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Effect of filtrates *of Serpulina(Treponema) hyodysenteriae* on the epithelium in colonic loops in swine

Boo-hyon Kang, Olander, Harvey J*
Toxicology Research Center, Korea Research Institute of Chemical Technology
Department of Pathology, School of Veterinary Medicine,
*University of California, Davis, California, USA**
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Serpulina(Treponema)hyodysenteriae 배양여과액의 돼지 공장 계제 점막에 미치는 영향

강부현 · Olander, Harvey J*
한국화학연구소 안전성연구센터
California, Davis 수의과대학*
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초록 : *Serpulina(S) hyodysenteriae* B 204 strain이 생산한 세포독소에 의한 장점막의 변화를 알아보기 위하여 일반사육조건에서 사육된 8주령, 수컷 잡종돼지 2마리의 결장을 외과적으로 결찰한 계제(loop)를 이용하여 실험하였다. *Serpulina*균을 trypticase soy broth(TSB)에 배양하여 결찰한 결장계재 4곳에 각각 멸균한 *Serpulina*균 TSB, 여과한 *Serpulina*균 TSB, 세척한 *Serpulina*균, 무처치 *Serpulina*균 TSB를 접종하였다. 점막조직을 접종(p.i.) 24, 48시간 후에 채취한 후 처리하여 주사전자현미경과 투과전자현미경으로 관찰하였다. 여과한 *Serpulina*균 TSB를 접종한 경우는 변화가 관찰되지 않았으나 세척한 *Serpulina*균을 접종한 경우는 무처치 *Serpulina*균 TSB를 접종한 부위에서 나타난 것과 유사한 초기변화가 관찰되었다. 본 실험의 결과는 *Serpulina*균의 세포독소가 실험적 감염시 초기병변형성에 별로 기여하지 않음을 시사하였다. *Serpulina*균의 독소가 병변을 야기하는데 관여하는 기전은 밝혀지지 않았다.

Key words : swine, *Serpulina hyodysenteriae*, TEM, SEM, intestine, cytotoxin.

Introduction

Swine dysentery(SD) is a mucohemorrhagic colitis caused by *Serpulina hyodysenteriae* uaually in young pigs.[4,21] SD was first described in 1921 by Whiting et al.[25] In 1971 Taylor and Alexander[21] reported the successful propagation of a pathogenic anaerobic spirochete. In 1972 Harris et al.[4] named *Treponema hyodysenteriae*. It is now classified in the genus *Serpulina*, because the organism is not typical of the genus *Treponema* in RNA homology, DNA-DNA reassociation and SDS-page profiles of whole cell proteins.[20] Pathogenesis of the disease has been suggested by several investigators which Wilcook & Olander[26] classified into three categories. These included epithelial invasion by spirochetes,[2,3,18,22,27] cytotoxin production by spirochetes or associated colonic anaerobes[5,6,11] and mucosal infarction initiated by spirochetal or other bacterial toxins.[11] The theories of cytotoxic effects and mucosal infarction were not supported by the work of

Wilcock & Olander.[26,27] They described no visible effect on the cell cultures, erythrocyte suspensions or the mucosa of ligated colonic segments by broth cultures or by infected colonic content sterilized by filtration or disruption with ultrasound.[27] They also demonstrated no evidence of obstruction to mucosal vascular perfusion in acute swine dysentery experimentally induced by using ligated colonic segments.[26] The theory of epithelial cell invasion has been partially supported by previously reported experiments in which large *Serpulinas* were demonstrated within degenerative colonic epithelial cells.[2,3,22] Kang & Olander[7,8] also suggested that spirochetes invade, without initial attachment, primarily through areas of the colonic mucosa where cohesion between epithelial cells had been physiologically or pathologically lost. Invasion dose not take place through healthy epithelial cells either from the microvillous surface or where normal intercellular or basement membrane attachment is intact. After penetration and disruption of the epithelial membrane, these spirochetes may continue to invade adjacent healthy or degenerative epithelial cells, the lamina propria and sometimes the muscularis externa. When the epithelial cells are invaded by spirochetes, they are disrupted by the physical activity and possibly cytotoxins of spirochetes.

Hemolysin and lipopolysaccharide of *S hyodysenteriae* have been shown to be associated with pathogenicity of the organism. The hemolysin was originally described by Saheb et al.[19] and purfied by Kent & Lemcke.[10] Kinyon et al.[12] demonstrated that the hemolytic activity is closly correlated with the enteropathogenicity of *S hyodysenteriae*. The lipopolysaccharide was originally described by Baum & Joens[1] and has a biological activity similar to that of endotoxin of gram negative bacteria as demonstrated *in vitro* by Nuessen et al.[15] It has been suggested to be an important antigen in the stimulation of host defense against the *Serpulina* by Nuessen & Joens[16] and to be involved in the pathogenicity of *S hyodysenteriae* by Nuessen et al.[17]

The purpose of this experiment was to investigate whether cytotoxins in the filtrate of the culture broth of *S hyodysenteriae* cause damage to the mucosal surface and initiated disease in surgically prepared ligated loops of the colon[6,24] which is detectable by transmission(TEM) or scanning electron microscopy(SEM).

Materials and Methods

Two conventional male pigs, 8 weeks old, maintained at the Animal Resources Service of the University of California, Davis were used for the experiment. They were born and raised in the animal sciences swine facility of the University of California, Davis. They were healthy, had no history of diarrhea. The study subjects had not been immunized with any commercial vaccine preparation. They were fed a 16% protein corn-soy meal ration free of antibiotics *ad libitum* and given access to clear fresh water *ad libitum*. Feed was withheld from them 48 hours before surgery and thereafter. The pigs were anesthetized with 1.0% halothane during surgery which lasted about 3 hours. The spiral colon was exposed via an incision in the midline of the abdominal wall. In each pig, four colonic segments(test loops), approximately 8 cm long, were separated by interloops, approximately 5 cm long, by tying silk ligatures around the colon.[6,24]

S hyodysenteriae strain B 204 was grown in modified Lemcke medium. Trypticase soy broth(TSB;BBL Microbiology System, Cockeysville, Md.) was prepared while exposed to the ambient atmosphere and was dispensed into anaerobe tubes containing stir bars before autoclaving. At the time of inoculation, 10% fetal calf serum, 0.2%(wt/vol) sterile sodium bicarbonate, 0.05%(wt/vol) sterile cysteine hydrochloride monohydrate and 0.5%(wt/vol) sterile glucose were added to the tubes under a flow of 10% CO_2-N_2. The medium was inoculated under a flow of 10% CO_2-N_2 and incubated on a magnetic stirrer which rotated at 500 rpm for 48 hours at 38℃.[13] The number of spirochete in the TSB culuture was 1.5×10^8 cells/ml. Quantitations of growth was assessed by total cell counts which were determined by dilution of culture in phosphate buffered saline(PBS;0.01M, pH 7.2) solution and visually counted microscopically using a bacteria counting chamber(Hausser Scientific, Horsham, PA). The supernatant was removed after the TSB cultures were centrifuged at 5,000× g at 4℃ for 30 minutes to prepare filtrate and washed cells. The pellet was washed twice in PBS by resuspension and recentrifugation. The washed cells were suspended in sterile TSB at a concentration of 2.0×10^8 cells/㎖. The cells were quantitated by the

method described above.

The supernatant was sterlized by passage through a 0.22um cellulose acetate filter(Nalge Company, Rochester, N.Y). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) was performed on the concentrated filtrate according to the method of Laemmli.[14] SDS-PAGE slabs were stained in 0.25% Coomassie blue stain for 24 hours 37℃ and overnight at room temperature.

Either sterile TSB culture, the filtrate of *Serpulina* culture, the washed cells of *Serpulina* culture or the TSB culture of *Serpulina* was placed in a loop, until the loop become turgid, which required 15~20㎖, using a 35㎖ syringe and 22-gauge needle. The loop inoculated with sterile TSB was used as a negative control. The colon was put into the abdominal cavity and the abdominal incision was closed. The pigs were put in recovery cages, they were killed by electrocution 24 and 48 hours postinoculation(p.i.).

Immediately after electrocution two 20×20mm square pieces of colonic mucosa, which contained all layers of the wall, were taken from a central area of each colonic loop and fixed in Karnovsky's fixative for TEM and SEM. After fixation, three 5×3×1mm pieces of colonic tissues for TEM and two pieces of 5×5×3mm tissues for SEM were trimmed from specimens of each loop and put in Karnovsky's fixative. For TEM, the tissue were washed in S-collodine buffer, post-fixed in 1% cacodylate-buffered osmium tetroxide, dehydrated through ethanol/propylene oxide and embedded in Epon-araldite. Semithin sections were cut and stained with toluidine blue. Two to five ultrathin sections containing the mucosa and a small parts of the submucosa from each loop were then cut and stained in 2% aqueous uranyl acetate. They were examined using Zeiss EM 10(Carl Zeiss, West Germany). Tissue for SEM were washed three times in S-collidine buffered solution for 1 hour each time, postfixed in S-collidine buffered 1% OsO_4 for 1 hour at 4℃, dehydrated in graded alcohol solutions for 30 minutes each step and dried for 2 to 3 hours by a critical point technique, using CO_2 as transitional fluid. The dried specimens were mounted on aluminum sample holders using silver paint adhesive and lightly coated with gold under a vacuum evaporator. The coated specimens were examined with an ISI-SS 60 SEM (International Scientific Instrument Inc. USA). Micrograghs were taken on Polaroid type 665 film.

Results

Negative control loops-loops- : The tissues from loops 24 and 48 hours after inoculation with sterile TSB generally had similar ultrastuctures. There usually were no or only minor changes in microvilli, mitochondria, endoplasmic reticulum, nucleus and intercellular junctions of epithelial cells in the mucosal surface. However, effete cells were often found undergoing extrusion between healthy epithelial cells. There was loss of cohesion, mild edema and sometimes leukocytes present in areas surrouning necrotic effete cells(Fig 1). Goblet cells appeared intact on the mucosal surface. No recognizable change in a ultrastructural morphology of the crypt was seen. Mild to moderate edema was frequently seen in the lamina propria.

Negative control loops-SEM : There were some variations in shapes of the crypt opening and the density, size and distribution of surface, depending on a degree of contraction of epithelial cells on the mucosal surface. Microvilli and goblet cells generally appeared intact. Effete cells were often present partially embedded in the mucosal surface(Fig 2).

Filtrate exposed loops collected 24 and 48 hours p.i. : Several protein bands were observed by SDS-PAGE which was performed on the filtrate of *S hyodysenteriae* culture. In general, the tissue did not differ from the control in ultrastructure in loops of 24 and 48 hour p.i.(Figs 3~5).

Washed *Serpulina* cell exposed loops collected 24 and 48 hours p.i. : The lesions varied in severity from focus to focus in loops of 24 and 48 hours exposure. They were generally more severe in loops of 48 hours than 24 hours p.i.

Washed *Serpulina* cell exposed loops collected 24 hours p.i.-TEM : Ultrastructural changes were minimal except for degenerative changes at sites of cellular extrusion. At these locations, epithelial cells were frequently separated and necrotic cellular debris and a few emigrated leukocytes were frequently present. Spirochetes were often found in intercellular gaps, in the cytoplasm of epithelial cells and in the lamina propria. There was a mild intracellular and intercellular edema in the superfi-

cial mucosa. Most of goblet cells appeared intact in the mucosal epithelium(Fig 6). Sometimes, there was a diffuse distribution of large numbers of spirochetes in the superficial mucosa. They were mostly present in edematous intercellular gaps between epithelial cells, in the lamina propria and often in cytoplasm of epithelial cells. Some epithelial cells, usually adjacent to sites of entry by spirochetes, had moderate to severe intracellular changes such as enlarged mitochondria, vacuolated endoplasmic reticulum, condensed nuclei and increased electron density of cytoplasm in this area. Others appeared normal, even though they had spirochetes in their cytoplasms(Fig 7). No ultrastructural alteration was recognized in the crypts of mucosa, but mild edema was present in the lamina propria. Small numbers of spirochetes were found in the cytoplasm of crypt epithelial cells and in the lamina propria. Large numbers were occasionally present in the crypt lumen(Fig 8).

Washed *Serpulina* cell exposed loops collected 24 hours p.i.-SEM : The mucosal surface had an increased amount of fibrin and mucous strands, comparing with negative control loops, frequently attached to the crypt. The morphology and numbers of microvilli were not different from normal. Goblet cells did not seem to increase in number. Fissures, which were often present at extrusion zones, were wider than normal and contained small numbers of fibrin clots, variable numbers of spirochetes and other bacteria. There was a diffuse distribution of spitochetes, usually trapped by fibrin clots and mucous clusters and more often found in fissures at extrusion zones and in intercellular gaps on the mucosal surface(Fig 9, 10).

Washed *Serpulina* cell exposed loops collected 48 hours p.i.-TEM : Degeneration and desquamation of epithelial cells, intracellular edema, evacuation of goblet cells, infiltration of leukocytes and severe extracellular edema in the epithelium and the lamina propria were prominent but variable in the superficial mucosa. Ultrastructural changes of epithelial cells included enlargement of mitochondria, vacuolation of endoplasmic reticulum, increased electron density of cytoplasm, condensation of nuclei and irregular loss, sparseness or shortening of microvilli. Variable numbers of spirochetes were present in intercellular gaps, in the cytoplasm of epithelial cells and in the lamina propria of superficial mucosa. In the colonic lumen, the numbers of emigrated leukocytes, mainly neutrophils and disrupted epithelial cells which often contained a few spirochetes(Fig 11, 12) were greater than in the 24 hours specimen. The crypts had similar but milder diffuse ultrastructural changes in epithelial cells and widening of lumens. However, the microvilli of absorptive cells appeared intact. The lamina propria had severe edema and infiltration of leukocytes, mainly neutrophils. Spirochetes were observed in small numbers in the cytoplasm of the crypt epithelial cells, sometimes in large numbers in the cytoplasm of evacuated goblet cells. Organisms were also found in variable numbers in the lamina propria(Fig 13).

Washed *Serpulina* cell exposed loops collected 48 hours p.i.-SEM : There was a mixture of lesions varying from initial to advanced stages. In the initial stages there was the formation of uneven ridges with a roughly polygonal pattern by disrupted epithelial cells on the mucosal surface usually over extrusion zones. In the advanced stages raised plaques composed of disrupted, detached epithelial cells and other debris covered the surface(Fig 14). Spirochetes were present on and beneath disrupted epithelial cells in variable numbers and partially embedded in the tissue in mild lesions. They were also frequently found in the crypt openings, mixed with clustered strands of mucus and fibrin clots. Microvilli were sparse on disrupted epithelial cells, while those on adjacent attached epithlial cells appeared unchanged(Fig 15). In severe lesions, there was a diffuse distribution of the spirochetes in large numbers, mixed with disrupted epithelial cells, mucus clusters, fibrin clots, erythrocytes and other bacteria.

Whole culture exposed loops collected 24 and 48 hours p.i. : Lesions observed by TEM and SEM were similar to those in loops inoculated with washed *Serpulina* cells, including the variation in severity and distribution of lesions, the location and number of spirochetes and ultrastructural changes in epithelial cells.

Discussion

In the present study, tissues from loops exposed to filtrates of TSB cultures of *S hyodysenteriae* had ultrastuctural features similar to those from negative controls, having no alteration recognizable by TEM or SEM. Our observations support findings by previous workers that

cytotoxins in culture filtrates had no visible effect on the mucosa of ligated colonic segments in pigs.[27] Although a single bolus of a culture filtrate cannot be expected to mimic continual local production of a toxin by organisms at sites of invasion and replication, other features of the developing lesions futher lessen the likelihood of a role for a toxin in the pathogenesis.

Scanning EM revealed that washed *S hyodysenteriae* were able to produce an irregular mucosal surface ridges associated with disrupted epithelial cells at extrusion zones similar to those produced by whole *S hyodysenteriae* cultures. This change was characteristic of an early stage in the lesions of swine dysentery described by other investigator.[23] Although the washed organisms probably would have been able to produce cytotoxins after inoculation, they usually did not invade the mucosal surface other than at areas where cohesion between epithelial cells had been lost. This discretely localized invasion suggests that any cytotoxin which is produced has little or no effect on the intact mucosal surface and probably is not important in the initial invasion. This indication weakens the hypothesis that such a toxin may reach a pathogenic concentration only near the mucosal surface which experiences the greatest overgrowth of bacteria including *S hyodysenteriae*.[2,6,27]

As previously reported in early stages of the lesions,[7,8] spirochtes were frequently observed at extrusion zones of the superficial mucosa between the crypt openings. They were also present in small numbers in the lamina propria and in epithelial cells of the crypts. The locations of the spirochtes in this study were partially or entirely in correspondence with those of others by TEM[2,3,5,7,22] and SEM.[8,23]

From observations in the present study, it is suggested that spirochetes, either whole cultures or washed cells, primarly invade the areas where cohesion between epithelial cells has been lost as well as necrotic cells on the mucosal surface. Once the surface epithlium has been breached, the organisms appear to invade largely by physical motion into adjacent epithelial cells by invagination of the cytoplasmic membranes and into interstitial spaces along lines of least resistance. Although not demonstrated in this study, and seemingly unlikely, locally secreted cytotoxins after invasion might play a role in altering cell membranes and interstitial tissues to enhance the invasiveness of the spirochetes.

In conclusion, the absence of significant changes by the filtrate allows us to suggest that the filtrate may play a minor role in the pathogenesis of swine dysentery at least at the early stage of the disease.

Summary

The cytotoxic effects of *S hyodysenteriae* strain B 204 on the mucosal surface were studied in surgically prepared ligated colonic loops in two male convenitonal mixed-breed pigs. In each one of four loops was inoculated with either sterile trypticase soy broth(TSB) of *Serpulina*, filtrate of *Serpulina* TSB culture, washed *Serpulina* cells or whole culture of *Serpulina*. Mucosal tissues were examined by transmission and scanning electron microscopy 24 and 48 hours after inoculation(p.i.). The filtrate did not induce any significant effect on the mucosal surface. The washed cells produced early lesions similar to those caused by the whole culture. These observations suggest that cytotoxins of the culture do not play a significant role in invasion of the epithelium in this experimental infection. The possible role of toxins associated with the organism at the site of interaction with the epithelial cells was not elucidated.

Legends for figures

Fig 1. Transmission electron micrograph(TEM) of the mucosal epithelium of a colonic loop from a pig 48 hours p.i. with sterile TSB. Epithelial cells(E) generally have minimal changes and mild intercellular and intracellular edma. Notice the effete cell(Ef) undergoing extrusion and several leukocytes(L) infiltrating in the epithelium. Lu ; colonic lumen. Bar=6um.

Fig 2. Scanning electron micrograph(SEM) of the mucosal surface of a colonic loop from a pig 48 hours p.i. with sterile TSB. Microvilli appear generally unchanged on the mucosal surface. Notice an effete cell(Ef) and evacuated goblet cells(G). Bar=2.9um.

Fig 3. TEM of the mucosal epithelium of a colonic loop from a pig 48 hours p.i. with *Serpulina* culture filtrate. The epithelium is necrotic and degenerate at an extrusion zone(EZ). Moderate edema is present in the lamina propria(LP). Epithelial cells(E) otherwise have minimal changes in ultrastructure. BV:Blood vessel, G:goblet cell, Lu:colonic lumen. Bar=6.8um.

Fig 4. TEM of the crypt of a colonic loop from a pig 48 hours p.i. with *Serpulina* culture filtrate. No change in ultrastructure is seen. There is moderate edema in the lamina propria(LP). Most of goblet cells(G) appear unchanged in the crypts. CL; crypt lumen. Bar=5.8um.

Fig 5. SEM of the mucosal surface of a colonic loop from a pig 48 hours p.i. with *Serpulina* culture filtrate. Most of microvilli appear unchanged on the mucosal surface. Bar=2.9um.

Fig 6. TEM of the superficial mucosa of a colonic loop from a pig 24 hours p.i. with washed *Serpulina* culture. An extrusion zone(EZ) appears separated and has a few leukocytes(L). Several spirochetes(arrow heads) are found in intercellular gaps and in the cytoplasm of epithelial cells(E). Epithelial cells have minimal changes in ultrastructure. Bar=4.4um.

Fig 7. TEM of the superficial mucosa of a colonic loop from a pig 24 hours p.i. with washed *Serpulina* cultures. Streams of spirochetes(arrow heads) are present in large numbers in the epithelium(Ep) and the lamina propria(LP). There is minimal intracellular and intercellular edema. Some epithelial cells have severe changes in ultrastructure while others have mild changes. Ef:effete cell, Lu:colonic lumen. Bar=6.7um.

Fig 8. TEM of a crypt near the mucosal suface in a colonic loop from a pig 24 hours p.i. with washed *Serpulina* culture. Epithelial cells(E) of the crypt have no recognizable changes in ultrastructure, although large numbers of spirochetes(arrow heads) are present in the crypt lumen(CL). Most of goblet cells(G) appears unchanged. A few spirochetes are observed in mucus of a goblet cell and in the lamina propria(LP). Bar=6.6um.

Fig 9. SEM of the mucosal surface of a colonic loop from a pig 24 hours p.i. with washed *Serpulina* culture. There are irregular cleavages usually at extrusion zones, mucus strands(MU) in the crypt openings(CO) and fibrin clots distributed diffusely over the mucosal surface. Bar=58.8um.

Fig 10. A higher maginifications of the area in Fig 9. Large numbers of spirochetes(arrow heads) appear on the fibirin cloted surface of a widened cleavage(Cl) at an extrusion zone with a few other bacteria. Microvilli generally appear unchanged. Bar=2.9um.

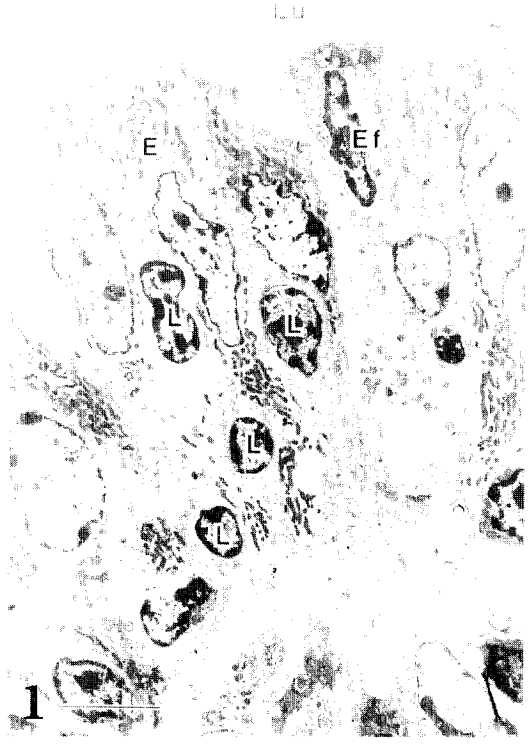
Fig 11. TEM of the epithelium of a colonic loop from a pig 48 hours p.i. with whole *Serpulina* culture. The epithelium(Ep) has prominent intercellular and intracellular edema, sparseness and shortening of microvilli, vacuolation of endoplasmic reticulum, enlargement of mitochondria. Notice spirochetes(arrow heads) in intercellular gaps and the cytoplasms of epithelial cells, in the lamina propria and in the cytoplasm of neutrophils(N) in the colonic lumen(LU). Bar=4um.

Fig 12. TEM of *S hyodysenteria* . A longitudinal section of the organism shows S-shape. Periplasmic flagella(pf) are situated between the outer membrane(OM) and cytoplasmic membrane(CM) in the cross and logitudinal sections of spirochetes. Bar=530nm.

Fig 13. TEM of a crypt in a colonic loop from a pig 48 hours p.i. with washed *Serpulina* culture. Spirochetes(arrow heads) are present in small numbers in a widened lumen of crypt(CL) and in the evacuated goblet cells(G) and in large numbers in the adjacent edematous lamina propria(LP). Epithelial cells(E) have mild to moderate changes in ultrastructure. Bar=7.6um.

Fig 14. SEM of the mucosal surface in a colonic loop from a pig 48 hours p.i. with washed *Serpulina* culture. A mixture of lesions from an initial stage(I) to an advanced stage(A) is present. The initial stage change is an uneven polygonal ridge overlying the extrusion zones. The advanced stage has raised plaques composed of disrupted epithelial cells, mucus strands, fibrin clots and spirochetes. Bar=58.8um.

Fig 15. A higher magnification micrograph of the area of Fig 15. Spirochetes(arrow heads) are frequently found around disrupted epithelial cells(E) mixed with mucus and fibrin clots, which form ridges over extrusion zones. Microvilli are almost completely lost on disrupted epithelial cells, while they appear unchanged on the remaining epithelial cells. CO:crypt opening. Bar=7.8um.



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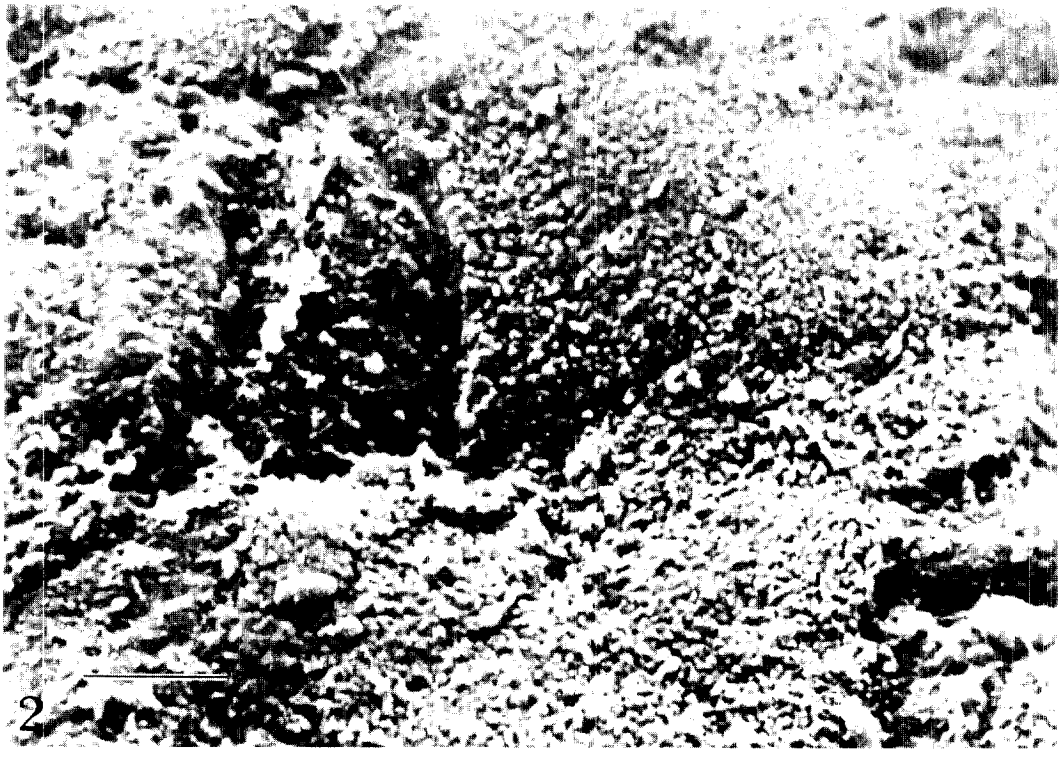

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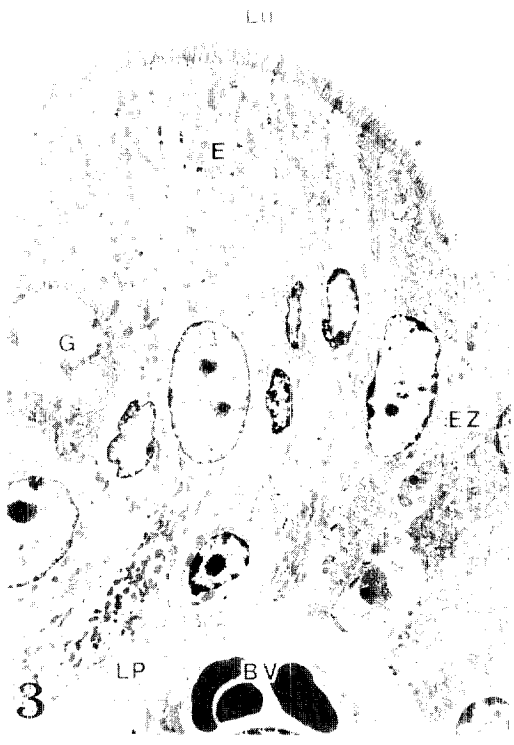

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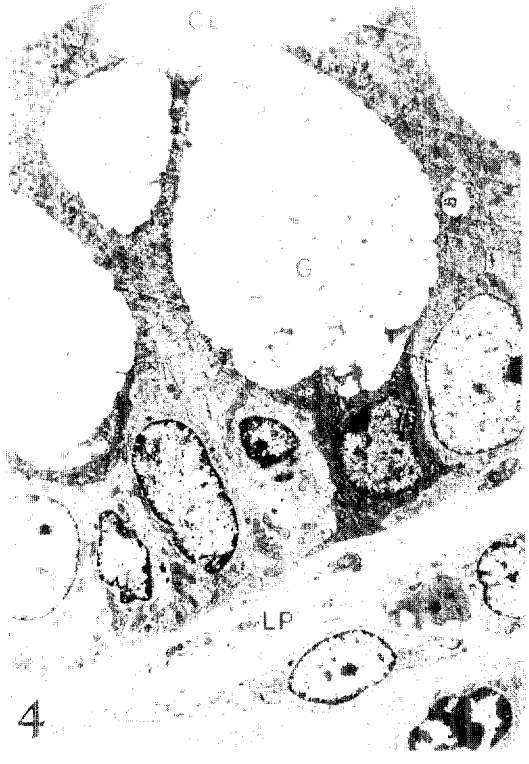

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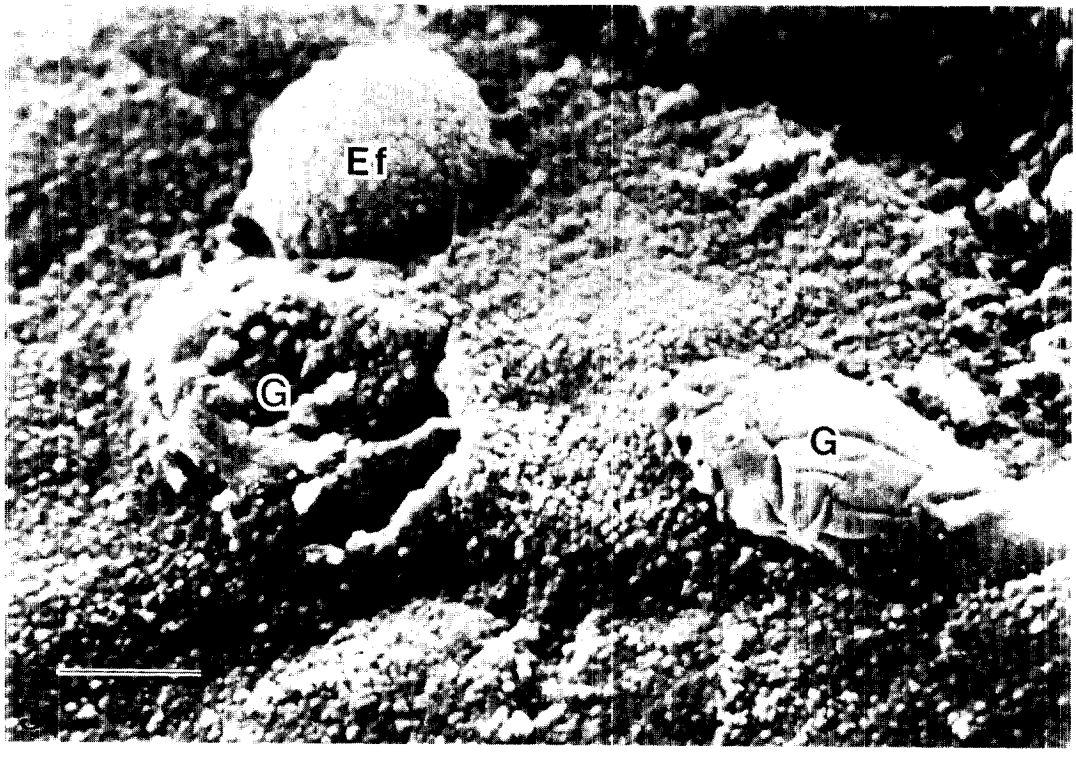

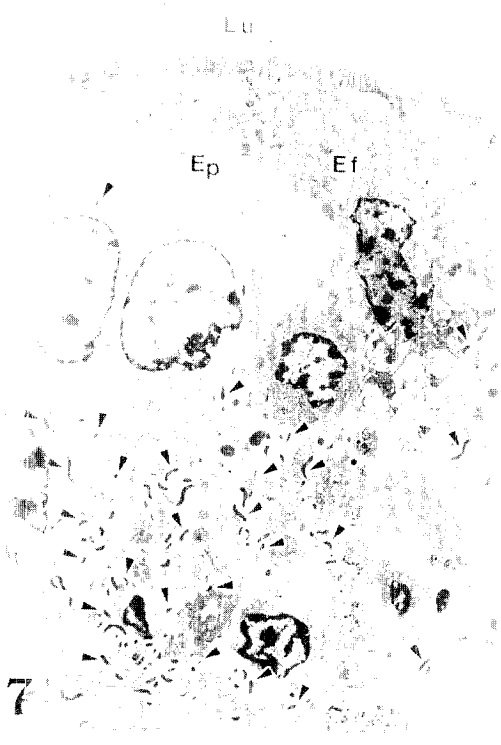

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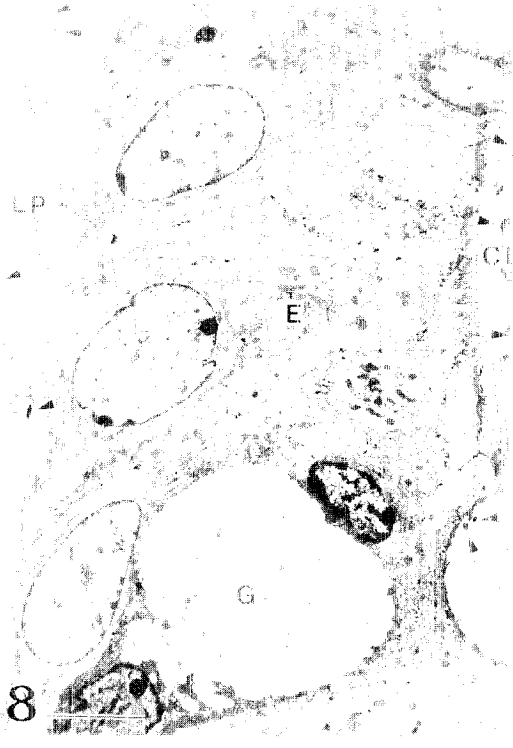

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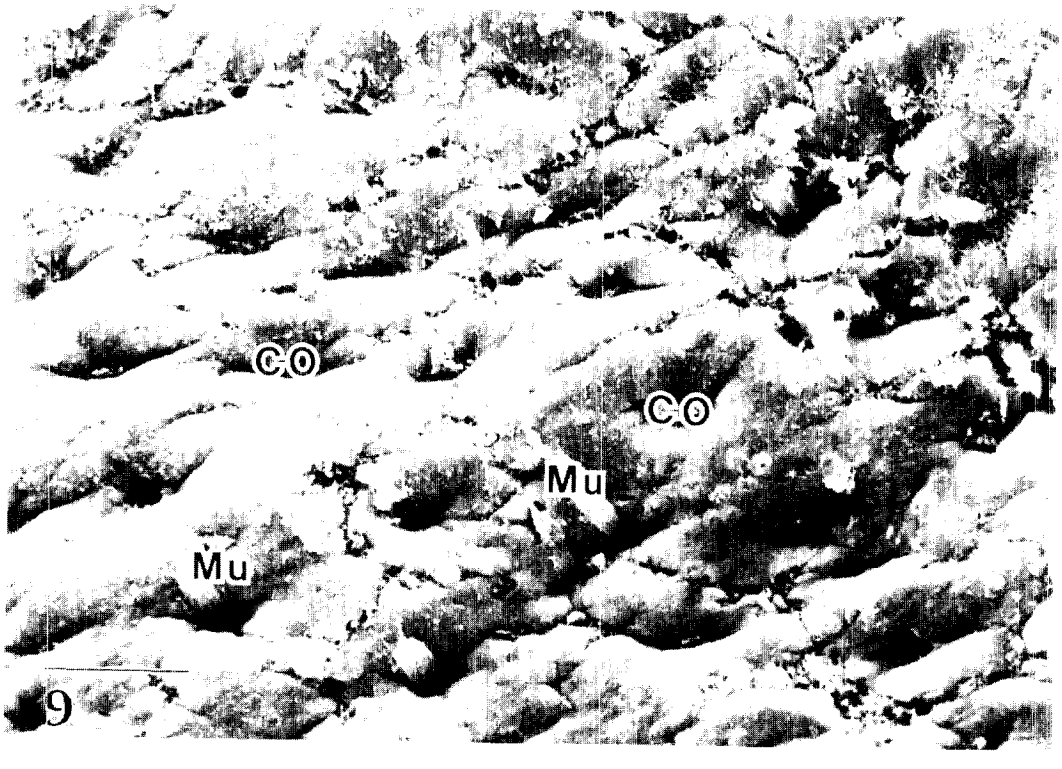

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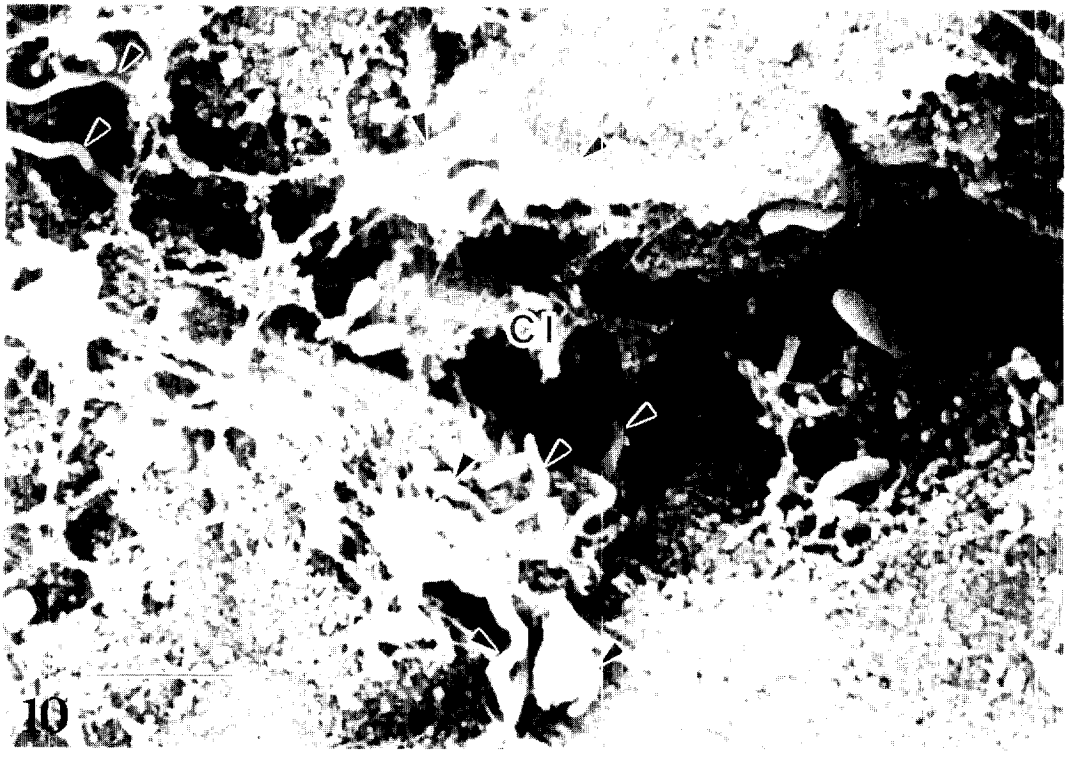

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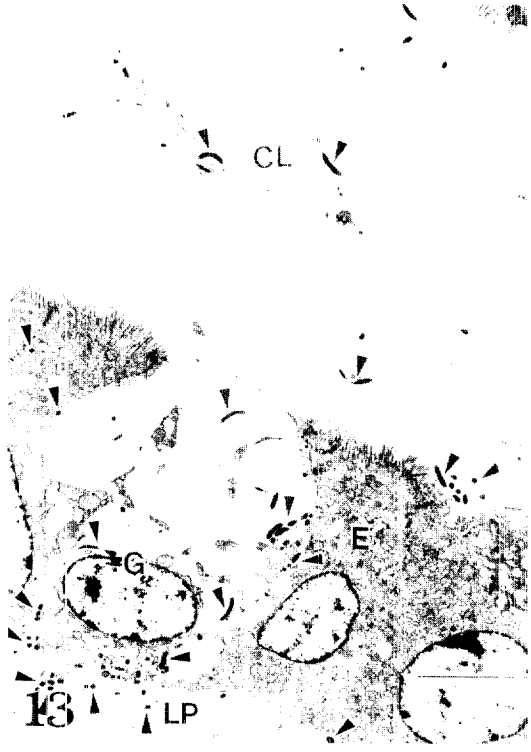

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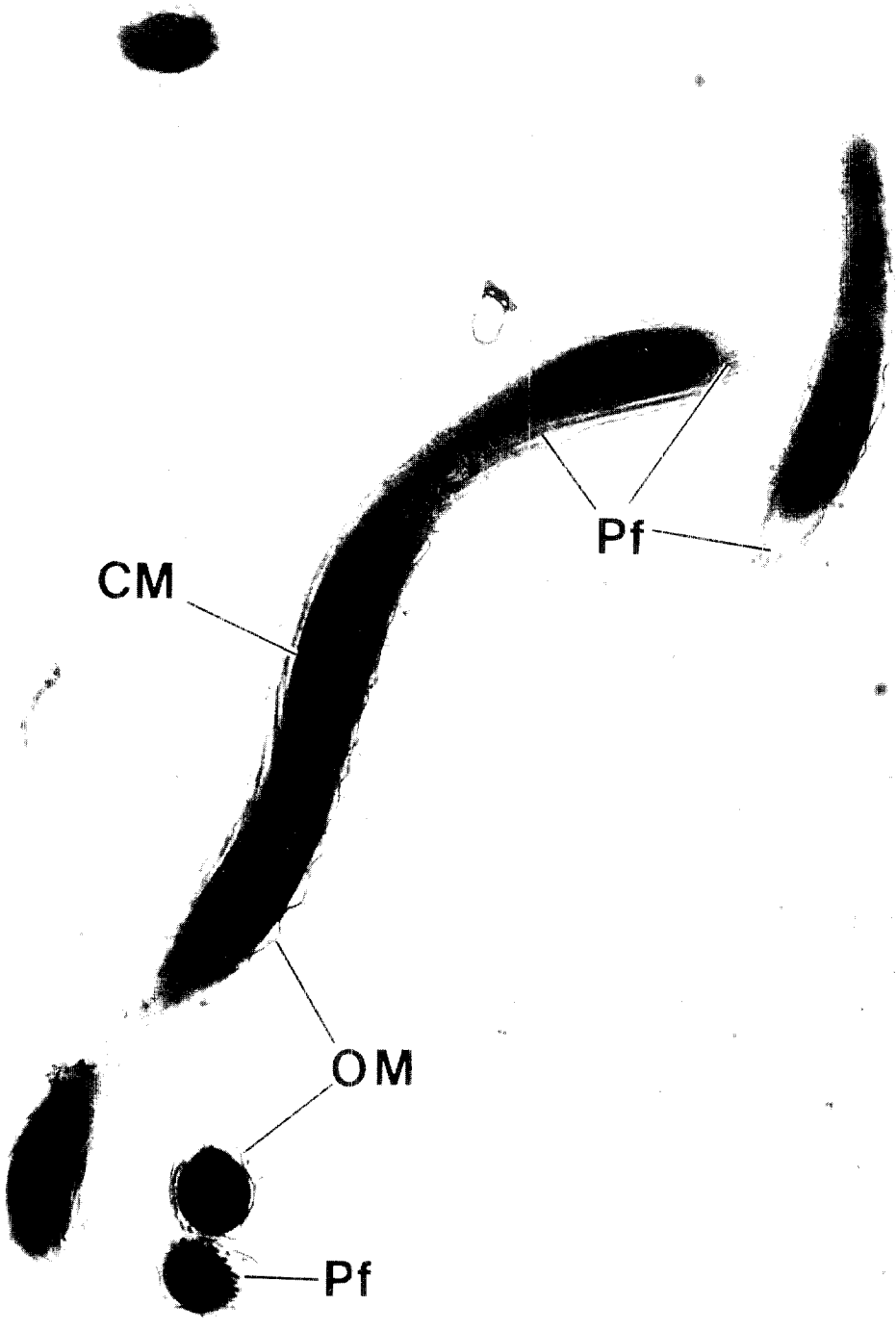

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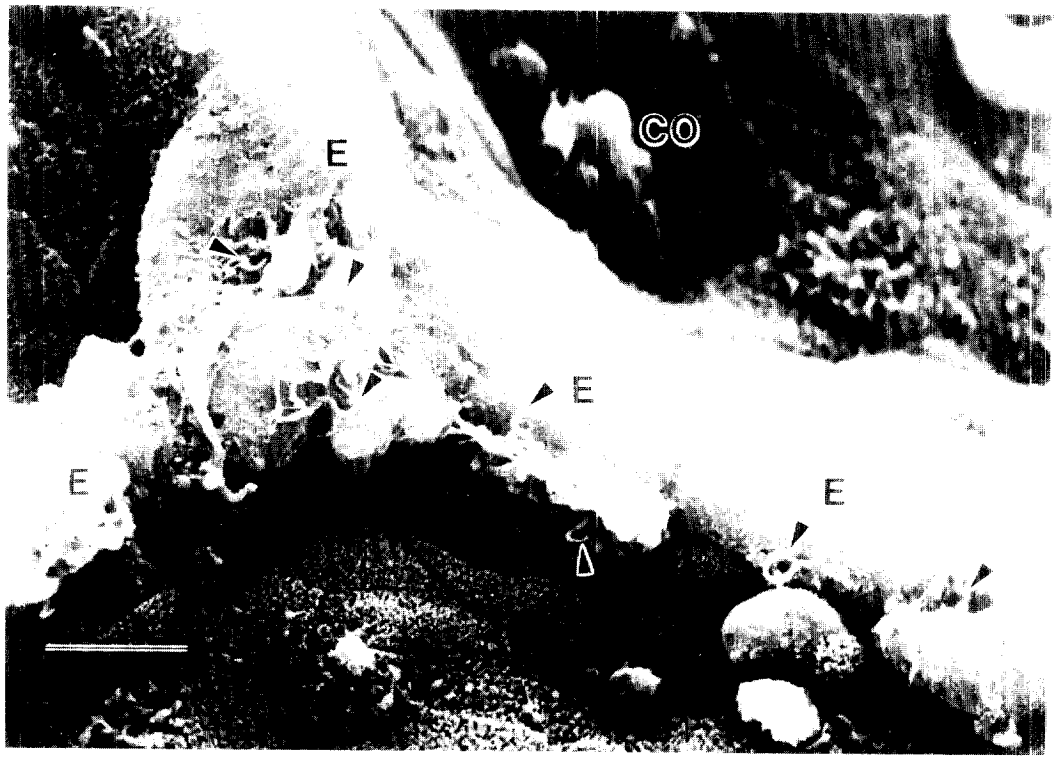
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