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Reovirus-Like Agent (Rotavirus) from Lambs

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Rotavirus particles were demonstrated by electron microscopy in the feces of lambs with diarrhea. Rotavirus antigen was synthesized in cell cultures infected with filtrates of the diarrheic feces, but the virus was not adapted to grow serially in cell cultures. An antigenic relationship between rotaviruses from lambs, pigs, and calves was demonstrated by immunofluorescence. Colostrum-deprived lambs were infected with the lamb rotavirus, and the virus was passaged in lambs. Viral replication occurred in the villous epithelial cells of the small intestine, and the virus was excreted in the feces up to 78 h postinfection. Diarrhea was not observed in the experimentally infected lambs.

Viruses resembling reoviruses and orbiviruses have been associated with gastroenteritis in a variety of animal species (2). The names duovirus (6) and rotavirus (7) have been proposed for this group of viruses, and rotavirus will be used in this paper. To date there has been only one report of a rotavirus in lambs (13). In this paper we report the detection of rotavirus particles in the feces of lambs from Northern Ireland and describe the experimental infection of colostrum-deprived lambs with the virus.

MATERIALS AND METHODS

Preparation of specimens for inoculation into cell cultures. Approximately 20% suspensions of feces were made in Eagle (BHK) medium (Wellcome Laboratories) with 15% fetal calf serum and 10% tryptose phosphate broth, and containing 1,000 U of penicillin, 1,000 μg of streptomycin, 5 μg of amphotericin B, and 80 μg of tylosin per ml (ETPB). These suspensions were clarified by centrifugation at 3,000 × *g* for 30 min, and the resulting supernatants were filtered through cellulose ester filters (0.45-μm pore size; Millipore Corp.). The resulting fecal filtrates were used to infect cell cultures.

Cell cultures. Primary cultures of lamb, pig, and calf kidney cells were prepared as described previously (9) and grown in Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate and 10% serum, with 100 U of penicillin and 100 μg of streptomycin per ml. Lamb kidney cells were grown in ox serum, whereas lamb serum was used for pig and calf kidney cells.

Continuous bovine kidney (MDBK), baby hamster kidney (BHK-21), monkey (Vero), and pig kidney cells [PK(15)] were grown in Eagle (BHK) medium with 10% fetal calf serum and 10% tryptose phosphate broth.

Cell cultures were infected by diluting freshly trypsinized cells at least 10-fold with fecal filtrates made up in ETPB. The diluted cells were then seeded into tubes containing flying cover slips, which were incubated stationary at 37°C.

Preparation of antisera for immunofluorescence. Antiserum to calf rotavirus, which was partially purified from feces, was prepared in a rabbit, as described elsewhere (11). Antisera to the pig and lamb rotaviruses were also prepared in rabbits. Fecal filtrates from rotavirus-infected animals were inoculated intramuscularly, together with Freund complete adjuvant. After 1 month the rabbits were inoculated intravenously with fecal filtrates alone and then were bled out 2 weeks later. Sera were fractionated and conjugated with fluorescein isothiocyanate as described by Clarke et al. (5).

Immunofluorescence. Cover slips were harvested from infected cell cultures 24 h postinfection, fixed in acetone for 10 min, and then stained for 30 min at room temperature with fluorescein-labeled rotavirus antisera. They were washed in several changes of phosphate-buffered saline for at least 1 h, mounted in buffered glycerol, and examined in a Leitz Ortholux microscope using ultraviolet illumination.

Frozen sections of intestine were stained overnight at 4°C; otherwise they were treated as described above.

Electron microscopy. Suspensions of feces and intestinal contents were made in ETPB and clarified by centrifugation as described above. This material was mounted on carbon-Formvar grids and stained with 4% sodium phosphotungstate, pH 7.1.

Pieces of the gastrointestinal tract from experimentally infected lambs were fixed for 2 h at 4°C in 4% glutaraldehyde in sodium cacodylate buffer and postfixed in 1% osmium tetroxide for 2 h at room temperature. Dehydration was performed in a graded series of ethanol, and the tissues were embedded in araldite.

Ultrathin sections were cut on an LKB Ultratome III, stained with uranyl acetate and lead citrate, and examined in an AEI EM6B electron microscope.

Virus inocula. Fecal suspensions (20 to 30%) were

made in ETPB from feces specimens demonstrated by electron microscopy to contain rotavirus particles. These suspensions were clarified and filtered as described above for cell culture inoculation; the resulting filtrates were used to infect colostrum-deprived lambs. We were unable to culture bacteria from these fecal filtrates. Similarly, no viruses other than rotaviruses were detected in the filtrates by electron microscopic examination or by inoculation into secondary cultures of lamb, pig, and calf kidney cells.

Experimental infection of lambs. Lambs were removed from the dam immediately after birth, so that they were completely colostrum deprived. They were housed in isolation units and fed on a mixture of equal parts of unsweetened condensed milk and 1% dextrose.

Lambs were inoculated orally with 2.5 ml of fecal filtrate approximately 12 h after birth. Samples of feces, abomasum, small intestine, and colon were removed during a postmortem examination. Tissues were processed for electron microscopy as described above, and also fixed at −70°C in 2-methylbutane to provide frozen section material.

RESULTS

Detection of rotavirus in feces of lambs with diarrhea. In the course of an investigation into acute disease in an intensive lamb-rearing unit, six sick 8-month-old lambs were sent to the Veterinary Research Laboratories for examination, where clostridial enterotoxemia was diagnosed. Three of these lambs also had diarrhea, and intestinal contents collected from them during postmortem examination were shown by electron microscopic examination to contain large numbers of rotavirus particles. The remaining three lambs did not have diarrhea, and we were unable to demonstrate the presence of rotavirus particles in the contents of their intestines.

The rotavirus particles observed in the intestinal contents of the lambs with diarrhea (Fig. 1) were morphologically identical to those described in Scotland by Snodgrass et al. (13) and to those observed by us in the feces of pigs and calves with diarrhea (McNulty et al., Vet. Microbiol., in press). Virus particles with both single- and double-shelled capsid structure were seen, but single-shelled particles were rare.

Double-shelled particles were approximately 74 nm in diameter, whereas single-shelled particles measured 60 nm.

Cell culture studies. Freshly trypsinized lamb kidney and PK(15) cells were infected with filtrates of feces in which rotavirus particles had been detected by electron microscopy. Specific intracytoplasmic fluorescence was ob-

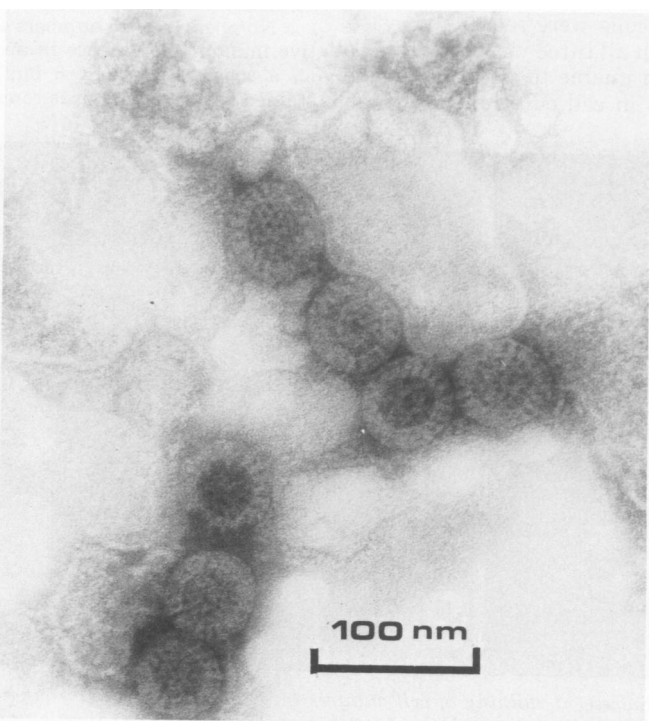

FIG. 1. *Electron micrograph of double-shelled rotavirus particles in the intestinal contents from a diarrheic lamb.*

served in a small number of cells when the cover-slip cultures were stained with the lamb rotavirus conjugate (Fig. 2a). Positive immunofluorescence was also obtained when antisera to the calf and pig rotaviruses were used (Fib. 2b, c). It was not possible to distinguish between the different antisera as regards the pattern of staining, the intensity of fluorescence, and the number of cells containing viral antigen. No fluorescence was observed when cell cultures were infected with filtrates made from feces in which we were unable to detect rotaviruses using electron microscopy. More cells were infected, as judged by immunofluorescence, when freshly trypsinized cells rather than established monolayers were used.

The ability of the lamb virus to infect a variety of cell cultures was compared with that of isolates of the pig and calf viruses which were not adapted to grow serially in cell cultures (Table 1). The degree of infection was measured by counting the number of cells showing positive immunofluorescence in an infected cover-slip culture. The lamb and calf rotaviruses infected lamb, calf, and pig kidney cells, but were best able to infect PK(15) cells. Overall, the number of fluorescing cells in cultures infected with the pig virus was less than with the lamb and calf viruses, and little or no pig viral antigen was synthesized in bovine kidney cells. BHK-21 and Vero cells were relatively resistant to infection with all three virus isolates. To date, we have been unable to serially passage the lamb rotavirus in cell cultures more than twice, and in most instances immunofluorescence was lost after the first passage. Prolonged initial passage periods were used in the successful adaptation of the calf rotavirus to grow in cell cultures (4, 11a, 12). However, with the lamb virus the number of infected cells was greatest approximately 24 h postinfection and no infected cells were detected 7 days postinfection.

Experimental infection of lambs. Two colostrum-deprived lambs were infected orally with a bacteria-free fecal filtrate containing lamb rotavirus. Rotavirus particles identical to those in Fig. 1 were first detected by electron microscopy in the feces of both lambs 24 h postinfection.

Lamb 1 became dull and disinclined to feed about 30 h postinfection and was moribund

TABLE 1. *Infection of cell cultures with lamb, calf, and pig rotaviruses*

Cell culture	Lamb rotavirus	Calf rotavirus	Pig rotavirus
Lamb kidney	70[a]	15	13
Calf kidney	15	32	2
MDBK	4	110	0
Pig kidney	82	13	13
PK(15)	260	200	30
Vero	4	3	0
BHK-21	0	0	0

[a] Numbers refer to numbers of cells showing positive immunofluorescence in an infected monolayer on a cover slip (22 by 6 mm). Cover slips were stained with calf rotavirus conjugate.

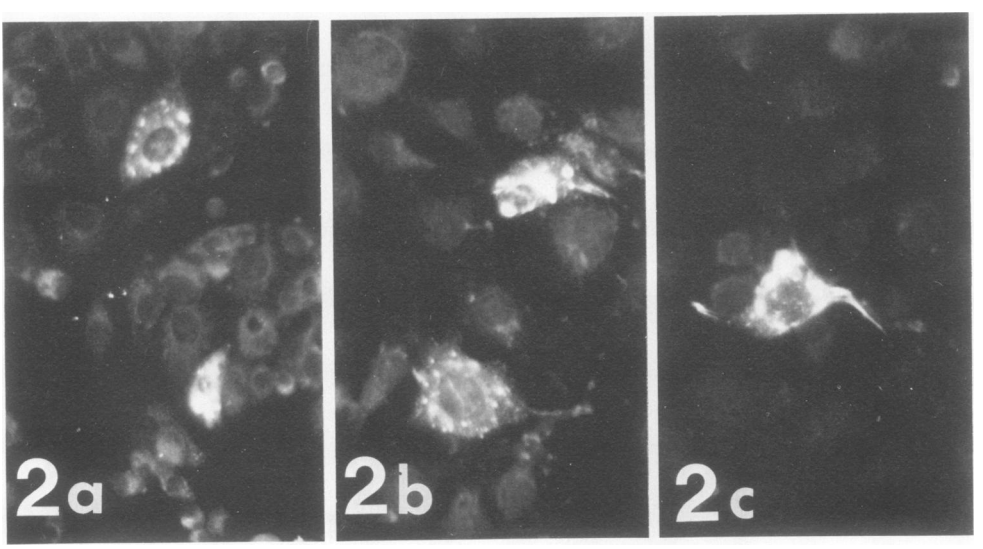

FIG. 2. *Immunofluorescent staining of cell cultures infected with the lamb rotavirus. (a) Infected lamb kidney cells stained with antiserum to the lamb rotavirus; (b) infected PK(15) cells stained with antiserum to the calf rotavirus; (c) infected PK(15) cells stained with antiserum to the pig rotavirus.*

when killed 48 h postinfection. Feces from this lamb were soft from 24 h postinfection, but were never diarrheic. Many rotavirus particles were observed in the contents of the small and large intestines and in feces collected during postmortem examination.

Lamb 2 remained clinically normal for the duration of the experiment. Virus was present in a feces specimen collected 78 h postinfection, but was not detected in intestinal contents or feces when the lamb was killed 117 h postinfection.

A filtrate was prepared from feces taken from lamb 1 48 h postinfection, and this was used to infect a third lamb (lamb 3). Rotavirus was present in feces specimens collected from this lamb 23, 43, 54, and 69 h postinfection. This lamb also remained clinically normal and was killed 69 h postinfection, when virus particles were detected in contents of the small and large intestines. Cover-slip cultures of PK(15) cells were infected with filtrates of feces obtained from lambs 1, 2, and 3, which contained rotavirus particles. Specific intracytoplasmic fluorescence was observed when these cover-slip cultures were stained with the calf rotavirus conjugate.

Frozen sections of abomasum, small intestine, and large intestine from lambs 1, 2, and 3 were stained with the calf rotavirus conjugate. Viral antigen was detected in villous epithelial cells in the distal third of the small intestines of lambs 1 and 3 (Fig. 3). No fluorescence was observed in stained sections of abomasum and colon from these two lambs nor in any of the tissues taken from lamb 2.

Ultrathin sections were cut from areas of the small intestine from lamb 1, which contained viral antigen, and these were examined in the electron microscope. Virus particles were observed in villous epithelial cells, located within distended cisternae of the endoplasmic reticulum (Fig. 4). Matrices of granular or finely fibillar material were often found near developing virus particles. This was probably viral precursor material or viroplasm, as it sometimes appeared to condense to form virus particles (Fig. 5). Two types of virus particles were observed. Particles measuring 55 to 60 nm in diameter and consisting of an electron-dense nucleoid surrounded by a single, less electron-dense shell were commonly seen. The second type of particle was 73 to 80 nm in diameter and was enclosed within an additional outer electron-dense shell, which in some instances appeared to be acquired by budding through the endoplasmic reticulum (Fig. 5). These two particle types probably correspond to the single and double-shelled virus particles, respectively, which were observed in negatively stained preparations of feces and intestinal contents.

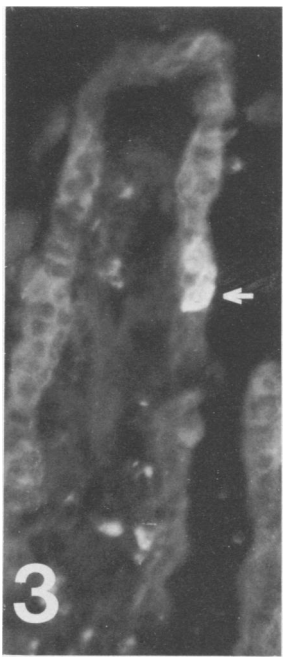

FIG. 3. *Immunofluorescent staining of frozen section of ileum of lamb 3, which was infected orally with lamb rotavirus 69 h previously. Viral antigen is present in a few villous epithelial cells (arrow).*

DISCUSSION

This paper reports the detection of rotavirus particles in the feces of lambs with diarrhea in Northern Ireland. Negatively stained preparations of virus particles were morphologically indistinguishable from those described in lambs in Scotland (13) and from those we observed in the feces of pigs and calves with diarrhea (McNulty et al., in press).

The ability of fluorescein-labeled antisera prepared against pig and calf rotaviruses to stain lamb rotavirus antigen shows that the calf, pig, and lamb rotaviruses share a common antigen. An antigenic relationship between the calf and lamb viruses was demonstrated by Snodgrass et al. (13).

The lamb rotavirus infected and synthesized viral antigens in lamb, pig, and calf kidney cell cultures. However, since we were unable to passage the virus more than twice, it is possible that this was a defective rather than a productive infection. A greater number of cells was infected when freshly trypsinized cells rather than established monolayers were inoculated with the lamb rotavirus. This requirement for

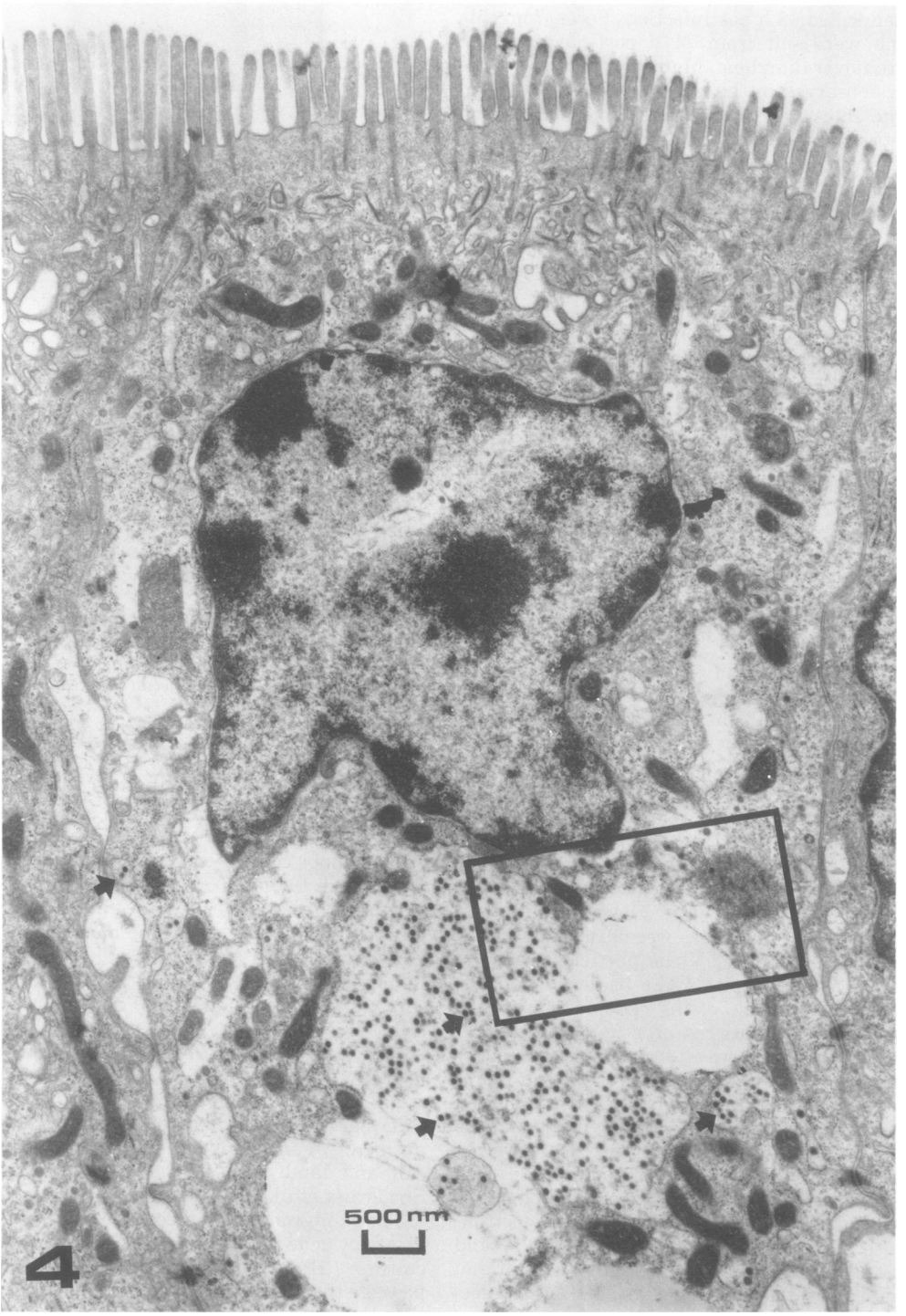

FIG. 4. *Electron micrograph of villous epithelial cell from ileum of lamb 1, which was infected orally with lamb rotavirus 48 h previously. Virus particles (arrows) are present within distended cisternae of the endoplasmic reticulum. The area within the rectangle is shown at higher magnification in Fig. 5.*

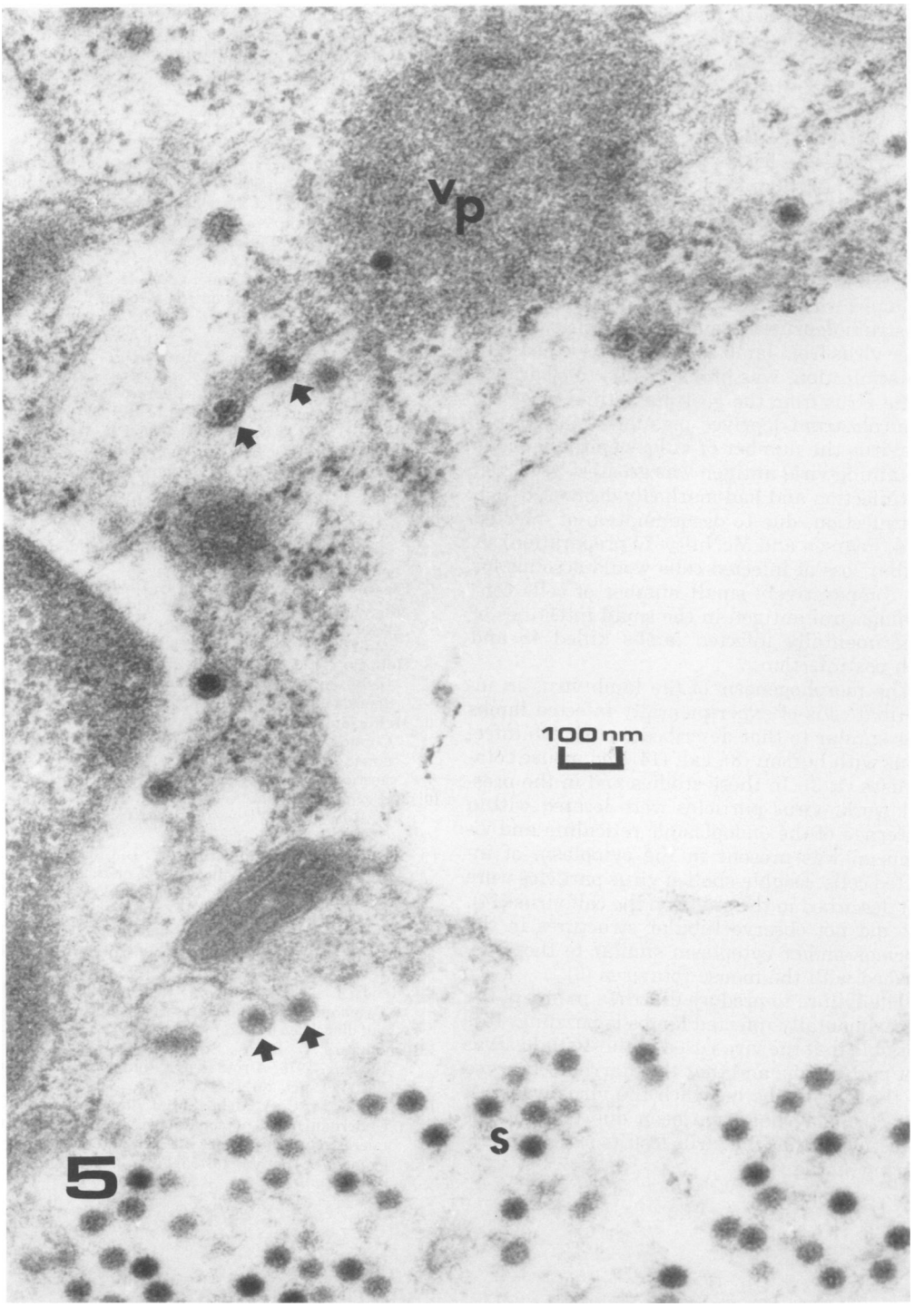

FIG. 5. *Detail of rotavirus-infected villous epithelial cell, showing viroplasm (Vp), single-shelled virus particles (s), and virus particles which are acquiring an additional outer shell by a process of budding (arrows).*

actively dividing cells has also been noted with other sheep viruses (10).

Colostrum-deprived lambs were infected with fecal filtrates from the diarrheic lambs. Evidence that viral replication occurred in these lambs was provided by the excretion of the virus in the feces up to 78 h postinfection, the demonstration of rotavirus antigen in villous epithelial cells of the small intestine, and the observation of developing virus particles in these cells by electron microscopy. Rotavirus excreted in the feces of one of these experimental lambs was successfully passaged in another colostrum-deprived lamb. The inability to recover virus from lamb 2 when it was killed, 117 h postinfection, was probably due to clearance of the virus from the gastrointestinal tract.

In colostrum-deprived pigs infected with pig rotavirus the number of villous epithelial cells containing viral antigen was greatest 24 to 30 h postinfection and had markedly decreased 48 h postinfection, due to desquamation of infected cells (Pearson and McNulty, in preparation). A similar loss of infected cells would account for the comparatively small number of cells containing viral antigen in the small intestines of experimentally infected lambs killed 48 and 69 h postinfection.

The morphogenesis of the lamb virus in intestinal cells of experimentally infected lambs was similar to that described for in vivo infections with human (8), calf (14) and mouse rotaviruses (1, 3). In these studies and in the present work, virus particles were located within cisternae of the endoplasmic reticulum and viroplasm was present in the cytoplasm of infected cells. Double-shelled virus particles were not described in the study on the calf virus (14). We did not observe tubular structures in the nucleus and/or cytoplasm similar to those described with the mouse rotavirus (3).

The failure to produce diarrhea in any of the experimentally infected lambs is puzzling. It is possible that the virus used in these studies was not pathogenic, and that the diarrhea observed in the sick lambs in which the virus was first detected may not have been due to rotavirus infection or that the virus lost its pathogenicity during storage.

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