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THE PATHOGENESIS OF AN ENTERIC INFECTION IN PIGS, EXPERIMENTALLY INDUCED BY THE CORONAVIRUS-LIKE AGENT, CV 777

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ABSTRACT

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Sixteen 2–3-days-old caesarean-derived, colostrum-deprived piglets were each dosed oro-nasally with 2 ml of a bacteria-free filtrate containing 10^4 pig-infectious-doses of CV 777. The piglets were killed at intervals of 12 to 120 h after infection. The coronavirus-like agent caused a local infection of the intestinal tract which resulted in villous atrophy, malabsorption and diarrhea. The pathogenesis of this infection was similar to that of transmissible gastroenteritis (TGE), a known coronaviral infection of pigs. However, there were some differences. By immunofluorescent staining, CV 777 antigens were not only detected in the epithelial cells covering the small intestinal villi, but also in the cells of the colonic surface epithelium. Occasional fluorescence was also seen in the small intestinal crypt epithelium, but the regenerative capacity of the crypts was not affected. The progress of intestinal epithelial cell infection by CV 777 was much slower than that in TGE, resulting in a longer incubation period and in less drastic epithelial cell destruction. The infection of regenerating cells occurred to a much higher degree during the late stage of a CV 777 infection than has been observed in TGE.

INTRODUCTION

Viruses commonly associated with diarrhea in pigs are transmissible gastroenteritis virus (TGEV), a member of the Coronaviridae, and the porcine rotavirus. The pathogenesis of the intestinal infection due to these viruses is well documented (Hooper and Haelterman, 1966, 1969; Pensaert et al., 1970a,b; Butler et al., 1974; Pearson and McNulty, 1977; Theil et al., 1978). Both viruses replicate in the cytoplasm of the absorptive epithelial cells on the villi of the small intestine. Subsequent destruction and detachment of these epithelial cells leads to shortening of the villi. The morphological and functional alterations induced by virus replication produce diarrhea, especially in baby pigs. The crypt epithelium is not affected and this enables a quick regeneration of the villi. In spite of this regeneration, death results from dehydration, particularly in TGE-infected piglets.

Recently another virus was described as a cause of diarrhea in swine (Pensaert and Debouck, 1978). It was designated coronavirus-like agent (CVLA) since the particle has a typical coronaviral morphology. Although CVLA particles cannot be differentiated from TGEV on the basis of morphology, it was demonstrated that these viruses are antigenically different. Experimental infections with the CV 777 isolate of the CVLA have shown that this virus is a primary etiologic agent of diarrhea in pigs (Debouck and Pensaert, 1980). In the same study it was shown that the CVLA replicates in the villous epithelial cells of the small and large intestine.

The present work was designed to study the pathogenesis of CVLA infection in more detail and to compare it with that of other enteric infections in pigs, especially transmissible gastroenteritis.

MATERIALS AND METHODS

Virus

The CV 777 isolate of the coronavirus-like agent (CVLA) (Pensaert and Debouck, 1978) was used for the experimental inoculation of pigs. A virus stock was made by perfusion of the small intestinal lumen of a pig inoculated orally with a bacteria-free filtrate of a 20% intestinal homogenate containing CV 777 (Debouck and Pensaert, 1980). The infectivity titer of the virus stock was 10^4 pig-infective-doses (PID) per 2 ml.

Experimental design

Seventeen caesarean-derived, colostrum-deprived (CDCD) piglets were obtained and kept in isolation as earlier described (Debouck and Pensaert, 1980). Sixteen pigs were inoculated oro-nasally with 2 ml of the virus stock on the second to the third day of life. The pigs were killed for collection of specimens at different time intervals after the inoculation (Table I). One pig served as an uninoculated control. It was sacrificed when 4 days old. Immediately after death, annular segments were taken from the duodenum (5 cm distally to the pylorus), the jejunum (five pieces taken at 50-cm intervals: j1, j2, j3, j4 and j5), the ileum (2 cm prior to the ileo-cecal valve), the middle of the colon and the rectum. Samples of the pyloric gland region of the stomach, mesenteric lymph nodes, tonsils, lungs, spleen, liver and kidneys were also collected. Intestinal contents for electron microscopical examination were collected from the cecum.

Immunofluorescence and histopathology

All tissues were processed for immunofluorescent staining. Small blocks of lung, spleen, liver and kidneys were frozen in liquid nitrogen. The other tissues were embedded in Tissue Tek II (Lab-Tek Products, Miles Laboratories

Inc., Naperville, IL, U.S.A.) and frozen in a dry ice-alcohol bath. The direct immunofluorescent staining procedure on cryostat sections has been described (Pensaert et al., 1968). The conjugate was prepared from a hyperimmune serum as earlier reported (Debouck and Pensaert, 1980).

For histopathological examination, small intestinal tissues, adjacent to the segments collected for immunofluorescence, were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections of 6 μm were cut and were stained with hematoxylin and eosin. Measurement of the length of intestinal villi and crypts was made with an ocular micrometer according to Pearson and McNulty (1977).

Electron microscopy

Intestinal contents were diluted 1 to 5 in phosphate-buffered saline (PBS) and clarified at $3,000 \times g$ for 30 min. The supernatant was layered on top of a 20% sucrose cushion and centrifuged at $150,000 \times g$ for 40 min. The resulting pellet was resuspended in a few drops of distilled water, mounted on formvar-coated grids and stained with 2% potassium phosphotungstate, pH 6.1. The grids were examined at $28,000 \times$ in a Zeiss EM 9 S-2 electron microscope.

RESULTS

Clinical observations

Four inoculated pigs showed no clinical signs at the time of killing (Table I). The first signs of vomiting and diarrhea were seen after an incubation period varying from 22 to 36 h. Sick pigs had a decreased appetite. The two pigs killed at 120 h after inoculation still had diarrhea and were severely dehydrated. The uninoculated control pig remained healthy until it was sacrificed.

Immunofluorescence

Small intestine. Specific fluorescence was seen throughout the entire small intestine and was confined to the cytoplasm of the epithelial cells covering the villi (Fig. 1). Occasionally a few epithelial cells lining the crypts also showed bright fluorescence. Villi covering the Peyer's patches in the ileum contained only a few fluorescent epithelial cells. In the control pig, all sections were negative for CVLA antigens.

The distribution of CVLA antigens with the course of the disease is shown in Table I. In the pig killed at 12 h after the inoculation, no fluorescence was seen except in one absorptive epithelial cell in the jejunal segment no. 2. Some single fluorescing epithelial cells were detected in the duodenum and jejunum of one of the pigs killed at 18 h after inoculation. In the two

TABLE I

Villous shortening and pattern of fluorescence in pigs experimentally inoculated with the coronavirus-like agent CV 777

Pig no.	Killed on postinocul. hour	Hours of diarrhea at killing	Villous shortening	Pattern of fluorescence in intestinal epithelium				Fluorescence in mesenteric lymph nodes
				duodenum	jejunum (j3)	ileum	colon	
688	12	none	none	neg	neg	neg	neg	neg
736	18	none	none	+ ^a	neg	neg	neg	neg
711	18	none	none	+	+	+	neg	neg
734	30	none	none	+++	+	+	neg	neg
744	32	1	none	+	++	+++	neg	neg
689	24	2	extensive ^b	+	+++	+++	neg	neg
710	30	6	extensive	+	++	++	+	neg
691	36	6	moderate	+	+	++	+	pos
737	41	6	extensive	+++	+++	+	+	pos
748	38	12	extensive	+	+	+	+	pos
696	48	21	extensive	+	+	+	+	pos
690	48 ^c	none	none	neg	neg	neg	neg	neg
693	60	36	moderate	+	+	+	+	pos
695	72	45	moderate	+	+	+	+	pos
694	96	60	moderate	++	+	+	+	pos
692	120	84	moderate	+	++	+	+	pos
745	120	96	extensive	+++	+++	+++	+	pos

^a + : 1-29% of epithelial cells fluorescing.⁺⁺ : 30-59% of epithelial cells fluorescing.⁺⁺⁺ : 60-100 % of epithelial cells fluorescing.^b extensive: villous length less than 1/3 of normal length.^c moderate: villous length between 2/3 and 1/3 of normal length.^c control pig.

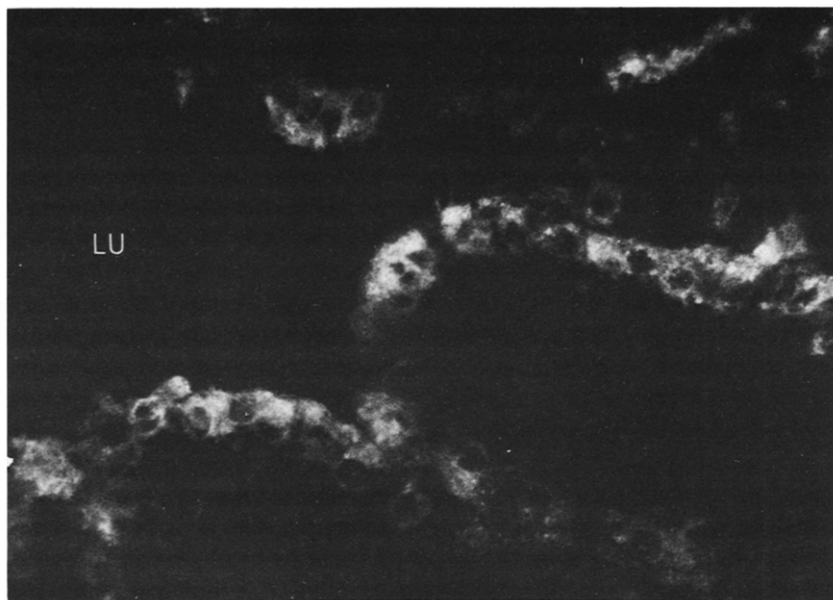


Fig. 1. Detection of CVLA antigen in the cytoplasm of the epithelial cells covering the small intestinal villi. Immunofluorescent staining of jejunum of a piglet infected orally with CVLA 96 h previously. (LU = lumen). 700 \times .

remaining pigs sacrificed during the incubation period fluorescence was seen throughout the small intestine and as many as 30–50% of the absorptive epithelial cells were positive. Although all villous epithelial cells were equally susceptible to CVLA infection, a higher number of fluorescing cells was sometimes observed at the base of the villus.

Five pigs were sacrificed within 6 h of the start of diarrhea. During this early stage of disease, the percentage of fluorescing epithelial cells reached a peak of 90–100% in the jejunum and ileum and 70–80% in the duodenum. This peak of fluorescent cells was reached earlier in the ileum and distal jejunum (j5) than in the proximal jejunum (j1) and duodenum. In some jejunal segments of two pigs (nos. 698 and 737) fluorescence had a very unusual appearance, showing abundant fluorescence that was not clearly confined to the cytoplasm of the epithelial cells (Fig. 2). Brightly fluorescing cells were occasionally seen in the lumen of the small intestine, and in one section entire rows of fluorescing cells were exfoliating from a villus top.

The number of fluorescing absorptive cells decreased sharply in pigs killed between 12 and 45 h after the onset of diarrhea. In pigs sick for 24–36 h, only 1–10% of the epithelial cells were infected throughout the small intestine.

It was observed that the number of fluorescing cells again increased in all parts of the small intestine in later stages of the disease. A second peak with 90–100% of the cells fluorescing was observed in one of the pigs (no. 745) killed 4 days after the onset of the clinical signs.

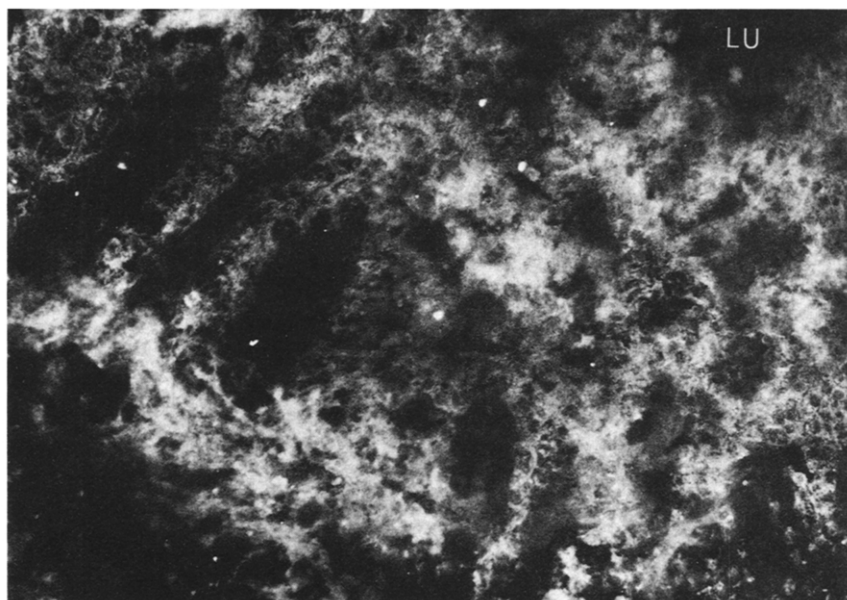


Fig. 2. Very high amount of fluorescence as seen in the jejunum of a piglet infected orally with CVLA 24 h previously and killed 2 h after onset of diarrhea. (LU = lumen). 128 \times .

Large intestine. Viral antigens were also detected in epithelial cells covering the colonic villi. Fluorescence was never detected in the cells lining the crypts. Fluorescence in the colon was only observed in pigs with a diarrhea of 6 h or longer and remained limited to a few scattered cells. Rectum was always negative.

Other tissues. Patchy fluorescence was often found in the mesenteric lymph nodes but only of pigs that were sick for 6 h or longer (Table I). This fluorescence, found within the lymph nodulae, was not always clearly confined to the cellular cytoplasm. On no occasion could CVLA viral antigens be detected by immunofluorescence in the pylorus, tonsils, lungs, spleen, liver and kidneys.

Intestinal mucosal measurements

The length of the small intestinal villi of the control pig varied between 700 and 900 μm . Normal values, as found in the control pig, were also detected in the pigs that were still in the incubation period and in the pig (no. 744) that had diarrhea for only 1 h. A decrease of the villus length was observed in all other inoculated pigs. Villous atrophy was most severe in the jejunum (j2 to j5) of the pigs killed from 6 to 21 h after the start of the diarrhea. The length of the villi in these pigs was about 200–300 μm . A gradual increase in the length of the villi was seen in the pigs killed during later stages of the disease, but in the pig sacrificed at 96 h after the start of the diarrhea, the villi were shorter again. Throughout the experiments, crypt

depths in the inoculated pigs remained approximately the same as those in the control pig, with values between 100 and 130 μm . The villus length : crypt depth ratios in the jejunum of the control pig and of the five inoculated pigs without villous atrophy ranged from 6.5 to 8.0 whereas the ratio decreased in the most severely affected pigs to an average of 2.3. The lowest ratio was 1.5.

Electron microscopy

The morphology of the CVLA particles has been fully described (Pensaert and Debouck, 1978). CVLA particles were detected in the intestinal contents of all pigs with the exception of the control pig, the pig killed 12 h after inoculation, and two pigs killed in the late stage of the disease (nos. 693 and 694). It should be mentioned that all samples were processed and examined twice. Three samples scored positively on only one of the two examinations.

DISCUSSION

The coronavirus-like agent CV 777 caused a severe diarrhea in 2–3-days-old piglets, resulting in dehydration. Following oro-nasal inoculation at this age, the coronavirus-like agent (CVLA) passed directly to the small intestine without replicating in tonsils or stomach. Viral antigens were detected only in the villous epithelium of the small and large intestine and in the mesenteric lymph nodes. Even though CVLA seemed to leave the intestinal tract via the lymphatics, viral replication in other organs was not found. Similar findings were reported by Theil et al. (1978) in porcine rotaviral infection. In contrast, transmissible gastroenteritis virus (TGEV) has been shown to replicate in tissues other than the intestinal tract (Woode, 1969), but the pathogenic significance of its presence in those organs remains unknown.

With CVLA, fluorescence appeared at about the same time in all parts of the small intestine. There was a positive correlation between the viral replication and the development of histologic and ultrastructural lesions in the intestines (to be reported in separate papers). A first peak in the number of fluorescent cells, observed shortly after the onset of diarrhea, coincided with a shortening of the villi. Infection of villous epithelial cells by the CVLA apparently led to destruction or sloughing of the cells into the intestinal lumen, resulting in a certain degree of villous atrophy. Diarrhea was a consequence of the malabsorption due to this loss of absorptive cells. However, diarrhea may also be caused by functional disorder of these infected cells, since the onset of diarrhea was seen before the appearance of villous atrophy. A similar sequence of events was reported with porcine rotaviral infection (Theil et al., 1978) and with transmissible gastroenteritis (TGE), but in the latter infection events proceed much more quickly.

CVLA antigens could be detected by immunofluorescence in the epithelial cells of the small intestinal villi from the onset of diarrhea until the end of

the observation period, which was about 4 days later. This implies that the IF technique is reliable for diagnosis of CVLA outbreaks in the field provided that young pigs with diarrhea for only a few days can be supplied to the laboratory.

Although the pathogenesis of a CVLA infection is very similar to that of TGE, there were some differences. It has never been observed with TGE that fluorescence was present in epithelial cells lining the crypts of Lieberkühn, and that fluorescence was located almost exclusively at the base of the villi as seen in two pigs killed during the early phase of CVLA infection. Although this has also been described by Pomeroy et al. (1978) in turkey embryos infected with turkey enteritis coronavirus, its pathogenic importance is unclear. In CVLA infection, fluorescence in the crypt epithelium was distinct but remained limited to a few scattered areas throughout the infection, and histologic data did not suggest epithelial damage (W. Coussement et al., to be published). Furthermore, the regenerative capacity of the crypts of Lieberkühn was not affected as indicated by the presence of newly produced undifferentiated cells on the regrowing villi (R. Ducatelle et al., to be published).

The progression of epithelial cell infection by the CVLA was much slower than that in TGE. In the latter infection, fluorescent cells can be detected as early as 6 h after inoculation (Pensaert et al., 1970a), whereas in the CVLA infection, fluorescence was almost absent at 12 h and only distinct at 18 h after inoculation. The longer incubation period observed in CVLA infection is a result of this slower infection rate.

The infection of regenerating cells occurred to a much higher degree in CVLA infection than in TGE. This was indicated by the second peak of fluorescence involving 90–100% of the epithelial cells as seen in a pig killed 4 days after the onset of diarrhea.

The most striking difference from the pathogenesis of TGE was the infection of the colon with the CVLA. Although the number of fluorescing cells remained rather low, this colonic infection might contribute to the malabsorption syndrome. Virus replication in the large intestine has also been described in calves infected with bovine coronavirus (Mebus et al., 1973).

The present study has shown that the coronavirus-like agent CV 777 causes a local infection of the intestinal tract in young pigs. The pathogenesis of this CVLA infection resembles not only that of other enteric viral infections in pigs (TGE and rotavirus) but also that of enteric infections caused by coronaviruses in other animal species such as calves (Mebus et al., 1973, 1975), dogs (Keenan et al., 1976) and turkeys (Pomeroy et al., 1978).

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