Different Particle Types in Tissue Culture and Intestinal Epithelium Infected with Rotavirus

By DAVID CHASEY

Central Veterinary Laboratory, New Haw, Weybridge, Surrey, KT15 3NB, U.K.

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SUMMARY

Rotavirus-infected intestinal epithelial cells in vivo and calf kidney cells in vitro have been examined by electron microscopy. Morphogenesis takes place in the cytoplasm and several particle types are observed. These can be classified broadly into two groups, one of which appears to represent the characteristic normal development of infectious virus and the other, distinctly different, which may be a non-infectious form. Two of the particle types are tentatively identified as the 'single' and 'double' capsid rotavirus particles seen typically in negatively stained preparations.

INTRODUCTION

Neonatal diarrhoea in a number of mammalian species is commonly associated with 'reovirus-like' agents. Virus particles have been identified in diarrhoeic faeces from, for example, children (Flewett, Bryden & Davies, 1973; Flewett et al. 1974b; Bishop et al. 1974; Kapikian et al. 1974), monkeys (Els & Lecatsas, 1972), calves (Woode et al. 1974), foals (Flewett, Bryden & Davies, 1975), lambs (Snodgrass et al. 1976) and mice (Much & Zajac, 1972) and on the basis of their distinctive wheel-like morphology in negatively stained electron microscope preparations they have been generally termed 'rotavirus'. Although rotaviruses from different sources show similar morphological characteristics in negative stain and exhibit various serological cross-reactions (Flewett et al. 1974a; Kapikian et al. 1974; Woode et al. 1976), both 'single' and 'double' capsid particles have been described (Els & Lecatsas, 1972; Flewett et al. 1973, 1974a, b; Bishop et al. 1974; Holmes et al. 1975) and it is not clear whether these represent two distinct particle types or are merely different aspects of the same structure. Moreover, the limited number of published observations on rotavirus in thin sections has not enabled further conclusions to be drawn (Bishop et al. 1973; Stair et al. 1973; Holmes et al. 1975; Hall et al. 1976).

In the present study rotavirus particles have been examined by both thin section and negative stain electron microscopy, and some morphological comparisons have been made.

METHODS

Virus strains. Two strains of rotavirus were used in this study. A strain of calf virus obtained from Dr G. Woode, IRAD, Compton, Newbury, Berks, underwent passages at Weybridge and had a final titre of $10^5.5$ TCID$_{50}$/ml. A pig virus, isolated at Weybridge by Miss M. Lucas, was obtained from a faecal sample from a 3-week-old animal with diarrhoea.

Experimental animals. A 2½-h-old colostrum-deprived calf was inoculated orally with 20 ml of the third tissue culture passage material of the Compton strain of virus. The calf was
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killed 24 h later and the small intestine was removed immediately and prepared for electron microscopy.

A 5-day-old piglet from a minimal disease herd was inoculated with the porcine rotavirus and the small intestines from this and an uninfected control animal were prepared for electron microscopy. The preparative methods were essentially similar to those used for the calf and have been described in a previous report (Chasey & Lucas, 1977).

Tissue culture. Primary calf kidney (CK) cells were grown in 2 oz plastic bottles (Nunc A/S Denmark) using Hanks' balanced salt solution supplemented with 0.01% yeastolate (Difco), 0.5% lactalbumin hydrolysate (General Biochemicals) and 5% foetal calf serum (Gibco-Biocult). After 3 days the medium was replaced with Earle's balanced salt solution, also supplemented but with 2% foetal calf serum. At 6 days the confluent monolayer of cells was inoculated with 0.25 ml of material from the fifth tissue culture passage of the calf virus. After an adsorption period of 1 h at 37 °C, the inoculum was removed and cultures, overlaid with 3 ml of Earle's medium, were incubated at 37 °C for 24 h before being prepared for electron microscopy. Control cultures were prepared in a similar manner but were not inoculated with virus.

Electron microscopy. The small intestines from each of the experimental animals were fixed in 3% glutaraldehyde in 0.1 M-sodium phosphate buffer (SPB), pH 7.2. Short sections taken at intervals along the length of the duodenum, jejunum and ileum were fixed for 1 h (18 h for material from the calf) at 4 °C and washed in SPB. Small cubes of tissue containing the epithelial layer of villi were post-fixed in 1% buffered osmium tetroxide for 1 h at room temperature before dehydration in graded alcohols and embedding in Araldite. Ultrathin sections, cut with glass knives, were stained with methanolic uranyl acetate followed by aqueous lead citrate and examined at 80 kV in either a Philips EM 300 or a Jeol 100B electron microscope.

The procedures for embedding tissue culture monolayers in plastic culture vessels and subsequent examination have been described previously (Chasey & Alexander, 1976).

The fluid recovered from the small intestines was examined by negative stain using 2% phosphotungstic acid, pH 6.6, with 0.003% bacitracin, on Formvar coated carbon stabilized grids. Before inoculation of the calf, faeces were also examined for virus particles.

RESULTS

Examination by negative stain of the contents of the small intestines from the infected piglet and calf revealed the presence of characteristic rotavirus particles in considerable numbers. These were 50 to 70 nm in diam. and they often exhibited dense hexagonal 'cores' approx. 45 nm in diam. Many particles lacked the typical smooth circular outline.

Three kinds of rotavirus particle were commonly observed in the cytoplasm of infected epithelial cells in the small intestine of the infected piglet and these have been described in a previous report (Chasey & Lucas, 1977). Type I particles, 25 to 35 nm in diam., were associated with dense inclusions, and Type II particles, 70 to 80 nm in diam., each consisting of a dense central core enveloped by a well-defined membrane, were found within dilated regions of rough endoplasmic reticulum. Type III particles, somewhat smaller in diameter and with indistinct outlines, were also seen within the endoplasmic reticulum; extracellular Type III particles were present near the tips of the microvilli. Virus-related particles similar to these three types were also found in infected cells from the calf small intestine and infected cells in primary CK cultures.

Examination of material from the calf revealed mild tissue damage in the epithelial layer,
Fig. 1. Dense inclusion containing Type I particles within a CK monolayer 24 h after infection. Predominantly Type II virus lies adjacent within rough endoplasmic reticulum. Magnification ×17000.

Fig. 2. Isolated focus of Type IV rotavirus particles within a large vacuole in the ileum of the infected piglet. Magnification ×10000.

Fig. 3. Enlargement of part of Fig. 2. Magnification ×46000.

Fig. 4. Type III and Type IV particles lying in close proximity within the same CK tissue culture cell 24 h after infection. Note the difference in size between the two types. Magnification ×82000.
similar to that observed in the infected piglet (Chasey & Lucas, 1977). Infected cells, occurring sporadically, appeared less dense than normal cells and the nuclei were swollen. They were found in the ileum and jejunum but none could be detected in the duodenum. Small numbers of Type II or III particles were seen throughout the cytoplasm of such cells, enclosed within ribosome-studded membranes, and electron-dense inclusions, occasionally containing Type I particles, were also present. The larger particles, although not so well-defined, were similar in appearance to those commonly observed in the pig intestine and their diameters were of comparable magnitude. It was, however, difficult to identify those with distinctive limiting membranes and it was not always possible to distinguish the two types unambiguously. Some suggestion of budding from the endoplasmic reticulum was observed but unequivocal evidence of this was not obtained.

Primary CK cultures, 24 h after inoculation with rotavirus, exhibited discrete foci of cytopathic effect covering approximately a third of the area of the monolayer. At the ultrastructural level, the architecture of infected cells differed little from adjacent uninfected cells or those in control cultures. In particular, mitochondria were not significantly damaged and the overall electron density was not markedly different. The cytoplasm of infected cells contained large numbers of virus-like particles, associated with dense unenveloped inclusions and rough endoplasmic reticulum, and Types I, II and III could be clearly identified (Fig. 1). Many cells at an advanced stage of degeneration contained numerous Type III particles bounded by rough endoplasmic reticulum and this type of particle was observed being released through broken plasma membranes. Many of the Type II particles exhibited a modified appearance in which the central core was of comparable density to the outer medium-dense annulus.

Virus-like particles of a distinctly different morphology were also observed in infected small intestine from the piglet and infected CK cultures. In the piglet these (Type IV) were not associated with endoplasmic reticulum or dense inclusions but were found in great numbers, together with cellular debris, within large vacuoles several microns in diameter (Fig. 2). The particles were circular in cross-section, with diam. in the range 50 to 65 nm. They were usually evenly electron-dense and although some ‘stippling’, indicative of subunits, was often evident, substructural details could not be clearly discerned (Fig. 3). Cells containing these particles never appeared to contain Types I, II and III.

Type IV particles were present in small numbers within many infected CK cells, often in areas containing the common forms. They were found generally within small vacuoles and were significantly smaller (50 nm) than the Type III particles for which they could occasionally be otherwise mistaken (Fig. 4). They were similar to the Type IV virus found in the intestinal epithelial cells of the piglet and short radial features, giving a ‘serrated’ appearance, could often be discerned around the periphery of each particle (Fig. 5). Small crystals of Type IV particles were also observed in CK cells (Fig. 6). Cells containing Type IV particles tended to exhibit a greater degree of organelle disorganisation and mitochondria were often swollen. An extreme cytopathic effect was observed in an infected monolayer examined 72 h after inoculation after an initial incubation period during which the cells were without maintenance medium. With few exceptions, infected cells contained Type IV particles exclusively in large numbers (Fig. 7). These were contained in vacuoles, several microns in diameter, and ‘empty’ capsids and small virus crystals were common. Small unbounded striated inclusions, with a periodicity of approx. 40 nm, were also found in this particular culture but were not associated with any virus-like particles.

An unusual feature was observed near the distal end of the ileum of the infected calf. This was a large vacuole in which lay a group of 50 nm particles (Fig. 8). Each particle
Fig. 5. Type IV virus within an infected CK tissue culture cell. A serrated outline is visible on many particles (arrows). Magnification ×112,000.

Fig. 6. Small crystal of Type IV particles within an infected CK tissue culture cell. Magnification ×80,000.

Fig. 7. Type IV particles within a large vacuole in a CK monolayer 72 h after infection. Magnification ×21,000.
DISCUSSION

The observations made in this study indicate the existence of a variety of rotavirus particles with different morphologies and they are listed in Table 1. The five types observed may be classified broadly into two groups, one of which appears to predominate in tissue culture and in vivo systems, and the other, not previously described, which is less common and distinctly different in character.

The first three types of particle constitute the first group and they probably represent different stages in the normal morphogenesis of the virions in vivo. Type III particles, although smaller than the Type II, may well represent the final stage in virus maturation; released extracellular virus of similar appearance and the large numbers of Type III particles seen in degenerating CK cells could suggest that this particle is the mature infective virion. The loss, or transformation, of the outer rim would thus be an integral process in the development of infective particles.

The second group of particles represents a generally less common and characteristically
### Table I. Morphology of rotavirus in thin sections*

<table>
<thead>
<tr>
<th>Particle</th>
<th>Morphology</th>
<th>Size (nm)</th>
<th>Position in cell</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td><img src="image" alt="Particle" /></td>
<td>25–35</td>
<td>Within dense inclusions</td>
<td>Characteristic of epithelial and tissue culture cells</td>
</tr>
<tr>
<td>Type II</td>
<td><img src="image" alt="Particle" /></td>
<td>70–80</td>
<td>Within cisternae of rough endoplasmic reticulum.</td>
<td></td>
</tr>
<tr>
<td>Type III</td>
<td><img src="image" alt="Particle" /></td>
<td>50–70</td>
<td>Type III also within lumen</td>
<td></td>
</tr>
<tr>
<td>Type IV</td>
<td><img src="image" alt="Particle" /></td>
<td>50–65</td>
<td>Usually within large vacuoles</td>
<td>Rarely found in normal cells. (Produced under adverse conditions?)</td>
</tr>
<tr>
<td>Type V</td>
<td><img src="image" alt="Particle" /></td>
<td>50</td>
<td>Within large vacuoles in calf intestinal cells</td>
<td>Rare</td>
</tr>
</tbody>
</table>

* Arrows denote formation of one particle type from another.

different rotavirus structure unrelated to the other forms. The particles of this group, represented principally by Type IV virus, are unenveloped and, apart from being smaller than Type III virus, do not possess a dense nucleoid. Furthermore, the morphogenesis of the second group appears to be distinctly different since the progeny particles are found within large vacuoles instead of within dilated endoplasmic reticulum, and no evidence of budding is apparent. Small crystals of particles, 'empty' capsids and striated paracrystalline inclusions are additional features not observed in association with viruses of the first group. Type IV particles are not considered to be a contaminating agent since they are associated with only the infected animals and tissue culture systems, each inoculated with one of two independently isolated rotavirus strains.

The significance of the type IV particles is unclear. Since Type III virus is produced characteristically in both intestinal cells in vivo and CK tissue culture monolayers, an obvious correlation between particle type and cell type is not evident. Type IV particles do, however, seem to be associated more commonly with damaged cells, and may represent a non-infectious form.

The characteristic appearance of particles in the first group corresponds closely with published reports of rotavirus in infected intestinal epithelium from other species. In particular, particles with and without the distinctive outer shell have been observed in mice infected with epizootic diarrhoea (EDIM) virus (Adams & Kraft, 1967; Banfield, Kasnic & Blackwell, 1968) and children infected with a non-bacterial gastroenteritis (Bishop et al. 1973; Holmes et al. 1975). Particles with a clear outline have not, however, been identified in piglets infected with a calf rotavirus (Hall et al. 1976), or in a previous study of calf intestine infected with a calf rotavirus (Stair et al. 1973). Large inclusions of membrane bound virus precursor material were observed in the latter study but were not seen in the present investigation. Striated inclusion bodies found in infected tissue culture cells resemble those previously described in intestinal epithelial cells from piglets infected with a calf rotavirus (Hall et al. 1976) but they were not identified in the piglet cells examined in the present study.

A recent investigation of MDBK cells in tissue culture infected with a cytopathic bovine
rotavirus (McNulty, Curran & McFerran, 1976) has indicated particles that can be identified as Types II and III, but the less common Type IV particle was not observed.

Distinctive particles interconnected by thin filaments (Type V) have not previously been described in calf intestines infected with rotavirus, but they have been reported in mouse tissue infected with EDIM virus (Adams & Kraft, 1967; Banfield et al. 1968). Filaments of similar appearance have also been observed in association with both orbivirus and reovirus grown in tissue culture (Lecatsas, 1968a, b). It has been suggested that these particles represent either degraded virions from which the RNA is leaking in filamentous strands or condensations of capsid material around incomplete nucleoprotein cores. There are no indications from the present study as to which of these interpretations is correct, but the clustered particles resemble most closely the appearance of Type IV viruses which are themselves probably aberrant or incomplete structures.

The question arises from previous observations of whether the 'single' and 'double' capsid particles, both characteristically seen in negative stain, are truly different morphological forms. A capsid composed of two separate layers of subunits has been described for rotavirus from children with gastroenteritis (Flewett et al. 1974b), but an ultrastructural study on similar material has indicated only one outer layer and differences in appearance have been ascribed to superposition artefacts (Martin, Palmer & Middleton, 1975). Nevertheless, the 'rim' of the larger particle has been related to an outer covering (Martin et al. 1975). The several distinct morphological forms of virus seen in the present work are compatible with the two structures commonly observed in negative stain. It has been suggested that the enveloped and unenveloped forms (corresponding to Types II and III) observed in infected MDBK cells correspond respectively to the negatively stained 'double' and 'single'-shelled particles (McNulty et al. 1976), but the results of the present study suggest an alternative hypothesis. The unenveloped Type III particles are the 'double' capsid structures seen in negative stain and the Type IV virus represents the smaller 'single' capsid forms; there is a close correspondence in appearance between the latter two structures and, in particular, the serrated outlines are comparable. Infectivity has been shown to be associated with the 'double' capsid particles (Bridger & Woode, 1976) and this observation is not inconsistent with such an hypothesis.

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REFERENCES


Rotavirus morphology


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