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**Light Microscopy and Ultrahistology of Intestinal Changes in Pigs
Infected with Epizootic Diarrhoea Virus (EVD):
Comparison
with Transmissible Gastroenteritis (TGE) Virus
and Porcine Rotavirus Infections**

By

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With 7 figures

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For almost three decades, transmissible gastroenteritis virus (TGE), a coronavirus, was the only known viral agent to cause severe neonatal diarrhoea in pigs, giving rise to considerable losses (1). Recently, a number of other viruses have been shown to be involved in the aetiology of diarrhoea in pigs, namely rotaviruses (9, 11), the epizootic diarrhoea virus (another coronavirus-like agent not related to TGE virus, 4, 8, 14), rotavirus-like, calici-virus-like and 23 nm virus-like particles (18). Present evidence suggests that in addition to TGE virus, rotaviruses and epizootic diarrhoea virus are widespread and of considerable economic importance. The clinical and epizootiological picture of infections with these three agents is similar (3, 5, 15) and differential diagnosis is difficult. Furthermore, histological findings in the small intestine, which is the target organ of these viruses, reveal remarkable similarities. Since the histological and ultrastructural changes of natural and experimental TGE and rotavirus infections are known from a number of studies (10, 12, 16, 17), it is of interest to investigate the pathological changes caused by epizootic diarrhoea virus and compare them with TGE and rotavirus infections.

Material and Methods

Animals

Six naturally infected piglets (2 piglets 5–6 days, and 4 piglets 4–6 weeks old) and four piglets (2–5 days old) infected experimentally with an intestinal filtrate from a confirmed EVD field case were examined. The experimentally infected animals were sacrificed 20–24 hours and 36–48 hours after infection. Clinical and epizootiological data on the naturally infected piglets have been reported elsewhere (8).

TGE lesions were studied in 5 naturally infected animals 3–9 days of age, and in 2 newborn piglets experimentally infected with 1,000 LD₅₀ of an intestinal suspension from a piglet infected with the Miller strain. These piglets were sacrificed 48 hours after infection. Rotavirus infected intestines were obtained by infecting 2 piglets at 2 days of age with the field strain Munich V 1274 and harvesting the material 48 hours later. Four non-infected healthy animals at 2–4 days of age served as controls.

The experimental piglets were colostrum-deprived and fed reconstituted dried cow milk supplemented with vitamins (Biofix-F, Milchhof, Munich). All infections were carried out orally. The animals were anaesthetized with Narcoren®, and jejunal tissues were taken from the cranial, middle and caudal parts. Colon tissue was sampled from the middle part. Regional lymph nodes were also investigated.

Immunofluorescence staining (FA)

Frozen sections were made from all intestinal samples for the demonstration of viral antigens by FA. The sections were fixed with acetone at 4 °C for 10 minutes and stained directly using EVD*, TGE-, and rotavirus antibody FITC conjugates.

Histology

After fixation in 7% neutral formalin, paraffin sections were stained with haematoxylin-eosin (HE) and periodic acid Schiff reagent (PAS). For the demonstration of lipids, frozen sections were stained with rhodamine (Merck, Darmstadt, FGR).

Electron microscopy

Tissues were obtained as described and fixed in 6.25% glutaraldehyde. The samples were washed for 4 hours in 0.1 M phosphate buffer, pH 7.2, containing 6.8% sucrose, post-fixed in 1% osmium-tetroxide and stained with 0.25% aqueous uranyl acetate. Semithin and thin sections were prepared following dehydration in acetone and embedding in Epon 812.

Ultra-thin sections were stained with uranyl acetate and lead citrate and investigated with a Zeiss EM 10.

Results

Immunofluorescence

Specific antigen was demonstrated using immunofluorescence methods with FITC antibody conjugates for epizootic diarrhoea virus, transmissible gastroenteritis virus and rotavirus. There was no cross-reaction between any of the three viruses, indicating that epizootic diarrhoea virus is antigenically not related to TGE virus.

Localization of EVD antigen in the villus epithelium is illustrated in Fig. 2. In some cases EVD antigen-containing cells were also found in crypt epithelium.

Infection with epizootic diarrhoea virus (EVD)

At the onset of diarrhoea (24 hours post infection) *histological* findings in the jejunum of *experimentally infected piglets* consisted of marked vacuolization (vacuoles contained fat and PAS-positive material) of the enterocyte

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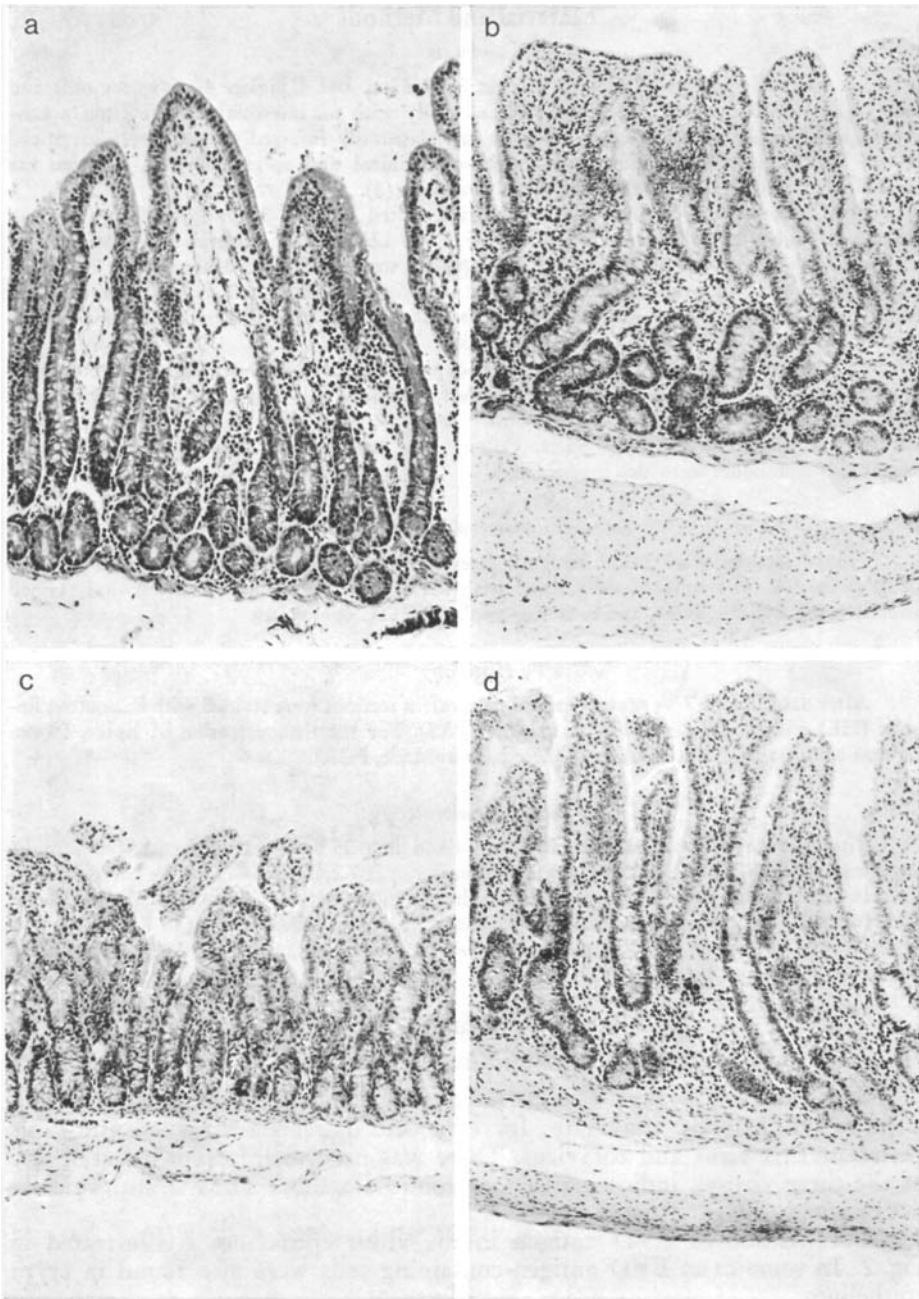
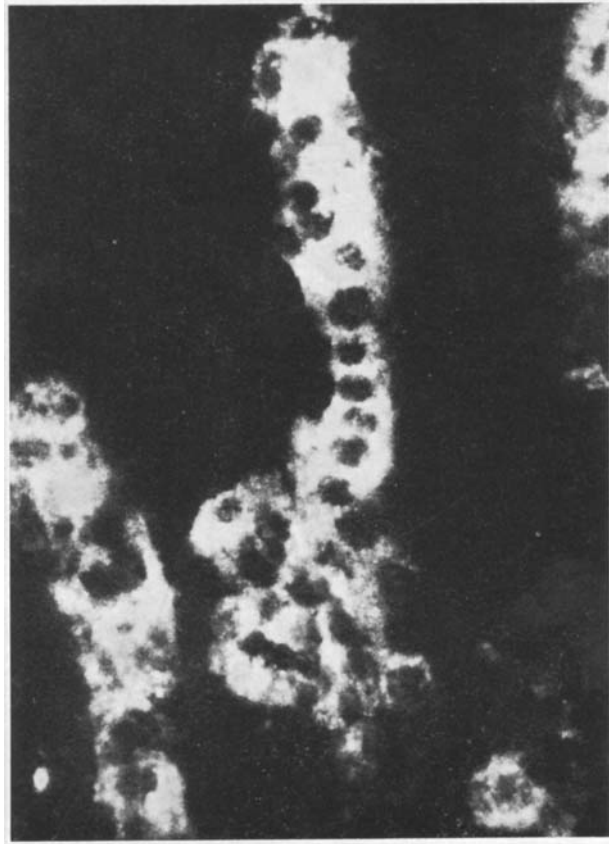


Fig. 1. Light microscopic changes in the jejunal mucosa of 4—7 day-old piglets experimentally infected with enteropathogenic viruses (HE staining). — a. Uninfected controls, 4 days old $\times 200$. b. EVD virus infection, 48 hours p. inf., $\times 200$. c. TGE virus infection, 48 hours p. inf., $\times 200$. d. Rotavirus infection, 48 hours p. inf., $\times 200$

cytoplasm from the tip of the villus to the villus/crypt border. Villous atrophy, absent at this time (Fig. 1 b), developed rapidly within the next 24 hours. The

Fig. 2. Immunofluorescent staining of intestinal sections from a piglet experimentally infected with epizootic diarrhoea virus using EVD virus antibody conjugate. $\times 370$



lesions could be detected throughout the jejunum and in the ileum. Morphology of enterocytes varied from cylindrical to cuboidal. With increasing degeneration of the brush border of the enterocytes, cuboidal cell morphology predominated.

Electron microscopy of the intestines from infected piglets demonstrated virus-containing enterocytes on the lateral wall of the villi. Occasionally, virus-infected cells were seen in or near jejunal crypts (Fig. 3).

Virus particles were seen in varying numbers in vesicles which were localized mainly in the supranuclear cytoplasm. The vesicles were irregular in morphology and size, measuring between 500 and 1,000 nm. Virus particles were highly pleomorphic. They appeared mainly as round or oval, but also bowl-like or elongated forms measuring between 50 and 105 nm. Open and closed rings could be distinguished. In round particles the diameter averaged about 73 nm. The inner structure consisted of concentrically arranged osmiophilic material, sometimes around a central electron-lucent space measuring about 32 nm in diameter (Fig. 4). The nucleocapsid was surrounded by a double membrane of about 8 nm width. Surface projections could not be demonstrated.

The mitochondria of infected cells were swollen and cristae were partly absent, partly disrupted or degenerated. Only a reduced number of cytoplasmic organelles, such as RER or Golgi apparatus were left. They tended to be concentrated more towards the microvillus border, leaving the basal parts

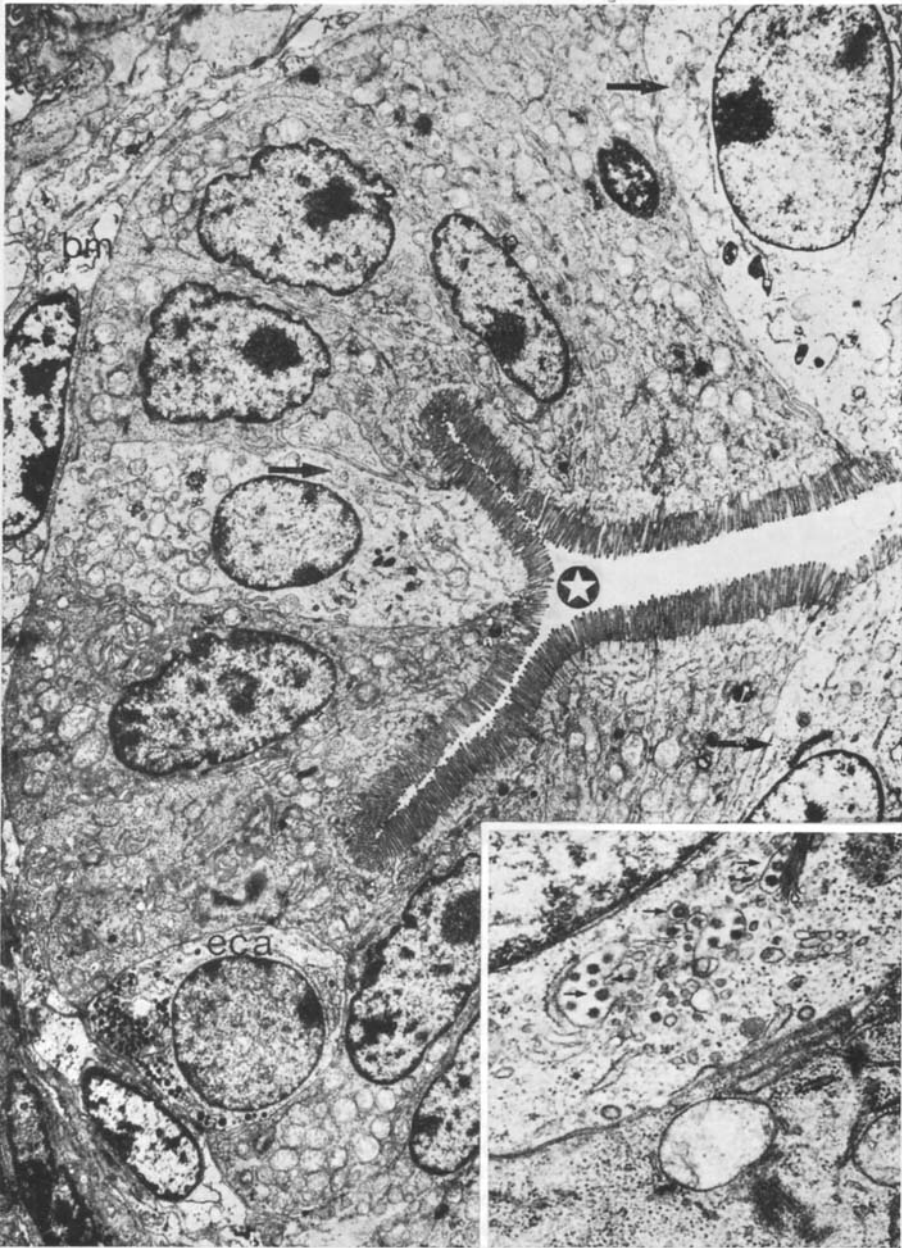


Fig. 3. EVD virus-infected epithelial cell in crypt region. Arrow indicates virus containing vesicles. Asterisk shows crypt lumen. eca: enterochromaffin cell, bm: basal membrane, $\times 2,300$

of the cytoplasm more or less empty. Absorptive vesicles were not found. Nuclei did not show morphological changes.

In cells containing large amounts of virus particles, microvilli appeared broad and short. Their number was considerably reduced (Fig. 5 a). In less infected cells, microvilli appeared intact; virus particles, however, could be

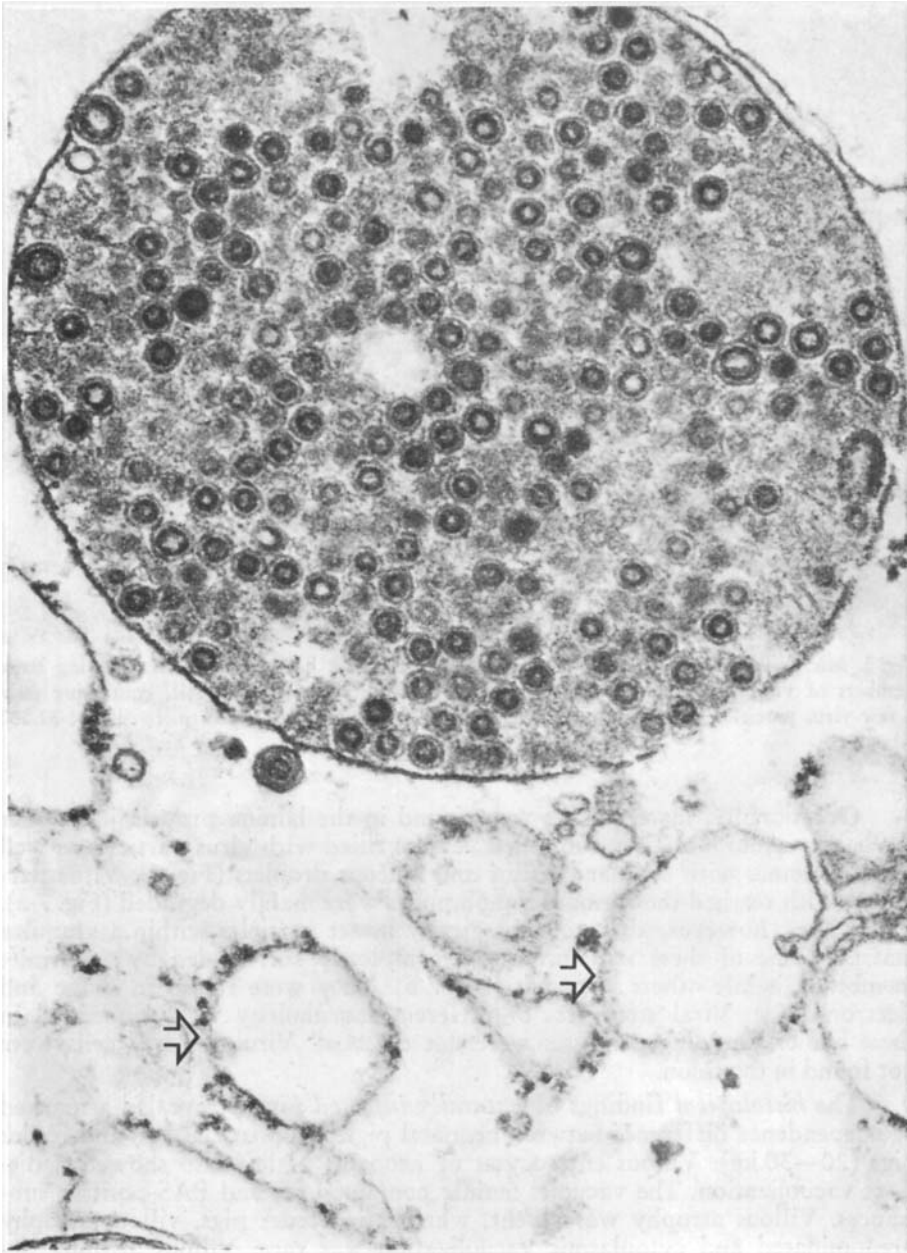


Fig. 4. EVD virus in cytoplasmic vesicle. Note concentrically arranged osmiophilic material around central electron-lucent space. The nucleocapsid is surrounded by a double membrane, arrows: dilated rough endoplasmic reticulum, $\times 130,000$

seen between microvilli (Fig. 5 b). In addition, virus particles were also present in intercellular spaces between adjacent jejunal enterocytes and near the basal membrane of infected cells. However, budding from the cytoplasmic membrane was not observed. Some degenerated, infected enterocytes shed from the lamina propria could be demonstrated in the intestinal lumen.

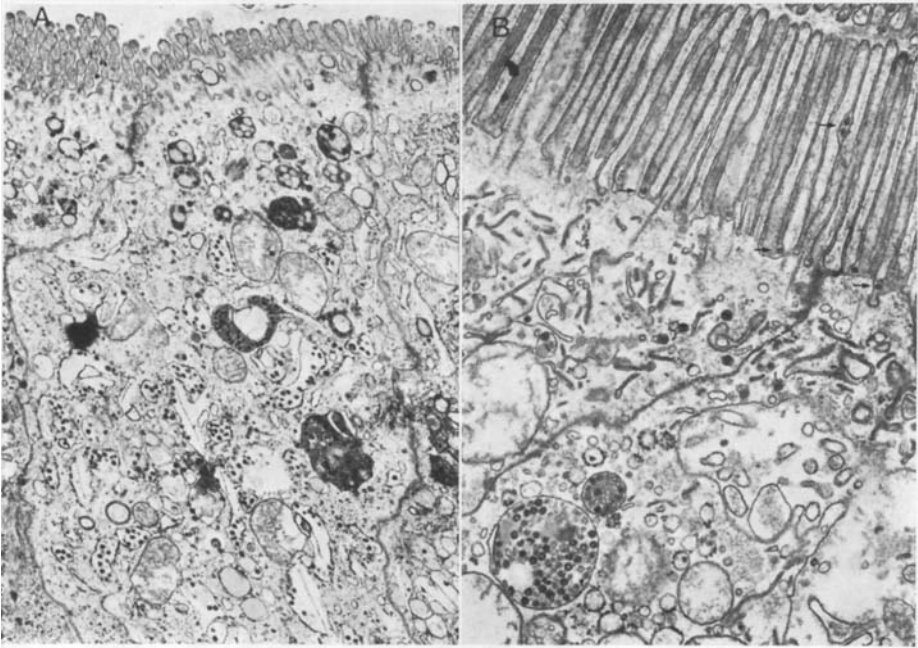


Fig. 5. Microvilli on EVD virus-infected cells. — A. Brush border on cells containing large numbers of virus particles, $\times 32,000$. B. Intact microvillus border on cells containing only a few virus particles. Arrow indicates virus particles between individual microvilli, $\times 32,000$

Occasionally, macrophages were found in the lamina propria just under the basal membrane. They contained vesicles filled with virus particles as well as phagosomes with cellular detritus and mucous droplets (Fig. 6). Virus particles which reached the regional lymph nodes were mainly degraded (Fig. 7 a). There were, however, also morphologically intact particles within a granular matrix. Some of these structures were completely surrounded by a definite membrane, while others were not (Fig. 7 b). They were round in shape and electron-dense. Viral structures of different morphology were embedded in these bodies. Budding processes were not detected. Virus infected cells were not found in the colon.

The *histological findings of naturally infected piglets* revealed a marked age dependence difference between neonatal piglets (up to 2,500 g) and feeder pigs (20—30 kg). Villous enterocytes of neonatal piglets also showed a distinct vacuolization. The vacuoles mainly contained fat and PAS-positive substances. Villous atrophy was absent, whereas in feeder pigs, villous atrophy predominated and cytoplasmic vacuolization was rare. Villous atrophy occurred throughout the jejunum and in the ileum.

Electron microscopy also demonstrated the presence of enterocytes containing virus particles, mainly on the lateral walls of the villi. The virus was found neither in enterocytes located at the tip of the villi nor in the crypts. Virus particles were seen in varying numbers in vesicles which were distributed over the whole cytoplasm. Further alterations in the cytoplasm were swollen mitochondriae and fragmentation of cristae. Remaining cytoplasmic organelles were concentrated near the apical cell membrane just as in experimentally infected piglets. There were no changes in the nucleus.

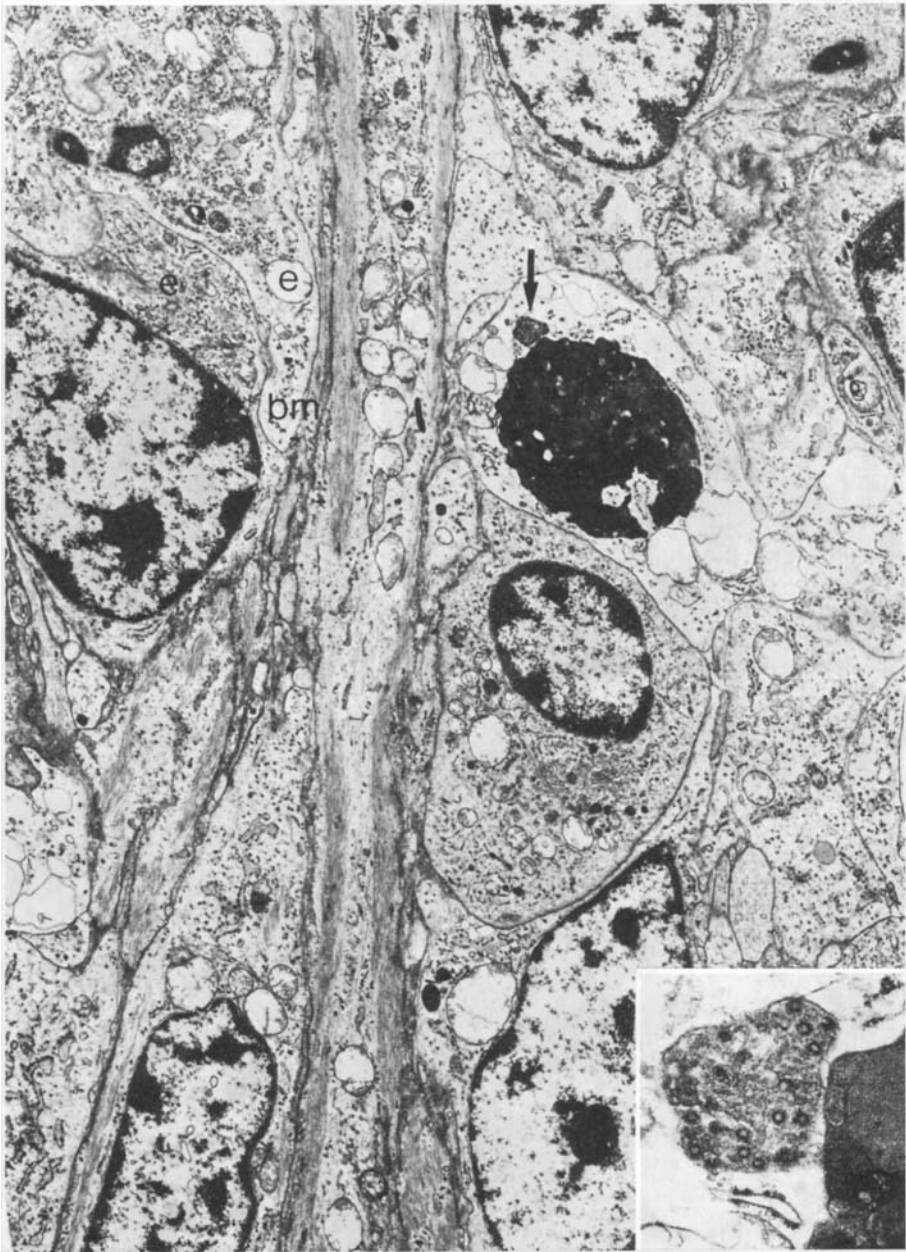


Fig. 6. EVD virus particles in a phagosome (arrow) of a macrophage containing mucous droplets in the lamina propria bm: basal membrane, e: epithelial cell, $\times 9,200$

The microvillous border of infected enterocytes containing large numbers of virions seemed to be morphologically intact. Only few virus particles were seen between the microvilli. Virus-containing detached cells, however, were found in the jejunal lumen.

In feeder pigs, ultrastructure and localization of alterations were comparable to those seen in the microvilli of neonatal piglets. However, microvilli

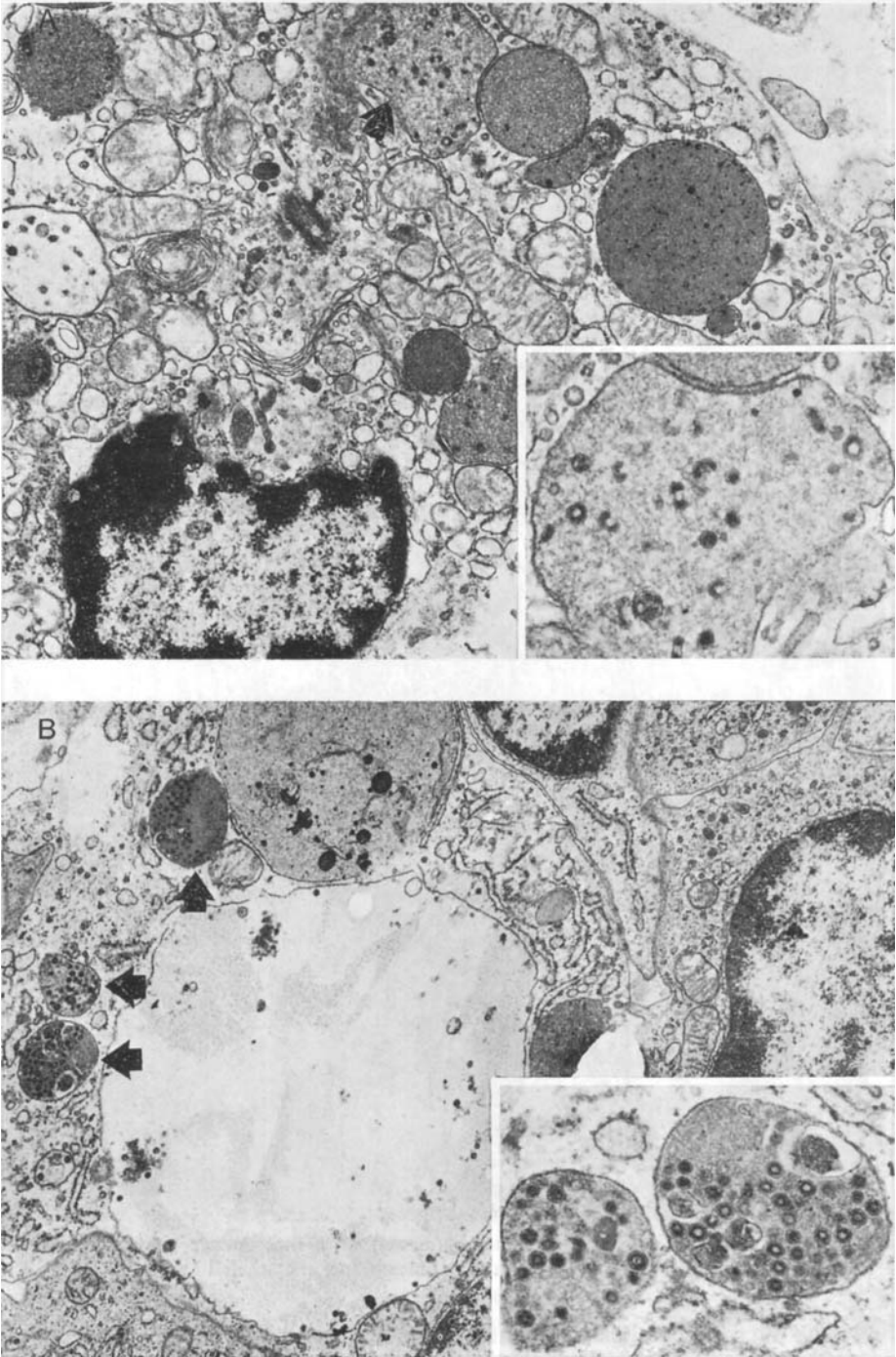


Fig. 7. EVD virus particles in phagocytes of a regional lymph node of the distal part of the jejunum. — A. Numerous virus particles (arrow) undergoing degradation $\times 18,000$. B. Morphologically intact EVD virus particles (arrows) within a membrane bound, electron-dense granular matrix, $\times 33,000$ (insets at higher magnification)

were markedly shortened and their number reduced. Free virus particles were found in the microvillous border. Infected enterocytes sporadically protruded into the jejunal lumen. Virus could not be demonstrated in the colon, epithelium or lymph nodes. The intestines of the non-infected control piglets showed well defined villi with cylindrical epithelial cells (Fig. 1 a). Enterocytes had deep basal nuclei with regular microvillus borders and well defined terminal webs. Cell organelles appeared unaltered when examined by electron microscopy.

Infection with Transmissible Gastroenteritis Virus (TGE)

In *experimentally* as well as in *naturally infected piglets*, *histological investigation* of the jejunum and ileum revealed the well-known distinct villous atrophy (Fig. 1 c). In the cytoplasm of infected enterocytes, vacuoles were sometimes present which contained mainly fat. In addition, there was a remarkable deposition of PAS-positive material in the lamina propria near the villus tips. The observed histological changes were similar, independent of age or weight of the piglets. Usually enterocytes were cylindrical to cuboidal in form, tending more towards cuboidal in villi with distinct histological lesions.

The *ultrastructural picture* of lesions in TGE virus-infected jejunal enterocytes is roughly comparable to that seen in EVD-virus infected cells. Virus particles were found in vesicles of enterocytes from the tip of the villus to the villus/crypt border. Virus-containing cells were not detectable in the crypt area.

The cytoplasm of infected cells revealed swollen mitochondria and partial fragmentation of the cristae mitochondriales. In experimentally infected piglets, the smooth endoplasmic reticulum showed a marked dilatation. Remaining organelles did not display any morphological alterations. The appearance of vacuoles of varying osmiophilia in infected enterocytes reaching nuclear size was striking. The brush border of cells containing virus appeared broadened, shortened, and microvilli were greatly reduced in number. Frequently viral particles could be demonstrated between the microvilli. Virus particles were neither detected in lymph nodes nor in epithelial cells of colon mucosa.

Infection with Porcine Rotavirus

Severe *histological lesions* were found in the middle and distal parts of the jejunum as well as in the ileum. They consisted of focal necrosis and epithelial cell desquamation, mainly at the tip of the villi. As a result, marked villous atrophy was present in the distal jejunum and the ileum (Fig. 1 d). No pathological lesions were detected in the proximal part of the jejunum or in the colon. Enterocytes appeared cylindrical in form regardless of whether the brush border was degenerated or intact.

Virus particles were mainly found in epithelial cells located at the tip of the villi. From large granular viroplasma areas or regions of convoluted membranes in the cytoplasm, mature virions budded into the dilated smooth (SER) and rough (RER) endoplasmic reticulum. These particles were double-shelled and measured 80–90 nm in diameter and contained an inner core 40 nm in diameter. Virus particles were also demonstrated in cytoplasmic vesicles derived from the SER or RER. These particles lacked the outer shell and measured approx. 65 nm in diameter. Both morphological types of virions were observed free in the intestinal lumen.

Cytoplasmic changes in infected cells began with a dilatation of SER and RER and an aggregation of ribosomes. Mitochondria were swollen and the

cristae underwent degeneration. Virus particles could not be detected in mitochondria. At this early stage the brush border did not reveal any alteration.

As soon as viroplasmic zones appeared in the cytoplasm, the microvilli formed blebs and then gradually disappeared. Finally, degenerated enterocytes desquamated and appeared free in the intestinal lumen. Intranuclear or intracytoplasmic tubular formations could not be found. The colon did not show any ultrastructural alterations or evidence for rotaviral replication.

Discussion

Comparison of histological intestinal lesions in EVD and TGE virus infected piglets did not reveal significant differences. Both virus infections lead to severe villous atrophy from proximal to distal jejunum and in the ileum, which is in contrast to the findings for rotavirus infection where villous atrophy was only found in the distal parts of the jejunum and in the ileum. The findings for TGE and rotavirus-induced intestinal lesions confirm earlier results obtained by others (9, 10, 11, 15, 16, 17, 20, 21). From the limited studies in experimentally infected piglets, it can be postulated that development of lesions in EVD virus-infected animals starts somewhat later than in TGE virus-infected piglets. PENSART et al. (15) reported cellular desquamation in TGE virus infection to begin around 12 hours post infection, whereas the first lesions in pigs infected experimentally with EVD virus appeared between 36 and 48 hours after infection. These results are in contrast to those reported by DUCATEL et al. (7) who detected the first intestinal changes around 18–24 hours p. inf. These differences may be dependent on the virulence of the strain used or on the virus dose given. The lack of villous atrophy in naturally EVD virus-infected newborn piglets compared to weanlings which were investigated only a few hours after the onset of diarrhoea may have been due to milk antibodies which could have a delaying influence on the development of morphological lesions in the intestine.

Electron microscopical findings in EVD virus-infected intestinal cells revealing swollen mitochondria, degenerated cristae, reduced numbers of cell organelles such as RER and Golgi apparatus, dilatation of the terminal web and irregular microvillus borders resembled those for TGE virus (16) and other enteropathogenic coronaviruses (2, 3, 6, 19). They are also consistent with the replication of coronaviruses in tissue culture cells (13).

Dense filamentous structures as demonstrated in intestinal infections with bovine and canine coronaviruses (6, 19) were, however, not found.

There were no apparent differences in the ultrastructural picture between naturally and experimentally infected piglets.

EVD virus-containing cells were mainly found on the lateral walls of the villi, leaving enterocytes on the tip of the villus uninfected. In some cases EVD virus-infected cells could also be demonstrated in or near Lieberkühn's crypts.

Such observations were not made with any of the other enteric coronaviruses studied *in vivo*, including TGE virus (2, 6, 16).

Whether these findings, which were also made using the fluorescent antibody assay (5, 8) are pathognomonic for EVD virus remains to be seen. If these results can be confirmed in future studies, the localization of virus replication could possibly be a property which could be used for differentiation of TGE virus from EVD virus in infected small intestines. In this respect rotavirus infection does not present difficulties because of its different morphology and mode of replication. The results obtained in this study on the infection of the small intestine with rotaviruses are well in accordance with data reported

by others (10, 11, 17, 20). The ultrastructural investigation of the lamina propria and regional lymph nodes revealed virus-containing cells in piglets experimentally infected with EVD virus. This suggests uptake of virus-containing cells by macrophages which are present in the lamina propria, and transport of the macrophages to the lymph nodes where virus degradation takes place (Fig. 7 a). Whether the granular inclusions in phagocytes containing intact virus particles resemble lysosomes, or are similar to structures described in connection with the replication of a human coronavirus in cell cultures (13), cannot be ascertained. Because replication of enteropathogenic coronaviruses has not been reported in other cells than differentiated small intestine enterocytes, one would be hesitant to compare these structures to the membrane-bound viral factories reported by DOUGHRI et al. (6) in a study on intestinal bovine coronavirus infection, although their resemblance is striking.

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Summary

Light microscopic and ultrahistological changes were investigated in the intestines of piglets naturally and experimentally infected with epizootic viral diarrhoea (EVD) virus and compared to those occurring during intestinal TGE and rotavirus infections in piglets. The course of the EVD virus infection is comparable to that of TGE. Villous atrophy develops throughout the jejunum and the ileum as in TGE. In this respect it differs from rotavirus infection. The ultrastructural picture of intestinal EVD virus infection resembles that of TGE and other enteropathogenic coronaviruses. Virus replication, however, was observed mainly in enterocytes located on the lateral walls of the villi extending to the villus/crypt border, and sometimes virus-infected cells were demonstrated in or near the Lieberkühn's crypts. EVD virus was also demonstrated in macrophages in the intestinal lamina propria and in phagocytes in regional lymph nodes.

Zusammenfassung

Lichtmikroskopische und ultrahistologische intestinale Veränderungen bei Ferkeln

nach Infektion mit dem Epizootischen Diarrhoe-Virus (EVD): Vergleich mit Übertragbaren Gastroenteritis (TGE) Virus- und Schweinerotavirusinfektionen

Lichtmikroskopische und ultrahistologische Veränderungen von Därmen natürlich und experimentell mit dem Virus der Epizootischen Virusdiarrhoe (EVD) infizierten Ferkeln wurden untersucht und mit denen nach Infektion mit TGE-Virus und Rotavirus verglichen. Der Verlauf der EVD-Virusinfektion ist vergleichbar mit dem der TGE. Eine Zottenatrophie tritt ähnlich wie bei der TGE im gesamten Jejunum und Ileum auf. In dieser Beziehung unterscheiden sich die Befunde von der Rotavirusinfektion.

Die ultrastrukturellen Darmveränderungen bei der EVD-Virusinfektion entsprechen denen der TGE und anderen enteropathogenen Coronaviren. Virusvermehrung war jedoch hauptsächlich auf die laterale Epithelschicht der Zotten begrenzt. Sie reichte bis in den Zotten/Krypten-Bereich. In einigen Fällen ließen sich infizierte Zellen in oder nahe den Lieberkühnschen Krypten nachweisen. EVD-Viruspartikel wurden weiterhin in Makrophagen der Lamina propria und in Phagozyten der regionalen Lymphknoten gefunden.

Résumé

**Lésions intestinales au microscope optique et ultrahistologiques
chez des porcelets
après une infection avec le virus diarrhéique épizootique (EVD):
Comparaison avec le virus de la gastro-entérite transmissible (TGE)
et des infections à Rotavirus chez des porcs**

On a examiné les lésions intestinales ultrahistologiquement et au microscope optique chez des porcelets infectés naturellement et expérimentalement avec le virus épizootique de la diarrhée (EVD). On a comparé cette infection avec celle du virus TGE et Rotavirus. Le déroulement de l'infection au virus EVD est comparable à celui de TGE. Une atrophie des villosités dans tout le jejunum et l'ileum est constatée comme avec TGE. Les résultats trouvés avec une infection à Rotavirus se différencient sur ce point.

Les lésions intestinales ultrastructurelles lors d'une infection avec le virus EVD correspondent à celles rencontrées avec TGE et d'autres virus Corona. La multiplication du virus fut cependant principalement localisée à la couche épithéliale latérale des villosités. Elle atteint la région des villosités/cryptes. Des cellules infectées ont été mises en évidence dans quelques cas dans ou près des cryptes de Lieberkühn. On a trouvé des particules virales EVD dans des macrophages de Lamina propria et dans des phagocytes des ganglions régionaux.

Resumen

**Fotomicroscopía e ultrahistología de las modificaciones intestinales
en lechones
infectados con el virus de la diarrea virósica epizootica (DVE):
Comparación con el virus de la gastroenteritis transmissible (GET)
e infecciones con el virus rota porcino**

Se examinaron las modificaciones fotomicroscópicas y ultrahistológicas en los intestinos de lechones infectados de forma natural y experimental con el virus de la diarrea virósica epizootica (DVE), comparándose con las habidas tras la infección con el virus GET y el virus rota porcino. El curso de la infección con el virus de la DVE se puede comparar con el de la GET. La atrofia de villosidades aparece, tal y como en la GET, en la totalidad del yeyuno y del íleon. A este respecto se diferencian los hallazgos de la infección con el virus rota.

Las modificaciones entéricas ultraestructurales en la infección con el virus de la DVE corresponden a los de la GET y otros virus corona enteropatógenos. Sin embargo, la multiplicación de virus se hallaba limitada sobre todo a la capa epitelial lateral de las villosidades. La misma alcanzaba hasta el ámbito de las villosidades/criptas. En algunos casos se pudieron identificar células infectadas en las criptas de Lieberkühn o cerca de las mismas. Partículas virales DVE se hallaron además en los macrófagos de la lámina propia y en los fagocitos de los ganglios regionales.

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