Pathology of Experimental CV777 Coronavirus Enteritis in Piglets.

II. Electron Microscopic Study

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Abstract. Sixteen cesarean-derived, colostrum-deprived piglets were infected oronasally with CV777 coronavirus on the second or third day of life. Two uninfected piglets were controls. After an incubation period of 22 hours to 36 hours, all principals showed severe diarrhea. The piglets were killed at different time intervals. Viral particles were found in the jejunal villous epithelial cells from 18 hours after infection until four days after the beginning of diarrhea. In the colonic epithelial cells, viral particles and degenerative lesions were found only in the piglet killed 36 hours after onset of diarrhea.

Degenerative lesions in the enterocytes began at 18 hours after infection and were most pronounced in the jejunum at the onset of clinical signs. From 24 hours on after the onset of clinical signs, three cell types were found: degenerated virus-containing enterocytes; cuboidal cells; and columnar, highly vacuolated cells containing lipid droplets.

Outbreaks of viral enteritis in piglets usually are associated with transmissible gastroenteritis virus or porcine rotavirus.

Recently, a coronavirus-like agent antigenically different from the known porcine coronaviruses of transmissible gastroenteritis and hemagglutinating encephalomyelitis has been seen in the intestinal contents of piglets with diarrhea [7]. Some clinical and virological characteristics of this virus have been described [3].

In our previous article, we described the histological and histochemical effects of experimental oronasal infection of cesarean-derived, colostrum-deprived piglets [2]. Simultaneously, we studied the pathogenesis of the disease in the same piglets by immunofluorescence [4].

This study describes the ultrastructural lesions in the intestines of the same experimental piglets, with emphasis on the sequential effects of the viral infection on the intestinal epithelial cells.

Materials and Methods

Eighteen cesarean-derived, colostrum-deprived piglets were used in this experiment. The virus source, the mode of infection, and the duration of the experiment have been described [2]. Specimens for electron microscopy were taken adjacent to the specimens for histology [2] and for immunofluorescence [4].

After electrocution and exsanguination of the piglets, tissues were fixed immediately by immersion in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. Specimens from the middle of the jejunum of 18 piglets were studied, and from the beginning and the end of the jejunum of eight piglets as well. The colon also was studied from four piglets, selected on the basis of immunofluorescence studies [4], with diarrhea for 12 hours, 21 hours, 36 hours and 45 hours respectively.

Tissues were post-fixed in 1% osmium tetroxide, and blocks were stained with uranyl acetate. The specimens were dehydrated in acetone in a vacuum chamber [12] and embedded in Spurr low-viscosity medium [13]. Semi-thin sections were stained with toluidine blue [1]. Ultra-thin sections were contrasted with lead citrate [10] and examined in an electron microscope.

Results

Control piglets

The epithelial cells of villi and crypts showed no lesions in control piglets. The ultrastructure of the epithelium was similar to earlier descriptions of intestinal epithelia of newborn pigs [11, 16].

The microvillus border of the cells covering the villi consisted of long, regular microvilli (fig. 1), covered on the luminal side with a faint enteric surface coat. Apical pits or caveolae often were seen between the microvilli. Sometimes the central core filaments of three to 10 microvilli fused in the apical cell cytoplasm to form a dense bundle of filaments that penetrated far beneath the terminal web. The apical cytoplasmic region usually showed a well-developed vacuolar and tubular system [14]. Numerous free ribosomes and strands of endoplasmic reticulum gave an intensely stained aspect to the absorptive cells. The nucleus was oval and often in the apical half of the cytoplasm instead of in the basal third as in adult pigs.

The tripartite intercellular junctions in the apical region of the cells were pronounced. Intercellular spaces were sometimes very wide in the basal region between the cells, covering the tips of the villi.

Infected piglets

Virions: Only in the piglet killed during the incubation period at 12 hours after infection were no viral particles found in the intestinal epithelium. At 18 hours after infection, still in the incubation period, viral particles were seen only in absorptive cells covering the villi of the terminal part of the jejunum. From the onset of clinical signs until four days later, viral particles were seen in the jejunum of all infected piglets (fig. 2). Colonic epithelial cells contained many viral particles in one of four piglets studied, at 36 hours after the onset of clinical signs. In the other three piglets, no viral particles were found in the colonic epithelial cells by electron microscopy.



Fig. 1: a. Microvilli of absorptive epithelial cell of control piglet. Faint enteric surface coat (1); terminal web region (2). b. Microvilli of severely degenerated cell (41 hours after infection). Fragments of terminal web (1); short irregular microvilli (2). Uneven luminal cell surface. c. Microvilli of regenerated cell (36 hours after infection), still much shorter than in control. Incomplete terminal web area. Bar = $0.5 \mu m$.



Fig. 2: Many viral particles inside large cytoplasmic vacuoles. Absorptive epithelial cell, middle jejunum, at onset of clinical signs (24 hours after infection). Bar = $0.5 \mu m$.

Fig. 3: Early lesions in infected epithelial cell (24 hours after infection): Electron-lucent cytoplasm (1); swollen mitochondria with disrupted cristae (2); intracytoplasmic viral particles (3); normal brush border (4). Bar = $1 \mu m$.

Intracellular viral particles were present inside intracytoplasmic vesicles or between lamellae of endoplasmic reticulum. Extracellular viral particles ranged in diameter from 60 to 90 nm. They consisted of an outer unit membrane, separated by a narrow clear zone from an inner electron-dense core. This inner core often showed a central clear halo.

Cell lesions: Morphological changes in infected cells first appeared at 18 hours after infection in the terminal part of the jejunum. They were confined to swelling of mitochondria, distention of endoplasmic reticulum, and loss of electron density of the cytoplasm.

At the onset of clinical signs, ultrastructural alterations were pronounced, and differed greatly from one infected cell to the other. The following description refers mainly to the jejunal absorptive epithelial cells of piglets killed within six hours after the onset of clinical signs.

Some infected cells contained viral particles while not showing any distinct morphological alterations. Most of the infected cells, however, had an electron-lucent cytoplasm (fig. 3). There were fewer free ribosomes and endoplasmic reticulum strands. The cisternae of the endoplasmic reticulum often were dilated. Mitochondria often were rounded, with distorted cristae. The nuclei were slightly swollen. The brush border of these cells usually was still complete. In more severely affected cells (fig. 4), the cytoplasm was very light, with large vacuolar distentions of smooth endoplasmic reticulum, swollen mitochondria, and sometimes disrupted cellular membranes. The Golgi complex often was a multilaminated, onion-like structure, surrounded by numerous vesicles. Many lysosome-like structures were present. The microvilli were short and irregular, and the terminal web was fragmented (fig. 1b).

Sometimes the microvilli and the terminal web had completely disappeared. In these cells, the nucleus was round and pale, and situated in the middle of the cell. Part of the cytoplasm protruded into the intestinal lumen (fig. 4). The cell had lost its columnar shape and had an irregular outline. Sometimes the tripartite junctions also lost contact, and the cell was released from the epithelium into the gut lumen. These exfoliated cells were not very numerous. Many more cells, however, showed most of the above-described lesions, while still being firmly attached to the basal membrane and neighboring cells.

Starting at 12 hours after the first clinical signs appeared, some areas on the villi were covered with low cuboidal epithelial cells (fig. 5), which had short but regular microvilli (fig. 1c). Their terminal web was sometimes incomplete. These cells contained very few strands of rough endoplasmic reticulum, but many free ribosomes and mitochondria. The nucleus was round, and situated in the middle of the cells. Viral particles were not seen in these cells.

Twelve or more hours after the first clinical symptoms, exfoliation of cells had become rare. It was seen only occasionally at the tips of villi. From 24 hours after the onset of clinical signs on, three different types of abnormal cells were seen covering the villi. The first type was a cell containing viral particles, morphologically similar



Fig. 4: Severely damaged epithelial cell (36 hours after infection): Pale, round, swollen nucleus (1); electron-lucent cytoplasm with degenerated organelles (2); disrupted apical cell membrane and loss of microvilli (3). Cytoplasm of this cell protrudes into intestinal lumen. Normal epithelial cell (4). Bar = $2 \mu m$.

Fig. 5: Cuboidal cells covering villus (36 hours after infection): Round nucleus in middle of cell (1); cytoplasm containing few endoplasmic reticulum strands, but many free ribosomes and mitochondria (2); short but regular microvillus border (3). Bar = 1 μ m.



Fig. 6: a. Columnar lipid-containing cells (60 hours after infection): Lipid droplet (1); vacuolated cytoplasm (2). Bar = 2 μ m. b. Villous epithelial cell from control piglet showing the vacuolar and tubular system. Bar = 1 μ m.

to degenerating infected cells described above. The second was the low cuboidal cell type, also described above. The third type of cell was columnar (fig. 6), often with a highly vacuolated cytoplasm and short, sometimes irregular microvilli. The terminal web was incomplete. These cells usually contained many large lipid droplets. The nucleus was situated in the basal third of the cell. No viral particles were seen in these cells. On any one villus, one of these three types of cells usually predominated. This situation persisted until 84 hours after the first clinical symptoms. At 96 hours, more epithelial cells were infected again, with lesions as described above.

In the colon of the piglet killed at 36 hours after the onset of clinical symptoms, a number of absorptive epithelial cells lining the intestinal lumen showed marked ultrastructural lesions. The cytoplasm was pale, with occasional distentions of the endoplasmic reticulum. Free ribosomes were fewer. Mitochondria often were swollen. The microvilli sometimes were irregular. Most of these cells contained viral particles in various numbers. No exfoliation of cells was seen. In the colons of three other piglets, no ultrastructural changes were found.

Discussion

This study shows that the CV777 virus in piglets affects the intestinal cells covering the villi of the small intestine and lining the lumen of the large intestine.

The absorptive cells undergo degenerative ultrastructural changes, mainly alterations of cellular organelles, in direct relation to the number of viral particles present.

No viral particles were seen at 12 hours after infection, although this piglet was positive by immunofluorescence [4]. This means that viral antigen already was present at that time, but no complete particles were formed as yet, or they were not found with the electron microscope. This has been suggested for coronavirus enteritis of turkeys as well [9].

Loss of electron density of cellular cytoplasm has been described for other types of viral enteritis in pigs [6]. It has been suggested that this may be a sign that the cells are undergoing necrobiosis [6]. The relatively rapid degeneration of mitochondria may affect the ability of the cell to generate energy [16]. One of the important energy requiring cell functions is that of active transport [16], which plays a role in absorption.

Fat globules, as seen in the above-described lipid-containing cells, have been mentioned in other diseases of the intestine [2]. They usually are considered evidence of inability of the cell to transport or to metabolize fat [2], and are not necessarily a direct result of virus multiplication. Active fat transport may be inhibited because of energy deficiency due to mitochondrial degeneration, and the fat metabolism may be impaired by the alterations of the endoplasmic reticulum and Golgi structure [16].

Shortening and loss of microvilli is in accordance with the decreased alkaline phosphatase activity described in our histochemical study of these piglets [2]. This was seen in canine coronavirus infection as well [15].

The cuboidal cell type in this study resembles the regeneration cells described in transmissible gastroenteritis infection [8]. They are probably newly produced im-

mature cells. These cells did not contain viral particles, as seen by electron microscopy. This may account in part for the low immunofluorescence from 12 to 45 hours after the onset of diarrhea [4].

Of the four piglets selected for the study of colonic lesions, only one showed ultrastructural cellular changes and none showed histological lesions [2]. This means that infection of the colonic epithelial cells produces only slight changes.

In conclusion, the loss of microvilli reduces the intestinal absorptive surface [5]. This, together with the functional deficiency and exfoliation of many enterocytes, may result in decreased absorption and cause an osmotic diarrhea, as suggested for neonatal calf diarrhea coronavirus [5] and transmissible gastroenteritis [8]. Ultrastructural lesions in the colon may contribute to the severity of the diarrhea. The time of onset and the time of maximal ultrastructural lesions are comparable to those in porcine rotavirus [6], but they occur later, and are slightly less severe than in transmissible gastroenteritis [8].

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References

- 1 ALSOP, D.W.: Rapid single-solution polychrome staining of semi-thin epoxy sections using polyethylene glycol 200 as a stain solvent. Stain Technol **49**:265–272, 1974
- 2 COUSSEMENT, W.; DUCATELLE, R.; DEBOUCK, P.; HOORENS, J.: Pathology of experimental CV777 coronavirus enteritis in piglets. I. Histological and histochemical study. Vet Pathol 19:46-56, 1982
- 3 DEBOUCK, P.; PENSAERT, M.: Experimental infection of pigs with a new porcine enteric coronavirus, CV777. Am J Vet Res 41:219–223, 1980
- 4 DEBOUCK, P.; PENSAERT, M.; COUSSEMENT, W.: The pathogenesis of an enteric infection in pigs, experimentally induced by the coronavirus-like agent, CV777. Vet Microbiol 6:157-165, 1981
- 5 DOUGHRI, A.M.; STORZ, J.: Light and ultrastructural pathological changes in intestinal coronavirus infection of newborn calves. Zentralbl Veterinaermed [B] 24:367-385, 1977
- 6 PEARSON, G.R.; MCNULTY, M.S.: Ultrastructural changes in small intestinal epithelium of neonatal pigs infected with pig rotavirus. Arch Virol **59**:127–136, 1979
- 7 PENSAERT, M.; DEBOUCK, P.: A new coronavirus-like particle associated with diarrhea in swine. Arch Virol **58**:243–247, 1978
- 8 PENSAERT, M.; HAELTERMAN, E.O.; HINSMAN, E.J.: Transmissible gastroenteritis of swine: Virus-intestinal cell interactions. II. Electron microscopy of the epithelium in isolated jejunal loops. Arch Ges Virusforsch 31:335-351, 1970
- 9 POMEROY, K.A.; PATEL, B.; LARSEN, C.; POMEROY, B.: Combined immunofluorescence and transmission electron microscopic studies of sequential intestinal samples from turkey embryos and poults infected with turkey enteritis coronavirus. Am J Vet Res 39:1348–1354, 1978
- 10 REYNOLDS, E.S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208-213, 1963

- 11 SIBALIN, M.; BJÖRKMAN, N.: On the fine structure and absorptive function of the porcine jejunal villi during the early suckling period. Exp Cell Res 44: 165-174, 1966
- 12 SITTE, P.: Einfaches Verfahren zur stufenlosen Gewebe-entwässerung für die elektronenmikroskopische Präparation. Naturwissenschaften 17:402–403, 1962
- 13 SPURR, A.R.: A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31-43, 1969
- 14 STALEY, T.E.; WYNN, E.J.; MARSHALL, A.E.: The jejunal absorptive cell of the newborn pig: An electron microscopic study. Anat Rec 161:497–516, 1968
- 15 TAKEUCHI, A.; BINN, L.N.; JERVIS, H.R.; KEENAN, K.P.; HILDEBRANDT, P.K.; VALAS, R.B.; BLANDT, F.F.: Electron microscope study of experimental enteric infection in neonatal dogs with a canine coronavirus. Lab Invest 34:539-549, 1976
- 16 TRIER, J.S.: Structure of the mucosa of the small intestine as it relates to intestinal function. Fed Proc 26:1391-1404, 1967

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