Ultrastructural Characterization of Colonic Lesions in Pigs Inoculated with *Treponema hyodysenteriae*

M.A. Albassam, H.J. Olander, H.L. Thacker and J.J. Turek*

ABSTRACT

Twelve pigs were inoculated orally with pure cultures of *Treponema hyodysenteriae*. Pigs were necropsied at different time intervals postinoculation; colonic specimens were collected and prepared for light and electron microscopy. The earliest colonic lesion detected by electron microscopy consisted of superficial vascular congestion and dilatation, edema of the lamina propria and intercellular separation of the epithelial cells at the crypt shoulders. This lesion progressed to epithelial cell necrosis and extrusion into the lumen and extravasation of red cells. Large numbers of spirochetes were present and free, between, over and under necrotic epithelial cells whether in place or partially extruded. Spirochetal penetration of colonic enterocytes and intracytoplasmic multiplication were confirmed in this study. The spirochetes were found to invade the epithelial cells only from their lateral borders. The relationship between *T. hyodysenteriae* and the colonic anaerobes was not determined.

Key words: Swine dysentery, transmission electron microscopy, *Treponema hyodysenteriae*.

RÉSUMÉ

Cette expérience consistait à administrer des cultures pures de *Treponema hyodysenteriae* à 12 porcs, par la voie buccale, et à les abattre ensuite à divers intervalles, pour prélever des échantillons du côlon destinés à des examens aux microscopes photonique et électronique. Les premières lésions révélées par la microscopie électronique se caractérisaient par une congestion et une dilatation vasculaires superficielles, un oedème du chorion et une séparation entre les entérocytes du bord des cryptes. Ces lésions progressèrent jusqu'à la nécrose des entérocytes et leur desquamation dans la lumière, ainsi qu'à l'extravasation d'hématies. Plusieurs spirochètes étaient présents et libres, soit entre, en dessus ou en dessons des entérocytes nécrotiques encore en place ou partiellement desquamés. Au cours de l'expérience, les auteurs confirmèrent la pénétration des entérocytes du côlon par des spirochètes et la multiplication intracytoplasmique de ces derniers. Ils constatèrent aussi que les spirochètes envahissaient les entérocytes, seulement à partir de leurs côtés, mais ils ne déterminèrent pas la relation entre *T. hyodysenteriae* et les bactéries anaérobies du côlon.

Mots clés: dysenterie porcine, transmission, microscopie électronique, *Treponema hyodesenteriae*.

INTRODUCTION

Swine dysentery (SD) is a mucohemorrhagic diarrheal disease associated with proliferation of *Treponema hyodysenteriae* and other synergistic anaerobes within the large intestine of affected pigs (1,2). Despite the progress in the last decade in identifying the causative organism(s), only a few reports have been published that evaluate the pathogenesis and pathophysiology of the disease. By electron microscopy, *T. hyodysenteriae* has been seen within necrotic epithelial cells and in the lamina propria of the colon of pigs with SD but there was no evidence that invasion is essential for lesion production (3). It has been suggested that the colonic changes are due to infarction or superficial epithelial necrosis initiated by colonic anaerobes (4,5,6). More recently, in two reports, attachment of *T. hyodysenteriae* to animal cells *in vitro* was described (7,8). Cellular invasion and damage was not observed nor was it possible to demonstrate a toxin in supernatant fluid or sonically disrupted whole cultures of *T. hyodysenteriae* (8).

This study was undertaken to characterize the morphology and progression of ultrastructural lesions of swine dysentery.

MATERIALS AND METHODS

Fourteen pigs were used in this study. Ten pigs were born naturally, caught at birth, scrubbed with betadine solution (Purdue Fredric Company, Norwalk, Connecticut 06856) and administered 10 mL of sow's colostrum intragastrically via a stomach tube every 12 hours for 48 hours. Four pigs were cesarean derived and colostrum deprived. All fourteen pigs were placed in individual isolation cages in a controlled environment room for rearing and fed pasteurized cow's milk supplemented with mineral mix until five weeks of age. During the first two weeks the pigs were fed four times a day. At five weeks of age the pigs were fed a 16% protein corn-soy meal ration free of antibiotics *ad libitum*, given access to clear fresh water *ad libitum* and kept in the individual isolation cages.

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Submitted as journal series paper no. 10011, Purdue University Agricultural Experiment Station. Supported in part by USDA grant 0139-62-11735.

Submitted November 15,1984.

At four weeks of age, 12 pigs were fasted for 24 hours, then fed a small amount of milk mixed with the contents of 24 blood agar plate cultures on which 48 hours of *T. hyodysenteriae* isolate B204 was grown. The isolate B204 of *T. hyodysenteriae* was isolated from and known to be pathogenic for conventional pigs (9). On the following day, 240 mL of broth culture of isolate B204 of *T. hyodysenteriae* containing 1.4 x 10^8 organisms per mL, was mixed with milk and fed to the same 12 pigs. Two pigs were kept as uninoculated controls.

Rectal fecal samples were collected daily from all the pigs with plastic spoons that held approximately 0.5 g of feces. The fecal specimens were placed immediately into test tubes containing sufficient sterile phosphate buffered saline (PBS), pH 7.4 to an approximate dilution of 1:10. Dark field and culture examination of feces for *T. hyodysenteriae* were done as soon as samples were collected and returned to the laboratory.

Pigs were necropsied at different time intervals postinoculation. Three inoculated pigs were necropsied soon after spirochetes were observed in the feces and before they developed diarrhea (pigs #3, #6 and #13). These pigs were killed on days 5, 4 and 8 postinoculation. Three inoculated pigs were necropsied within 24 hours after onset of diarrhea (pigs #1, #11 and #12). These pigs were killed at days 5 and 8 postinoculation. One pig (pig #4) was necropsied 20 days postinoculation after three days of diarrhea. Two pigs (pigs #2 and #5) were necropsied 17 days postinoculation with five days of diarrhea. One pig (pig #7) was necropsied 26 days postinoculation after 16 days of diarrhea. Two pigs (pigs #9 and #10) that did not develop clinical signs of swine dysentery, but were shedding *T. hyodysenteriae*, were killed 50 days postinoculation (Table I). The first control pig (pig #8) was killed on day 1 of the experiment and the second control pig (pig #14) was killed on day 50 of the experiment. At necropsy, four specimens from the colon were placed in neutral buffered 10% formalin and 3% glutaraldehyde for processing for light and transmission electron microscopy. Formalin fixed tissues were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (HE). For electron microscopy, glutaraldehyde-fixed tissues were washed in Millonig's buffer, stained *en bloc* in 1% osmium tetroxide (two hours), rinsed in buffer, dehydrated in a series of graded ethanol, passed in propylene oxide and embedded in epoxy resins (Epon 812). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a Jeol JEM-100cx electron microscope operating at 60 or 80 kilovolts. Colonic mucosal scrapings were examined by dark field light microscopy and cultured for detection of *T. hyodysenteriae*. Colonic contents and mesenteric lymph nodes were cultured for *Salmonella* sp.

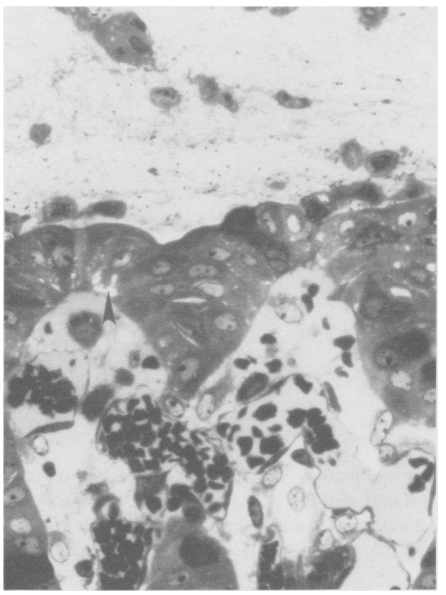

Fig. 1. Superficial colonic mucosa of a pig with diarrhea for less than 24 hours. The lumen contains a few necrotic cells and moderate number of spirochetes. The epithelial cells at the crypt shoulder appear separated from each other (arrow head) and vacuolated. The lamina propria is edematous and the blood vessels are congested. Methylene blue azure II. X560.

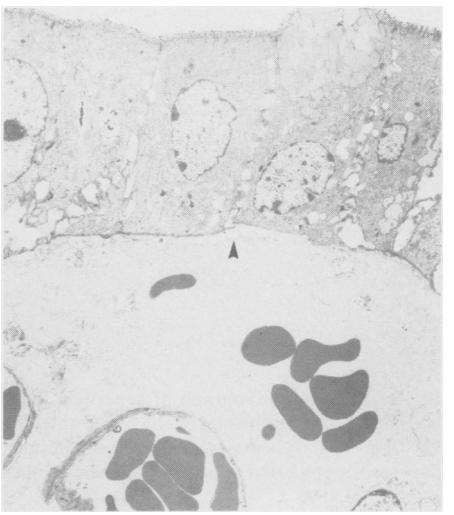

Fig. 3. Electron micrograph of superficial colonic mucosa from a pig with diarrhea for less than 24 hours. The intercellular spaces are widely separated and two epithelial cells are detached from their basement membrane (arrow head). The lamina propria is distended with plasma-like fluid and extravasated red cells. Uranyl acetate and lead citrate. X4,000.

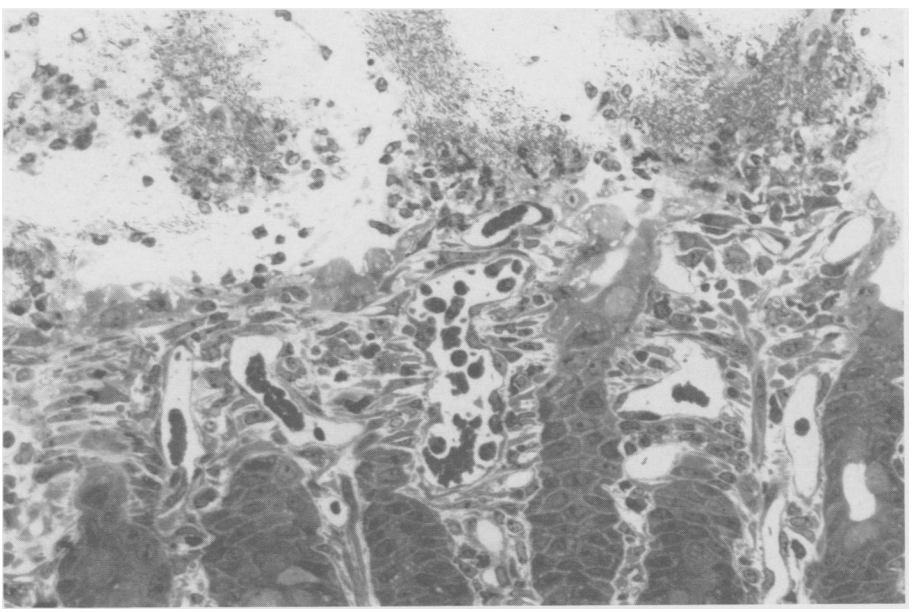

Fig. 2. Superficial colonic mucosa of a pig with diarrhea for 16 days. The lumen contains a mixture of spirochetes, bacilli, necrotic debris and inflammatory cells. The superficial epithelial cells are necrotic and the lamina propria is infiltrated with inflammatory cells. Methylene blue azure II. X350.

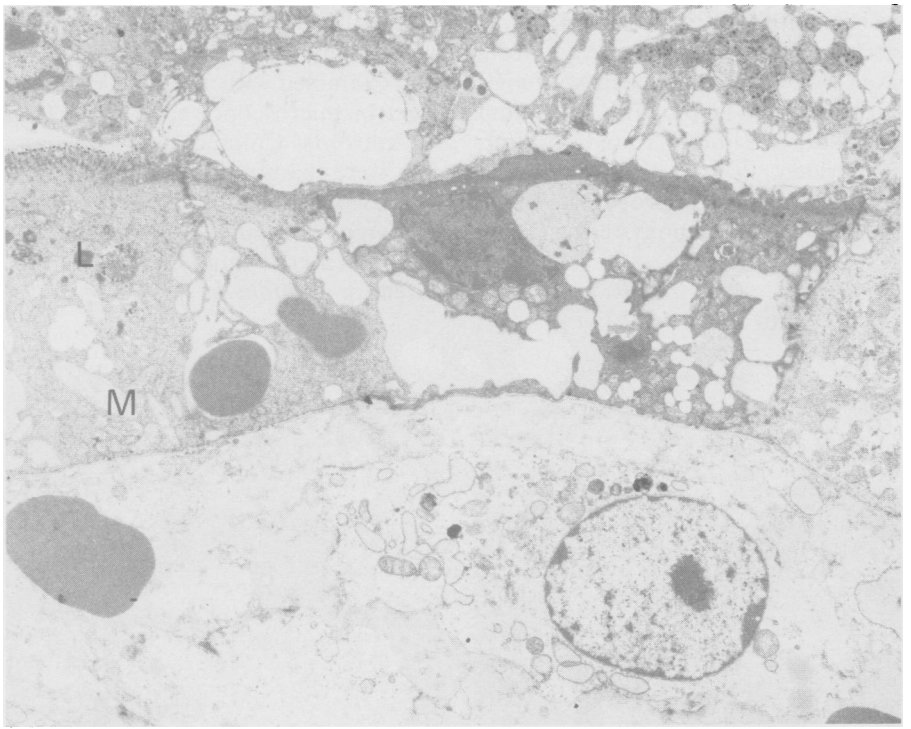

Fig. 4. Electron micrograph of superficial colonic mucosa from a pig which had diarrhea for 24 hours. Necrotic epithelial cells have disrupted microvilli and electron dense cytoplasm. (M) myelin-like figures; (L) lysosomes. The intercellular junctions are disrupted and red cells are passing between the necrotic epithelial cells. The lumen has abundant necrotic cellular debris. Uranyl acetate and lead citrate. X4,450.

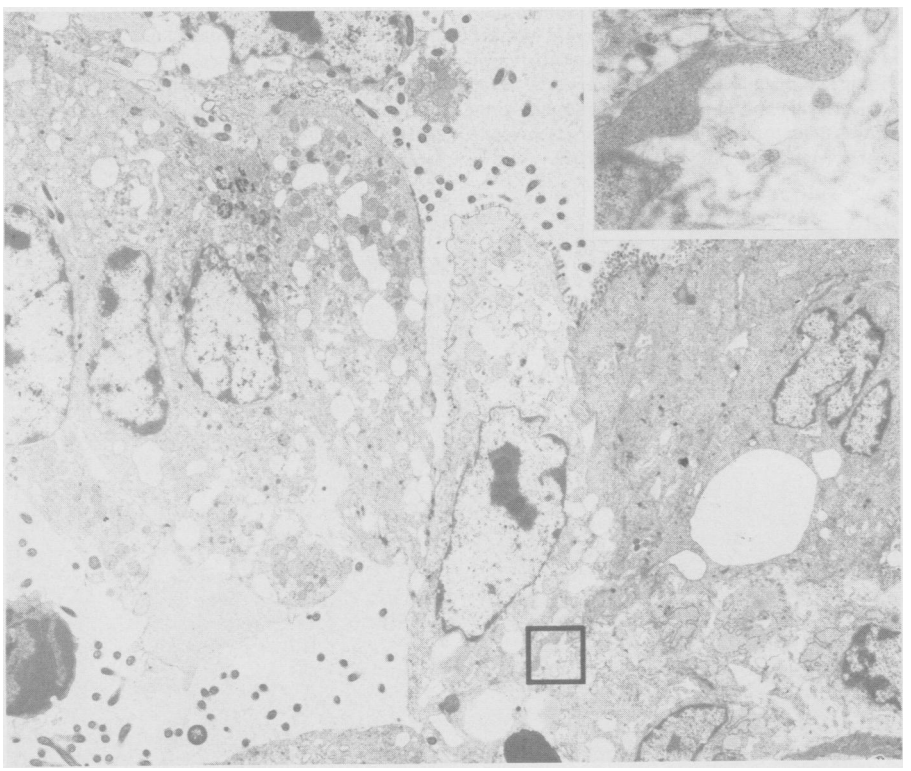

Fig. 5. Electron micrograph of superficial colonic mucosa from a pig which had diarrhea for 24 hours. Four necrotic epithelial cells are extruded into the lumen. Spirochetes are over, between and under extruded epithelial cells. *Inset*, a higher magnification of a partially detached basement membrane from unextruded but degenerate epithelial cell. Uranyl acetate and lead citrate. X4,000. Inset, X20,000.

RESULTS

One of the earliest lesions detected by light microscopy was the presence of large numbers of spirochetes in the colonic lumen mixed with a few necrotic epithelial cells and mucus. The subepithelial blood vessels were congested and dilated. The lamina propria was distended with fluid and a few extravascular erythrocytes (Fig. 1). A few cells at the shoulders of the crypts appeared vacuolated. Inflammatory cell response was minimal.

Animals with diarrhea of several days duration had more severe superficial colonic mucosal lesions. These lesions were characterized by large foci of necrosis of epithelium and superficial lamina propria infiltrated by a mixed population of inflammatory cells. The epithelial lining was markedly flattened or necrotic. Large numbers of bacilli were admixed with spirochetes at the sites of superficial mucosal necrosis (Fig. 2). The lamina propria was not markedly edematous but was infiltrated with a moderate number of mixed inflammatory cells.

The earliest lesion detected by electron microscopy was in the superficial mucosa. It was characterized by marked edema at the shoulders of the crypts which involved both the mucosa and the lamina propria. The mucosal edema was mainly intercellular and to a lesser degree intracellular. The intercellular fluid had the same electron density as the fluid in the lamina propria and the plasma of the subepithelial blood vessels (Fig. 3). A few extravasated red cells were seen in the plasma-like fluid in the expanded lamina propria. In more severe lesions individual or groups of necrotic epithelial cells were found at the mouths and shoulders of the crypts. The necrotic cells had irregular, sparse, short or disrupted microvilli. The apical surface of the cells bulged and blebbed into the lumen. The endoplasmic reticulum was markedly dilated, the mitochrondria were distended and the cristae were disrupted. There were also increased numbers of lysosomes, the formation of myelin-like figures and increased density of cytoplasmic gel. The nuclei were shrunken and condensed and the chromatin was marginated on or adjacent to the inner membrane of the

nuclear envelope. At this stage red cells were seen passing between the necrotic epithelial cells into the colonic lumen (Fig. 4). Spirochetes were more closely associated with necrotic cells than in less severe lesions; most were extracellular but a few were inside necrotic epithelial cells. In some areas many spirochetes were seen between the partially sloughed epithelial cells and the exposed lamina propria. The extrusion of epithelial cells was associated with loosening and detachment from their basement membrane (Fig. 5). A few neutrophils containing intracytoplasmic spirochetes were present in the necrotic areas. Epithelial regenerative changes were evident as immature cuboidal or low columnar colonic enterocytes present at the edges of necrotic areas.

More severe ultrastructural lesions were present in the superficial mucosa of animals with diarrhea of several days duration. The lesions were characterized by sloughing of large areas of necrotic epithelium into the lumen of the colon. The microvilli of the necrotic cells were disrupted and vacuolated and the endoplasmic reticulum was distended. The mitochondria were swollen with increased size and number of matrical dense granules (Fig. 6). The necrotic epithelium was covered by cellular debris and a large number of bacilli with moderate numbers of spirochetes (Fig. 7). The ultrastructural features of the bacilli were compatible with anaerobic bacilli (10). Macrophages and neutrophils were numerous and many contained different types of bacteria in various stages of degradation. Many neutrophils were degranulated. Large clots of fibrin were present between the inflammatory cells and the cellular debris on the surface. At this stage, the spirochetal association with the mucosal epithelium was prominent and observed as follows: 1) Spirochetes in moderate to large numbers were seen free, over, between and under necrotic epithelial cells whether in place or partially extruded. A few necrotic cells contained moderate numbers of spirochetes. 2) Occasionally, a few spirochetes with preserved normal ultrastructure were seen free in the cytoplasm of relatively normal enterocytes and goblet cells (Fig. 8); large numbers of spirochetes that appeared to be multiplying were associated with moderate to marked degenerative changes in the cytoplasm (Fig. 9). The microvilli of the penetrated epithelial cells were normal. 3) In a few instances individual spirochetes were present between two normal epithelial cells with minor or no damage to the adjacent plasmamembranes (Fig. 10). 4) Early stages of spirochete penetration into relatively normal epithelial cells were also occasionally seen. The spirochetes were found invading the epithelial cells only on the lateral borders of the cells. This lateral spirochetal penetration was clearly demonstrated in areas where an adjacent cell had recently been extruded or through an empty goblet

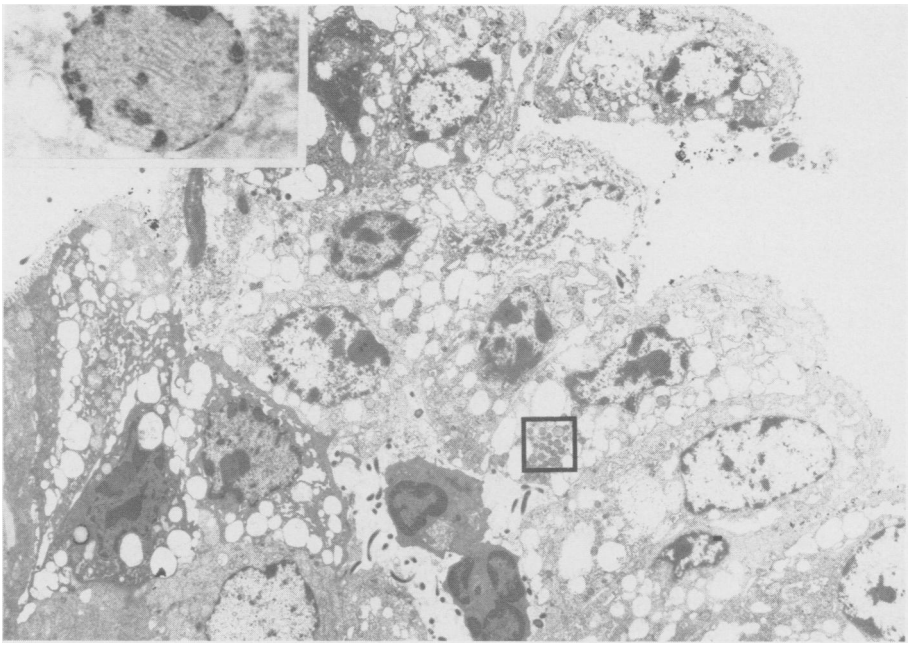

Fig. 6. Electron micrograph of superficial colonic mucosa from a pig which had diarrhea for three days. Epithelial necrosis is prominent; the nuclei have clumped chromatin and the endoplasmic reticulum is markedly distended. Spirochetes with two degranulated neutrophils are under the partially extruded epithelial cells. *Inset*, a higher magnification of a mitochondrion which has an increased number and size of matrical dense granules. Uranyl acetate and lead citrate. X3,600. Inset X51,300.

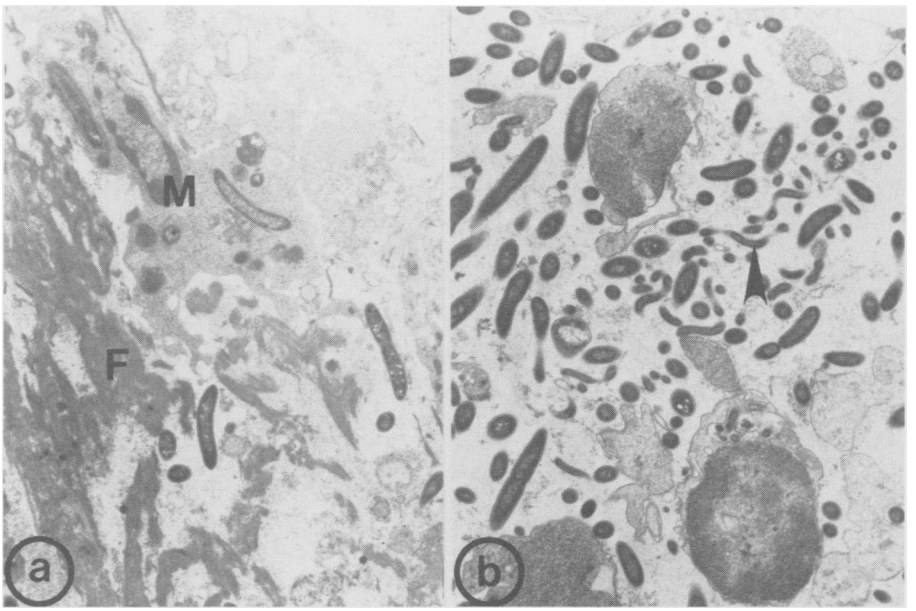

Fig. 7. Electron micrograph from two representative areas of cellular debris that cover an SD lesion. a, clots of fibrin (F) are between a macrophage (M) and several bacilli. Two bacilli are ingested by the macrophage. b, moderate number of spirochetes (arrow head) are mixed with bacilli, inflammatory cells and cellular debris. Uranyl acetate and lead citrate. X4,450.

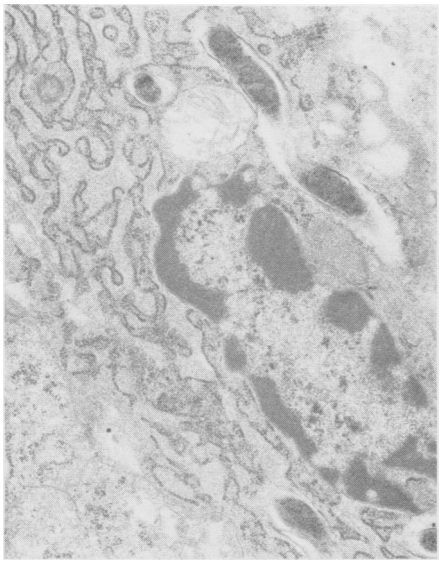

Fig. 8. Electron micrograph of a developing goblet cell from the superficial colonic mucosa. Five spirochetes are lying free between the rough endoplasmic reticulum. Uranyl acetate and lead citrate. X22,500.

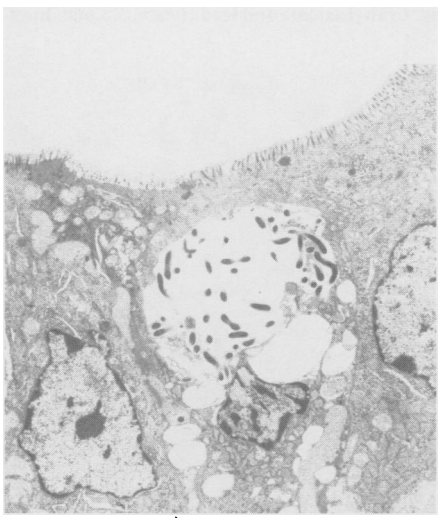

Fig. 9. Electron micrograph of superficial colonic mucosa from a pig with five days of diarrhea. Large numbers of spirochetes are present in the cytoplasm of a moderately degenerate epithelial cell. Uranyl acetate and lead citrate. X6,600.

cell (Fig. 11). 5) Spirochetes were never seen attached to or penetrating normal colonic enterocytes at the apical striated border. 6) Spirochetes were attached to the sparse and irregular microvilli of goblet and epithelial cells in the crypts.

The subepithelial lamina propria was less edematous and more cellular than at the early stages of the disease. It was infiltrated by macrophages, neutrophils and a few lymphocytes.

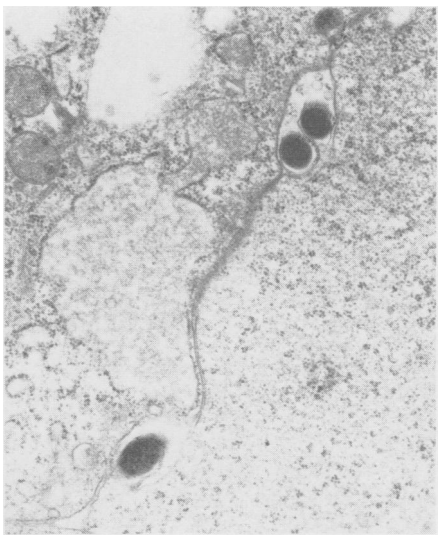

Fig. 10. Electron micrograph of three spirochetes present between the plasma membranes of two enterocytes. Uranyl acetate and lead citrate. X18,970.

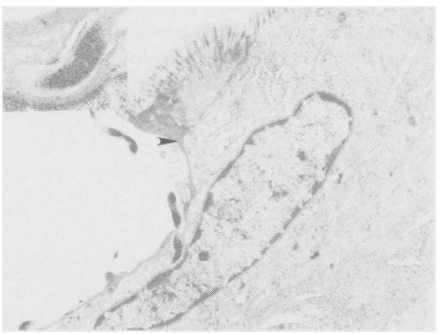

Fig. 11. Electron micrograph of a spirochete invading a colonic epithelial cell from the lateral border of the cell. The dense cytoplasm (arrow head) is a remnant of an empty goblet cell. *Inset*, higher magnification of the spirochete at the penetration site. Uranyl acetate and lead citrate. X11,000. Inset, X31,200.

Ultrastructural changes of endothelial contraction in the venules were observed in some of the subepithelial vessels in the acute stage of the disease. These changes included: a) bulging of the cell body into the vessel lumen, b) rounded nucleus, c) folded nuclear membrane and d) markedly folded luminal surface of the cell membrane (Fig. 12).

The progression and severity of swine dysentery lesions were similar in the caesarean derived colostrum deprived pigs and the naturally born pigs.

No significant lesions were detected by light or electron microscopy in colons from pigs that did not develop diarrhea, but were shedding *T. hyodysenteriae* or in colons from control pigs.

BACTERIOLOGICAL FINDINGS

Treponema hyodysenteriae was isolated at the time of necropsy from colons of pigs that had developed clinical signs of SD with typical diarrhea (pigs #1, #2, #4, #5, #7, #11 and #12). It was also isolated at the time of necropsy from the colons of three pigs that did not develop diarrhea but were shedding the organism (pigs #3, #6 and #13). Two pigs did not develop obvious clinical dysentery (pigs #9 and #10) but did shed the causative agent a few times during the experimental period (Table I); *T. hyodysenteriae* was not isolated by bacteriological culture at the termination of the study.

Salmonella organisms were not isolated from the tissues or colonic contents of any of the principal or control pigs.

DISCUSSION

The results of this study indicate an early and important manifestation of swine dysentery to be characterized by cellular separation with marked edema of the lamina propria and vascular congestion in the colonic mucosa. This event is followed by degeneration, necrosis and extrusion of superficial colonic enterocytes at the shoulders of the crypts. A similar sequence of events occurs in experimental *Shigella* dysentery of primates (11) where it was postulated that the mucosal necrosis was not caused by direct action of the causative *Shigella* bacteria but as the indirect effect of bacterial toxin which produced disturbance in circulation and eventually cellular maladjustment and hypoxia. In swine dysentery, circulatory disturbance in the superficial colonic mucosa was characterized by the presence of intercellular edema and by endothelial contraction of the subepithelial venules. Experimentally, histamine-type mediators induced ultrastructural changes in blood vessels highly suggestive of endothelial contraction (12). Histamine-type mediators in the dysenteric colon could have been produced by local mast cells. From the findings of this study, it was postulated that a spiro-

TABLE I. Experimental Infection of Pigs with *Treponema hyodysenteriae*

Pig No.	Source	Type of Inoculum	Day Killed Post-inoculation	Isolation of *T. hyo.* in Fecal Samples or Colonic Contents Postinoculation	Days of *T. hyo.* Shedding	No. Times *T. hyo.* Shed During Exposure	SD[e] Days
1	Natural birth	BAP[a] and BC[b]	4	Day 4	1	1	1
2	Natural birth	BAP and BC	17	Days 9,10,11,12,13,14,15,16,17	9	1	5
3	Natural birth	BAP and BC	5	Day 5	1	1	0
4	Natural birth	BAP and BC	20	Days 5,17,18,19,20	5	2	3
5	Natural birth	BAP and BC	17	Days 5,13,14,15,16,17	6	2	5
6	Natural birth	BAP and BC	4	Day 4	1	1	0
7	Natural birth	BAP and BC	26	Days 11,12,13,14,15,16,17,18,19 20,21,22,23,24,25,26	16	1	16
8	Natural birth	None — Control	—[c]	—	—	—	—
9	Natural birth	BAP and BC	50	Days 4,21,22,23,24	5	2	0
10	Natural birth	BAP and BC	50	Days 3,13,14,32	4	3	0
11	Cesarean	BAP and BC	5	Day 5	1	1	1
12	Cesarean	BAP and BC	8	Day 7	1	1	1
13	Cesarean	BAP and BC	8	Day 8	1	1	0
14	Cesarean	None — Control	—[d]	—	—	—	

[a]BAP = Blood agar plates
[b]BC = Broth culture
[c] = Killed on day 1 of experiment
[d] = Killed on day 50 of experiment
[e]SD days — Days pigs had swine dysentery diarrhea

chetal toxin induced an acute inflammatory response in the colonic mucosa with histamine release by the local mast cells with ensuant vascular changes.

This study and previous studies (13,14) have indicated that a characteristic lesion of SD is epithelial necrosis which invariably affects the most superficial epithelium first and never penetrates beyond the outer one-third of the mucosa. It has been postulated that the restricted superficial necrosis of the colonic enterocytes was due to direct action of a poorly diffusable toxin which reached toxic concentration only at the mucosal surface (13). The observations of the present study suggest that both direct and indirect action of a toxin contributes to the lesions of swine dysentery.

Penetration of spirochetes into normal superficial colonic enterocytes was confirmed in this study. It appeared that the most common portal of entry was through the lateral borders of the cells. This study indicates that penetration is not essential for the initiation of swine dysentery lesions. Early lesions were observed before penetration was seen. As the disease progresses, mucosal necrosis may be attributed to the following mechanisms. First, cellular penetration of the spirochetes occurs through the lateral borders of the cells with ensuant intracytoplasmic multiplication and eventual destruction of the enterocytes. Second, cytotoxin(s) are produced by *T. hyodysenteriae* with or without the interaction of other colonic anaerobes on the epithelial surface of the colon. The second mechanism appeared to be of major significance in the induction of cellular necrosis since there were no intracytoplasmic spirochetes in the large number of necrotic colonic enterocytes that were examined.

These results suggest that *T. hyodysenteriae* has a low invasive capability in comparison to other invasive enteric bacteria. In salmonellosis, the most common portal of entry is through the brush border. *Salmonella typhimurium* penetration is usually preceded by close contact of the bacteria to the brush border (less than 35 nm), followed by degeneration and disruption of the microvilli (15). This type of interaction was not seen between *T. hyodysenteriae* and the colonic epithelium. It was reported previously (16,17), and observed in this study, that penetration through the apical surface occurs in degenerate colonic enterocytes where the microvilli are sparse and irregular. It appeared that the brush border of normal mature colonic enterocytes served as a physical barrier against penetration of *T. hyodysenteriae*. The intercellular presence of spirochetes could be attributed to direct and active penetration through the junctional complex or entrapment by enterocytes as they moved together in closing gaps after extrusion of degenerate cells.

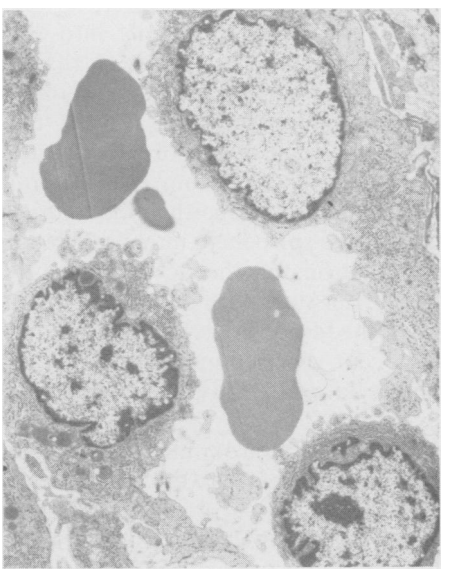

Fig. 12. Electron micrograph of a subepithelial venule from a dysenteric pig. The endothelial cells have cell bodies bulging into the lumen, rounded nuclei and folded nuclear and plasma membranes. Uranyl acetate and lead citrate. X8,250.

According to the concept of intracellular parasitism, an interaction must exist between two living cells, that is, a living bacterium and a living epithelial cell. In the present study, this has been shown by electron microscopy. The well preserved normal

ultrastructure of the cell and the bacteria with multiplication of *T. hyodysenteriae* suggest that the epithelial cell and the bacterium were alive. However, when there was extensive bacterial multiplication in the cytoplasm of the host cell, it was destroyed.

Spirochetes were observed free in the cytoplasm of morphologically normal epithelial cells. The absence of a phagosomal membrane around the bacteria may have been due to disintegration of the membrane. Disintegration of the phagosomal membrane has been observed in intestinal epithelial cells infected with *Shigella* and *Listeria* bacteria (11,18).

Intraepithelial spirochetes would be protected from phagocytosis by polymorphonuclear leukocytes and macrophages and also against humoral defenses of the host. Extensive intracellular spirochetal multiplication resulted in cellular destruction and eventual extrusion of cells into the colonic lumen. The cellular extrusion created a gap between remaining colonic enterocytes and allowed the liberated spirochetes to repenetrate the adjacent cells from their lateral borders. The intraepithelial propagation of the spirochete may provide an explanation for the development of the carrier state with or without organism shedding which has been shown to occur both experimentally and under natural conditions with swine dysentery (19).

The physical proximity between the colonic enterocytes, the spirochetes and the anaerobic microflora in the early lesions of the disease was very limited. Later in the disease epithelial necrosis and vascular leakage created conditions favoring the overgrowth of opportunistic anaerobic bacteria in close proximity to the epithelial surface. This overgrowth may be a contributing factor in the pathogenesis of the disease by toxin production and cellular damage.

ACKNOWLEDGMENTS

The advice of Dr. R.M. Claflin in the preparation of this manuscript was very much appreciated.

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