Ultrastructural Study of Rotavirus Replication in Cultured Cells

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SUMMARY

A systematic ultrastructural analysis of the replication cycle of the simian rotavirus SA11 in permissive MA104 cells was performed under reproducible conditions. At 8 h p.i., small areas of viroplasm were seen adjacent to swollen vesicles of the rough endoplasmic reticulum (rer) containing a few 80 to 90 nm virus particles. At later times, the size and number of these inclusions increased and the rer contained large numbers of the 80 to 90 nm particles as well as 52 to 65 nm particles. Infected cells eventually lysed, releasing progeny virus. Other cytological alterations included virus particles sequestered in lysosome-like bodies, 15 to 20 nm tubular structures in the nucleus and/or cytoplasm, convoluted membranes within the rer, filament bundles associated with virus particles, and mitochondria containing 1 to 5 virus particles. In addition, SA11 replication was studied in several less permissive cell lines. The results were similar to those with MA104 cells except that a smaller percentage of the cells were productively infected.

INTRODUCTION

Rotaviruses are widespread in nature and have been identified as a cause of non-bacterial gastroenteritis in a wide variety of young animals (McNulty et al. 1978). Previous efforts to study the morphogenesis of these viruses in tissue culture have been performed with relatively few time points and/or at unknown m.o.i., resulting in a variety of divergent findings. The use of the tissue-culture-adapted simian rotavirus SA11, which can be grown to high titre (Estes et al. 1979a) and quantified by plaque assay (Smith et al. 1979), has overcome many of these difficulties. This paper reports experiments in which rotavirus development and its effect on cellular architecture were studied at frequent intervals throughout the replicative cycle in a highly permissive cell line. Rotavirus growth in several less permissive cell lines was also investigated.

METHODS

Virus and cell lines. Stocks of the simian rotavirus SA11 were prepared from cultures of the foetal Rhesus monkey kidney cell line MA104 (Microbiological Associates, Bethesda, Maryland, U.S.A.) infected at < 0.01 p.f.u./ml as previously described (Estes et al. 1979a). All virus stocks were prepared in media without foetal calf serum. The MA104 cells, green monkey kidney (Vero), primary African green monkey kidney (AGMK) and primary Rhesus monkey kidney (RhMK) cells (Microbiological Associates) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), 5% tryptose phosphate broth, 0.2% vitamins, 0.03% glutamine, 0.25% glucose, antibiotics and 0.075% sodium bicarbonate.

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**Electron microscopy.** Cells were grown to confluent monolayers on 35-mm plastic culture dishes. The cultures were washed three times in tris buffer (TBS), pH 7.2, which contained 20 mm-tris, 140 mm-NaCl, 5 mm-KCl, 0.4 mm-Na2HPO4, 6 mm-glucose, 0.5 mm-MgCl2 and 0.7 mm-CaCl2, and were inoculated with SA11 at 5 p.f.u./cell. After a 90 min adsorption period at 37 °C, the cells were washed three times with TBS to remove unadsorbed virus and incubated at 37 °C in Eagle's MEM without serum. At various times after virus adsorption, the cells were washed twice in 0.1 M-1,4-piperazine-diethane sulphonic acid (PIPES) buffer, pH 7.2, fixed at room temperature for 1 h in 0.1 M-PIPES containing 3 % glutaraldehyde and stored overnight at 4 °C. They were then washed in 0.1 M-PIPES, post-fixed for 1 h in 0.1 M-PIPES-buffered 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded *in situ* in Epon 812. The embedded cells were separated from the culture dish, mounted on blank Epon capsules, and sectioned parallel to the growth substrate. After staining with a saturated solution of uranyl acetate in 50% ethanol for 5 min followed by alkaline lead citrate for 2 min, sections were examined in an Hitachi HU11B electron microscope operated at 75 kV.

**Virus production assay.** Duplicate cultures to those processed for electron microscopy were harvested 26 h p.i. to measure virus production. Sonicated, clarified supernatants were assayed for infectious virus by the plaque technique under an agar overlay containing pancreatin and DEAE-dextran as previously described (Smith *et al.* 1979). Plaques were counted after 4 days incubation at 37 °C. Virus antigen synthesis was monitored on coverslip cultures fixed in 100 % ethanol by indirect immunofluorescence (IF) microscopy. Staining with hyperimmune guinea pig anti-SA11 serum by fluorescein-conjugated goat anti-guinea pig globulins was performed as previously described (Estes & Graham, 1979).

**Immunoperoxidase microscopy.** Cells infected with SA11 were rinsed in TBS, fixed for 10 min in 100 % ethanol at −20 °C and air dried. The fixed cells were treated with 10 % FBS (free of rotavirus antibody) in 0.01 M-tris-HCl, pH 7.6, containing 0.15 M-NaCl (TN). Coverslips were stained sequentially with hyperimmune guinea pig anti-SA11 serum diluted 1:100, goat anti-guinea pig heavy and light chain IgG (Cappell Laboratories Inc., Cochranville, Pennsylvania, U.S.A.) diluted 1:10, and guinea pig peroxidase-antiperoxidase (PAP; Cappell Laboratories Inc.) diluted 1:50. Each antiserum was diluted in 2 % FBS in TN. All incubations with antisera were at 37 °C for 30 min in a moisture chamber. Extensive washings in 2 % FBS in TN followed each antiserum incubation. The coverslips were washed in TN without FBS and treated with a solution of 0.05 % 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St Louis, Missouri, U.S.A.) freshly prepared in TN for 5 min in the presence of 0.01 % hydrogen peroxide. The coverslips were rinsed in TN without serum, counterstained for 1 min in Gill’s haematoxylin no. 3, rinsed in tapwater, immersed in saturated lithium carbonate for 1 min and rinsed again in tapwater. The coverslips were dipped in distilled water, dehydrated through a graded series (15, 50, 75, 95 and 100 %) of ethanol and mounted in Permount (Fisher Scientific Co., Fair Lawn, New Jersey, U.S.A.).

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**Fig. 1.** Cytoplasm of SA11-infected MA104 cell, 8 h p.i. A small area of viroplasm (V) is located near vesicles of the rer which contain 80 to 90 nm virus particles (arrowheads).

**Fig. 2.** Perinuclear region of SA11-infected MA104 cell, 24 h p.i. Viroplasms (V) are associated with rer cisternae which contain mostly 50 to 65 nm virus particles. A few 80 to 90 nm particles (arrowheads) are also present. One mitochondrion (∗) contains five virus particles. Inset is a light micrograph showing perinuclear distribution of virus antigens in MA104 cells, 24 h p.i., labelled by the PAP procedure with antisera to SA11.

**Fig. 3.** Virus within rer of lysed MA104 cell, 20 h p.i. Fine threads extend from many of the particles (arrowheads). Several virus particles appear to be budding through the rer membrane.
Rotavirus replication in cultured cells
RESULTS

SA11 replication in MA104 cells: time course of virus infection

The MA104 line was chosen for a detailed study of SA11 morphogenesis, as SA11 grows to a higher titre in MA104 cells than in a variety of other primary and continuous cell culture lines. Samples of SA11-infected MA104 cells were fixed for electron microscopy at 4, 8, 12, 16, 20, 24, 36 and 48 h following the end of the 90 min absorption period.

Evidence of virus replication was first observed at 8 h after absorption, corresponding well with the first synthesis of infectious virus detected in growth curves of SA11 in MA104 cells (Estes et al. 1979 b). At this time, a few of the cells contained small cytoplasmic accumulations of dense granular material or viroplasm. These were not membrane-bound, but were distinct from the surrounding cytoplasm. They occurred in close proximity to vesicles of the rough endoplasmic reticulum (rer) which usually contained a few virus particles within a fibrous matrix (Fig. 1). The virus particles produced at this time were 80 to 90 nm in diam. with a sharply defined outer layer and contained an electron-dense core 25 to 35 nm in diam. As the infection progressed, the size and number of viroplasmic inclusions increased. Inclusions were typically concentrated in the perinuclear region of the cytoplasm, a site where virus antigens were also localized by immunoperoxidase microscopy (inset, Fig. 2). The nuclei of SA11-infected cells are unstained and are surrounded by the dense peroxidase reaction product. Simultaneously, the cisternae of the rer grew progressively more swollen and contained vast numbers of virus particles, many of which were smaller (52 to 65 nm) and appeared to lack the distinct outer layer (Fig. 2). Eventually, the infected cells lysed, releasing virus into the culture medium. At no time were particles observed budding through the plasma membrane. Lysed cells were first observed at 12 h after absorption and were increasingly seen in the 16, 20 and 24 h samples. Many of the virus particles associated with the lysing cells seemed to be extruding fine fibres from the region of the nucleoid (Fig. 3). After 36 h, lysed cells were rarely seen, probably because with the in situ fixation and embedding procedure cells detached from the substrate are lost.

Virus within lysosome-like inclusion bodies

At all of the time points examined, structures resembling lysosomes were found which contained one or more virus particles. The frequency of observation and the number of particles found in individual lysosome-like bodies were greater in cells fixed at the later time points, especially the 36 and 48 h samples. These were found in cells containing other rotavirus particle types and also in cells which were otherwise uninfected. The virus particles seen within lysosome-like bodies were 52 nm in diam. and often appeared to be hexagonal in shape. When several particles were present, they were found in crystalline arrays (Fig. 4).

Morphogenesis of SA11 virions

The assembly of the virus particles occurred near the periphery of the areas of viroplasm where the nucleoids and the less-dense surrounding layers appeared to condense from the granular material (Fig. 5a). In approx. 1% of the viroplasmic inclusions, 50 to 55 nm shells empty of the dense core were produced (Fig. 5b). Following assembly, virus particles entered the cisternae of the rer, although the method by which this occurred is not entirely clear. In some sections, virus particles appeared to bud through an area of the membrane devoid of ribosomes with the simultaneous acquisition of a distinct outer layer (Fig. 3 and 6). However, as noted above, large numbers of particles lacking this layer were also found in these vesicles. In general, the larger, sharply delineated particles remained near the periphery of the rer while the smaller particles occurred in the interior regions (Fig. 2, 6, 10 and 11). In a
Fig. 4. Lysosome-like structure containing crystalline array of virus particles in the cytoplasm of an MA104 cell, 20 h p.i.

Fig. 5. Formation of virus particles near the periphery of viroplasmic inclusions. (a) Condensation of particles with dense core (arrowheads) in MA104 cell, 16 h p.i. (b) Rare formation of empty particles (arrowheads) in MA104 cell, 24 h p.i.

Fig. 6. Virus particles budding from viroplasm (V) into the rer of MA104 cell at 20 h p.i.

Few cells, areas of highly convoluted, membrane-like material were seen within cisternae of the rer. Virus particles with distinct outer layers were seen adjacent to this material (Fig. 7a and 11).

Other cytological alterations

In samples of SA11-infected MA104 cells fixed between 8 and 24 h p.i., rotavirus particles were frequently observed within the mitochondria (Fig. 2 and 7a to d). In all cases the virus particles associated with mitochondria possessed the dense outer layer. The particles were always seen between the cristae, never within the denser matrix. They apparently entered the mitochondria by budding through both the inner and outer mitochondrial membranes together (Fig. 7a, b). Dense granules, such as the one in Fig. 7(e), were also seen in mock-infected cells and therefore were probably not related to virus replication. Many mitochondria contained two or more virus particles; as many as five were seen within a single mitochondrion (Fig. 2). In addition, the mitochondria of infected cells seemed to have a
Fig. 7. Rotavirus particles within mitochondria. (a) Cytoplasm of MA104 cell, 16 h p.i. Arrowhead indicates virus particles budding into mitochondrion. Also note membrane-like material (*) within rer. (b to d) Mitochondria containing virus particles in MA104 cells, 24 h p.i.

denser matrix material than those of uninfected cells and the inter-cristal space appeared to be swollen (Fig. 2 and 7). This was also true of mitochondria that did not contain virus particles. Although such mitochondrial alterations can be artifacts of fixation, this was unlikely since mitochondria of uninfected cells seen in the same section with infected cells appeared normal.

A number of other cytological alterations were noted in MA104 cells infected with SA11. Structures which appeared to be tubular in longitudinal section were observed in the cytoplasm (Fig. 8b) and/or the nucleus (Fig. 8a) of a few cells. Their diam. was 15 to 20 nm – smaller than cellular microtubules and larger than membranes. These structures were the only evidence of nuclear involvement in SA11 replication seen in our experiments. In two cells, we observed an accumulation of viroplasm and virus-containing cisternae associated with closely packed bundles of cytoplasmic filaments approx. 10 nm in diam. (Fig. 9). The virus particles themselves were also surrounded by a meshwork of kinky filamentous structures similar to those seen in reovirus replication. The majority of such particles appeared to be 52 to 65 nm, although close spacing made definitive interpretation difficult.
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Fig. 8. Tubular structures in the nucleus (a) and cytoplasm (b) of MA104 cells, 24 h p.i. V indicates small area of viroplasm.

Fig. 9. Cytoplasm of MA104 cell, 20 h p.i., showing bundles of filaments closely associated with virus particles. Viroplasm (V) and several mitochondria are also present.
SA11 replication in other cell lines

The morphogenesis of SA11 virus was also examined in Vero, a continuous cell line, and in two primary monkey kidney cell cultures, AGMK and RhMK. Experiments performed under identical conditions using immunofluorescence microscopy showed that only 1 to 4% of the Vero cells and up to 10% of the AGMK and RhMK cells contained virus antigens compared to > 50% of the MA104 cells. The numbers of productively infected cells observed by electron microscopy were consistent with these data. It was impossible to make detailed observations on the replication of SA11 in Vero cells since so few produced virus. However, nearly all of the particles in the cisternae of these cells were of the smaller type. Coreless particles, although seen occasionally in MA104 cells, were not observed in these lines.

The general features of SA11 morphogenesis were similar in the primary AGMK (Fig. 10) and RhMK (Fig. 11) cell cultures and in MA104 cells. However, the more unusual cytological changes described in the MA104 cells (i.e. virus particles within mitochondria, intranuclear tubular structures, cytoplasmic filament bundles) were not seen in these cells. It is possible that one or more of these changes could have been missed in the primary cell cultures, since fewer infected cells were examined. The matrix material within the rer cisternae was consistently denser in infected AGMK cells than in RhMK cells (Fig. 10). In addition, the virus particles in RhMK cells were sometimes associated with multi-vesicular bodies within the endoplasmic reticulum (Fig. 11), a feature not seen in AGMK cells but observed occasionally in MA104 cells. Also shown in Fig. 11 are tubular structures resembling an elongated virus particle and a normal virus particle budding into the rer.

DISCUSSION

The ability to grow SA11 to high titre and to quantify infectivity has allowed a systematic analysis of rotavirus replication to be performed under reproducible conditions. Electron microscopic examination of samples taken at 4 h intervals confirmed that SA11 follows the pattern considered typical for rotavirus morphogenesis. All of the five particle types described by Chasey (1977) in cells infected with calf and pig rotaviruses were seen in these experiments. Although the relationships between these particle types are not yet completely understood, our data suggest several possibilities.

The earliest particles formed following the eclipse period (8 and 12 h samples) were bounded by an electron-dense outer layer, whereas smaller particles lacking this layer were more common late in infection. The smaller particles were usually seen in cisternae containing large numbers of virus particles. These observations confirm those made by Saif et al. (1978) using porcine rotavirus and suggest that the availability of one or more components of the outer layer may be limited in areas of rapid virus morphogenesis. Alternatively, the smaller particles could be derived from the larger type through loss of the outer layer. The observation that particles without a well-defined outer layer are typically seen in the interior of the cisternae is consistent with the latter hypothesis. Our experiments (Fig. 3, 6 and 11), as well as those of several previous investigators (Adams & Kraft, 1967; Lecatsas, 1972; Holmes et al. 1975; McNulty et al. 1976; Saif et al. 1978), have shown that rotavirus particles apparently acquire their outer layer by a budding process when they enter the endoplasmic reticulum. Thus, this layer is derived from host cell membrane and could be considered an envelope. Similar observations have been made for several members of the orbivirus group (Murphy et al. 1968; Bowne & Richie, 1970). As rotavirus infectivity, like orbivirus infectivity, is resistant to chloroform and ether extraction, it seems more appropriate to refer to
Fig. 10. Viroplasm (V) and virus particles within swollen rer in AGMK cell, 20 h p.i. Note the fibrous matrix material between particles.

Fig. 11. Multivesicular membranes within rer of RhMK cells, 20 h p.i.; 80 to 90 nm virus particles are closely associated with these membranes. Arrowhead denotes elongated virus-like particle budding through the rer membrane.
this outer layer as a 'pseudoenvelope'. In addition, some virus particles may obtain their outer layer from inclusions of membrane-like material located within vesicles of the endoplasmic reticulum (Fig. 7a and 11; Holmes et al. 1975; McNulty et al. 1976, 1978; Saif et al. 1978).

The type of virus particle seen within lysosome-like structures in SAI-infected cells (Fig. 4; Lecatsas, 1972) is similar in appearance to the 'type IV' particles seen in cytoplasmic vacuoles in cells infected with calf or pig rotaviruses (Chasey, 1977). These particles are slightly smaller than the unenveloped particles seen within the endoplasmic reticulum, appear to be hexagonal in shape and are the only type found in crystalline arrays (Fig. 4; Chasey, 1977). These particles (type IV) have been interpreted as either a stage of virus morphogenesis (Chasey, 1977) or as the breakdown products of progeny or incoming particles (Lecatsas, 1972). The finding of a few such particles in cells before the appearance of either typical virus factories or particles within the endoplasmic reticulum (4 h time point) favours the latter hypothesis. The particles seen within membrane-bound inclusions at the late time points might result from uptake of progeny virus by cells which were not productively infected by the initial inoculum. A sequestering of incoming virus particles into lysosomes has been reported previously for reovirus (Dales, 1973) and members of the orbivirus group (Lecatsas & Erasmus, 1967).

Although several investigators have reported alterations in the mitochondria of rotavirus-infected cells (Lecatsas, 1972; Hall et al. 1976) and orbivirus-infected cells (Lecatsas & Erasmus, 1967), the frequent observation of virus particles within the mitochondria of MA104 cells was quite unexpected. The functional significance, if any, of intramitochondrial virus particles is unknown. Maul et al. (1978) have reported the occurrence of enveloped SV40 within the mitochondria of pre-implantation mouse embryos, but these apparently were incoming particles rather than newly synthesized ones. The rotavirus-containing mitochondria were almost always seen in close proximity to viroplasmic inclusions, suggesting that maturing virus particles might sometimes enter the mitochondria instead of the rough endoplasmic reticulum. Preliminary experiments have shown that the outer mitochondrial membranes in SAI-infected MA104 cells, as well as those of the endoplasmic reticulum, contain virus-specific antigens (Altenburg et al. 1979). The observations of virus in the process of entering mitochondria were insufficient to determine whether the outer layer was derived from mitochondrial membrane or was acquired prior to entry.

Nearly all of the less frequently observed cytological features reported by others in rotavirus infections in animals or in cell culture were seen in the SAI-infected MA104 cells. The single exception was the large (54 to 62 nm) tubules seen in the nuclei of murine rotavirus-infected cells (Banfield et al. 1968) and porcine rotavirus-infected cells (Saif et al. 1978). The tube-like structures seen in both the nucleus and cytoplasm (Fig. 8a, b) by ourselves and Lecatsas (1972) appear to be unique to SAI rotavirus infections. Although termed 'membranous elements' by Lecatsas (1972), the size and general appearance of these structures are not characteristic of cellular membranes. Similar structures have been reported in nuclei of cells infected by orbiviruses such as Colorado tick fever (CTF; Murphy et al. 1968) and in the cytoplasm of cells infected with CTF and African horse sickness virus (Lecatsas & Erasmus 1967). Whether they contain virus-specified proteins remains to be determined. Another unusual feature, not previously reported in rotavirus infected cells, was the finding of bundles of closely packed fibres in areas of virus assembly (Fig. 9). The appearance of these fibres is very similar to that of the 10 nm or intermediate filaments seen in a wide variety of normal cell types. However, it is not known whether the filament bundles seen in our cells represent a virus-induced aggregation of cell-specified structures or the synthesis of a different type of fibre.
The replication cycle of SA11 was found to be similar in a variety of primary and continuously cultured cell lines which vary several thousand-fold in their ability to support the production of infectious virus (Estes et al. 1979a). In contrast to the results with rotaviruses of pig and lamb origin (Pearson & McNulty, 1979), obviously defective particles lacking the inner, dense core were not observed in any of the less permissive SA11-infected cell lines, although small groups of them were occasionally seen late in the infection cycle in MA104 cells. Experiments are under way to determine the point(s) at which the replication of SA11 is blocked in the less permissive cell lines.

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