Multiplication of Human Rotavirus in Cultured Cells: an Electron Microscopic Study

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(Accepted 20 November 1979)

SUMMARY

Human rotaviruses were capable of efficient multiplication in LLC-MK2 cells when the inoculum was pre-treated with trypsin, centrifuged on to the cell monolayer and the infected cells maintained in a medium containing trypsin. However, not all of the human rotavirus isolates used to infect cells resulted in efficient virus production. The ability of human isolates to multiply in cultured cells was studied by direct observation of virus in the electron microscope, by radioactive labelling with ³H-uridine of the newly synthesized virus and by electron microscopic examination of thin sectioned infected cells. With one of the specimens used (F-617) only 5 to 10% of the cells showed evidence of virus multiplication, with the great majority of the infected cells showing numerous complete (double-capsid) virus particles scattered in the cytoplasm. When cells were inoculated with another human specimen (SIB-I), infected cells were more abundant, reaching a maximum of 60%; however, a variety of particle types, probably representing different subviral structures or different steps of rotavirus morphogenesis, were commonly observed. The presence of these aberrant or incomplete virus structures may represent a manifestation of the defectiveness of this virus and may explain the difficulties encountered in its serial passage.

INTRODUCTION

Rotaviruses are now considered to be a major cause of neonatal diarrhoea in a variety of animal species, including humans (Flewett & Woode, 1978; McNulty, 1978). Human rotaviruses have a worldwide distribution and we have previously reported that they were the most common pathogen identified in hospitalized Venezuelan children with gastroenteritis (Esparza et al. 1977; Viera de Torres et al. 1978).

Since rotaviruses are frequently found in great quantities in the faeces of infected individuals and the purification procedures are very well developed, a great deal of work has been done on the characterization of the rotavirus particle. Numerous papers have been published on its structure (Martin et al. 1975; Woode et al. 1976; Stannard & Schoub, 1977; Esparza & Gil, 1978), genomic RNA (Kalica et al. 1976; Schnagl & Holmes, 1976), structural polypeptides (Rodger et al. 1977) and virion-associated RNA polymerase (Cohen, 1977; Hruska et al. 1978). However, very little is known about the replicative cycle of rotaviruses.

Studies on the multiplication of human rotaviruses have been hampered by the difficulties encountered in obtaining efficient in vitro cultivation and particularly its serial passage in cell cultures. Infectivity of human rotaviruses was demonstrated by Banatvala et al. (1975)
and Bryden et al. (1977), when the inoculation of the culture was made by centrifuging the inoculum on the cell monolayer, with further detection of virus growth by immunofluorescent staining.

An additional advance in the in vitro multiplication of rotaviruses is the use of mild proteolysis to enhance virus infectivity. Theil et al. (1977) reported the successful isolation and passage of porcine rotavirus, when virus suspensions were treated with pancreatin prior to inoculation on to cell monolayers. Furthermore, Babiuk et al. (1977) and Almeida et al. (1978) demonstrated that the incorporation of trypsin in the maintenance medium results in a dramatic increase of the in vitro production of bovine rotaviruses. Almeida et al. (1978) also suggested that this procedure may be effective for the propagation of human rotaviruses.

In the present study we report on the morphogenesis of human rotavirus after infection of monkey cells by the combined use of centrifugation and trypsin treatment.

**METHODS**

**Virus.** Human rotavirus was obtained from stools collected from patients admitted with gastroenteritis to the “J.M. de Los Ríos” Children’s Hospital in Caracas, Venezuela and to the “Hospital Policlínico” in Los Teques, Venezuela. A 5% suspension of faecal material was made in phosphate-buffered saline (PBS), pH 7.35, containing ten times the usual amount of antibiotics, to give final concentrations of 1000 units of penicillin per ml, 1000 μg of streptomycin per ml and 25 μg of amphotericin B per ml. The specimens were homogenized and clarified by centrifugation at 4000 g for 30 min at 4 °C. Supernatants were used for inoculation immediately after being processed. Since excretion of enteroviruses is frequent in Venezuelan children (Esparza et al. 1977; Viera de Torres et al. 1978) the possible presence of contaminating viruses was properly checked and found to be negative.

**Cell culture and inoculation procedure.** After the results of Bryden et al. (1977) we chose to use LLC-MK2 cells, a continuous line of monkey cells, provided by R. Walder. The cells were grown and maintained at 37 °C in disposable tissue culture flasks with a 25 cm² growth area (Falcon Plastics, Oxnard, Calif., U.S.A.), using Eagle’s minimum essential medium. In all the experiments described, confluent monolayers in 30 ml tissue culture flasks were inoculated with 0.1 ml (approx. 10⁷ physical particles) of the human rotavirus suspension, which had been previously treated for 1 h at 37 °C with 100 μg per ml of trypsin. Immediately after inoculation, the virus was centrifuged on to the cell monolayer at 1200 g for 30 min at room temperature. After centrifugation, 2 ml of media, containing 10 μg/ml of trypsin and 2% gamma globulin (GG)-free foetal calf serum, were added to each culture, and then incubated at 37 °C for various times.

**Radioactive labelling and virus purification.** Confluent monolayers of LLC-MK2 cells were inoculated with human rotavirus as described before, using in the media extensively dialysed GG-free foetal calf serum. Three hours after inoculation, 3H-uridine was added to a final concentration of 50 μCi/ml and incubation was allowed to proceed for 44 h at 37 °C. For virus purification, infected cells were frozen and thawed twice and then clarified by centrifugation at 4000 g for 30 min at 4 °C. The cell pellet was resuspended in PBS and extracted twice with half volumes of trifluorotrichloroethane (Freon-113) and pooled with the aqueous phase of a Freon-113 extraction of the supernatant. The virus suspension was layered over a 2 ml cushion of 45%, w/v, sucrose in PBS and ultracentrifuged in an SW-41 Spinco rotor at 29000 rev/min (100000 g) for 2 h at 4 °C. The virus pellet was resuspended in PBS and further purified by isopycnic banding in a CsCl gradient. Virus was resuspended in 12 ml of PBS containing 5.68 g of CsCl adjusted to a refractive index of 1.367 to give a
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Fig. 1. Isopycnic banding in CsCl of 3H-uridine labelled human rotavirus particles produced in LLC-MK2 cells after inoculation with specimen SIB-I. • — •, Infected cultures; ○ --- ○, uninfected cultures; ■—■, density.

final density of 1.35 g/ml. Centrifugation was carried out in an SW-41 Spinco rotor at 29000 rev/min for 14 h, at 15 °C. Fractions were collected by bottom puncturing and the density of each fraction was determined by refractometry. The radioactivity of each fraction was determined after precipitation with 5% cold trichloroacetic acid (TCA) in the presence of 0.1 mg/ml of BSA used as carrier. Precipitates were collected on nitrocellulose filters (Millipore, Type DA, 0.65 µm; Millipore Corp., Bedford, Mass.), washed three times with cold 5% TCA, air dried and counted in a toluene-based scintillation fluid (Aquasol, New England Nuclear, Boston, Mass.). As a control, uninfected cells were treated identically and processed as described for those which were infected.

Electron microscopy. At the appropriate times after inoculation, maintenance medium was removed from the cultures and the cell monolayers fixed overnight in glutaraldehyde (2%, v/v) in cacodylate buffer (pH 6.7, 0.1 M). The cells were then scraped from the plastic surface, washed by centrifuging three times in the same buffer and post-fixed in osmium tetroxide (2%, v/v) for 3 h. After washing in buffer, the suspension was centrifuged at 4000 g, resuspended in agar (2%, v/v) at 50 °C and immediately centrifuged at 5000 g. The agar block was cut into 1 mm³ pieces which were dehydrated in an ethanol series and embedded in Epon (1A : 1B) (Luft, 1961). Thin sections were cut with a diamond knife in a Porter Blum MTI ultramicrotome, and then stained with aqueous uranyl acetate (2%, w/v) for 10 min at 60 °C and lead citrate for 10 min at room temperature. Sections were examined at 80 kV with a JEOL JEM-100B electron microscope.

RESULTS

Ability of different isolates of human rotavirus to multiply in tissue culture

Six different isolates were initially tested for their ability to multiply in LLC-MK2 cells inoculated as described in the section of methods. The initial screening was done by pelleting of the supernatants by ultracentrifugation and direct observation of the pellets in the electron microscope, after staining with phosphotungstic acid (Esparza et al. 1977). Even when all
Fig 2 to 5. Electron micrographs of LLC-MK2 cells inoculated with specimen F-617 of human rotavirus.

Fig. 2. Mature rotavirions scattered in the cytoplasm. Inset, higher magnification of rotavirus showing the presence of spikes which project from the central structure to the periphery.

Fig. 3. Membranous vesicle containing numerous densely packed subviral structures.
samples were inoculated at the same m.o.i., great quantities of morphologically intact rotavirus particles were found in only two of these specimens, identified as F-617 and SIB-I. This suggested to us that virus particles observed in the pellets were not simply unadsorbed particles derived from the inoculum but represented newly produced viruses.

**Radioactive labelling of in vitro synthesized human rotaviruses**

The ability of human rotavirus to multiply efficiently in LLC-MK2 cells was further confirmed by radioactive labelling of the *in vitro* synthesized virus particles. One 25 cm² tissue culture flask of inoculated or control cells was processed and analysed in CsCl gradients as described in Methods and the results are shown in Fig. 1. Infected cultures gave a sharp band of radioactivity with a density of 1.365 g/ml, in a region where double-shelled rotavirus particles band. A small shoulder of higher density (~ 1.39 g/ml) was also observed, probably corresponding to single-shelled particles. Uninoculated cultures did not show any of these radioactivity peaks, with all the label being pelleted to the bottom of the centrifuge tube, probably corresponding to cellular ribonucleic acid.

**Virus morphogenesis of in vitro cultured human rotavirus**

The first series of experiments was done with the specimen identified as F-617. Cells were inoculated with a high m.o.i. (in the order of 10⁴ physical particles per cell) and processed for electron microscopy at 12, 24 and 48 h p.i. Virus particles were observed only in cells processed at 48 h p.i., and even in this case not more than 5 to 10% of the cells showed evidence of virus multiplication. The majority of the infected cells showed numerous virus particles scattered in the cytoplasm, with no alteration of the cell nuclei (Fig. 2). These particles appeared to be mature rotavirus, having a sharply defined outline with an overall diam. of 83 nm and a more densely stained central structure from which spikes appeared to project towards the periphery (Fig. 2, inset). Occasionally, vesicles containing densely packed particles were observed (Fig. 3). These particles were smaller than those just described (with a diam. between 40 and 50 nm) and may represent immature or subviral particles. The diam. of these particles was similar to that of the dense central structure of the rotavirus. Tubular structures with an approximate diam. of 70 nm were occasionally present in the vicinity of virus particles (Fig. 4). Extracellular viruses were also observed (Fig. 5), but compared with the intracellular particles, their diam. was 70 nm, probably corresponding to single-shelled virions (Esparza & Gil, 1978).

The other rotavirus specimen used in the present study (SIB-I) gave slightly different results from those just described. Infected cells were more abundant than those in the first experiment, reaching a maximum of approx. 60% between 48 and 60 h p.i. However, the major difference was the presence in the cytoplasm of a variety of particle types, representing different subviral structures or different steps of the rotavirus morphogenesis (Fig. 6). A higher magnification of an area of the same photograph illustrates some of the morphological particles observed (Fig. 7). Particles with a diam. of 83 nm, interpreted as double-shelled mature virions, were observed, with a dense central region, probably containing the genomic RNA (Fig. 7, Df), or as empty particles (Fig. 7, De). Subviral particles or ‘cores’, with a diam. of 50 nm, were also observed either as full (Fig. 7, Cf) or empty (Fig. 7, Ce) structures. It is of interest to note that ‘cores’ are separated from other cytoplasmic structures by a relatively electron translucent halo, indicating the existence of a poorly staining virus structure, probably corresponding to the subunits of the outer shell (see arrow in Fig. 7). In the same particles it is even possible to note a rudiment of what appears to be the outer virus capsid. Fig. 8 shows several particles at the time at which assembling of the outer capsid may take place by a process similar to that of budding.
Fig. 4. Tubular structures (arrowhead) in the vicinity of virus particles.

Fig. 5. Aggregates of extracellular virus particles.
Fig. 6 to 10. Electron micrographs of LLC-MK2 cells inoculated with specimen SIB-I of human rotavirus.

Fig. 6. Different subviral structures present in the cytoplasm of infected cells.

Fig. 7. Higher magnification of Fig. 6 showing different virus particle types: Df, double-shelled full virions; De, double-shelled empty particles; Cf, full 'cores'; and Ce, empty 'cores'. The arrow indicates an electron translucent halo surrounding some cores probably corresponding to the subunits of the outer shell.

Fig. 8. Single-shelled particles at the time of acquisition of the outer capsid.
Fig. 9. Vesicles containing numerous subviral particles with complete virions lining the limiting membrane. The arrow indicates a virus particle in what appears to be a budding process.

Fig. 10. Single-shelled particles and cores accumulating either as full or empty structures.

True budding of particles through cellular membranes was never observed, even when some micrographs, such as that of Fig. 8, seem to suggest it. Another example is shown in Fig. 9, in which double-shelled particles are lining the inner side of a virus-containing vesicle, some in close contact with the membrane, similarly to a budding process (Fig. 9, arrow). However, incomplete particles are only observed in the interior of the vesicle. In
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Fig. 11. Schematic diagram of rotavirus morphology as deduced from the electron microscopic study of thin sectioned infected cells.

some cells, subviral particles never acquire the outer capsid and are accumulated either as full or empty subviral structures (Fig. 10).

DISCUSSION

Human rotavirus was capable of efficient multiplication in LLC-MK2 cells when the inoculum was pre-treated with trypsin, centrifuged on to the cell monolayer and the infected cells maintained in medium containing trypsin. All these procedures have been reported as useful in obtaining infectivity with human and animal rotavirus (Banatvala et al. 1975; Babiuk et al. 1977; Bryden et al. 1977; Theil et al. 1977; Almeida et al. 1978). The mechanism by which centrifugation and trypsin treatment enhance the infectivity of rotavirus is not yet understood (Almeida et al. 1978; Babiuk & Mohammed, 1978; Theil et al. 1978). It is important to mention that the isolates used in the present study were shown to be of human origin by the segmentation pattern of their genomic RNA when analysed by polyacrylamide gel electrophoresis (Kalica et al. 1976; our unpublished observation).

Electron microscopic observations of the rotaviruses produced in cultured cells showed the presence of some morphologically intact double-shelled particles (Esparza & Gil, 1978). Uridine-labelled virions banded at a density of 1.365 g/ml (Fig. 1) similar to the density of complete virions (Rodger et al. 1975; Kapikian et al. 1976; Elías, 1977), suggesting that a normal amount of genomic RNA was present in the virus particles. These facts suggest, but do not prove, that complete infective virions are formed (Nonoyama et al. 1970; Spandidos & Graham, 1976). The possibility that defective viruses are formed is an interesting one, since serial passage of human rotavirus, as well as of animal rotaviruses (Babiuk et al. 1977; McNulty et al. 1978) has proved difficult.

Bearing in mind that human rotaviruses may grow in tissue culture with different degrees of defectiveness and putting together the observations made with the two isolates we used
in the present study, we wish to propose the ultrastructural model depicted in Fig. 11 for human rotavirus. This model suggests that the complete virion is a double-shelled structure with a thin-section diam. of approx. 83 nm. This particle could correspond to the double-capsid viruses seen in negative stained preparations (Esparza & Gil, 1978). In Fig. 7 a more densely stained peripheral rim, 5 to 7 nm thick is particularly evident, constituting the outer shell of the rotavirus particle. A similar observation has been reported with purified viruses, where the outer capsid gave to rotaviruses their characteristically sharp outline (Esparza & Gil, 1978).

Several subviral structures can now be identified in rotaviruses. Many particles show a more densely stained central structure, probably corresponding to the genomic RNA. This central structure, designated by us ‘nucleoid’ has a diameter of approx. 37 nm and is enclosed within a zone with a diam. of 50 nm, which constitutes the rotavirus core. Rotavirus cores have been obtained by Palmer et al. (1977) by treatment of purified virions with versene-trypsin; these particles possess a limiting structure named by the authors, very appropriately, as the inner layer.

Between the core wall, or inner layer, and the outer capsid exists a more electron translucent region which may correspond to the subunits of the inner capsid. In fact, in some instances (Fig. 2, inset, and 7), spikes appear to project towards the periphery of the particles. One of the interpretations of the structure of the inner capsid has suggested that it is composed of 320 trimeric morphological subunits which form an icosahedral arrangement around 162 holes, with a pattern characteristic of T = 16 (Esparza & Gil, 1978). Those morphological subunits are approx. 15 nm long and are located on top of a clearly defined baseline, which may correspond to the inner layer just described. The fact that the inner capsid is an open mesh structure may explain its low density, a feature also reported by other authors (McNulty et al. 1976; Chasey 1977; Saif et al. 1978). As indicated before, the existence of the inner capsid sometimes can only be discerned by the presence of an electron translucent halo which separates cores from the cytoplasmic structures (Fig. 7, arrow).

The virus structures reported in this paper are not markedly different from those described by other authors (Holmes et al. 1975; McNulty et al. 1976; Chasey, 1977) and the model proposed by us does account for most of the different morphological types of particles observed with human and animal rotaviruses. An important point for discussion and further investigation is that of the acquisition of the outer capsid. Most authors agree that the outer capsid is acquired by a budding process through membranes of rough endoplasmic reticulum or at the level of the virus factories (Holmes et al. 1975; McNulty et al. 1976; Saif et al. 1978). In our study we did not observe true budding of particles through cellular membranes, even when the acquisition of the outer capsid seems sometimes to be associated with membranous structures (Fig. 7 and 8). Fig. 9 shows an image which has been more frequently interpreted as the strongest evidence in favour of budding. However, as indicated before, single-shelled particles are only observed in the interior of the vesicle and not outside, as would be expected if budding through a membrane were necessary for the acquisition of the outer shell. In our opinion, the micrograph suggests that the initial stages of virus morphogenesis take place in the nearby virus factories or in the cisternae of the rough endoplasmic reticulum. At a late stage of maturation, the single shelled particles are covered by the outer capsid and migrate to the periphery of the vesicle or, alternatively, the single shelled particles migrate from the interior of the vesicle to the membranes of the rough endoplasmic reticulum to be coated by the outer capsid protein synthesized at that level.

The clarification of this point awaits further study, mainly serial observations of a well defined single-step cycle of multiplication, an experiment which is at present difficult to perform in view of the relatively low infectivity of rotavirus in tissue culture.
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The ultrastructural study of rotavirus morphogenesis in human biopsy specimens revealed an accumulation of numerous virus particles in cytoplasmic vesicles from differentiated intestinal epithelial cells, with both single and double-shelled particles being present (Holmes et al. 1975). Occasional particles lacking the dense core were also reported. In our study, human rotavirus induces in tissue culture a number of incomplete or aberrant structures, including tubules, which may represent a manifestation of the defectiveness of this virus and may explain the difficulties encountered in its serial passage. A similar observation and proposition was recently made by McNulty et al. (1978) for animal rotaviruses.

We wish to thank Dr L. Montet for giving us access to her little patients; Lic. Aleida Sánchez, Bio. Carmen I. Piña and Mrs Mirta Romano for excellent technical assistance; Mrs Renée Lira and Diane Urwin for helping in preparing the manuscript; and Mr M. Díaz for his cooperation with the photographic work.

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(Received 3 July 1979)