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ELECTRON MICROSCOPIC INVESTIGATIONS OF ROTAVIRUS MORPHOGENESIS IN CELL CULTURES

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Summary. — The replication of simian rotavirus SA11 in GMK cells and of bovine rotavirus in calf kidney cells was studied by electron microscopy. By 30 min post-inoculation (p.i.) SA11 virus was absorbed to the cell membrane in the absence of trypsin and became engulfed into the cell; clusters of viral particles were internalized also into cytoplasmic vacuoles. At 2 hr p.i., viral particles were seen in lysosomes and 6 hr p.i., the first progeny virus was found in the cisternae of endoplasmic reticulum (ER). Precursor virus particles budded from viroplasm into the cisternae of endoplasmic reticulum, where they became enveloped reaching a diameter of 80—90 nm. There was no difference between SA11 virus and bovine rotavirus. Although the budding of rotavirus particles is essential for acquiring of glycoproteins, the envelopment of capsids was transient. After stripping off the envelope, mature particles were formed 65—70 nm in diameter, consisting of either smooth or rough capsids. The final cytocidal stage of cell infection is described.

Key words: SA11 virus; bovine rotavirus; cell culture; electron microscopy

Introduction

The detection of human or animal rotaviruses in small intestine epithelial cells was always associated with demonstration of different stages of viral morphogenesis as first described with human rotaviruses (Bishop *et al.*, 1973). Cell cultures were used for electron microscopic investigations on morphogenesis of human rotaviruses (Drozdov *et al.*, 1979, 1983; Esparza *et al.*, 1980; Wyatt *et al.*, 1980; Roseto *et al.*, 1981), bovine rotaviruses (Chasey, 1977; McNulty *et al.*, 1978; Carpio *et al.*, 1981; Schulze *et al.*, 1983), ovine rotaviruses (McNulty *et al.*, 1978), porcine rotaviruses (McNulty *et al.*, 1978), Saif *et al.*, 1978) and simian rotavirus SA11 (Lecatsas, 1972; Altenburg *et al.*, 1980; Estes and Graham, 1980). Despite of overwhelming literature, some questions concerning early stages of the replication cycle have remained unclear and the interpretation of virus assembly seems contro-

versial. The present paper deals with the morphology of rotavirus absorption on and penetration into the cell, with transient envelopment and assembly of virus particles and with the lysis of infected cells.

Materials and Methods

Viruses and cells. Bovine cell-adapted rotavirus and SA11 virus were used. The number of their passages in cell cultures was unknown. The bovine rotavirus was grown in secondary calf kidney cells; the SA11 virus was propagated in secondary cells of African monkeys and in GMK cells. (The cell suspensions were kindly provided by Dr. M. Hilgenfeld, Institute of Applied Virology, Berlin.) Calf kidney cells were cultured in Eagle's basal medium, monkey cells in Hank's solution containing lactalbumin hydrolysate and supplemented with 2% calf serum. After 3 times washing, the cells were inoculated with 0.5 ml of virus suspension. The inoculum was once frozen and thawed. The absorption interval took 1 hr at 37 °C. Then maintenance medium was added and cells were further incubated at 37 °C.

Electron microscopy. GMK cells inoculated with SA11 virus were fixed at 30, 60 and 90 min as well as by 2, 3, 4, 6, 8, 12, 24 and 48 hr post-infection (p. i.) The monkey kidney cells infected with SA11, the calf kidney cells infected with bovine rotavirus and uninfected controls were fixed at 24, 48 and 72 hr p.i. The interval of fixation was calculated starting from the beginning of virus absorption. The cells were fixed in 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.5), postfixed with 1% osmium tetroxide and embedded into Epon. Cells harvested 48 hr p.i. were pelleted, 3 times frozen and thawed and resuspended in distilled water. The cell debris were fixed for 1 min with 2.5% glutaraldehyde in cacodylate buffer and contrasted with 2% phosphotungstic acid (pH 6.0). The samples were examined in Tesla BS-500 microscope.

Results

The SA11 virus inoculum consisted mainly of cell debris with attached rotavirus particles and of some free virus particles. Single virions or their clusters attached to the cell surface. The virus became absorbed to the cytoplasmic membrane already within 30 min (Fig. 1). The following penetration phase was started with engulfment of virus by the cellular membrane. This was observed at sites adjacent to the bottom of microvilli (Fig. 2). As the inoculum was not washed off, absorption and engulfment of virions into vesicles were found also at later intervals. As early as 30 min p.i. some virions have been seen in a phagosome. Lysosomes frequently contained virus particles since 2 hr p.i. (Fig. 4) to the later intervals p.i. (Fig. 5). Large virus clusters could also be engulfed into vacuoles (Fig. 6).

The assembly of rotavirus particles begins in viroplasm (Holmes *et al.*, 1975) along to the rough ER. In our case the viroplasmic matrix was small and hardly to recognize, but already by 6 hr p.i., the SA11 virus-infected cells contained slightly widened cisternae of ER with the first newly formed progeny virus (Fig. 7). Later on, the viroplasm was more marked and contained core particles about 40 nm in diameter (Fig. 8). in bovine as well as in SA11 rotavirus-infected cells. These were surrounded by a less elctron-translucent zone and aligned along the membranes of the rough ER. The particles gained access to the rough ER by budding, being enveloped by the membrane of the ER so that their diameter reached 80—90 nm (Fig. 8). Such 80 nm particles were seen exclusively in the rough ER, and were never found

in the cytoplasmic matrix. Between the core and the envelope radially arranged rod-like protrusions were seen. The outer envelope was stripped off within the ER cisterna (Figs 9, 10). The resulting particles were 65—70 nm in diameter and the envelope fragments were situated within the cisterna mainly in opposition to the viroplasm (Fig. 10). The larger particles had a 5 nm wide rim around the rod-like structures. Occasionally incomplete particles lacking a nucleoid were found (Fig. 10). By the progress of infection the 65—70 nm particles were found also in cytoplasm, but they were not seen in the nucleus.

Among the debris of lysed cells either ,,smooth'' rotavirus particles 70 nm in diameter with an outer rim (Fig. 12) or rough forms lacking the rim (Fig. 13) were seen in negatively stained specimens. The enveloped capsids measured 80—90 nm throughout (Fig. 12).

The virus-containing cells showed cytopathic changes. The cisternae of ER were widened and filled with 65—70 nm particles (Fig. 14). The lysosomes were lacking of their membranes and the continuity of the rough ER membranes was lost too. This way the patricles were located into the cytoplasmic matrix. After complete destruction of the infected cells the virions were released and detected among the cell debris.

Discussion

After a single cycle of freezing and thawing so much virus still remained cell-associated that further procedures of cell lysis were necessary.

The engulfment of rotavirus particles into cells is accomplished by viropexis. This corresponds to the statements of Dales (1973) concerning reoviruses. The virions are decomposed in lysosomes, which represent probably the uncoating process. Lecatsas (1972) and Altenburg *et al.* (1980) found virus particles in lysosomes either as decomposition products of the virus inoculum or as progeny virus. Trypsin enhances the infectivity of rotavirus particles (Bryden *et al.*, 1977; Drozdov *et al.*, 1979). It acts on the structural polypetides of the double-shell capsid and enhances uncoating of the particles after their engulfment. Nevertheless, only a part of virus population requires trypsin for infection (Graham and Estes, 1980; Clark *et al.*, 1981). A second part of the virus population is infectious also in the absence of trypsin. Our experiments with inoculum lacking trypsin have shown that trypsin did neither affect the infectivity of such particles nor the course of their penetration; Carpio *et al.* (1981) found bovine rotavirus progeny after trypsin tratment of the inoculum alread yat 6 hr p.i., at the same time as we did without trypsin treatment. Estes *et al.* (1979) detected SA11 virus antigens by immunofluorescence and Altenburg *et al.* (1980) also found virus particles by electron microscopy 8 hr after absorption.

The core-particles formed in viroplasm are precursor structures, i.e. particles of type I according to Chasey (1977). The intracisternal membrane--bound particles, 80 nm in diameter, formed by budding correspond to the type II according to Chasey. Several authors (Holmes *et al.*, 1975; Altenburg *et al.*, 1980; Esparza *et al.*, 1980; Carpio *et al.*, 1981; Coelho *et al.*, 1981;

Suzuki *et al.*, 1981; Subara *et al.*, 1982) interpreted the 80 nm particles as virions corresponding to the smooth double-shell rotavirus capsids in negatively stained specimens and to L-particles in centrifugation gradient. The smaller 65—70 nm particles (type III according to Chasey) would correspond to D-particles and represent premature "incomplete" particles. In contrast to this interpretation, we think in accordance with Chasey (1977), Saif *et al.* (1978) and Korolev *et al.* (1981) that the 65—70 nm particles represent the end-phase of virus assembly. The main components of the outer capsid layer of rotaviruses are glycoproteins (Rodger *et al.*, 1977). The viruses gain them in the course of budding across the rough ER membrane (Carpio *et al.*, 1981; Suzuki *et al.*, 1981). The outer shell is not identical with the envelope of the 80 nm particles. The latter is transient in nature and becomes stripped off from the virus particles. The glycoproteins seem to get retained on the particle durface. The 65—70 nm particles comprise of both, D- and L-capsids. During budding of the rough D-capsids glycoproteins are not incorporated into the particle. Therefore, they cannot be regarded for precursors of L-capsids. Negative staining of the same specimen showed the presence also of 80—90 nm particles which were still enveloped. These could be occasionally seen in stool specimens of children with rotavirus infection (Schumacher *et al.*, 1983).

The infrequently present incomplete coreless particles are related to multiplicity of infection (McNulty *et al.*, 1978; Drozdov *et al.*, 1983). In general, rotaviruses cause lytic infection. Only a few authors described the release of rotavirus particles (Carpio *et al.*, 1981; Suzuki *et al.*, 1981). A special case is the inapparent or persistent rotavirus infection in vitro (Babiuk and Misra, 1980; Estes and Graham, 1980). Rotavirus is usually released after cytolysis, which seems to begin by destruction of the menbrane of lysosomes. The lysosomal enzymes can disrupt the membranes of the rough ER, so that the virus gets access into the cytoplasmic matrix. Next step is the disruption of the cell membrane. Due to the intracisternal localization of the particles, much virus remains bound to cell debris.

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Explanation of Electron Micrographs (Plates XXXII—XXXV):

Fig. 1. Absorption of SA11 virus to the cell membrane at 30 min p.i. (× 90 000)
Fig. 2. SA11 particle on the basis of microvillus is being engulfed by the cellular membrane at 30 min p.i. (× 128 000).
Fig. 3. SA11 particle within the phagosome, 30 min p.i. (× 96 000).

Fig. 4. SA11 virus in the lysosomes, 2 hr p.i. (× 72 000).
Fig. 5. SA11 virus particles in a lysosome, 24 hr p.i. (× 72 000).
Fig. 6. Giant phagosome containing clusters of SA11 particles, 2 hr p.i. (× 36 000).
Fig. 7. Progeny virus SA11 in cisterns of rough ER, 6 hr p.i. (× 48 000).
Fig. 8. Enveloped particles are formed by budding across the membrane of rough ER from viroplasm to the cisterna; bovine rotavirus 48 hr p.i. (× 72 000).
Fig. 9. Enveloped 80 nm particles lying within the cisterna of rough ER; several capsids inside of the same envelope, fragments of the envelope and 70 nm particles. SA11 virus in GMK cells 24 hr p.i. (× 48 000).
Fig. 10. In the left enveloped bovine rotavirus particles after budding, in the right 65—70 nm particles and envelope remnants; a few coreless particles (× 72 000).
Fig. 11. SA11 virions and "smooth" capsids, negative staining (photographed by Ch. Liebe, × 200 000).
Fig. 12. Non-enveloped and enveloped SA11 particles 90 nm in diameter negative staining (photographed by Ch. Liebe, × 200 000).
Fig. 13. Rough bovine rotavirus particles surrounded by the membrane of rough ER; compare to Fig. 9 (negative staining, Ch. Liebe, × 200 000).
Fig. 14. Beginning of cytolysis. 70 nm particles gain access into cystoplasmic matrix from the cisterna of rough ER; SA11 virus in GMK cells, 24 hr p.i. (× 54 000).

Addendum. After completing our studies we became aware of following recently published papers:

Quan, C. M., and Doane, F. W. (1983): Ultrastructural evidence for the cellular uptake of rotavirus by endocytosis, *Intervirology* **20**, 223—231.
Suzuki, H., Konno, T., Kitaoka, S., Sato, T., Ebina, T., and Ishida, N. (1984): Further observations on the morphogenesis of human rotavirus in MA-104 cells. *Arch. Virol.* **79**, 147—159.