ELECTRON MICROSCOPIC STUDY
OF THE CYTOPATHOLOGY OF ECHO VIRUS
INFECTION IN CULTIVATED CELLS

O. NÚÑEZ-MONTIEL, M.D., J. WEIBEL, and J. VITELLI-FLORES

From the Laboratorio de Estudio de la Gastroenteritis, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela

ABSTRACT

The cultivated monkey kidney cell is subject to changes when infected with ECHO viruses 6, 9, and 19. The electron microscope reveals three stages of infection: (a) initial stage. The nucleus appears granular with chromatin condensation on the nuclear envelope. The cytoplasm contains electron transparent vesicles and vacuoles forming nests. (b) Intermediate stage. The nucleus seems to diminish, appearing more pyknotic and displaced toward the periphery. The cytoplasm is filled with electron transparent vacuoles and vesicles, and dense masses as well as some spiral bodies are seen. The mitochondria retain their shape. Dense particles are seen, which are possibly of viral nature. (c) Final stage. The nucleus is contracted to a narrow strip close to the cellular membrane or is completely destroyed. The cytoplasm shows no apparent changes. Crystals are frequently observed in cells infected with ECHO viruses 6 and 19, consisting of dense particles with an average diameter of 14.4 mμ ranging from approximately 13.2 to 15.6 mμ for ECHO virus 6, and 14.5 mμ ranging from approximately 12.5 to 16.5 mμ for ECHO virus 19. These particles are clustered in hexagonal packages forming angles of 75° and 105°. The particles in most crystals are arranged in rows separated by a constant distance, the latter varying from one crystal to another and being approximately 1.5 and 2.5 times the distance between particles. Other particles were observed which, however, are not considered to be of viral nature.

As a part of a general study of electron microscopic changes induced in tissue cultures by enterovirus infection, the alterations in cultivated monkey kidney (MK) cells infected with three types of ECHO virus are here presented.

The changes induced in cultivated kidney cells by polioviruses have been studied previously, by both electron (1) and light (2, 3) microscopy. There are reports on the findings of crystals in polioviruses (4, 5), Coxsackie virus (6), ECHO virus 19 (a preliminary communication by us, reference 7), and recently also a presumably ECHO virus (8) and ECHO virus 9 (9).

MATERIAL AND METHODS

Tissue Cultures: Kidney cells of Macaca mulatta Zimmermann (1780) and M. irus Cuvier (1818) were prepared by trypsinization, following the technique described by Bodian (10). The cells were grown in 103 medium.

Stock Viruses: ECHO virus 6, D'Amori strain; ECHO virus 9, HEV-3 strain; and ECHO virus 19, Burke strain.

1 According to Hartmann and Strauss, we are dealing with Macaca mulatta Zimmermann (1780) and M. irus Cuvier (1818) rather than the species erroneously listed in our previous publication (7).
Procedures: Cells were massively infected and samples collected in diverse stages of infection. The first samples were taken when initial signs of infection appeared in some cells, which generally occurred 4 to 5 hours after inoculation. The last samples were collected when pathologic signs appeared and many cells had become detached from the glass, usually within 48 hours.

The material was fixed with a buffered 1 per cent osmium tetroxide solution (pH 7.3) made isotonic with sucrose. Fixation time was 1 hour or more. The material was embedded in methacrylate and sectioned by means of a diamond knife in a Fernández-Morán type ultramicrotome (11). Observations were made under a Siemens Elmiskop I electron microscope.

Observations

Normal Cell

Fig. 1 shows a normal MK cell used as a control. The nucleoli are small and clear cut. The nuclear envelope is normal. Mitochondria of various sizes and shapes are seen in the cytoplasm. Structures resembling the Golgi complex are sometimes visible, occupying mostly a small zone formed by vesicles and vacuoles. Fat droplets are frequently seen.

Cytopathological Changes

In the infected cells three stages of infection can be distinguished.

Initial Stage: Early in the infection, the nuclei become more granular, tending to condense at the periphery. The nucleoli appear larger. No reduction in nuclear volume or changes in nuclear envelope are evident at this stage (Fig. 2).

The cytoplasm shows few alterations. Groups of electron transparent vesicles and vacuoles of various sizes and densities are more numerous than in normal cells. Occasionally the vesicles appear elongated as they do in the Golgi complex. The mitochondria appear to be normal (Fig. 2).

Intermediate Stage: Flattened and displaced nuclei are located at the periphery. Their volume decreases and the membranes of the nuclear envelope appear to be pulled apart, forming lacunar spaces. A remarkable condensation of the chromatin can be seen on the nuclear envelope. The nucleolus is enlarged (Figs. 3, 4, and 7).

The cytoplasm becomes filled with electron transparent vacuoles and vesicles, which sometimes form a central mass in association with normal and abnormal organelles (Figs. 3 and 4). Vacuolization commences at foci (Fig. 2) and increases gradually, eventually encompassing the whole cell. The vesicles are usually difficult to detect with precision because they are small and are scattered among electron transparent vacuoles (Figs. 3 and 4).

Dense masses of varying size, as well as spiral bodies, dispersed in the cytoplasm, which do not appear during the initial stage, are also seen at this period (Figs. 3 to 7). A spiral body is apparently a cytoplasmic mass which is isolated in a cavity and undergoes spiraling but which remains connected to the rest of the cytoplasm by a bridge. These bodies are devoid of organelles (Figs. 3 to 7).

The mitochondria appear to be generally normal, except for some swelling and rupture of a few cristae, even during late stages of cellular disintegration (Figs. 3, 4, 7, and 10).

During the intermediate stage, groups of particles or crystals in process of formation appear in the cytoplasm (Figs. 6, 7, and 10).

Advanced Stage: The nuclei are destroyed by karyolysis, or are reduced to a very dense mass close to the cellular membrane. Cells shrink to

Figure 1

Normal monkey kidney cell. In the center is a nucleus (N) with a small nucleolus (n). Dispersed mitochondria (M) and a few small vesicles and vacuoles are visible. X 12,000.

Figure 2

Initial stage of a cell infected with ECHO virus type 19. The nucleus (N) presents a slightly dense chromatin with a tendency to condense under the nuclear envelope, which appears normal. Mitochondria (M) can be seen in the cytoplasm, as well as an accumulation of vesicles and a few electron transparent vacuoles (Vp). The size of the vesicles is variable; some vesicles are denser than others. X 59,000.
rounded masses, retaining no structure at all. Most of the crystals are seen during this stage (Figs. 8, 9, and 11). The progressive degeneration of the cytoplasm is manifested by rupture of the mitochondrial cristae and disappearance of vesicles, masses, fat droplets, and spiral bodies (Fig. 8).

**Particles**

Three kinds of particles may be found in the cytoplasm of cells infected with ECHO viruses, the most specific being of uniform size and organized into crystals. These particles were seen in cells infected with ECHO viruses 6 and 19 (7), but not in cells infected with ECHO virus 9. Each rounded or elongated particle has an osmiophilic central dense mass, the average size of which for ECHO virus 6 is 14.4 μm ranging from approximately 13.2 to 15.6 μm, and for ECHO virus 19 is 14.5 μm ranging from approximately 12.5 to 16.5 μm (Figs. 6 to 11). These particles are surrounded by an electron lucid zone. The distance between particles ranges from 20 to 30 μm, being constant in each crystal, but varying from crystal to crystal in both ECHO viruses 6 and 19.

Careful observations of crystals having similar densities, formed by equidistant particles, showed them to be clustered into hexagonal packages forming angles of 75° and 105°, never angles of 90°. The particles in most crystals are arranged in rows separated by a constant distance, the latter varying from one crystal to another. This distance was found to be approximately 1.5 times (Figs. 7, 8, and 11) and 2.5 times (Fig. 10) the distance between particles. Occasionally some crystals were observed among disintegrated cells, in association with debris (Figs. 9 and 11).

There are two other kinds of particles, one of which is smaller and the other larger than the virus particles. The smaller of these is about 10 μm or less in diameter and is found in clusters, but never forms crystals (Fig. 7). The larger particle, which is very rarely seen, varies in size from 20 to 50 μm. It has a rounded or elongated shape (Fig. 10) with very clear borders, is quite dense, and usually occurs singly.

**DISCUSSION**

The nucleus is apparently the first part of the cell to respond to the viral attack. Most probably the nucleoprotein which forms the virus within the cytoplasm comes from the nucleus (12). Maassab and Ackermann (13), who studied the metabolism of HeLa cells infected with polioviruses, found that the nuclear RNA increases during the first hours and decreases thereafter, while the RNA in the cytoplasm begins to increase rapidly during the second hour after infection and shows a marked accumulation between the fifth and the twelfth hour. No particle was ever observed inside the nucleus, contrary to the observations reported in certain other viruses (14-20).

The significance of the spiral bodies is at present obscure. A lamellar structure similar to the spiral bodies has been described by Adams and Prince (21), in the cytoplasm of Ehrlich ascites tumor cells infected with Newcastle disease viruses. Thus, the spiral bodies may be related to the defense mechanism of the cell.

The early appearance of electron transparent vesicles and vacuoles in the infected cells is of interest. Although these structures do not have the typical form of the Golgi complex, we believe them to be of the same nature. These structures are reminiscent of those observed in normal tissue (22-24). In experiments with other viruses—Reovirus (25) and VEE virus (26)—similar changes have been noted.

During the infectious process, there appears to be a hyperproduction of vesicles in the Golgi complex (27). Changes in the Golgi zone have

---

**Figure 3**

Intermediate stage of a cell infected with ECHO virus type 6. The nucleus (N) is pushed toward the periphery. The chromatin is condensed under the nuclear envelope. Apparently the nucleus has begun to diminish in volume, leaving empty spaces between the membranes of the nuclear envelope (+). The cytoplasm is filled with a central mass of electron transparent vacuoles (Ve) of different sizes and shapes. Vesicles (Ve), five spirals (S), and some dense masses (DM) are seen. Normal mitochondria (M) lie between them. × 43,000.
FIGURE 6
Intermediate stage of a cell infected with ECHO virus type 6. A bridge of a spiral (S) can be seen touching a viral crystal (C). The particles are equidistant. Mitochondria (M) and electron transparent vacuoles (Va) are also visible. X 79,000.

FIGURE 4
Intermediate stage of a distorted cell infected with ECHO virus type 19. The nucleus (N) is completely distorted and displaced and its chromatin condensed at the periphery. Apparently the nucleus (N) has started to diminish in volume, leaving lacunar spaces between the membranes of the nuclear envelope (+). The center of the cytoplasm is occupied by a mass of electron transparent vacuoles (Va), vesicles (Ve), spirals (S), dense masses (DM), and normal mitochondria (M). X indicates an unknown structure. X 23,000.

FIGURE 5
Intermediate stage of a cell infected with ECHO virus type 6, showing a spiral in the process of formation. The arrow points to a false bridge. Mitochondria (M) are also visible. X 44,000.
also been described in Ehrlich tumor cells infected with Newcastle disease, West Nile, and Anopheles A viruses, as well as in chicken lymphoma cells infected with St. Louis encephalitis virus and HeLa cells infected with Cossackie B3 virus (28). Also, Adams and Prince (21) found an increased number of Golgi vesicles in Ehrlich ascites cells infected with Newcastle disease virus, and they speculated that this might represent a "participation in a type of intracellular defense mechanism." On the other hand, the Golgi complex might perhaps play a role in the formation of new viral particles.

The particles which form crystals in the infected cells are believed to be of viral nature because of their homogeneous appearance and uniform size. They have never been found in normal cells, and their size is in good agreement with that determined by other techniques (29). Hanzon and Phillipson (30) observed crystalline formations, similar to those described here, in highly purified material which was shown to have a high ECHO virus 7 infectivity.

More recently the finding of cytoplasmic crystals in cells infected presumably by ECHO virus (8) and ECHO virus 9 (9) has confirmed further the assumption that they are of viral nature.

Particles 10 m\(\mu\) in diameter or less are considered to be nucleoprotein granules. The largest particles (20 to 50 m\(\mu\)) are thought to be unrelated to viruses and may possibly be a product of cellular destruction.

The authors would like to express their gratitude to Dr. M. Roche for his stimulating advice and criticism, as well as to the Photographic and Translation and Publication Department of the Instituto Venezolano de Investigaciones Científicas for their help and cooperation in connection with this work.

Received for publication, May 14, 1961.

BIBLIOGRAPHY


Figure 7

Intermediate stage of a cell infected with ECHO virus type 19. The flattened nucleus (N) is seen with the chromatin resting on the nuclear envelope. The crosses (+) indicate the empty spaces between the membranes of the nuclear envelope. An accumulation of particles (P), 10 m\(\mu\) or less, is seen in the cytoplasm. Mitochondria (M), electron transparent vacuoles (Va), and a crystal (C) formed by elongated particles are also visible. On the left side of the nucleus is a mass resembling a spiral (S). X 51,000.

Figure 8

Advanced stage of a cell infected with ECHO virus type 19, showing a crystal (C) at the left. A spiral-like mass (S) is visible on the right. The particles are elongated and the distance between them is well defined. The distance between the rows is 1.5 times the distance between the particles. X 39,000.

Figure 9

An elongated crystal adhering to cellular remnants appears among amorphous masses of cellular origin. The cell was infected with ECHO virus type 19 and was in the advanced stage. The particles are rounded and equidistant, forming angles of 75° and 105°, never right angles (90°). X 75,000.

Figure 10
A crystal in the cytoplasm of a cell infected with ECHO virus type 19, in the intermediate stage. There are vacuoles (Va), dispersed particles, and a mitochondrion (M). To the left of the crystal is a large dense particle (arrow). All particles in the crystal are round. The distance between the rows of particles is 2.5 times the distance between particles. Careful observation will reveal fairly dense strips, separated by clear ones, between the rows. X 108,000.

Figure 11
A crystal, apparently disintegrating, in cellular debris during the advanced stage of infection. No structure is discernible in the mass of cellular remnants. The particles are round. The distance between the rows is 1.5 times the distance between the particles. X 60,000.