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Tissue Culture to Assess Bacterial Enteropathogenicity

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1. Introduction

It is quite clear nowadays that the pathogenesis of infectious disease, although determined by pathogenic features of their causative agents, cannot be fully comprehended without structural (morphologic) analysis of immediate interaction between these agents and cells, tissues, and defense systems of the host. In diseases caused by agents adapted to certain cellular targets, the pathogenesis is, in addition, influenced by histophysiological features of these targets which are utilized or distorted or both by the pathogen.

Acute diarrheal diseases caused by Gram-negative bacteria: *Vibrio cholerae*, diarrheagenic *Escherichia coli*, *Shigella*, *Salmonella*, *Aeromonas* and enteropathogenic *Yersinia*, are examples of this type of infection. All of these agents target the same cell type, the enterocyte, and produce potent exotoxins. [1]

The morphologic method either produced an impetus to bacteriologic studies or served as a tool in the evaluation of bacterial pathogenicity, including that of genetically altered microorganisms. In particular, morphologic studies have revealed that enteric bacteria either colonize enterocytes while remaining epicellular (i.e., bound to the cell surface) with or without affecting cellular architecture, or they invade the cell with or without its destruction. On the other hand, biochemical studies have shown that prevalence of the secretory or destructive inflammatory disturbances in the gut is, for the most part, determined by bacterial exotoxins. Although exotoxins affect enterocytes by a variety of mechanisms, they can be combined into two groups that are referred to as cytotoxic and cytotonic or respectively, as enterotoxins and cytotoxins. It has also been established that each step in host-pathogen interaction is governed by multiple determinants encoded in bacterial plasmid and chromosome genes. [1,2,3]

The genus *Aeromonas* comprises gram-negative bacteria that can be isolated from water and a diversity of foods. Some strains are important diarrhea producers, particularly in children under five years and in older adults [4,5].

The clinical manifestations of diarrhea vary from autolimited symptoms to severe cases with presence of mucus and blood in faeces, suggesting that, as in *Escherichia coli* pathogenic types, *Aeromonas* virulence is multifactorial [6,7]. In recent years *Aeromonas* spp. have emerged as an important human pathogen, with increasing incidence among travelers (causative agent of traveler's diarrhea), due to their presence in food as well as in treated water for human consumption [8].

The aim of this chapter, is to show that with the development of an *in vitro* animal model, to explore the mechanisms related to the colonization of the digestive tract, as well as the determination of the mechanisms of interaction with the host epithelium, provide a valuable tool in the study of bacterial enteropathogenicity that the genus *Aeromonas*

2. Interaction host/bacterial pathogen

The *Aeromonas* enteropathogenic capability is usually underrated [7,9,10]. Information about decisive diarrhea virulence factors is limited, due to lack of appropriate animal models for the study of *Aeromonas* genus. [7,10,11]). With the models developed up to now, it has obtained scarce information about the interaction between the host and *Aeromonas* spp. [10,11,12,13,14].

In this regard, the development of an *in vitro* model to explore the mechanisms related to the colonization of the digestive tract by *Aeromonas* spp. as well as the determination of the mechanisms of interaction with the host epithelium, provides a valuable tool in the study of *Aeromonas* pathogenicity. On the other hand, the critical steps in the pathogenesis of virulent strains involve the adhesion and colonization of the intestinal mucosa by *Aeromonas*.

The present focuses in the analysis of the mechanisms causing the pathogenicity of *Aeromonas* strains in co-cultures of isolated bacteria with intestinal cells. Ultrastructural aspects of mouse small intestinal tissue cultures infected with *Aeromonas* strains are described using a novel experimental procedure which allows to culture *Aeromonas* inside a previously sterilized short cylinder of mouse's small intestine.

3. Materials and methods

3.1. Bacterial strains and growth conditions

Two strains of *Aeromonas caviae* were used, one (A) isolated from an asymptomatic patient and the other one (D) from a patient with diarrhea in which the isolated *Aeromonas* was the only enteropathogen. The strains were inoculated in trypticase soya agar (HIMEDIA laboratories Ltd., Bombay, India 400 086) at a concentration of 1.5×10^8 CFU/ml and incubated for 24h. After incubation the media were gently filtered and the corresponding *Aeromonas* strain resuspended in 1ml Basal Medium Eagle (BME).

3.2. Tissue culture procedure

Segments of the small intestine were removed from the abdominal cavity of young adult NMRI mice to prepare small intestine cylinders of ~3cm in length that were sterilized with a 10% chlorine solution. One end of each intestinal cylinder was tied with sterile surgical thread and the cavity was filled with 1.5×10^8 CFU/ml of a given one of the two *Aeromonas* strains suspended in BME. After filling, the cylinders were tied close with surgical thread. The whole preparations were co-cultured in 50ml of tissue culture media containing 90% BME with Earle's salts and L-glutamine, 10% horse serum, 5000IU/ml penicillin and 5mg/ml streptomycin, 360mOsm, pH 7.2, at 37°C, under constant rotation at 70rpm, for 24h and 48h. During the incubation period, the media were oxygenated every 4h during daytime. Control small intestine cylinders were cultured for 48h under the same conditions but without *Aeromonas* inside. Once the programmed culture time was accomplished, the intestinal cylinders were removed from the flask and immediately immersed in a fixing solution containing 3% glutaraldehyde and 3% formaldehyde in 0.1M cacodylate buffer, pH 6.3 [15] during 6h at 4°C.

The intestinal cylinders were cut in small sections of approximately 3mm³, washed in 0.1M cacodylate buffer, pH 7.2, and postfixed for 24h in 1% osmium tetroxide prepared in the same buffer. The tissue was then dehydrated in ascending concentration ethanol solutions, followed by propylene oxide and finally embedded in Epon 812. Sections of 1µm were stained with 1% toluidin blue and observed under a high resolution light microscope. Sections of 90 nm were contrasted with uranyl acetate [16] and lead citrate [17] using a modification of this classic method [18] and were analyzed using a Hitachi-7000 transmission electron microscope. [19,20].

4. Effects of *Aeromonas caviae* co-cultured in mouse small intestine

The experimental desing herein presented offers appropriate conditions for the physiopathologic study of the interrelationship between the intestinal wall and bacteria under *in vitro* conditions very similar to *in situ* conditions.

On the other hand, this experimental model employed using co-cultures of mouse intestinal mucosa with *Aeromonas caviae* revealed important information on the pathogenicity of this species in gastrointestinal infections. The strain A, isolated from asymptomatic patient, produce minor to mild alterations of the intestinal wall, while more pronounced alterations were found at both periods of incubations when strain D, from the patient with diarrhea, was used to prepare the co-cultures.

The damage produced was demonstrated by the alterations of the integrity of intestinal microvilli, disruption of the epithelium and presence of mucosal microulcerations, which were brought about by the fact that this strain is an high cytotoxin producer and may possess other virulence factors [4,21]

These strains belong to the *A. caviae* species and were considered of lesser virulence than other strains of this species [22].

Light microscopical observations revealed a varying degree of histological alterations of the intestinal wall, according to the severity of the damage. The major tissue damage is shown in Figure 1, where a large bacterial cluster can be seen occupying the crypts between intestinal folds. In most cases, the intestinal wall showed a high degree of generalized cellular atrophy with tissue lysis when the diarrhea producing strain (D) was used. These images were seen at 24h as well as at the 48h samples of incubation.

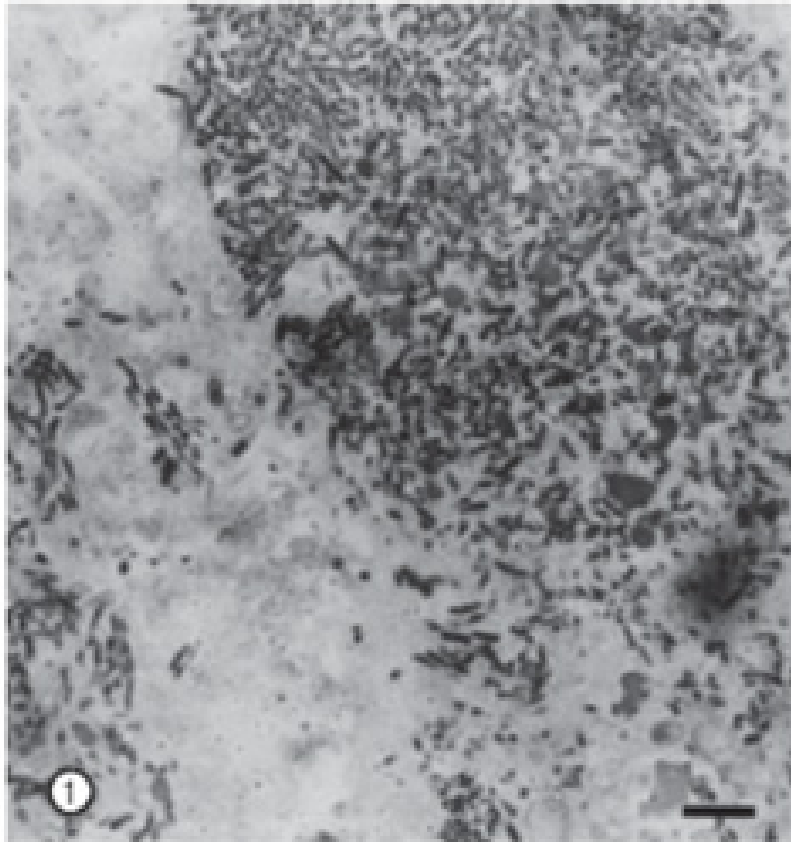


Figure 1. Large clusters of *Aeromonas* occupied the intestinal crypts when the co-cultures were prepared using the strain D, which comes from a symptomatic patient. Note: the high degree of tissular lysis. Bar: 9.4 μ m.

In cases of moderate damage, the basic cytoarchitecture of the intestinal mucosa was preserved and it was possible to recognize enterocytes, mucous cells and germinative or mother cells. The villi as well as the majority of the crypts were seen, although some of them were shorter and thicker (Figures 2 and 3).

These mild damages were caused when the strain A from the asymptomatic species was employed for the co-culture. Microvilli were seen forming part of the apical surface of the enterocyte as well as cytoplasmic protrusion that come from the apical portion of the enterocytes (Figure 4).

Some segments of the intestinal cylinder co-cultured with *Aeromonas* strains showing a higher degree of alterations contain clusters of spheroidal cells associated to bacterial elements (Figures 3 and 6).



Figure 2. A case of mild damage of the small intestinal epithelium produced when the asymptomatic strain (A) was co-cultured during 48h. Well-aligned enterocytes can be seen, with a few of them exhibiting a dark content indicating cellular atrophy. The arrow points at an exocytic fragment. Bar 24.0µm

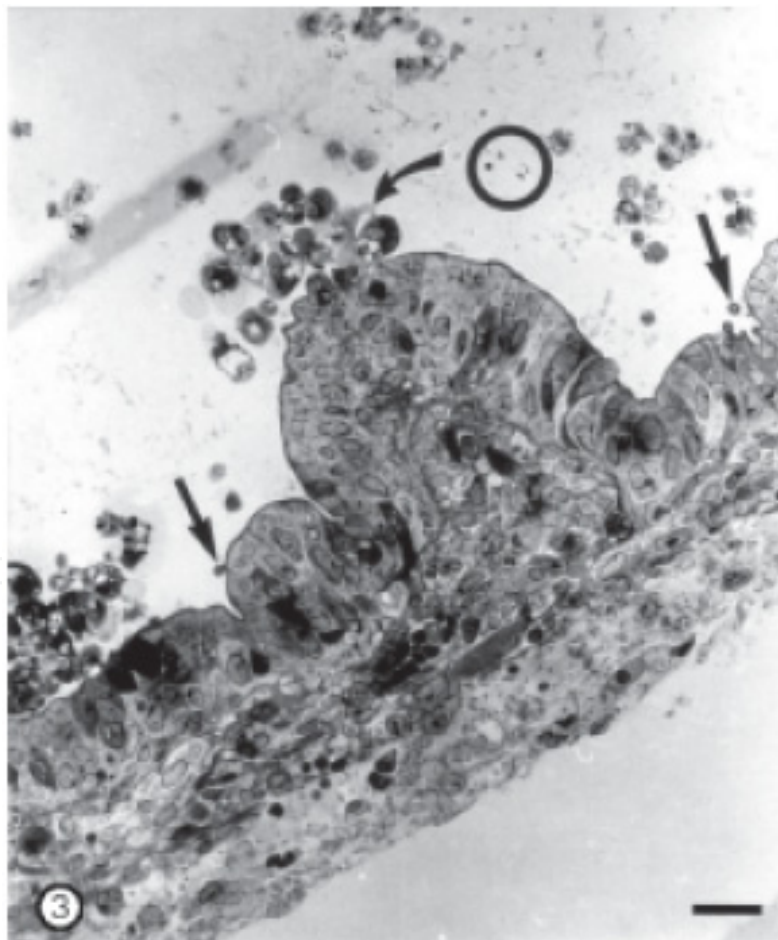


Figure 3. In addition to the exocytic detachment fragments (straight arrows), clusters of extraintestinal cells close to the epithelium are also seen (curved arrow). Some *Aeromonas* (circle) are present. Note that bacterial cells are not in contact with the enterocytes. Strain A was used co-cultured. Bar: 24.0µm.

Transmission electron microscopy revealed minor alterations of the intestinal mucosa when the strain from asymptomatic patients (A) was co-cultured. Most enterocytes were seen with typical ultrastructural characteristics, however, they showed numerous apical protrusion detachments (Figures 2 and 4). In more damaged regions, there was a progressive atrophy of the epithelial cells showing loss of microvilli and large cellular vacuoles loaded with cellular detritus (Figure 5).

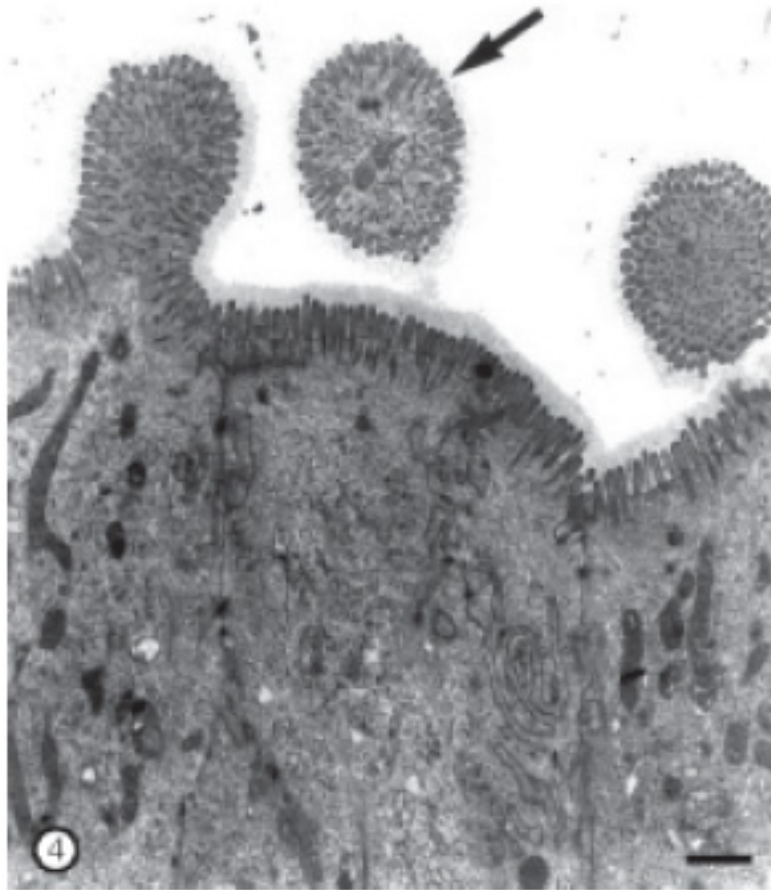


Figure 4. Enterocytes show almost normal ultrastructural characteristics at 24h of co-culture when the asymptomatic strain (A) was employed. The main pathological feature observed in these cultures is the presence of exocytic detachments (arrow). The enterocytic epithelial cell, cytoplasm and microvilli also have normal ultrastructural features. Bar: 1.0 μ m.

In the intestinal regions having intermediate epithelial alterations, globular cells identified as blood and lymphatic cells were observed in the gut lumen associated to *Aeromonas* and cellular debris (Figures 3 and 6). Eosinophils were seen with multilobular nuclei and their characteristic lysosomes with crystal-like structures (Figures 7 and 8). In Figure 8 phagocytated *Aeromonas* within the eosinophilic cytoplasm are clearly visible.

Cells identified as lymphocytes (Figure 7) and others as plasma cells (Figure 9) were also observed as part of the clusters of globular cells found in the small intestine cylinder lumen after two days of culture.

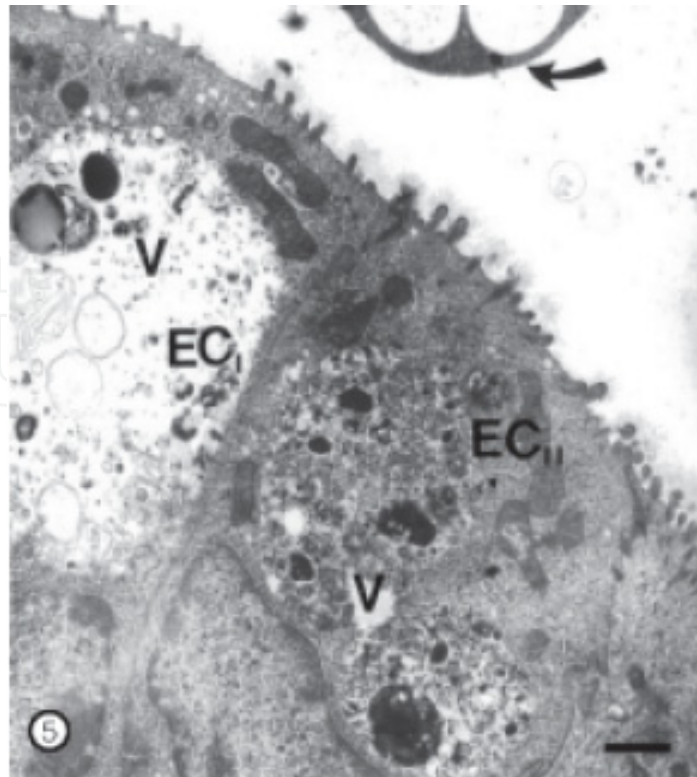


Figure 5. After 48h, the co-cultures prepared with strain A, the enterocytes showed large vacuoles (V) loaded with cytoplasmic debris ('EC_I,' EC_{II}') as seen in this photograph. Note the significant reduction of the microvilli. Bar: 1.0µm.

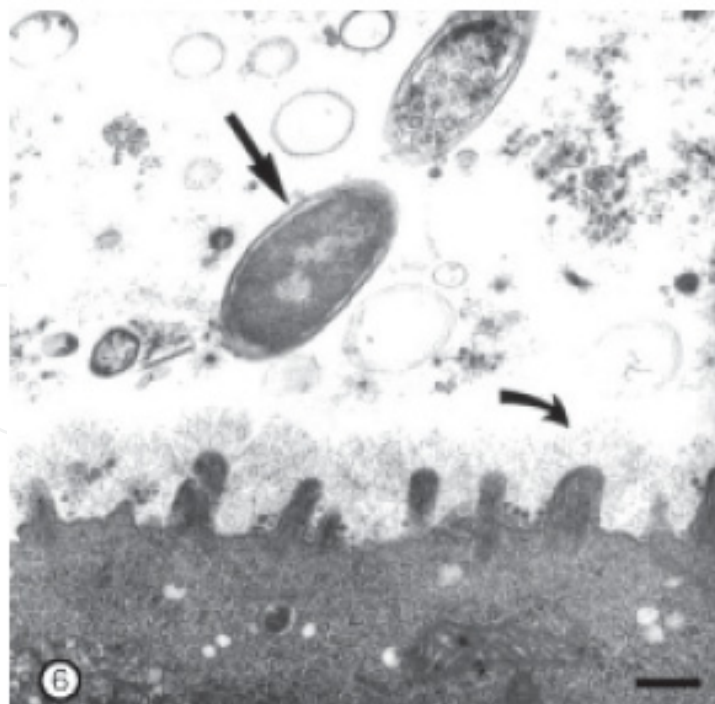


Figure 6. In all analyzed cases, the *Aeromonas* (straight arrow) are not seen attached to the surface of enterocytes that, while showing a significant reduction of microvilli, have preserved their glycocalyx cover (curved arrow). Bar: 0.4µm.

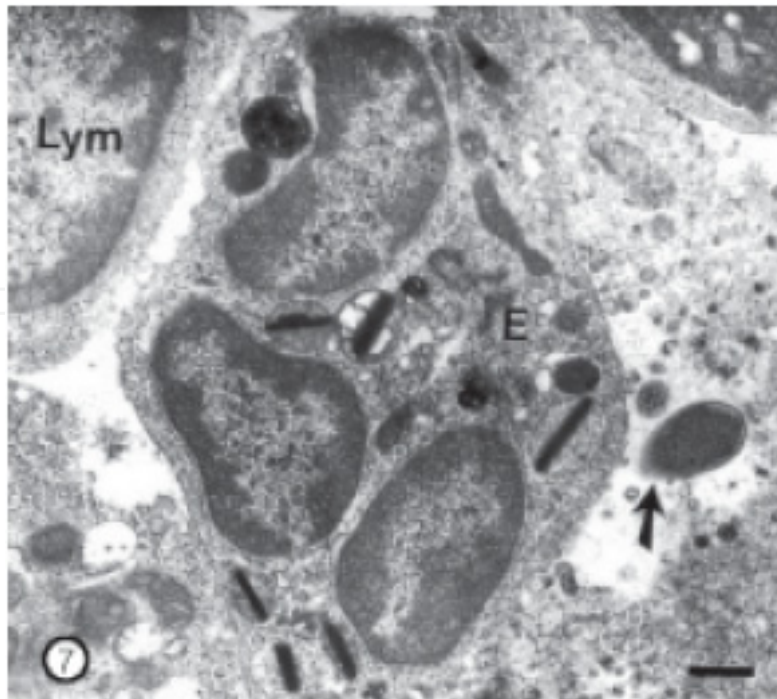


Figure 7. Among the globular cells located in the intestinal lumen of the co-cultures prepared using strain A from the asymptomatic patient, abundant eosinophilic granulocytes (B) were found. Numerous cytoplasmic lysosomes containing crystal-like inclusions characterize these cells and nuclear lobules are also visible. Profiles of *Aeromonas* (arrow) are detected outside the eosinophilic cells. Lym: lymphocyte. Bar: 0.5 μ m.



Figure 8. Within the cytoplasm of eosinophils, *Aeromonas* ('A') could be observed in association with the lysosomes, indicating the phagocytic capability of these cells. N. nucleus. Bar: 0.7 μ m.

In Figure 10 an image is shown of a cylinder of small intestinal segment incubated for 48h using the same culture conditions but without *Aeromonas* in its lumen, with the purpose to compare the control cylinders to the ones co-cultured with the bacteriae. No atrophic cells are seen and there are no visible epithelial protrusions, extraepithelial cells nor microulcerations of the intestinal wall. The villi and crypts show minor changes due to the culture conditions.

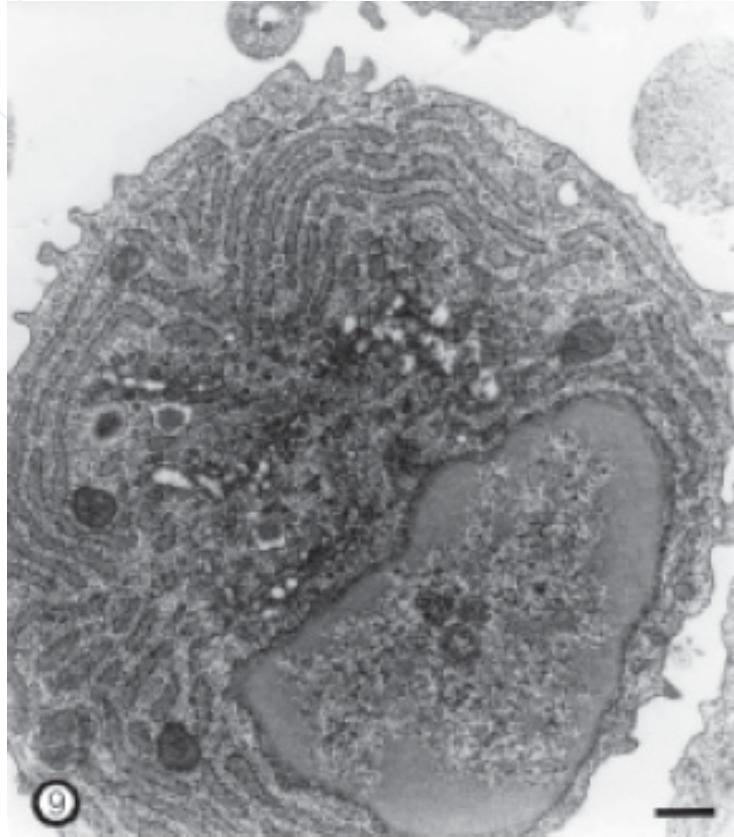


Figure 9. Plasma cells were other lymphatic elements frequently observed in the lumen of clustered cells. In this particular case, note the proliferation of the rough endoplasmic reticulum. Bar: 0.4 μm .

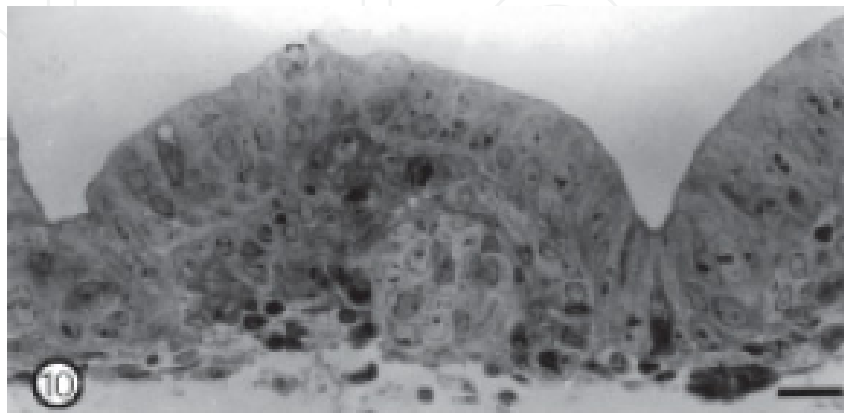


Figure 10. A control small intestine cylinder incubated without *Aeromonas* during 48h. Note the well preserved villi and cryptae as well as the absence of atrophic enterocytes and epithelial protrusions. Extraepithelial cells are not seen. Bar: 24.0 μm .

The cylinders of small intestinal cultures incubated for 24h and 48h revealed good preservation. The glycocalyx was clearly observed covering the microvilli (Figure 11). No vesicular chains were observed in or between the microvilli, nor was any type of bacteria observed in the intestinal lumen.

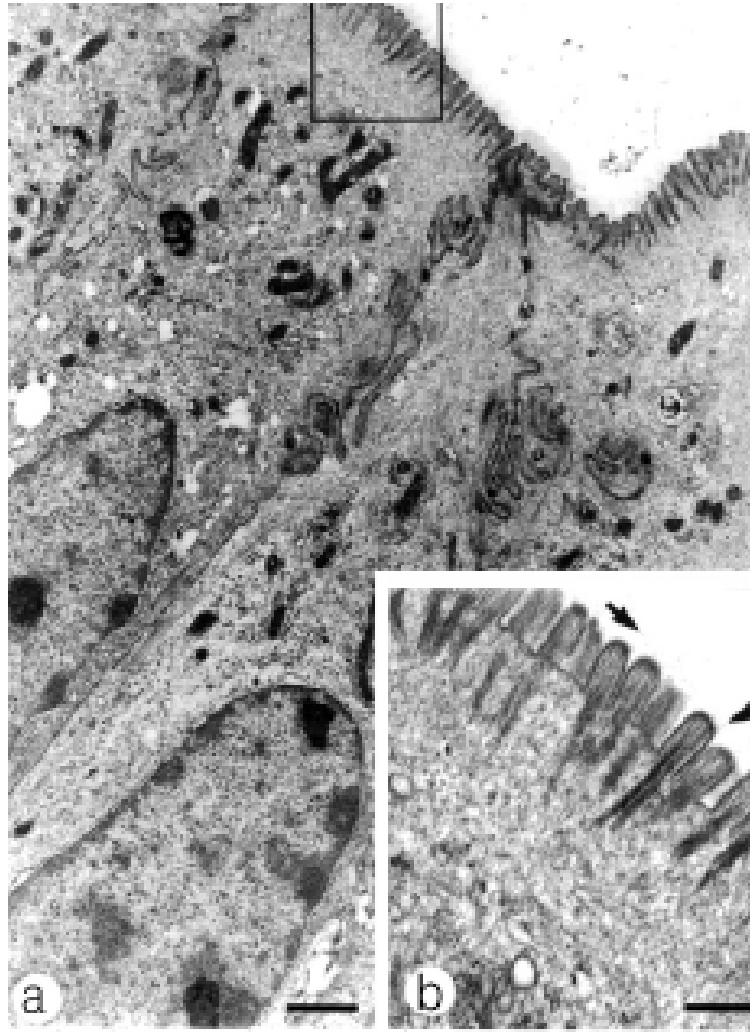


Figure 11. a: Segments of enterocytes from a control sample with normal ultrastructural characteristics after 48h of culture. Bar: 1.5 μ m. b: detail of the normal ultrastructural features of an intestinal epithelial cell. Note the absence of vesicles between microvilli. Arrows, glycocalyx. Bar: 0.5 μ m

After 24h de incubation, the *Aeromonas* spp. strain cultured with the intestinal tissue showed vesicular elements attached to the bacterial outer membrane (Figure 12). These vesicles were seen alone, in pairs, or in groups of five or more vesicular units organized in chains emerging from the external membrane. Adjacent to these vesicles some complex membranous structures were observed (Figure 12).

The small intestine tissue cultivated with *Aeromonas* spp. and incubated for 24h did not present any alteration or modification in its cellular structure. The only outstanding observation was the presence of vesicular chains composed of four to ten vesicular units, which were aligned between microvilli spaces (Figure 13).

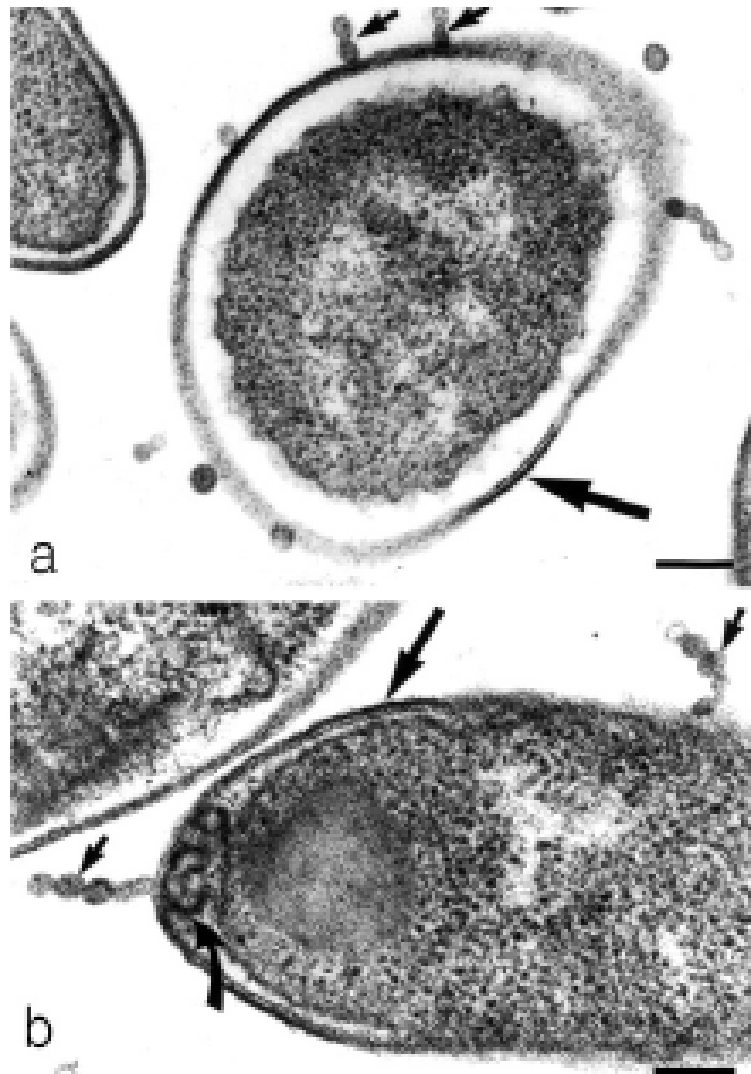


Figure 12. Cross sections of *Aeromonas* spp. cultured for 24h. Short chains of vesicles (in a) and large chains of vesicles (in b) can be seen emerging from the external bacterial membrane. Notice adjacent complex membranous structures (curved arrow) to these chains. Thick arrows: bacterial external membrane; short arrows: chains of vesicles. Bars: 0.17 μm in a, 0.15 μm in b.

When the segments of the small intestine were cultivated with *Aeromonas* spp., and then incubated for 48h, severe damage of the enterocytic epithelial surface was seen, with regions of tissular lysis on the surface of the intestinal microvilli (Figures 14 and 15). Numerous defensive cells such as lymphocytes were also observed (Figure 15), as well as abundant eosinophils showing their typical crystal-like structures inside lysosomes (Figure 16), and phagocytic mononuclear cells (Figure 17). Inside the cytoplasm of both of these cell types, *Aeromonas* spp. could be observed (Figures 16 and 17)

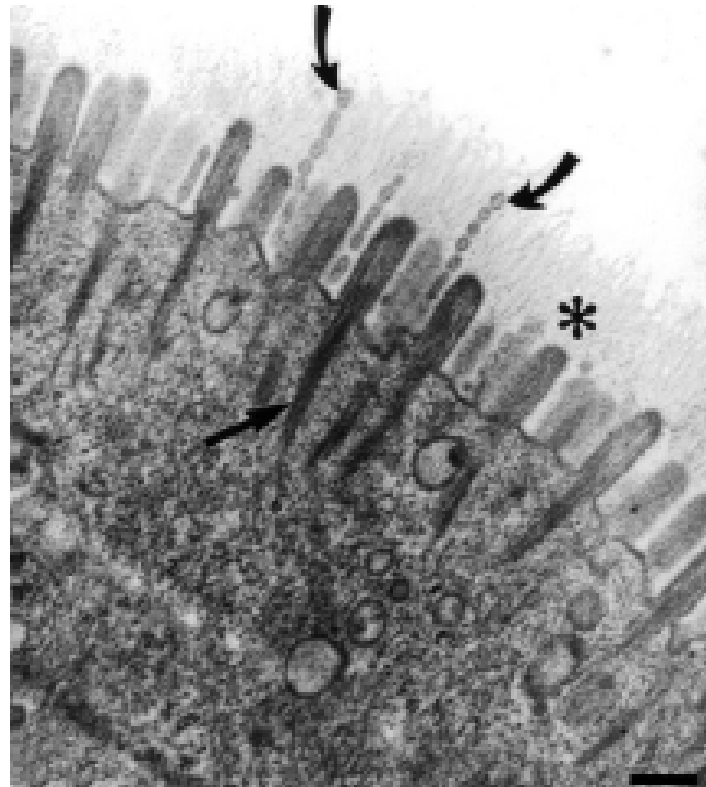


Figure 13. At a higher magnification, it is possible to identify the glycocalyx(asterisk) of the microvilli, and the particular vesicular chains (curved arrows) appearing between microvilli after 24h of culture with an *Aeromonas* spp. strain. Some microvilli fibrillary roots (straight arrow) are clearly seen. Bar: 0.35µm

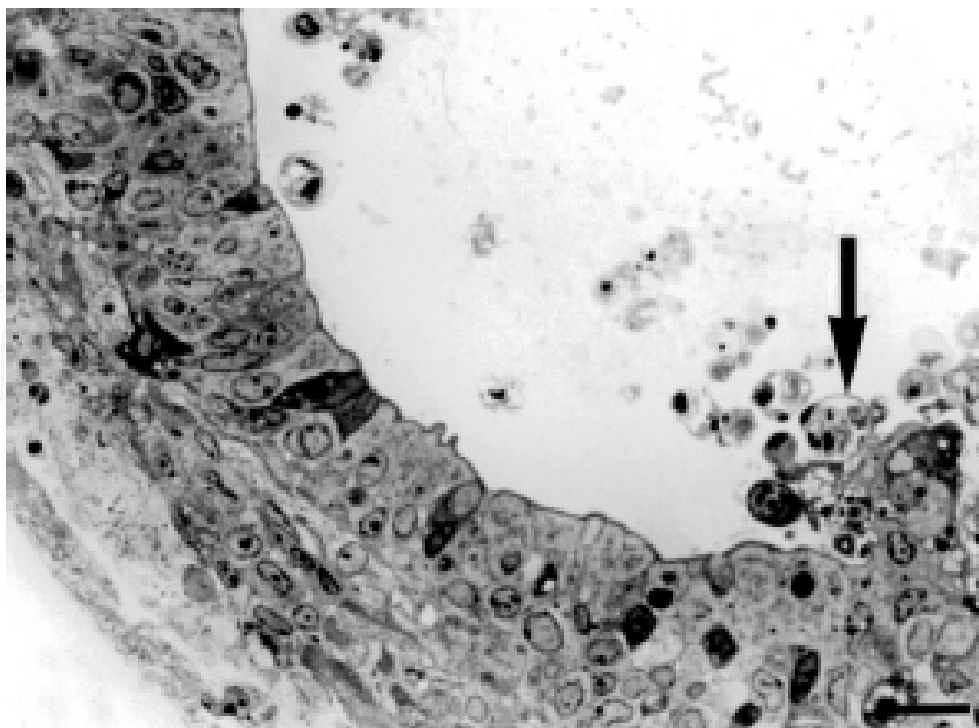
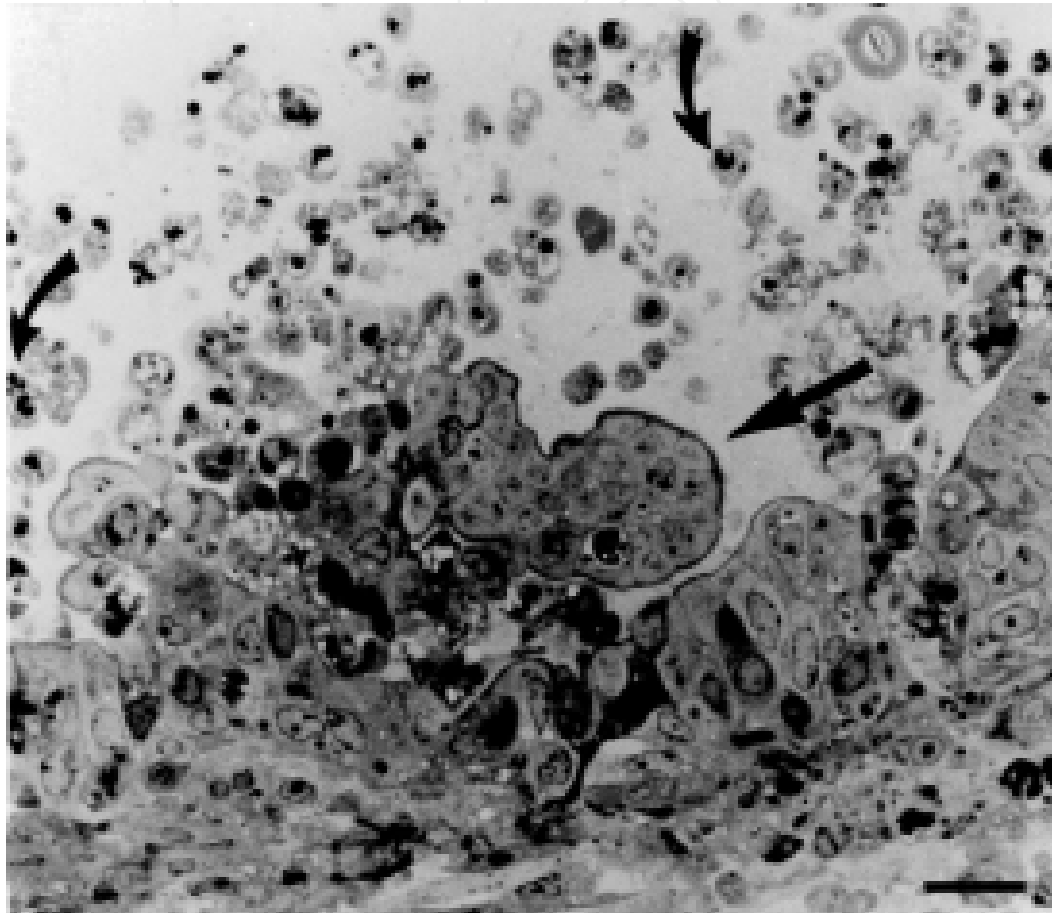


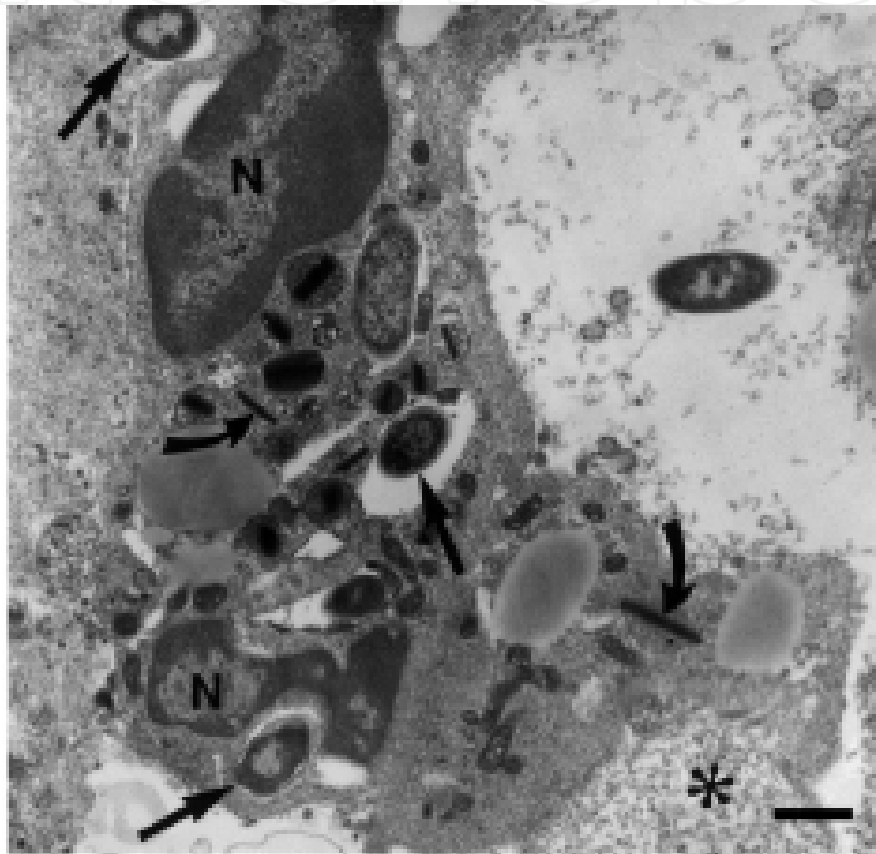
Figure 14. Light microscopic image of a segment of a small intestinal cylinder cultivated with *Aeromonas* spp. for 48h. The enterocytic epithelial surface shows regions with tissular lysis (arrow). Bar: 7.0 µm.



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Figure 15. Notice the lysis (straight arrow) of the surface of the small intestine and the presence of defensive cells or lymphocytes (curved arrows) after 48h culture with *Aeromonas* from the symptomatic patient. Bar: 4.4 μm .

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Figure 16. Eosinophils containing numerous phagocytosed *Aeromonas* spp. (straight arrows) from symptomatic strain, are a common feature of the 48h intestinal *Aeromonas* cultures. These eosinophils show lysosome crystal-like structures (curved arrows). N: nucleus, *: cellular debris. Bar: 0.95 μm .

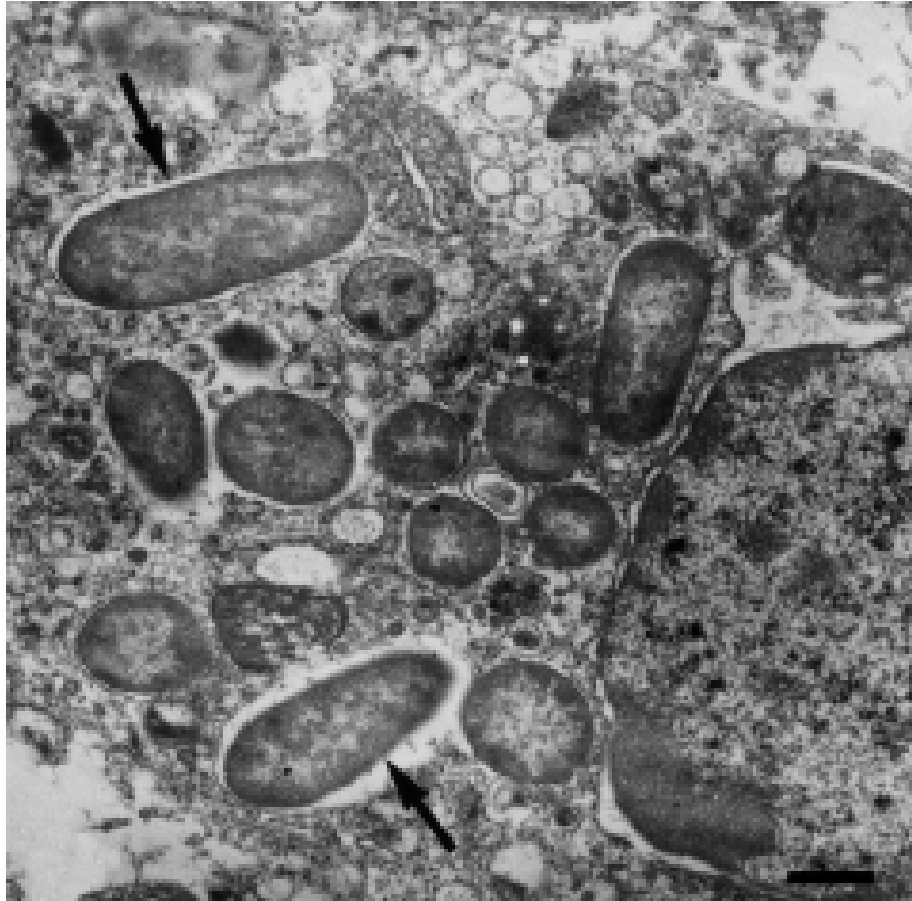


Figure 17. After 48h culture of *Aeromonas* spp. with small intestine tissue it is possible to see, within cellular clusters adjacent to the enterocytes, numerous phagocyte mononuclear cells. Inside the cytoplasm of these cells, phagocytosed *Aeromonas* (arrows) can be identified. Bar:0.65 μm .

The pathogenicity of *Aeromonas* has been confirmed with this procedure and its virulence has been corroborated as strain-specific and not species-specific.

When the intestinal epithelium lesions were observed no bacteriae adhered to the cells were seen, indicating that direct bacteria-epithelial cell contact was nor required for tissue alterations: on the contrary, it seems that *Aeromonas* triggers a chemotactic response which activates migratory actions to the lymphatic cells from the adjacent lymphatic plaques.

The lymphatic submucous plaques and the autonomous defensive structures are able to act in culture conditions even in the absence of the circulatory blood elements, bone marrow cells or other lymphatic organs. Among the defense elements that migrate in answer to the chemotactic stimulus are the eosinophilic granulocytes, which have been reported to participate only in parasitic diseases. In this investigation their capability to defend intestinal cells against bacterial aggressions [23,24] is ultrastructurally documented.

Under the conditions of the experiment, no alteration of the intestinal epithelium was observed in the cultures from the small intestine with *Aeromonas* spp. incubated during 24h,

except for vesicular chains aligned between microvilli spaces (Figure 13), which were not distinguished from those vesicular elements seen attached to the bacterial outer membrane (Figure 12) in the same cultures.

On the other hand, in cultures with 48h incubation, *Aeromonas* initially induced important tissue damage, including necrosis and lysis of the intestinal microvilli (Figures 14 y 15).

The vesicles produced on the surface of the bacterial outer membrane are also constituted by a double membrane unit. The production of these vesicles was observed in all the cultures analyzed. However, the more important vesicular formation was detected in the cultures incubated for 24h, whereas in the samples incubated for 48h, the vesicles were found where the intestinal damage was more severe.

The ultrastructural analysis suggest that *Aeromonas* spp. produce vesicles that form chains and can be seen between the microvilli of the intestinal epithelium. The contact between them could be one the mechanisms that trigger virulence factors produced by *Aeromonas* spp., contained in the vesicles, and that could subsequently initiate mechanisms that attract eosinophils and mononuclear cells into the intestinal lumen.

The vesicles do not appear to be intestinal exocytic vesicles because they are formed on the surface of the bacterial outer membrane. Moreover, no clathrin-like outer skeleton is seen, which suggest that this vesicular system is a part of the pathogenic mechanism of action that induces the migration of eosinophils and macrophages.

In the cultures with a 24h incubation period, the vesicular chains were found adhered to the bacterial outer membrane as well as occupying the free spaces between microvilli. In cultures incubated for 48h, the vesicles were absent, whereas some regions of the enterocytic epithelial surface lost their organization, leading to tissue lysis. The vesicles could constitute a carrier tool that facilitates the approximation and the interchange of enterotoxins, immunologicals material or any other natural element responsible for the virulence and pathogenicity of *Aeromonas* spp., similar to what has been described by [25].

The strain isolated from the patient with diarrhea produced important alterations in the intestinal mucosa, indicating its enteropathogenic potential. This strain is a cytotoxin producer and, as has been pointed out for *Shigella*, a correlation exists between the severity of the lesions of the mucosal membrane and the toxigenicity of a particular strain [1].

5. Conclusion

In conclusion the genus *Aeromonas* has great enteropathogenic potential, although additional studies are necessary to understand more about the pathogenicity of other *Aeromonas* strains, this procedure also may useful to determinate currently unknown interactions between a variety of other enteropathogenic bacteria and, the results obtained in these studies give a valuable contribution for the future application of this procedure in the bacterial patogenicity research.

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