Morphogenesis of Porcine Rotavirus in Porcine Kidney Cell Cultures and Intestinal Epithelial Cells

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SUMMARY
The morphology of porcine rotavirus was similar in vitro in porcine kidney (PK) cell cultures and in vivo in porcine epithelial cells as examined by electron microscopy. Infected cells contained cytoplasmic, non-membrane-bound viroplasm and accumulations of virus particles within cisternae of the rough endoplasmic reticulum (RER). Three types of virus particles were noted: double-shelled or complete particles which averaged 77 nm in diam.; single-shelled or naked particles which ranged from 50 to 55 nm in diam.; and electron-dense nucleoids, or cores, 31 to 38 nm in diam. Virus particles acquired outer shells by budding through either matrices of granular, electron-dense viroplasm or membranes of distended RER. Accumulation of numerous single-shelled particles was observed only in PK cell cultures containing a high percentage of infected cells. In these cells, virus release occurred through disruption of the plasma membrane. Tubules, similar in diameter to the single-shelled particles, were observed in the nuclei of a few infected PK cells.

INTRODUCTION
A group of morphologically similar and antigenically related reovirus-like agents, often referred to as rotaviruses (Flewett et al. 1974), have been associated with diarrhoea in mice (Much & Zajac, 1972), calves (Mebus et al. 1969; Woode et al. 1974), humans (Flewett et al. 1973; Bishop et al. 1973; Kapikian et al. 1974), pigs (Woode et al. 1976; Saif et al. 1977) and lambs (McNulty et al. 1976a; Snodgrass et al. 1976). The simian virus SA 11 and O agent (Els & Lecatsas, 1972; Lecatsas, 1972) have also been grouped with these viruses.

Whereas rotaviruses have been included in the family Reoviridae on the basis of physicochemical characteristics (Davidson et al. 1975), they are antigenically distinct from reoviruses (Kapikian et al. 1975; Woode et al. 1976; Saif et al. 1977) and certain orbiviruses (Kapikian et al. 1974). Further characterization of rotaviruses has been hampered by the difficulty of propagating most rotaviruses in cell culture (Rubinstein et al. 1971; Flewett et al. 1974; Kapikian et al. 1974).

The recent adaptation of porcine rotavirus to growth in porcine kidney (PK) cell cultures (Theil et al. 1977) permitted comparative studies of its morphogenesis in vitro in cell culture and in vivo in intestinal epithelial cells.
METHODS

*Porcine rotavirus.* This agent was isolated from a 2-week-old conventional pig (OSU herd) and passaged in gnotobiotic pigs as described previously (Bohl et al. 1977; Saif et al. 1977).

**Cell culture.** Primary porcine kidney (PK) cell cultures were grown in 2 oz prescription bottles containing coverslips, and infected with porcine rotavirus as described previously (Theil et al. 1977). Briefly, confluent monolayers, washed three times with Hank's balanced salt solution (HBSS), were inoculated with 0.8 ml undiluted porcine rotavirus (OSU isolate) suspension. This virus had been passaged 13 times in PK cell cultures using pancreatin treatments between passages. Pancreatin treatment consisted of incubating the virus suspension with pancreatin (25 μg/ml) for 1 h at 37 °C prior to inoculating cell cultures. Control cultures were inoculated with 0.8 ml HBSS containing pancreatin (25 μg/ml). After adsorption for 1 h at 37 °C, the inoculum was decanted. Monolayers were washed once with HBSS to remove residual pancreatin and then re-fed with serum-free maintenance medium. This time was designated as 0 h post-exposure (p.e.).

Two separate experiments were conducted using PK cells derived from the kidneys of two different pigs. PK cells from the first (Lot 1) and second (Lot 2) pigs were used in Experiments 1 and 2, respectively. Inoculation procedures for each experiment were identical. At selected intervals in each experiment (Table I: Experiment 1, 15 h, 24 h and 40 h p.e.; Experiment 2, 10 h and 18 h p.e.) coverslips were removed for immunofluorescent staining and the cell cultures prepared for electron microscopy. Monolayers from Experiment 1 (15 and 24 h) were rinsed with HBSS and then submersed in the fixative (3% glutaraldehyde, 2% paraformaldehyde, 1.5% acrolein in 0.1 M-collidine buffer, pH 7.3) for 24 to 48 h at 4 °C. The other monolayers (Experiment 1, 40 h, and Experiment 2) were processed by scraping the cells from the glass, washing them once in HBSS and resuspending them in the above fixative.

*Gnotobiotic pigs.* Two 10-day-old gnotobiotic pigs were inoculated orally with 1 ml of a 10% bacteria-free suspension of a seventh gnotobiotic pig passage of porcine rotavirus. A third littermate pig was maintained in a separate isolator as an uninoculated control. One inoculated pig was sacrificed shortly after developing a light tan watery diarrhoea at 16.5 h p.e. The second inoculated pig did not have such marked clinical signs and did not have diarrhoea when sacrificed at 18 h p.e. The control pig remained clinically normal and was sacrificed at the same time as the second pig.

The intestines from the infected and control pigs were removed immediately after death. For electron microscopy, small sections were cut from the jejunum and ileum and immersed in the same fixative as used for PK cells. Scrapings from the mucosa of the mid-jejunum and ileum were fixed in a similar manner. Mucosal smears from similar regions were prepared on microscope slides (Bohl et al. 1977) for subsequent immunofluorescent staining.

*Immunofluorescent microscopy.* Coverslips removed from virus-inoculated and control cell cultures and mucosal smears were fixed for 10 min in acetone at room temperature and stained with fluorescein-conjugated anti-porcine rotavirus globulin prepared from hyperimmune pig serum (Theil et al. 1978). Stained preparations were examined by immunofluorescent microscopy as described previously (Frederick et al. 1976).

*Electron microscopy.* The fixed PK cells and intestinal scrapings were pelleted by centrifugation, washed with collidine buffer and then post-fixed in 2% osmium tetroxide for 3 h. Small blocks of the intestinal sections were post-fixed in a similar manner. Samples were then washed in buffer and stained overnight in 1% uranyl acetate. Dehydration was performed in an ethanol-acetone graded series, and specimens were embedded in Spurr plastic.
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After polymerization, blocks were sectioned on a Sorvall MT-2B ultramicrotome using glass and diamond knives and thin sections were picked up on Formvar-coated grids. Sections were stained with 0.5% uranyl acetate followed by 0.1% lead citrate. Preparations were viewed and photographed with a Philips 201 electron microscope.

RESULTS

Light and immunofluorescent microscopy

Porcine rotavirus c.p.e. was moderate in PK cells and generally was not demonstrable until approx. 15 to 18 h p.e. (Table I). This c.p.e. was characterized initially by the appearance of rounded or spindle-shaped cells scattered at random throughout the monolayer, but as the infection progressed, many cells detached from the glass. The number of cells within the monolayer with c.p.e. remained fairly constant until 24 h p.e. and then diminished while the number of detached cells floating in the medium appeared to increase steadily after 15 h p.e. Control cultures remained normal throughout.

Although the source of porcine rotavirus inoculum was the same for Experiments 1 and 2, immunofluorescent staining indicated that more cells were infected in Experiment 2 (Table I). Immunofluorescent cells were detected in all infected monolayers but not in the control monolayers. Infected cells contained extensive immunofluorescent antigen in large cytoplasmic granules and thick, thread-like aggregates of virus antigen were present either superimposed on or within the nuclei.

Rotavirus infection of inoculated gnotobiotic pigs was confirmed by the demonstration of numerous immunofluorescent villous epithelial cells in the jejunum and ileum (Table I). Immunofluorescent cells were not observed in the control gnotobiotic pig. Because the rotavirus infection was more extensive within the jejunum, samples of this region were selected for examination by electron microscopy.

Electron microscopy of infected cell cultures

Throughout Experiment 1, about 5 to 10% of the cells examined by electron microscopy appeared infected. Cytopathology or other evidence of virus infection was not seen in control cultures. At 15 h p.e., marked ultrastructural changes were generally not observed within the infected cells. However, rough endoplasmic reticulum (RER) cisternae were distended and often filled with virus particles (Fig. 1a). Scattered polyribosomes were seen still attached to the endoplasmic reticulum membrane and many virus particles were budding through matrices of granular, electron-dense material into the RER cisternae (Fig. 1a). This dense, granular virus precursor material, referred to as viroplasm (Holmes et al. 1975), was not membrane-bound and was seen in a wide range of sizes.

Numerous virus particles were in various stages of acquiring an outer shell by budding through membranes of the RER in regions devoid of ribosomes (Fig. 1a, b). These budding virus particles and those within the cisternae were 74 to 80 nm in diam. (average diam. of 77 nm) and had complete outer shells or envelopes. Many contained distinctive electron-dense nucleoids, or cores, which measured 31 to 38 nm in diam. (Fig. 1b). Furthermore, a number of these nucleoids were evident in the cisternae within a filamentous matrix.

By 24 h, and particularly 40 h p.e., pronounced ultrastructural changes were evident in the infected PK cells in Experiment 1 (Fig. 1c). The cytoplasm was rarefied and contained scattered fine filaments along with swollen mitochondria possessing disrupted cristae. Numerous ribosomes were attached to the distended membranes of the RER and aggregates of what appeared to be ribosomes were observed free within the cytoplasm. The layers
of the nuclear membrane were separated and invaginated. Chromatin was often marginated with decreased density of interchromatic material.

At this stage, virus particles were seen less frequently within the RER cisternae, but occasionally were found scattered throughout the cytoplasm. Accumulations of what appeared to be virus cores were sometimes seen within membrane-bound vesicles in the cytoplasm. The principal type of virus particle encountered throughout this experiment was the 77 nm double-shelled particle. Neither large numbers of smaller single-shelled particles nor the release of large numbers of virus particles from the cells was evident in Experiment 1.

In contrast, in Experiment 2, a greater percentage of PK cells was infected (Table 1). As early as 10 h p.e., a few cells were beginning to lyse and large numbers of virus particles had accumulated within distended cisternae of the RER and in the cytoplasm (Fig. 2a, b). In these cells, single-shelled (naked) and double-shelled (enveloped) particles were present with the former particles predominating (Fig. 2d). These smaller particles averaged 50 to 55 nm in diam. (Fig. 2b), whereas the larger double-shelled ones were 75 to 78 nm. Details of virus morphology were evident in many of the smaller particles (Fig. 2c) including: spoke-like peripheral capsomeres, hexagonal rings surrounding the cores, and pentagonal structures possibly representing tangential sections through fivefold axes of icosahedral symmetry. Viroplasm and budding virus particles were also noted but not as frequently as in Experiment 1.

### Table 1. Sequential changes observed in PK cells and small intestines of gnotobiotic pigs infected with porcine rotavirus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time p.e. (h)</th>
<th>Positive cells (%)</th>
<th>Location of virus antigen</th>
<th>Light microscopy: c.p.e. (%)</th>
<th>Electron microscopy: infected cells (%)</th>
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<tr>
<td>PK cells, Lot 1</td>
<td></td>
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<tr>
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<td>30</td>
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<td>5-10</td>
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<tr>
<td></td>
<td>24</td>
<td>30</td>
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<tr>
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<td>Ileum</td>
<td>Control‡</td>
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</table>

* Collected at the same time as the 24 h p.e. culture.
† Collected at the same time as the 18 h p.e. culture.
‡ Collected at the same time as the 18 h p.e. samples.
§ NA, Not applicable.
Fig. 1. Electron photomicrographs of PK cells 15 h and 40 h p.e. to porcine rotavirus (Experiment 1). (a) At 15 h p.e., infection was marked by formation of viroplasm (VP) and budding of virus particles through viroplasm and RER membranes (arrows) into distended cisternae of the RER. (b) High magnification of a budding virus particle containing a core, with other core particles (arrow) free within the cisternae of RER. (c) By 40 h p.e. the rarefied cytoplasm contained fine filaments and aggregated ribosomes (arrow). Note absence of virus particles within distended RER cisternae. Nucleus (N) contains margined chromatin and convoluted nuclear membrane.
Fig. 2. Electron photomicrographs of PK cell 10 h p.e. to porcine rotavirus (Experiment 2). (a) Accumulation of virus particles both within distended cisternae of the RER and free in the cytoplasm of a vacuolated degenerating cell. (b, c) High magnification of single-shelled virus particles (50 to 55 nm in diam.) within distended RER cisternae. Some detail of virus morphology is evident in particles in (c), including peripheral spoke-like capsomeres (single arrow), hexagonal ring surrounding the core (double arrows), and a pentagonal structure possibly representing a tangential section through fivefold axes of icosahedral symmetry (treble arrows). (d) Numerous single-shelled virus particles within RER cisternae. Few double-shelled particles are evident.
Fig. 3. Electron photomicrographs of PK cell 18 h p.e. (Experiment 2) to porcine rotavirus. Note release of (a) mostly double-shelled and (b) single-shelled virus particles, many within membrane-bound vesicles, through breaks in the plasma membranes (arrows). (c) High magnification of accumulations of 35 nm core particles within a filamentous matrix in the cisternae of the RER. A few complete virus particles are present. (d) Tubules were present within the nucleus (arrow) of a few infected cells.
By 18 h p.e. in Experiment 2, most cells were lysing (Fig. 3a, b) and virus particles, many still within membrane-bound vesicles, were being released through breaks in the plasma membrane. Whereas accumulations of the 55 nm single-shelled particles were prevalent (Fig. 3b), large clumps of the 77 nm double-shelled forms were only observed occasionally (Fig. 3a). Also, commonly seen at this time within the cisternae of the RER were numerous core particles which averaged 35 nm in diam. (Fig. 3). Many of these, as well as the other virus particles, contained an electron-dense central dot which measured about 15 nm in diam.

At this stage of infection, intranuclear tubules were observed in a few infected cells (Fig. 3d). These tubules averaged about 50 nm in diam. which was about the same diameter as the single-shelled virus particles.

![Electron photomicrographs of villous epithelium from gnotobiotic pigs infected with porcine rotavirus. Infected cells contain virus particles within distended cisternae of the RER and viroplasm (VP). (a) Infected cell with rarefied cytoplasm between two apparently normal cells. (b) Infected cell with shortened, irregular microvilli and a break in the brush border (arrow). Viroplasm (VP) within inset is shown at higher magnification in Fig. 5(c).](image)

**Electron microscopy of intestinal sections**

Similar to the rotavirus-infected PK cells in Experiment 1, only about 5% of the columnar epithelial cells from infected pig intestines examined by electron microscopy appeared infected (Table 1). Infected cells were dispersed among normal cells such that cells adjacent to the infected cells often appeared normal (Fig. 4a). Few differences were noted between jejunum sections from the pig sacrificed at 16.5 h p.e. and those from the 18 h p.e. pig. Occasionally, infected cells from the latter pig had rarefied cytoplasm (Fig. 4a) and breaks in the brush border (Fig. 4b). Except for these alterations, marked ultrastructural changes
were rare. Microvilli were generally normal in length on cells containing virus particles and only on an occasional infected cell were they shortened and irregular (Fig. 4b). In contrast, more advanced cytopathology was observed in infected cells from jejunum mucosal scrapings including vacuolated and disintegrating cells, that were mostly devoid of virus particles.

Intracellular location of virus particles and cytopathology were similar to those observed in infected PK cells. Most virus particles were found within distended cisternae of the RER (Fig. 5a), often budding through the RER membrane or out of granular electron-dense viroplasm into the RER cisternae. These particles ranged from 75 to 78 nm in size and were double-shelled. In addition, particles were frequently observed which contained several virus forms within a single outer layer.

Besides the dense, granular viroplasm (Fig. 5a), a second type of viroplasm, consisting of convoluted smooth membranous material (perhaps smooth ER), was observed in infected cells from the intestinal sections (Fig. 5b, c). Virus particles occurred within the matrix of this material (Fig. 5b, arrow), and this viroplasm, unlike the other type, was seen only within the distended cisternae of the RER. Virus was also observed budding at the periphery of this material into the cisternae (Fig. 5c, arrow).

The epithelial cells from the jejunum of the control pig were normal and evidence of virus particles or virus-induced cytopathology was not observed.

**DISCUSSION**

The morphogenesis of porcine rotavirus was similar in vivo in epithelial cells from the jejunum of infected pigs and in vitro in infected PK cell cultures. In both studies, virus accumulated predominantly within cisternae of the RER, and non-membrane-bound viroplasm or virus precursor material was observed in the cytoplasm of infected cells. These two distinctive characteristics have been reported for other rotaviruses, including an in vitro infection with bovine rotavirus (McNulty et al. 1976b) and in vivo infections with porcine (Chasey & Lucas, 1977; McNulty et al. 1976c), human (Bishop et al. 1973; Holmes et al. 1975), bovine (Stair et al. 1973), ovine (McNulty et al. 1976a) and murine (Adams & Kraft, 1967; Banfield et al. 1968) rotaviruses.

Generally, the intracellular localization of porcine rotavirus seen by electron microscopy was in accordance with the distribution of virus antigen detected by immunofluorescence. Also, both the type and distribution of immunofluorescence noted in cell culture and epithelial cells agree with investigations by others of reoviruses (Rhim et al. 1962) and various rotaviruses (Mebus et al. 1971; McNulty et al. 1976a, c). At present, it is not known if the immunofluorescent thread-like aggregates of virus antigen observed in the nuclear region of infected cells correspond to the intranuclear tubules seen in thin sections.

In studies on the morphogenesis of rotaviruses, various sizes were reported for the virus particles and this information has been summarized by McNulty et al. (1976b). Three types of virus particles were noted in many of these studies: the largest, double-shelled or enveloped particles; single-shelled particles; and smaller cores or nucleoids. Chasey & Lucas (1977) distinguished three morphological types of virus particles within the small intestinal epithelial cells of infected pigs. These included 25 nm electron-dense cores, 60 nm naked particles and 70 to 75 nm membrane-bound particles. These results are similar to ours, except that the smaller, single-shelled naked particles, seen only in PK cells, were 50 to 55 nm in diam. and the nucleoids averaged 35 nm. This discrepancy in the size of the naked particle may be due to the inability to measure them accurately because of their indistinct
Fig. 5. Electron photomicrograph of villous epithelium from gnotobiotic pig infected with porcine rotavirus. (a) Note virus particles budding from viroplasm into the distended cisternae of the RER. Only double-shelled particles (75 to 78 nm) were present. (b) Enveloped virus particles are situated within (arrow) and at the edge of convoluted smooth membranous material. (c) Higher magnification of viroplasm shown within inset in Fig. 4(b). Arrows indicate virus particles budding at the periphery.
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outer margins or may represent particles in a different stage of the replicative cycle. Absence of such particles from the intestinal sections and PK cells from Experiment 1 may be related to the time of sampling with loss of cells containing these particles into intestinal fluids or cell culture medium.

Because of the difficulty in adapting most rotaviruses to propagate in cell culture, there are few reports on their morphogenesis in vitro (McNulty et al. 1976b; Wyatt et al. 1976). Recent propagation of porcine rotavirus in PK cells by using pancreatin treatment of the virus (Theil et al. 1977a), allowed us to follow the development of the virus in these cells at different stages post-infection. The m.o.i. could not be determined in this study since there was no means to quantify virus effectively or determine how many particles were actually infectious following pancreatin treatment. Thus, the percentage of infected cells varied with each experiment. This may also have been influenced by the susceptibility of the different PK cell cultures used. However, it is possible that all culture experiments probably represented single cycle replications of rotavirus since enough active residual enzyme would probably not remain in the medium to activate released virus for a second cycle of infection, and rotavirus propagation in the absence of enzyme was minimal (Theil et al. 1977). Also, by electron microscopy, most infected cells appeared to be in a similar stage of replication at each of the different sampling periods.

Rotavirus infection of PK cells progressed rapidly. In Experiment 1, by 15 h p.e. many budding virus particles were seen within cisternae of the RER. Then by 24 and 40 h p.e. most of these vesicles were devoid of virus particles, possibly because the virus particles were already released into the medium by disruption of the plasma membrane of infected cells.

In Experiment 2, which had a higher percentage of infected cells, a few cells in the early stages of lysis were evident by 10 h p.e. and cells were filled with numerous virus particles. These effects were even more pronounced in cells at 18 h p.e. The accumulation of large numbers of naked virus particles in the cisternae of the RER and free within the cytoplasm was seen only in cells in the process of disruption or showing signs of pronounced cytopathology. Such naked virus particles may represent a final stage of virus assembly for which the availability of RER membranes is limited, thus precluding the accumulation of large numbers of enveloped forms. This observation is in contrast to the study of McNulty et al. (1976b) which showed an accumulation of naked particles prior to the appearance of larger enveloped forms. The reason for such a discrepancy is uncertain, but may be influenced by such variables as m.o.i., sampling time, type of rotavirus (porcine instead of bovine) and cell culture system employed. Since in our study the infection progressed rapidly, perhaps these naked particles would have been noted in samples collected at an earlier stage of infection. Thus earlier sampling times and shorter intervals between samplings would be needed to resolve more fully the early events in the replicative cycle of this virus.

In infected cells from jejunum sections of pigs, little synchrony of infection was evident and cells in various stages of infection were widely scattered among normal cells. Few cells in these samples were filled with large numbers of virus particles or were seen in the process of lysis, possibly because such infected cells would be rapidly desquamated into the intestinal lumen. Supporting this idea was the observation that cells scraped from the jejunum had more advanced stages of cytopathology with many vacuolated and lysed cells devoid of virus.

Based on similarities in both morphology and morphogenesis, rotaviruses have been grouped in the family Reoviridae which includes the genera reoviruses and orbiviruses (Davidson et al. 1975). A common characteristic of these viruses is their association with
viroplasm in the cytoplasm of infected cells. Whereas reoviruses form intracytoplasmic crystalline arrays of virus particles and their maturation is associated with spindle tubules (Harford et al. 1962; Rhim et al. 1962; Dales et al. 1965; Lecatsas, 1968b), this has not generally been observed with rotaviruses either in the present study or studies of others (Adams & Kraft, 1967; Lecatsas, 1972; Stair et al. 1973; Holmes et al. 1975; McNulty et al. 1976b) with the exception of the O agent (Lecatsas, 1972). Some orbiviruses (Murphy et al. 1968; Breese et al. 1969) and most rotaviruses, except the O agent acquire an envelope from the RER membrane by a budding process and mature within the RER cisternae. However, unlike orbiviruses (Lecatsas, 1968a; Tsai & Karstad, 1970; Murphy et al. 1971), most rotaviruses have not been observed to form tubules (McNulty et al. 1976b). An exception, however, is the EDIM virus which formed tubules in the nuclei and cytoplasm of infected mouse epithelial cells (Banfield et al. 1968). Similar tubule formation was observed only in the nuclei of PK cells in our study, but occurred rarely. Whether such tubules are actual virus material or represent a physiological change associated with virus-induced cytopathology, similar to that reported for other pathologic conditions (Borgers et al. 1977), has not been determined. Also, most rotaviruses except the SA 11 virus (Lecatsas, 1972) do not form viroplasm in the nucleus.

The authors agree with McNulty et al. (1976b) that on the basis of morphogenesis, rotaviruses more closely resemble orbiviruses than reoviruses. However, because of the heterogeneity within the rotavirus and orbivirus groups, further clarification of physical and biochemical properties is needed before classification of these viruses can be determined.

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