observed in any part of the cells from infected cultures. The significance of the structures observed has not been determined.

Methods for the cultivation of rubella virus in cell culture have been well established (19, 20). The course of events following rubella virus infection of cell cultures (22, 29, 30) and the occurrence of naturally infected carrier cultures (9, 23) have been described. Light microscopy of rubella virus-infected tissue culture cells by use of fixed and stained preparations (28, 30) or by techniques employing immunofluorescence (2, 31) indicate the presence of virus material in the cytoplasm without involvement of the nuclei of the cells. Cytological investigation by electron microscopy has been restricted to examination of cell-free extracts and partially purified preparations of virus (16, 21). A viruslike particle has recently been reported to be present in rubella-infected material after concentration on a sucrose density gradient (16).

The work presented here describes the changes in morphology and the structures observed in rubella-infected LLC-MK₂ cells by electron microscopy of ultrathin sections.

MATERIALS AND METHODS

Cell cultures. Stock LLC-MK₂ (continuous rhesus monkey kidney) cells (obtained from R. Hull, Eli Lilly & Co.) were maintained in Eagle's minimal essential medium (MEM) containing 10% hypogamma calf serum, penicillin (100 µg/ml), streptomycin (100 µg/ml), and nystatin (10 µg/ml). Cultures for virus infection were prepared by trypsinizing stock

cultures with 0.05% trypsin (Difco) and seeding 200-ml dilution bottles with about 2×10^6 cells in MEM-10% hypogamma calf serum. The cultures were used after 4 to 5 days, when the cell sheet was complete.

Rubella virus. Two rubella virus strains isolated in this laboratory were selected for this study. One strain was isolated in African green monkey kidney cells and subsequently passaged in LLC-MK₂ cells. Both strains were neutralized by rabbit anti-M33 antibody (kindly supplied by P. D. Parkman of the National Institutes of Health, Bethesda, Md.). In the present experiments, both viruses were used after their eighth cell culture passage.

LLC-MK₂ cell cultures in 200-ml dilution bottles were infected by the addition of MEM-2% hypogamma calf serum containing 100 interfering doses of rubella virus per ml. The cultures were incubated at 36°C, the medium was changed after 4 days, and the cells were harvested at 6 and 9 days after infection. Uninfected control cultures were run simultaneously with all test cultures.

Electron microscopy. LLC-MK₂ cells from both infected and control cultures were freed from the surface of the glass by use of a rubber spatula and suspended in Hanks' balanced salt solution. To each of the suspensions was added an amount of 2% osmium tetroxide in *s*-collidine buffer (pH 7.4) to give a final osmium tetroxide concentration of 1%. After fixation for 1 hr at room temperature, the cells were centrifuged for 5 min at 800 rev/min, and the pellets were resuspended in fresh *s*-collidine buffer and recentrifuged. Each pellet was then gently resuspended in a small amount of molten, 2% agar (Difco bacterio-

FIG. 1. Ultrathin section of LLC-MK₂ control cells harvested at 6 days. N, nucleus. $\times 8,000$.

FIG. 2. LLC-MK₂ cells harvested 6 days postinfection. L = lipid body; D = desmosome. Arrays of annulate lamellae (arrow) are seen lying below the two lipid bodies. $\times 10,000$.

logical) and set in a cylindrical mold. The agar pieces were sequentially dehydrated, infiltrated, and embedded in Epon 812, according to the method of Luft (15). The infiltration time in the resin-propylene oxide mixture was extended to 5 hr.

Favorable cell concentrations in the Epon blocks were located by examining large, 1- μ sections stained with azure II methylene blue (25). Ultrathin sections were cut from the retrimmed Epon blocks, stained for 2 hr in a 3% aqueous solution of uranyl acetate, and observed with an RCA EMU-3G electron microscope.

RESULTS

LLC-MK₂ cells are epithelioid cells containing a large nucleus with a well-defined nucleolus. The cytoplasm of the cell contains numerous mitochondria, an occasional lipid body, and an occasional inclusion of undetermined nature (Fig. 1). No unusual features were noted in either the cytoplasm or nucleus of the uninfected cell. It was not uncommon in ultrathin sections of both control and infected cells to find desmosomes between adjacent cells, bridging the plasma membranes (Fig. 2).

Rubella-infected LLC-MK₂ cell cultures showed no changes in nuclear morphology, and no unusual structures appeared in the nucleus of the cells (Fig. 2). In the cytoplasm, however, of 15% of the cells from infected cultures at 6 and 9 days postinfection, annulate lamellae were present (Fig. 2 and 3). These cytoplasmic inclusions varied in complexity from parallel arrays of annulate lamellae to lamellar structures of complex morphology (Fig. 5 and 6).

In longitudinal section, the annulate lamellae consisted of parallel arrays of cisternae bounded by smooth membranes (Fig. 3). In the plane of section, the cisternae showed intermittently at regular intervals of 1,000 Å; in other planes, the membranes were parallel and continuous (arrows). The distance between rows of cisternae was also 1,000 Å. In the more oblique planes of sectioned cells, both partial longitudinal sections and partial cross sections were seen, as shown in Fig. 4 (arrows). In some cases, the ends of the smooth lamella membranes were observed to have attached ribosomes (Fig. 3a). Ribosomes, however, were not observed along other portions of the lamellar membrane or in the spaces between lamellae.

The position of the lamellar complex in the cytoplasm varied, but lamellar structures were never found in the nuclei of the cells, nor was there any morphological evidence for the presence of lamellae in the cytoplasm of the control cells.

The markedly more complex arrays of lamellae were seen in the cytoplasm of 10% of the cells from infected cultures harvested on the 9th day

postinfection (Fig. 5 and 6). At this time, maximal viral titers were present in the supernatant fluids. These complex arrays of lamellae formed organelles often measuring 5.0 by 3.0 μ . In Fig. 5, three distinct areas in cross section are seen radiating out in a fairly uniform circular manner. Measurements of a part of such a cross-sectional area at higher magnification (Fig. 7) indicate the diameter of these forms to be 1,000 Å, with an inner core of 600 Å. In a narrow peripheral band, 200 Å wide, are a number of equally spaced microcylinders. They are more evident in some areas than others (arrows). It was estimated that such a peripheral space could accommodate about 10 microcylinders. Another complex array of lamellae cut largely in a longitudinal plane is shown in Fig. 6. It appears to be composed of a series of plates of annulate lamellae in regular arrangement with a single small area in cross section lying exposed below (arrow). The individual plates were observed to have regular periodic constrictions at intervals of 1,000 Å, and the distance between plates was about 700 Å. These organelles, like the less complex arrays of annulate lamellae, were found neither in a special position nor in consistent association with any other component in the cytoplasm of the cells.

In addition to the annulate lamellae, the cytoplasm of an occasional cell from infected cultures contained a crystal lattice structure close to areas containing lamellae. In most cases, connections between the lattice structure and the lamellae were evident, as shown in Fig. 8 and 9. A high-magnification photograph of the lattice spacing is shown in Fig. 10. The distance between lattice centers was approximately 270 Å. The nature of the crystalline material is undetermined.

Finally, the cytoplasm of some cells contained large inclusions, often 5 by 2 μ in size, consisting of disorganized arrays of "unit" membranes. The inclusion as a whole was not bounded by a membrane (Fig. 11), and no obvious connections between it and the nucleus or components of the cytoplasm were seen.

Large lipid bodies (Fig. 2 and 11) containing numerous extremely electron-dense particles were also found, and were equally as numerous in the cytoplasm of control cells as in infected cells. In appropriate sections, individual lipid bodies were seen to be bounded by a unit membrane.

DISCUSSION

Our findings indicate that, associated with rubella virus infection of LLC-MK₂ cells, several types of inclusions are formed in the cytoplasm of the cells. No changes in the nuclei of the cells were observed. The findings are consistent with

FIG. 3. Portion of an infected cell showing two separate parallel arrays of annulate lamellae. In one area (arrows) the lamellae are parallel and continuous, whereas in other areas the lamellae have the appearance of cisternae formed at regular intervals. $\times 33,000$.

FIG. 3a. Area of an infected cell where the ends of the smooth annulate lamellar membranes have attached ribosomes (arrow). $\times 72,500$.

FIG. 4. Portion of an infected cell showing annulate lamellae in partial longitudinal section and cross section lying adjacent to the nucleus. $\times 40,500$.

FIG. 5. Complex array of lamellae in the cytoplasm of an infected cell harvested after 9 days. Three areas of annulate lamellae in cross section are seen, and, at the periphery of these areas, lamellae cut obliquely are found radiating out in a circular manner. $\times 45,000$.

FIG. 6. Portion of an infected cell showing an array of lamellae cut largely in a longitudinal plane with one area (arrow) showing cross-sectional morphology. $\times 54,000$.

FIG. 7. Enlarged area of annulate lamellae cut in cross section. Individual circular units show evidence of microcylinders equally spaced around the periphery (arrows). $\times 109,000$.

FIG. 8 and 9. Examples of infected cells containing cytoplasmic inclusions in the form of crystal lattice structures. Annulate lamellae are also present and show evidence of possible connections (arrows) with the lattice components. In Fig. 9, the annulate lamellae are seen in cross section. $\times 54,000$.

FIG. 10. Enlarged portion of the lattice structure shown in Fig. 8. $\times 127,000$.

FIG. 11. Area of an infected cell containing a large membranous inclusion (I) adjacent to two large lipid bodies (L). Part of the nucleus (N) of the cell is seen (upper left). $\times 13,000$.

the observation that specific immunofluorescence (2, 31) is restricted to the cytoplasm and that cytoplasmic but not nuclear inclusions (28, 30) are seen in rubella virus-infected cells in culture.

The two most prominent changes observed in the infected cells were the appearance of disorganized arrays of "unit" membranes (Fig. 11) and annulate lamellae of varying complexity. It is reasonable to assume that the lamellar structures are induced by infection with rubella virus, since they are not present in uninfected cell cultures. Although the appearance of a variety of membranous structures in tissue culture cells as a result of virus infection has been reported (1, 4, 5, 10, 12), the appearance of annulate lamellae is unusual. The laminated structures observed in tissue culture cells infected with herpes virus (18), adenoviruses (10, 19), and other viruses (1, 4, 12) do not appear to be of the type reported here. The possible exception is the complex laminated structure observed by Lapis (14), in the cytoplasm of KB cells infected with adenovirus 12.

It is interesting to note that annulate lamellae have been described for a variety of cells (3, 7, 8, 11, 26) and, in particular, are quite frequent in invertebrate eggs (24, 27). The lamellae reported in HeLa cells (8) and the mouse ascites tumors (3) are not as complex as those seen in rubella virus-infected cells. Although the origin and function of the lamellar structures have not been determined, it has been suggested, because of their high content of ribonucleic acid, that they are involved in protein synthesis (27).

The significance of the structures observed in rubella virus-infected LLC-MK₂ cells in the present study is also undetermined. It is difficult to make an interpretation solely on observations with the electron microscope (4, 17), and the interpretations here must be considered tentative. It is conceivable that the structures observed are involved in the synthesis of viral material, since they are observed only in the cells from rubella virus-infected cultures. The intimate association of the crystalline material with the annulate lamellae is suggestive of this function. The interpretation is consistent with the suggestion that annulate lamellae are involved in protein synthesis (26); however, that the crystalline material observed is viral material has not been established and, in fact, viruslike particles were not observed in association with the cytoplasmic inclusions or in any other part of the cells from infected cultures. That rubella virus was present and was capable of replication was demonstrated by a 3-log increase of virus in the supernatant fluids of infected cultures at the time of harvesting.

An alternative interpretation is that the observed structures may reflect a reaction of the

cells to viral infection rather than an active involvement of the structure in the synthesis of viral material. Both production of interferon (28) and cytopathic effects (28) have been reported to follow rubella virus infection of LLC-MK₂ cells. Certain chemicals and their toxic effects have also been reported to induce various membranous structures in cells growing in tissue culture (6, 13).

However, in this laboratory no cytopathic effects of LLC-MK₂ cells were observed with the rubella virus strains used; rubella virus-infected LLC-MK₂ cultures could be subcultured indefinitely, and the cells continued to shed virus. Other rubella-carrier cultures of LLC-MK₂ cells have been reported by Brown et al. (2).

Although it is reasonable to assume that the cytoplasmic structures observed are induced by infection with rubella virus, their significance and function still remain to be established. In this respect, ferritin-labeled antibody to the virus may offer a means to determine the extent of the involvement of the lamellated structure in viral synthesis. Such a label may also reveal viral precursors in the cytoplasm or sites of virus aggregation.

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