Ultrastructural Changes in the Small Intestine of Suckling Mice, Caused by *Vibrio cholerae* Hemagglutinin/Protease

E. A. Bardakchian, E. V. Monakhova, N. G. Kharlanova, S. R. Sayamov, and R. V. Pisanov

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Suckling mice aged 4-5 days were injected with *Vibrio cholerae* hemagglutinin/protease and ultrastructural changes in their small intestine were studied after 5 h. The preparation caused a statistically significant accumulation of fluid in the intestine, appearance of large gaps along cell-cell spaces in the villi and crypts, intense production and secretion of the mucus by goblet cells. The formation of interepithelial cavities was paralleled by vascular changes, supplemented by extravasal disorders caused by mast cell reaction. The role of enterochromaffin cells and lipofibroblasts, modulating the secretion in the intestine, is confirmed.

Key Words: *Vibrio cholerae hemagglutinin/protease; small intestine; ultrastructure*

Vibrio cholerae hemagglutinin/protease (HA/P) is characterized by proteolytic and hemagglutinating activities and causes accumulation of liquid in isolated loops of the rabbit intestine [9], activates cholera toxin (CT) [7] and *V. eltor* soluble hemolysin (HlyA) [11], and induces detachment of vibrios from the intestinal mucosa, which promotes propagation of the agent [8]. Similarly as zonula occludens toxin (Zot), HA/P can impair the epithelial barrier function by modifying the tight junction. Its target is occludin (protein) linked with ZO-1 protein in the inner surface of the membrane and regulating the organization and structure of F-actin cytoskeleton [14]. Published data on HA/P as an independent factor causing diarrhea are contradictory [8,10,13]. Further studies of HA/P biological activity *in vivo*, including studies at the ultrastructural level, are needed to clear out its role in the pathogenesis of cholera, particularly non-epidemic (caused by non-cholerogenic *V. cholerae* strains).

Laboratory of Pathomorphology and Pathophysiology, Plague Control Institute, Rostov-on-Don, Russia. ***Address for correspondence:*** unicorn54@mail.ru. E. V. Monakhova

We studied changes caused by HA/P preparation in the small intestine of suckling mice by electron microscopy.

MATERIALS AND METHODS

The HA/P preparation was obtained from recombinant *E. coli* Jm103pHP61 producer strain, constructed at our laboratory [5]. Hemagglutinin/protease in the mature form with molecular weight of 32 kDa retained all characteristics described for it: hemagglutinating activity towards chicken erythrocytes and proteolytic towards certain proteins, including the capacity to processing of CT, *E. coli* thermolabile toxin, and *V. eltor* hemolysin (pro-HlyA).

Experiments were carried out on 54 suckling mice aged 4-5 days (3-4 g) which received rectally 10 and 20 μg of the preparation in 100 and 200 μl saline, respectively [4]. Controls received isotonic saline. After 5 h the animals were sacrificed by chloroform and the proportion of the gastrointestinal tract weight to body weight was evaluated. The data were statistically processed using Primer of Biostatistics v.4.03 software.

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Seven animals from each group were taken for electron microscopy. Fragments of the small intestine were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C and postfixed in 1% OsO_4 in the same buffer for 1 h at 4°C. After dehydration in ascending alcohols, the material was embedded in epon 812. Common changes were studied on semithin sections stained with toluidine blue. For subsequent studies the sections from the same blocks, sliced by an LKB-8800 microtome, were contrasted with uranyl acetate and lead citrate and examined under a JEM-100 B electron microscope.

RESULTS

The ultrastructure of the small intestine in control suckling mice was largely similar to that of 10-day-old rabbits, which were the object of our previous study [2,3]. However, rare lysosomes, poorly developed Golgi complex, and tubules of the granular endoplasmic reticulum were seen in the mouse epithelial cells (EC) villi. Osmiophilia of the cytoplasm was associated with the presence of numerous free ribosomes and polysome complexes (Fig. 1, *a*). Apical EC plasmalemma had microvilli, poorly developed in the crypt cells, and interdigitations of

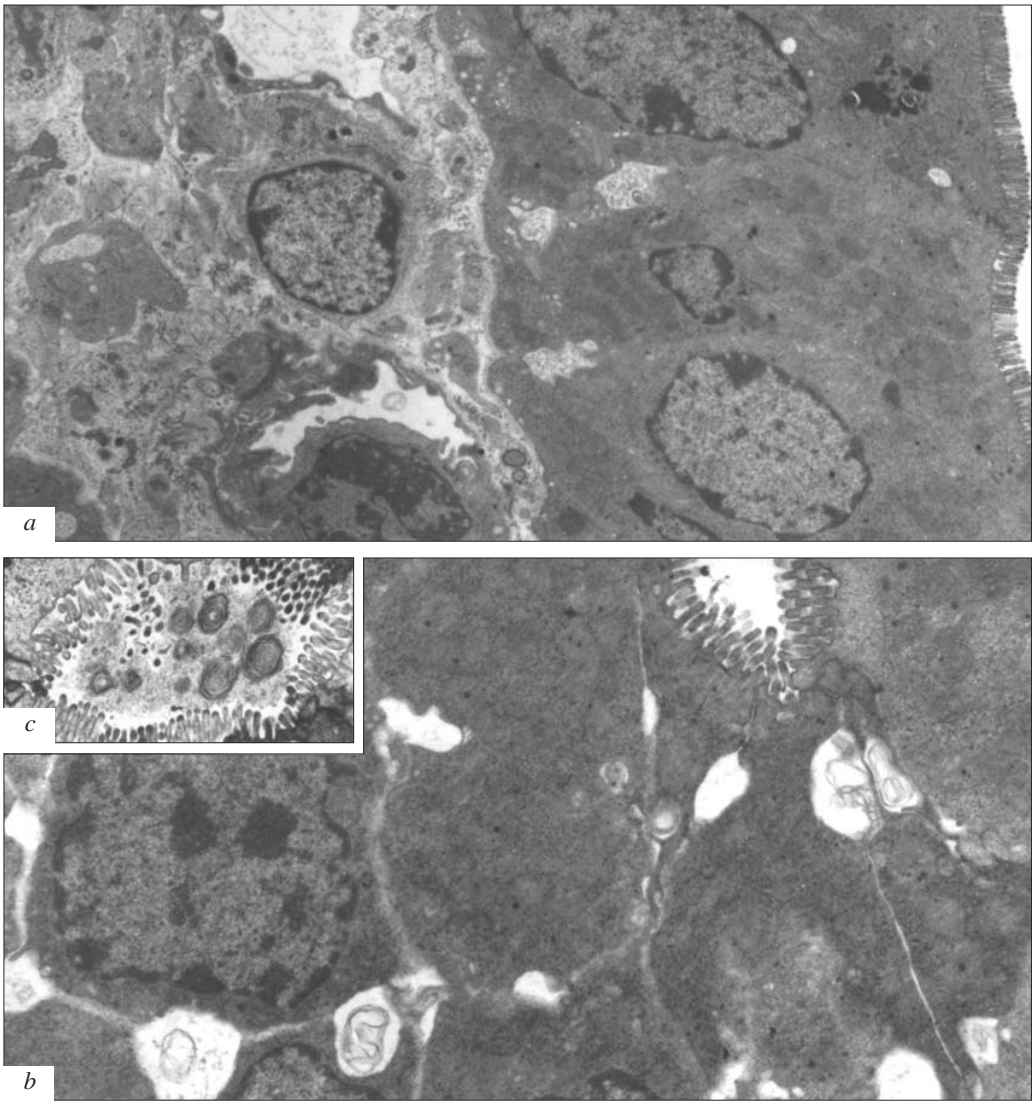

Fig. 1. Ultrastructure of suckling mouse small intestine in the control (*a*) and after 10 μg HA/P (*b*, *c*). *a*) local widening of cell-cell spaces in the villous epithelium (control), ×3000; *b*) formation of numerous cavities between EC crypts and appearance of myelin-like structures in them, ×5000; *c*) myelin-like structures in crypt lumen, ×5000.

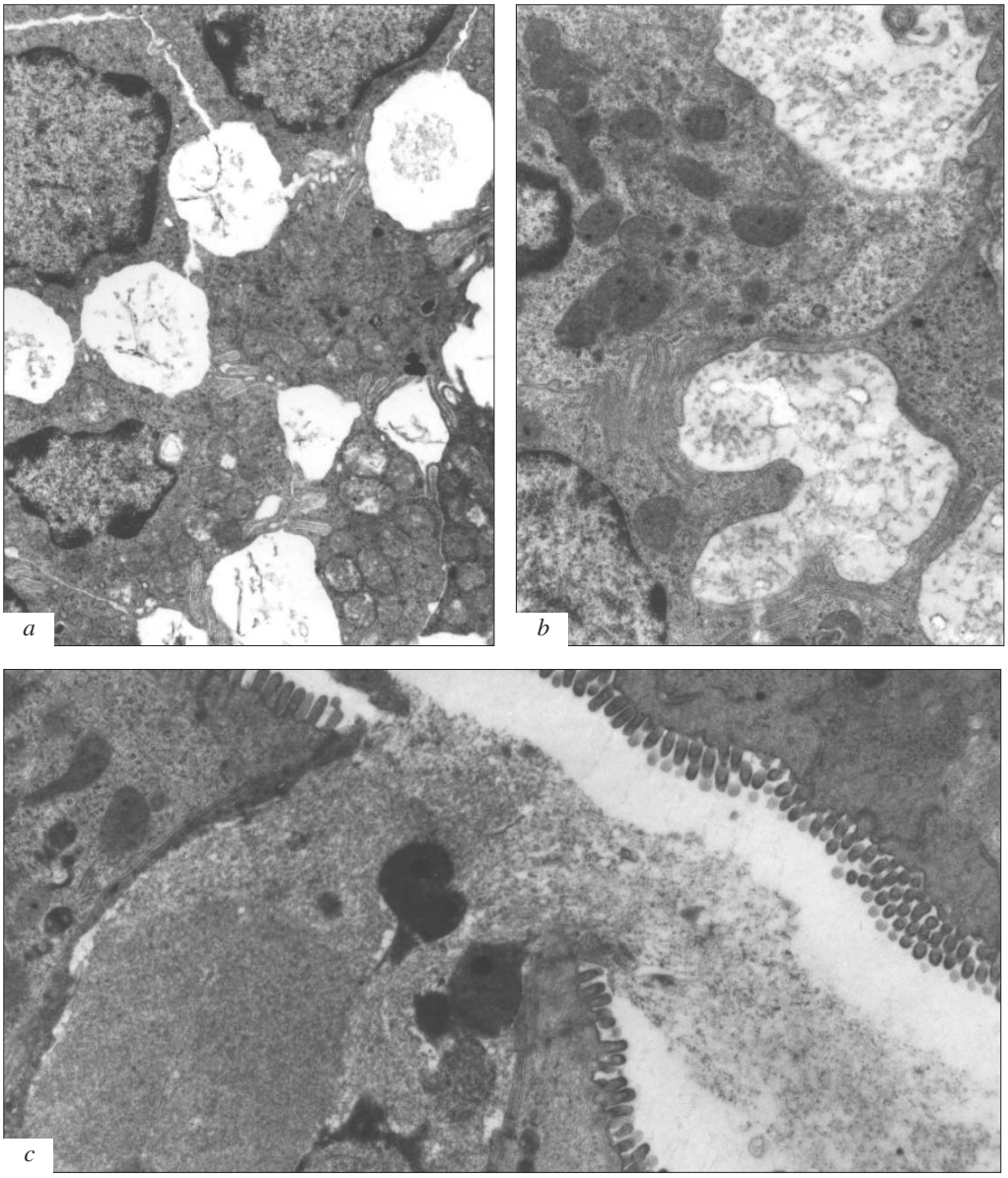

Fig. 2. Ultrastructural changes in the small intestine of suckling mice after 20 μg HA/P. *a*) numerous cavities between cryptic EC are connected to each other by dilated interepithelial spaces, ×5000; *b*) formation of large cavities between villous EC, ×8000; *c*) intense secretion from goblet cell, ×6000.

the adjacent erythrocyte membranes were in fact not discernible. Similarly as in suckling rabbits, villous and cryptic stroma was formed by connective tissue with blood capillaries, while stromal cells were represented by lymphocytes, plasma and mast cells; fibroblasts, macrophages, collagen fibers, and some nerve fibrils were also seen. Lipid interstitial cells (lipofibroblasts) which we previously described in suckling rabbits [2], were detected among the suckling mouse intestinal crypt cells. Slight dilatations of cell-cell spaces, characterized by apical basal orientation and located near the villous epithelial basal membrane (Fig. 1, *a*), were detected in animals of two species. These cavities (lacunae) were usually filled with homogenous material of different electron density.

The HA/P preparation in both doses caused statistically significant ($p<0.005$) accumulation of liquid in the intestine of suckling mice.

Remarkable that 5 h after administration of 10 μg HA/P to suckling mice, the main targets of injuries were cells of the intestinal crypts, but not of the villi, the majority of which looked intact. The formation of large gaps along the interface between

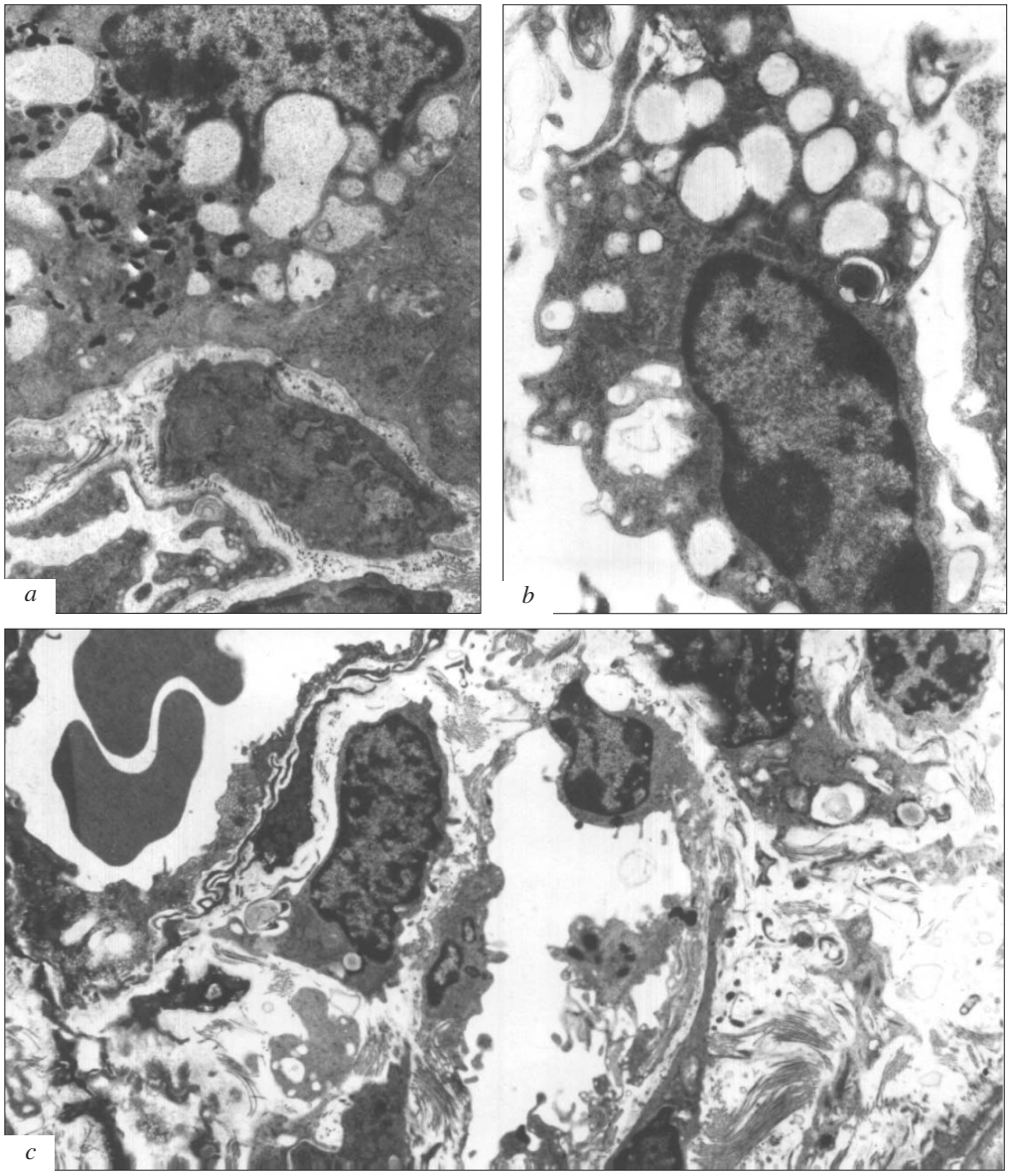

Fig. 3. Ultrastructural changes in the small intestine of suckling mouse after 20 μg HA/P. *a*) partial degranulation of enterochromaffin cell and pronounced vacuolation of the cytoplasm, ×8000; *b*) total degranulation of mast cell, myelin-like structures in the cytoplasm and stroma, ×5000; *c*) intensification of transendothelial micropinocytosis and emergence of microdefects in endothelial lining, ×3000.

adjacent EC and myelin-like structures in the lacunae (Fig. 1, *b*) and crypt lumen (Fig. 1, *c*) were the most obvious ultrastructural disorders in the crypts. As they formed in the absence of activation of the cellular lysosomal system (primary lysosomes in enterocyte cytoplasm are extremely rare), we can say that the degenerative changes were caused by the preparation.

Common and granulated goblet cells of suckling mice reacted similarly to HA/P: by intensification of mucus production and secretion of the mucus globules into the intestinal lumen. However, the dose of 10 μg had virtually no effect on the enterochromaffin cells and lipofibroblasts, while mast cells were just slightly degranulated. Small intestinal capillaries were highly sensitive to the preparation. The endothelium was flattened and contained numerous micropinocytous vesicles, microclasmatosis was constantly observed, as well as detachment of microvilli and larger cytoplasmatic processes.

A clear-cut dose dependent effect of the preparation was observed after the dose of 20 μg. The

number and size of lacunae between EC crypts increased (Fig. 2, *a*). Similar cavities formed between the villous enterocytes, and it was clearly seen that they formed (like in the crypts) as a result of repeated widening of cell-cell spaces and were filled with substance resembling the plasma (Fig. 2, *b*). Moreover, in the controls the cavities were elongated and located as a rule near the EC basal membrane, while after HA/P administration they rounded and could be seen anywhere between the neighboring cells. Close contacts were not impaired. Myelin-like structures were still seen in the enterocyte cytoplasm and in the lacune lumen. Nuclei with chromatin margination and condensation, phagocytosed apoptotic bodies were seen in solitary cases, indicating probable EC death by apoptosis. With increasing the preparation dose, mucin-like granules in goblet cells fused, lost their membranes, the secretion being somewhat cleared, the apical membrane ruptured, and the contents intensely released (Fig. 2, *c*). Liquefaction of the mucus seemed to be due to the mucinase activity of the preparation [5,8].

Virtually all enterochromaffin cells looked moderately degranulated with sharply vacuolated cytoplasm (Fig. 3, *a*). The vacuoles were large and so numerous, that they squeezed the nucleus to the periphery of the cell, compressed and deformed it. We previously observed similar cell reaction not only under the effect of *V. cholerae* cholerogenic strains, but also after infection with *Vibrio cholerae El Tor* devoid of CT genes [1]. In addition, the increase of the preparation dose led to modification of the lipofibroblast ultrastructure and led to reduction of the lipid material in their granules. We consider this fact particularly important, as the development of cholera in suckling rabbits was also associated with reduction of lipid incorporations in their cytoplasm [2] and coincided with many-fold increase of prostaglandin level in the stroma [12] and small intestinal capillaries [6].

The interepithelial cavities formed in the presence of vascular changes, developing after administration of HA/P in the capillary endothelium and could be paralleled by extravasal disorders, depending on mast cell reaction, because degranulation of these cells promotes the release of many bioactive substances (histamine, serotonin, heparin, Fig. 3, *b*). Micropinocytosis was intensified in EC, complex interdigitations formed at sites of their contacts, as well as numerous microvilli and cytoplasm swelling, subjected to microclasmatosis (Fig. 3, *c*). It seems that HA/P labilized the endothelial lining, as a result of which EC were desquamated and defects formed, promoting an increase of vascular permeability, development of interstitial edema, and release of blood cells.

Hence, experiments on suckling mice showed that HA/P led to emergence of large cavities (lacunae), connected to each other through a common system of cell-cell spaces, in the villi and crypts. The dose-dependent effect of ultrastructural disorders in the intestine was confirmed by the involvement of enterochromaffin cells, lipofibroblasts, mast cells, and development of vascular changes. These results present HA/P as a factor of *V. cholerae* virulence, involved in the pathogenesis of cholera.

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