Scanning Electron Microscopy of Intestine of Gnotobiotic Piglets Infected with Porcine Rotavirus

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ABSTRACT

The development of intestinal lesions caused by the porcine rotavirus were studied in six day old gnotobiotic piglets by scanning electron microscopy. The onset of diarrhea followed an incubation period of 17 to 31 hr. The first detectable lesion was observed in the ileum at 12 hr postinfection, a few hours before the onset of diarrhea. At this time enterocytes appeared swollen and began to separate from each other. Seventeen hours after the onset of diarrhea, lesions were quite severe in jejunum and ileum. Enterocytes were detaching from the lamina propria leaving denuded areas. Microvilli were sparse on the cell surfaces and there was marked villous atrophy. Regeneration of ileal mucosa was evident at 4.8 days after the onset of diarrhea. Nine days after recovery from diarrhea the intestinal villi had returned to near its normal structure but there remained some evidence of mucosal damage.

RÉSUMÉ

Cette expérience consistait à étudier le développement de lésions intestinales attribuables au rotavirus porcin, chez des porcelets gnotoxéniques et âgés de six jours, à l'aide de la microscopie électronique. La diarrhée débuta, après 17 à 31 heures d'incubation. On décela la première lésion intestinale, dans l'iléon, 12 heures après l'infection, i.e. quelques heures avant l'apparition de la diarrhée. À ce moment, les entérocytes étaient gonflés et commençaient à se séparer les uns des autres. Dix-sept heures après le début de la diarrhée, le jéjunum et l'iléon présentaient des lésions marquées. Les entérocytes se détachaient de la lamina propria, ce qui provoquait des érosions focales de la muqueuse. Les entérocytes accusaient une diminution de leurs microvillosités, tandis que les villosités présentaient beaucoup d'atrophie. La régénération de la muqueuse de l'iléon devint évidente à 4,8 jours après le début de la diarrhée. Neuf jours après la cessation de la diarrhée, les villosités intestinales étaient redevenues presque normales, mais la muqueuse présentait toujours une certaine évidence du dommage qu'elle avait subi.

INTRODUCTION

The importance of rotaviruses as a primary cause of diarrhea in pigs is now well established (1, 6, 8, 9, 19, 20, 27). Several studies have been conducted to determine the pathogenicity of porcine rotaviruses in conventional, colostrum-deprived, and gnotobiotic piglets (3, 5, 16, 17, 18, 22, 25). These studies have involved the use of immunofluorescent staining, examination of fecal samples by transmission electron microscopy (TEM), and histological observations by light microscopy and TEM. Mebus *et al* (11, 12, 13) reported on the pathogenicity of rotaviruses and coronaviruses in gnotobiotic calves using scanning electron microscopy (SEM), in addition to the above techniques. These SEM studies of

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the intestinal mucosa have provided more detailed information of the alterations on the intestinal mucosa caused by viruses due to the tridimensional observations of a much larger surface area of affected intestinal mucosa. The present project was undertaken to study the sequential changes on the intestinal mucosa of gnotobiotic piglets infected with the porcine rotavirus using SEM procedures. Detailed studies of the effect of rotaviruses on the intestinal tract of piglets are quite important, because this animal has also become a good experimental model for the study of rotaviruses from other animal species (2, 4, 14, 23, 24).

MATERIALS AND METHODS

VIRUS

A strain of porcine rotavirus (designated B-317) isolated in our laboratory from a field outbreak was used for these experiments. This strain of rotavirus was found to be free of transmissible gastroenteritis (TGE) coronavirus by both virus isolation attempts, direct fluorescent antibody (FA) staining of intestinal sections from infected gnotobiotic piglets and by electron microscopy study of fecal extracts. Infected intestinal sections were only FA positive using a calf rotavirus conjugate (13). Electron microscopy of fecal extracts revealed only the presence of numerous virions undistinguishable from other known rotaviruses of human or animal origin.

ANIMALS

Nine six-day-old cesarean-derived, colostrum-deprived gnotobiotic Yorkshire piglets were used in these experiments. Piglets were housed in sterile plastic isolators and maintained as previously described (26).

EXPERIMENTAL DESIGN

Seven gnotobiotic piglets were orally inoculated with 2.0 mL of the fecal filtrate. Two piglets were kept as uninoculated controls in a separate isolator. Infected piglets were killed at 0.5, 0.8, 1.5, 2.5, 6.5, 10.5 and 16.0 days postinoculation (PI). Control uninoculated piglets were killed simultaneously with the first and the last principal piglets (Table I).

NECROPSY

Piglets were killed by exsanguination under light chloroform anesthesia. The entire intestinal tract was immediately removed and 3-4 cm ligated segments were obtained from the upper (5 cm from the pylorous), lower (5 cm from the ileo-cecal valve) and middle (mid-way between upper and lower samplings) small intestine. One segment of spiral colon (3 cm) was also obtained. The ligated intestinal segments were fixed with 2.5% glutaraldehyde solution in Millonig's buffer at 4°C for three to four hours. Fixed samples were then washed twice in Millonig's buffer and stored in the same solution at 4°C.

TABLE I. Clinical Observations and Presence of Virus Particles and/or Viral Antigens in Samples From Rotavirus Infected Gnotobiotic Piglets

Piglet No.	Diarrhea	Onset of Diarrhea (Days PI)	Duration of Diarrhea (Days)	Time of Euthanasia (Days PI)	Rotavirus in Feces	Immunofluorescent Staining U	M	L[a]
1[b]	No			0.5	−	−	−	−
2	No			0.5	+	−	+	+
3	Yes	0.7	0.1[c]	0.8	++++	−	+	+
4	Yes	0.8	0.7[c]	1.5	++++	−	−	+
5	Yes	0.7	1.8[c]	2.5	++	−	−	−
6	Yes	1.7	4.8[c]	6.5	+	−	−	−
7	Yes	1.5	5.5	10.5	++	−	−	−
8	Yes	1.6	7.0	16.0	−	−	−	−
9[b]	No			16.0	−	−	−	−

[a]Upper (U), Middle (M) and Lower (L) small intestinal segments
[b]Control noninoculated piglets
[c]Diarrhea present at necropsy

Adjacent intestinal samples were also collected from the upper, middle and lower small intestine, and from the spiral colon and processed for routine histological examination and for frozen sectioning as described by Mebus *et al.* (13). Immunofluorescent staining was done using a fluorescein conjugated rabbit anti-calf rotavirus antibody (10). Intestinal contents were collected from cecum and rectum, stored at -60°C and then processed for virus screening by TEM[1] as previously reported (24).

SCANNING ELECTRON MICROSCOPY

Glutaraldehyde-fixed intestinal samples were cut lengthwise and sections (5 x 5 mm) were obtained from the intestinal wall opposite to the mesenteric attachment. These intestinal sections were washed twice in Millonig's buffer, postfixed by the osmium-thiocarbohydrazide-osmium method (8), critically point dried and mounted. Samples were observed and photographed with a scanning-electron microscope[2] at 20 kV at different magnifications.

RESULTS

CLINICAL OBSERVATIONS

Except for the principal piglet killed 12 hours PI, all inoculated piglets developed diarrhea, but none vomited. The incubation period for the onset of diarrhea was between 17 to 31 hours PI. Diarrhea lasted for 5.5 and seven days in those piglets allowed to run a complete course of the infection. Rotavirus particles were detected in all infected piglets except for the one killed on day 16 PI. Despite the severity of diarrhea, rotavirus infected piglets remained alert and had normal appetites. None died from the infection. Control uninoculated littermates remained healthy throughout the observation period (Table I).

[1]**Model EM 201, N. V. Philips' Gloeilampenfabrieken, Eindhoven, The Netherlands.**

[2]**Model AMR1000A, Advanced Metals Research Corporation, Bedford, Massachusetts.**

NECROPSY FINDINGS

In all cases the stomachs of the piglets were partially filled with milk, regardless of differences in times between last feeding and necropsy. The wall of the small intestine of piglets 3, 4, 5 and 6 was thin and translucent. The intestines were moderately dilated with liquid content. In these four piglets lacteals were filled with chyle indicating a continuous ability to absorb lipid soluble nutrients. Piglets 5 and 6 which had severe diarrhea for 1.8 and 4.8 days respectively, had signs of dehydration and severe loss of weight.

Specific rotavirus immunofluorescence was detected only in the middle and lower segments of the small intestine in piglets 2 and 3 and only in the lower small intestine in piglet 4. Fluorescence was confined to the cytoplasm of enterocytes of the upper third of the intestinal villi.

All principal and control piglets remained free of bacteria throughout the observation period.

SCANNING ELECTRON MICROSCOPY

Uninoculated control piglets 1 and 9 had uniform finger-like intestinal villi throughout the small intestine. The surface of upper and middle sections in these control pigs was smooth and crossed with numerous transverse furrows (Fig. 1). At higher magnifications the surface of the villi had a velvet-like appearance due to densely packed, well-developed microvilli which obscured individual cell boundaries (Fig. 2). The villi of the lower small intestine presented a similar overall shaped but their surface was rougher with more prominent enterocytes (Fig. 3). Individual cell limits were quite distinct (Fig. 4). Microvilli, although very dense, were not as tightly packed as in the more proximal intestinal segments (Fig. 5). There were also numerous openings of goblet cells in the villi of the lower segment of the small intestine.

Structural alterations in the intestinal mucosa of rotavirus infected gnotobiotic piglets were confined to the lower and middle portions of the small intestine. No visible changes were observed in the mucosa of either the upper small intestine or in the spiral colon. Lesions in the middle segments of the small intestine were observed only in piglets 3 and 4.

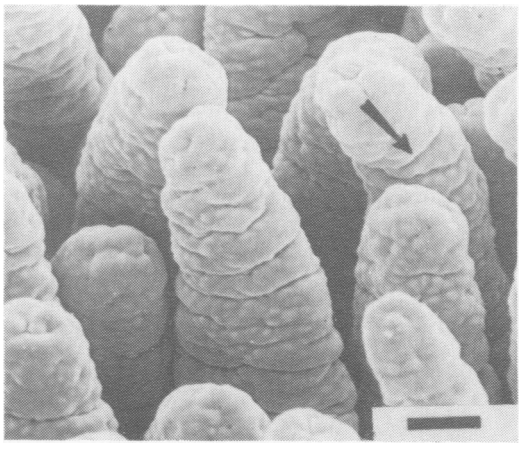

Fig. 1. Middle section, small intestine of piglet 1 uninoculated control. Normal appearance of villi with numerous transverse furrows (arrow). (Bar = 50 μm)

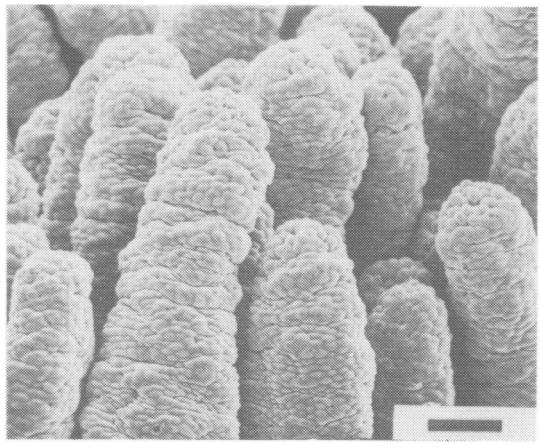

Fig. 3. Lower section, small intestine of piglet 1 uninoculated control. Enterocytes are very prominent giving a rough appearance to the villi. Numerous transverse furrows are present. (Bar = 100 μm)

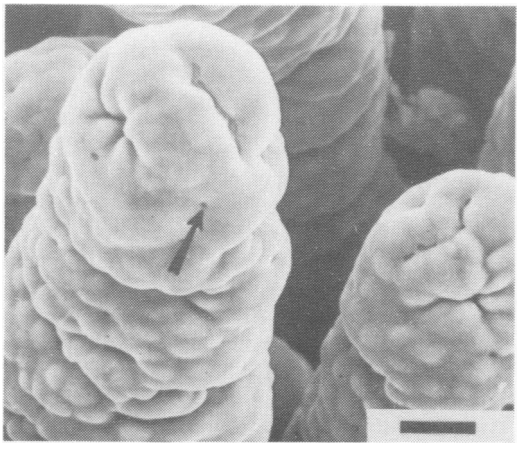

Fig. 2. Middle section, small intestine of control piglet 1. Individual cell boundaries obscured as a result of microvilli very densely packed together. Arrow points to an opening of a goblet cell. (Bar = 20 μm)

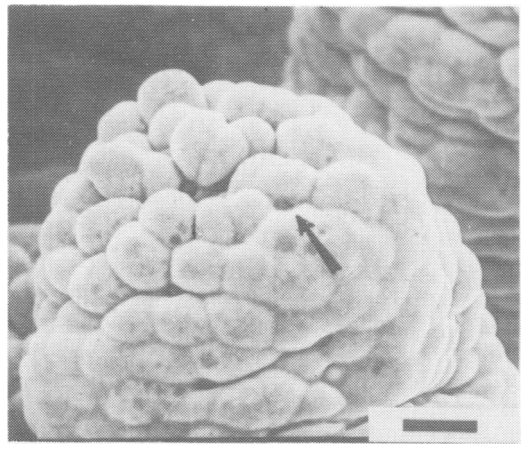

Fig. 4. Lower section, small intestine of control piglet 1. Individual cell limits quite prominent. Arrow points to an opening of a goblet cell. (Bar = 20 μm)

The first detectable lesions by SEM examination were observed in the lower small intestine of piglet 2 killed 12 hr PI. Enterocytes were swollen and occluded transverse furrows and openings of goblet cells (Fig. 6). Intercellular boundaries were more prominent and enterocytes started to separate from each other in the upper portions of the intestinal villi (Fig. 7).

Piglets 3 and 4 killed at 0.8 and 1.5 days PI had similar changes in the lower small intestine, although the severity of intestinal damage was more pronounced in piglet 4 which had diarrhea for about 17 hr prior to examination. At this time there was a significant villous atrophy (Fig. 8). Villi were about one-third to one-fourth in lentgh and about one-half in thickness as compared to normal lower intestinal villi. Enterocytes were swollen and detaching leaving denuded areas of lamina propria (Figs. 8 and 9). Microvilli of these degenerated rotavirus infected cells were sparse and short (Fig. 10). Villi of the mid-

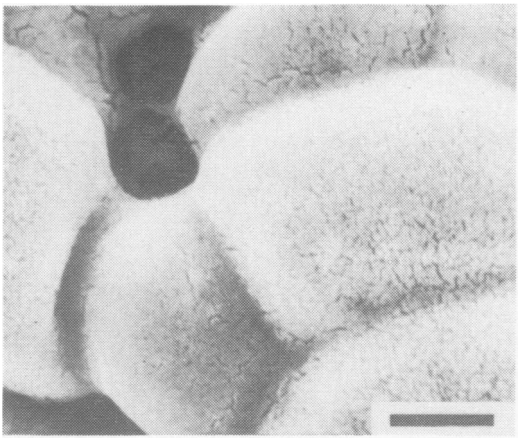

Fig. 5. Higher magnification of Fig. 4 illustrating the dense microvilli coat and two openings of goblet cells. (Bar = 5 μm)

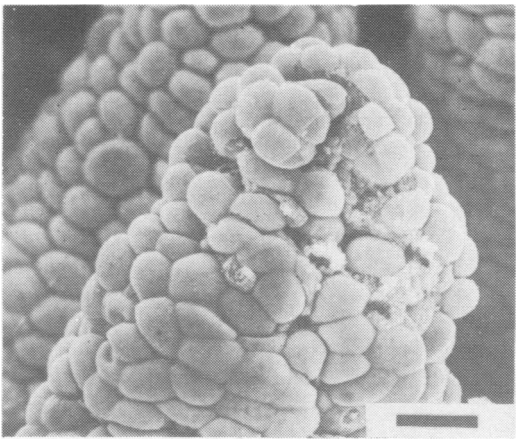

Fig. 7. Higher magnification of Fig. 6. Swollen enterocytes at the tips of the villus have begun to separate from each other. (Bar = 20 μm)

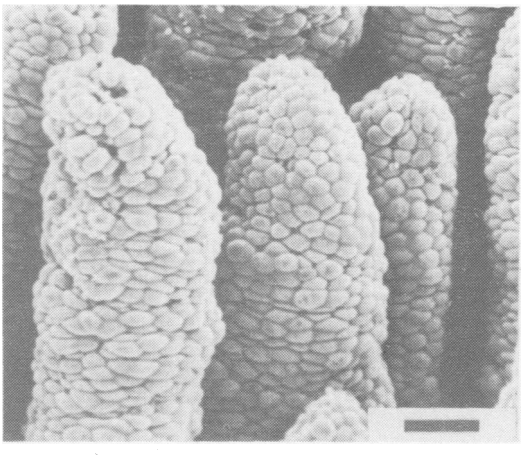

Fig. 6. Lower section, small intestine of piglet 2, killed 12 hours after rotavirus infection. Note the extensive swelling of enterocytes obscuring the openings of goblet cells, and the absence of transverse furrows. (Bar = 50 μm)

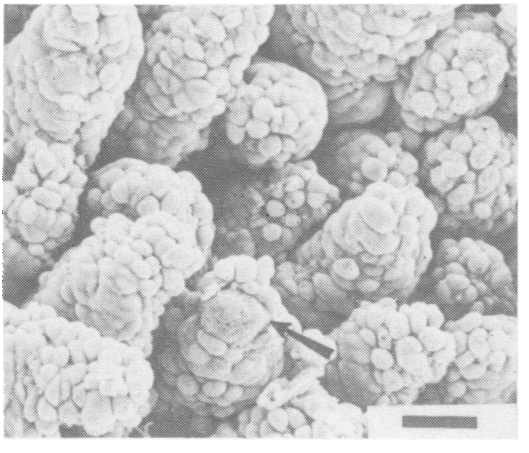

Fig. 8. Lower section, small intestine of piglet 4 killed at 1.5 days after rotavirus infection. There is extensive villous atrophy, with cell degeneration and detachment. Arrow points an area of denuded lamina propria. (Bar = 50 μm)

dle small intestine of piglets 3 and 4 presented similar alterations to those described for the lower intestine of piglet 2 although enterocyte swelling was not as marked.

The lower small intestine of piglets 5 and 6 killed at 2.5 and 6.5 days PI had a marked villous atrophy with blunting of the villus tips. In piglet 6 there was a marked fusion of adjacent villi (Fig. 11). At this stage newer cells with normal microvilli were covering most of the villi, although there were still some enterocytes completely devoided of microvilli (Fig. 12).

Lower small intestinal villi of piglet 7, killed five days after recovery of diarrhea were about half the length of normal villi and about two-thirds of their diameter. Villi were quite irregular in shape but there was no fusion of villi. A few enterocytes were still swollen and had occasionally detached from the upper half of the villi (Fig. 13). Microvilli were of normal appearance.

Piglet 8, killed at 16 days PI (nine days after recovering from diarrhea) had villi of almost normal dimensions in the lower small intestine. A few swollen enterocytes were

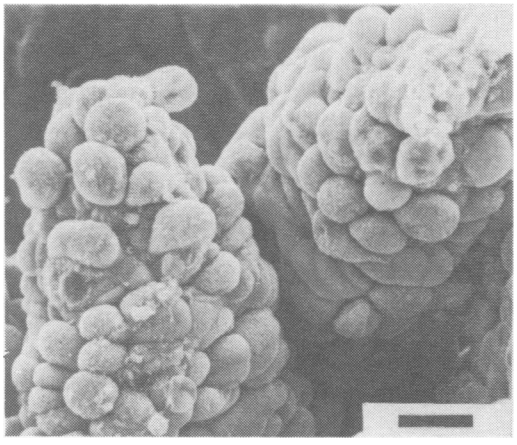

Fig. 9. Higher magnification of Fig. 8. Several stages of enterocyte degeneration and detachment from lamina propria. (Bar = 20 μm)

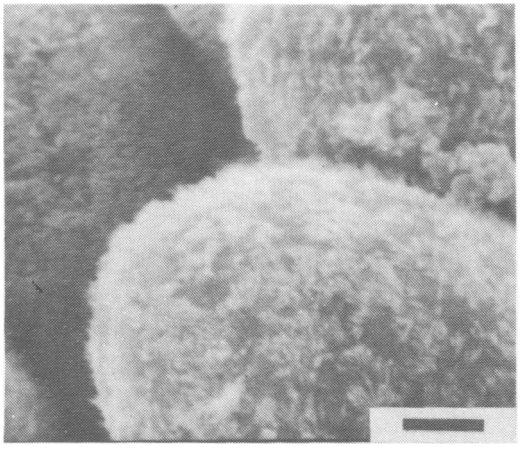

Fig. 10. A much higher magnification of Fig. 8. Coarse and short microvilli of degenerative rotavirus infected enterocytes. (Bar = 2 μm)

Fig. 11. Lower section, small intestine of piglet 6 killed at 6.5 days after rotavirus infection. Marked villous atrophy and villous fusion. Villi covered with new enterocytes except for some denuded areas towards the tips of villi. (Bar = 50 μm)

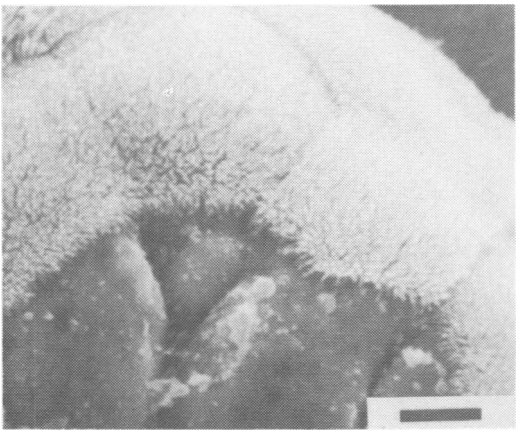

Fig. 12. Higher magnification of Fig. 11. Newer cells with normal microvilli adjacent to microvilli denuded area. (Bar = 3 μm)

noted in the upper one-third of the villi, apparently undergoing exfoliation (Fig. 14).

DISCUSSION

The incubation period, clinical observations and duration of diarrhea in the seven gnotobiotic piglets infected with strain B-317 of porcine rotavirus are in agreement with previous reports of natural and experimental infections of piglets with porcine rotavirus (1, 3, 5, 16, 18, 22, 25). However, no vomiting has ever been observed with our strain of porcine rotavirus in gnotobiotic piglets (Torres-Medina, A., and Underdahl, N. R.-unpublished results).

Shedding of rotavirus in feces was similar to the pattern observed previously by the authors in gnotobiotic piglets infected with the human rotavirus (24) and similar to other experimental infections of piglets with the porcine rotavirus (5, 9, 25). The results from the immunofluorescent staining were similar in location and distribution to those reported by Theil *et al* (22) with the exception that no viral antigen

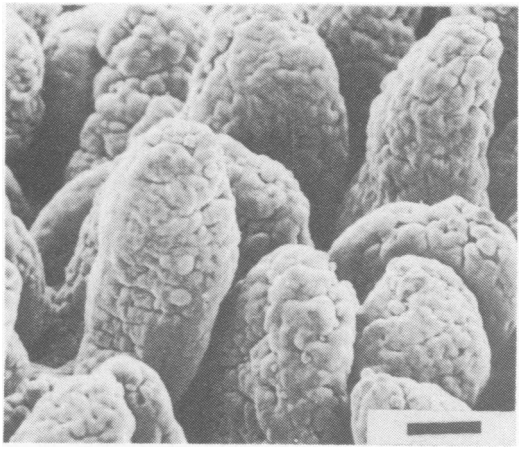

Fig. 13. Lower section, small intestine of piglet 7 killed 10.5 days after rotavirus infection. Irregular still shortened villi with some swollen enterocytes. (Bar = 50 μm)

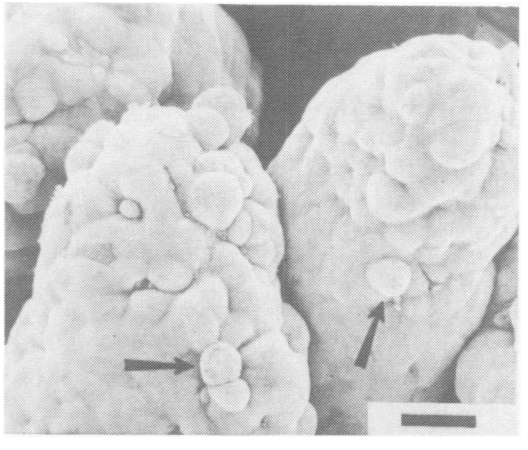

Fig. 14. Lower section, small intestine of piglet 8 killed 16 days after rotavirus infection. A few swollen enterocytes in the process of detaching from villi (arrows). (Bar = 20 μm)

was detected in the samples of upper small intestine of inoculated piglets. The combined results of viral antigen detection in feces by TEM and in intestinal cells by immunofluorescence correspond with the observations of other researchers (5, 6, 22, 25) and with our own experience that pigs and calves infected with rotavirus shed virions in the feces for longer periods of time than what the immunofluorescence of viral antigens in enterocytes indicates. The histopathological observations of this study were quite similar to those previously reported in gnotobiotic piglets (16, 22).

Detailed SEM studies of the enteric mucosa of gnotobiotic piglets infected with porcine rotavirus had not been available, except for a few SEM pictures published by Lecce *et al* (5, 6, 7). The results of our SEM studies illustrated the sequence of events that take place in the intestinal mucosa of pigs during rotavirus infection, complementing the information already available from histological, ultrastructural and immunofluorescent studies. The high susceptibility of the ileum of newborn piglets for rotavirus infection was clearly demonstrated with the use of the SEM. The earliest structural alteration of enterocyte swelling and separation observed at 12 hours PI in the upper third of ileal villi (Figs. 6 and 7) was not readily detectable by routine histopathological methods. Pearson and McNulty (17) in their ultrastructural studies with TEM described this initial swelling, which may be related to viral interference of enterocyte metabolism. These swollen enterocytes contained large amounts of viral antigen as detected by immunofluorescence. The detachment of the enterocytes was believed to be mechanical due to the swelling. The lesions observed between two to 17 hours of diarrhea (Figs. 8 and 9) were comparable to the ones observed in gnotobiotic calves infected with the bovine rotavirus (13). At this stage, microvilli were very sparse leaving denuded areas of cell membrane (Fig. 10). This type of lesion has also been observed with the TEM by other researchers in piglets infected with the porcine rotavirus (17, 21) as well as infected with a bovine rotavirus isolate (4). It is interesting to note that, except for close proximity of denuded cells of adjacent villi, no fusion of villi was found at this early stage of epithelial cell damage as it has been observed by Lecce *et al* (5, 6, 7) and by Theil *et al* (22). Villus fusion was only observed in piglet 6 killed after 4.8 days of diarrhea (Fig. 11). This result corresponds more closely to the observations of Pearson and McNulty (16). The fact that the enterocytes at this stage of healing and regeneration had an almost normal layer of microvilli (Figs. 11 and 12) — except for small areas on the tips of the villi — indicate that these were new cells that had migrated from the crypts. The observation of mucosal alterations still present in the ileum of piglet 8 (Fig. 14)

killed after 16 days PI (nine days after recovery of diarrhea) suggest that the time required for ileal epithelium to fully recover from the damage of rotavirus infection may be quite long. However, complete healing by replacing damaged villus enterocytes with newer ones from the crypts may be more rapid in conventional piglets since gnotobiotic piglets have a slower rate of epithelial cell replacement (15).

The present SEM study illustrated the severity of the mucosal alterations caused by rotavirus infection of piglets more clearly than by previous observations of thin sections by either light or transmission electron microscopy and thus contributes to a better understanding of the pathogenicity of rotaviruses in susceptible piglets.

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