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Ultrastructural Changes in Cultured Cells under the Effect of *Vibrio Cholerae* Hemagglutinin/Protease

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Electron microscopic study of changes in cultured cells caused by *Vibrio cholerae* recombinant hemagglutinin/protease (HA/P) showed significant structural changes, most pronounced in HeLa and L-929 cells not forming a compact monolayer with tight junctions between the cells: formation of numerous vesicles on the cell surface and clasmatosis, vacuolation of the cytoplasm, swelling of mitochondria, clarification of their matrix and crist distortions, and increase in the number of lysosomes. Cytoplasm vacuolation predominated in MDCK culture, while clasmatosis was less intense. Addition of HA/P to CaCo2 cells forming a differentiated polarized monolayer, led to extension of cell-cell spaces not impairing tight junctions, swelling of mitochondria, cytoplasm vacuolation, and clasmatosis on the apical surface. These changes virtually completely coincided with those caused by the so-called NMDCY factor (non-membrane-damaging cytotoxin), described as new *Vibrio cholerae* toxin. These findings confirm our previous hypothesis about the identity of these factors.

Key Words: *Vibrio cholerae; hemagglutinin/protease; non-membrane-damaging cytotoxin; cell cultures; ultrastructure*

Hemagglutinin/protease (HA/P) is an important factor of *Vibrio cholerae* virulence/persistence. In addition to stimulation of *V. eltor* toxin and soluble hemolysin (HlyA) [10] and promotion of vibrio detachment from the intestinal mucosa [6], HA/P causes fluid accumulation in the intestine of experimental animals [1,8]. In T84, MDCK-I, HT29, and CaCo2 cell cultures, HA/P caused significant changes [9,13]. We demonstrated rounding of CHO-K1, L-929, HeLa, HEp2, and McCoy cells and destruction of MDCK and CaCo2 compact monolayer paralleled by detachment of the culture from the well bottom [2]. This reaction could be reversible depending on the dose and duration of exposure. Ultrastructural changes caused by HA/P in cultured cells have not been described up to the present time.

Here we studied changes induced by HA/P preparation in L-929, HeLa, MDCK, and CaCo2 cells by electron microscopy.

MATERIALS AND METHODS

HA/P preparation obtained previously from recombinant *E. coli* Jm103pHP61 producer strain [4] was stored lyophilized in sealed ampoules. Working dilution in distilled water with total protein concentration of 1 mg/ml was prepared directly before the experi-

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ments. HA/P was in a mature form (molecular weight 32 kDa) due to autoprocessing and retained all the properties described for it: hemagglutinating activity towards chicken erythrocytes and proteolytic activity towards several proteins, including cholera toxin, *E. coli* thermolabile toxin, and *V. eltor* hemolysin (pro-HlyA) processing.

Biological activity of HA/P was evaluated on the following cell cultures: L-929 (mouse subcutaneous connective tissue), M-HeLa (cervix uteri epithelioid carcinoma), MDCK (dog kidney; NLB), and CaCo2 (human small intestinal epithelium, Institute of Cytology, Russian Academy of Sciences, St. Petersburg).

The cells were cultured in plastic flasks to confluence, HeLa and L-929 in RPMI-1640 (Sigma) with 10% FCS for 1-2 days, MDCK and CaCo2 in DMEM (Sigma) with 10% FCS for 2-3 and 5-7 days, respectively. Directly before the experiment, complete growth media were replaced with a fresh portion containing 1% serum and 100 μg/ml gentamicin.

The test preparation was added to the test cultures to a final concentration of 20 μg/ml and the cultures were incubated in a CO_2 incubator (Sanjo) at 37°C. Morphological changes were recorded every 0.5-1.0 h under an inverted phase microscope (Reichert). After complete rounding of L-929 and HeLa cells (30-40 min) and destruction of MDCK and CaCo2 cell monolayers (40-60 min), the culture fluid was carefully collected and 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) was added to the cells. The cells under the fixative layer were carefully detached from the flask bottom with a special silicon spatula, the suspension was transferred into centrifuge tubes, and the cells were precipitated by short (3-5 min) low-frequency (1000-2000 rpm) centrifugation. Total duration of fixation was 20 min. The material was postfixed in 1% OsO_4 in the same buffer for 1.5 h at 4°C. After dehydration in ascending alcohols, the material was embedded in epon 812. The sections sliced on an LKB-8800 ultramicrotome were contrasted with

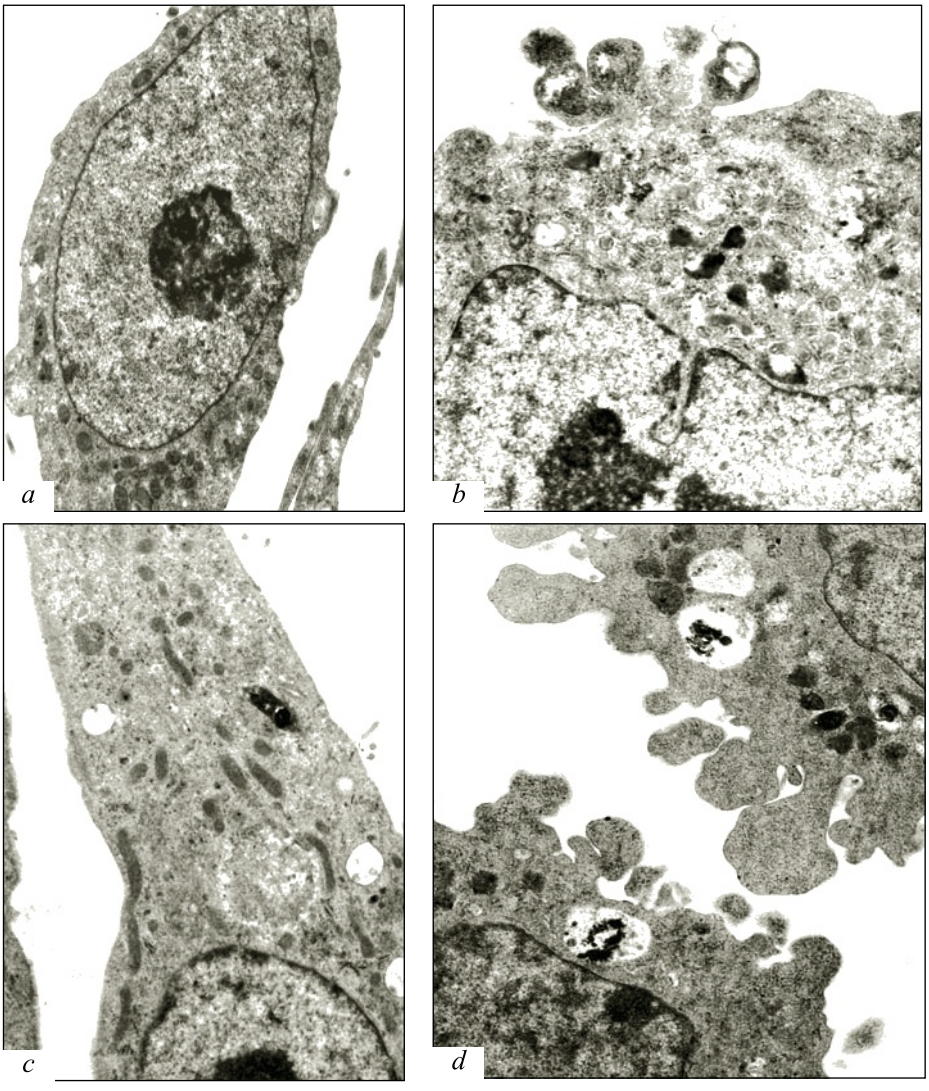

Fig. 1. Ultrastructural changes in cultured HeLa (*a*, *b*) and L-929 cells (*c*, *d*) under the effect of HA/P. *a*, *c*) control, ×8200; *b*, *d*) cell treated with HA/P: *b*) clasmatous vesicles on the surface, lysosomes more numerous than normally; ×9900; *d*) vesicle formation and detachment from the surface of rounded cells; swollen mitochondria, numerous lysosomes, and large phagolysosomes in the cytoplasm, ×8200.

uranyl acetate and lead citrate and examined under a Tecnai 12 electron microscope (Phillips).

RESULTS

We previously showed that HA/P caused significant morphological changes in cultured cells: destruction of compact monolayer and cell rounding. These changes depended on the dose and duration of exposure [2]. Electron microscopy showed structural changes which were not seen under a light microscope. These changes were most pronounced in HeLa and L-929 cells forming no true compact monolayer with tight junctions (tj) between the cells. In contrast to the control, the cells treated with HA/P had numerous vesicles containing the cytoplasm with destroyed organelles on their surface (Fig. 1). These clasmatous vesicles were later detached from the cells. However, this process did not lead to their lysis and, as we had shown before, the reaction was reversible at the specified dose and incubation time [2]. Other changes consisted in cytoplasm vacuolation, swelling of the mitochondria, loosening of their matrix and crist destruction, intense development of the Golgi complex, and appreciable increase in the number of lysosomes (Fig. 1, *b*) and (in some HeLa cells) of lipid granules. Some authors interpreted this picture as severe injuries in the cells resultant from intoxication [5].

Cytoplasm vacuolation predominated in MDCK culture (Fig. 2, *a-c*). Some vacuoles were particularly large and pushed the nucleus to the cell periphery. Many of them seemed to be phagolysosomes contai-

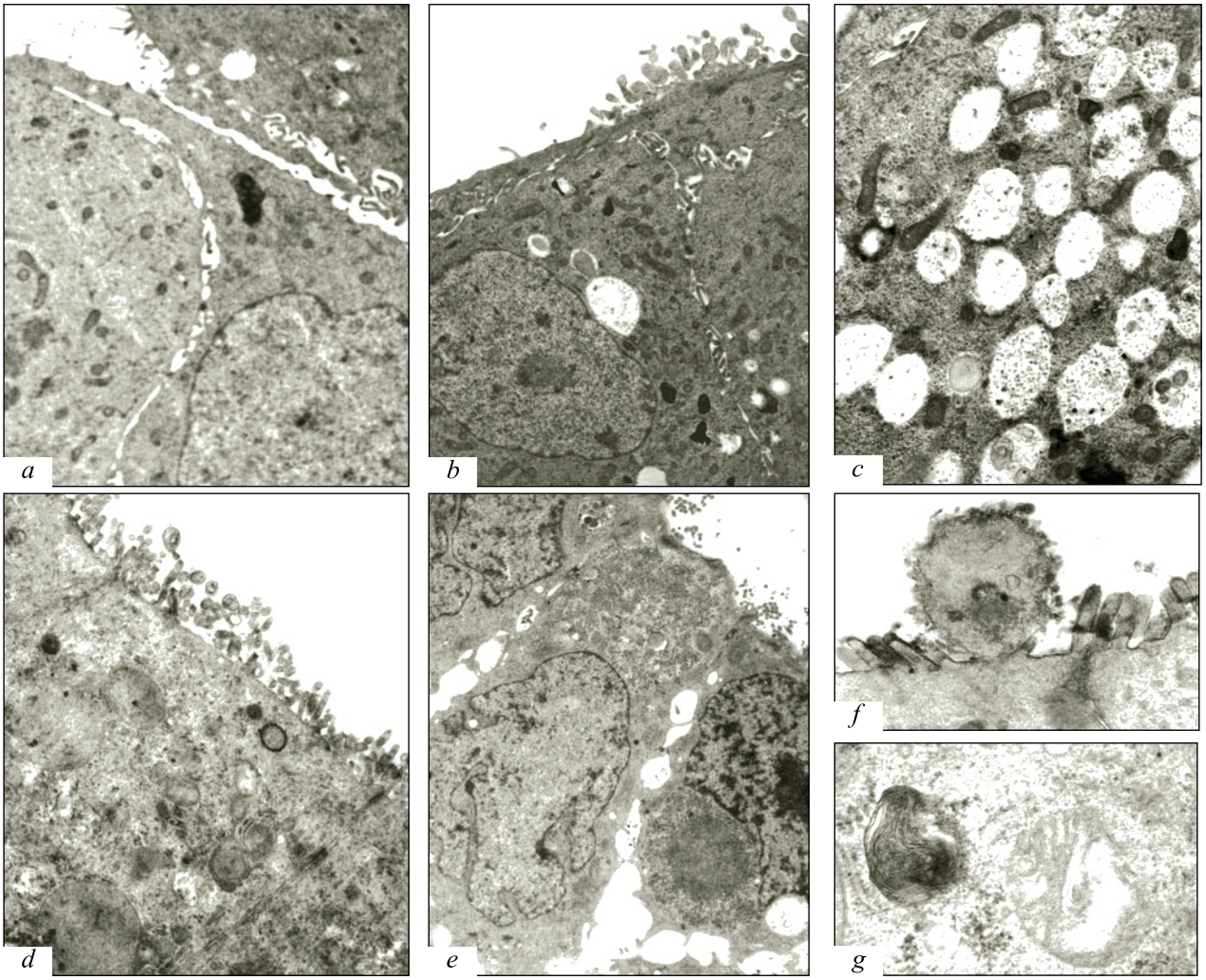

Fig. 2. Ultrastructural changes in cultured cells under the effect of HA/P. *a-e*) MDCK: *a*) control, ×4200; *b-e*) cells treated with HA/P: *b*) microclasmatosis on membrane surface, phagolysosomes in the cytoplasm, mitochondria virtually intact, ×6000; *c*) numerous phagolysosomes with fine granular contents, some have myelin-like structures, ×9900. *d-g*) CaCo2: *d*) control; microvilli on apical surface, tight junctions (tj) in subapical sites, ×8200; *f-g*) cells treated with HA/P: *e*) cell retraction and extension of cell-cell spaces in the monolayer with intact tj, ×4200; *f*) formation of a clasmatous vesicle with destroyed organelles; an intact tight junction to the right of it, ×20,000; *g*) myelin-like structure in the cytoplasm and swollen mitochondrion with damaged crists, ×43,000.

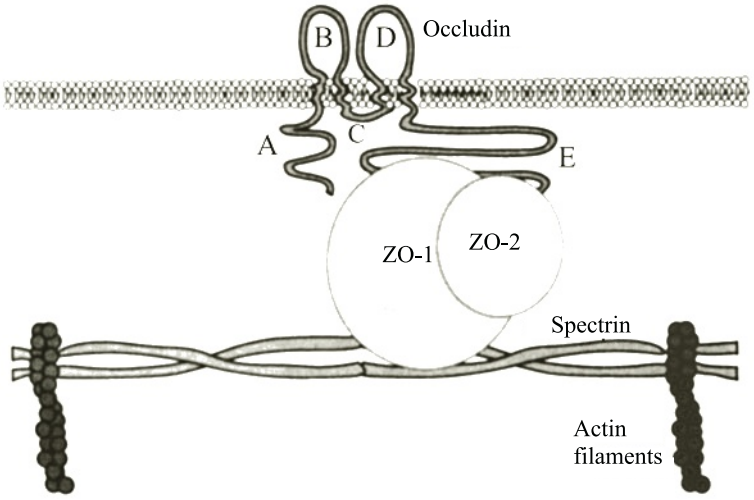

Fig. 3. Model of occludin structure in tj [7].

ning the cytoplasm and cell organelle degradation products, as well as myelin-like structures (Fig. 2, *c*). Clasmatosis was less intense and was found in just few cells (Fig. 2, *b*). The mitochondria also looked intact.

A characteristic feature of cultured CaCo2 cells (Fig. 2, *d*-*g*) was the formation of a differentiated polarized monolayer structurally similar to epithelial lining of the small intestine. The apical surface faced the culture medium, while microvilli and tj located closer to the apical domains were clearly seen in longitudinal (tangential) sections (Fig. 2, *d*). Clasmatosis on the apical surface was seen in cells treated with HA/P (Fig. 2, *f*). Cytoplasm vacuolation was less pronounced in comparison with MDCK culture. Swelling of the mitochondria, destruction of their crists, and formation of myelin-like structures in the cytoplasm were found in the cells (Fig. 2, *g*). Extension of the cell-cell spaces was seen in the monolayer, tj remaining intact (Fig. 2, *e*, *f*). Ultrastructural studies of HA/P effects on the small intestine of suckling mice *in vivo* also showed intact tj [1]; large lacunae appeared between the adjacent villous and cryptic epitheliocytes, not involving the tj. These results contradicted a previous report [13] according to which the target of HA/P activity was occludin, a transmembrane protein and a tj component, cleaved into two fragments by HA/P. The authors hypothesized that this event served as the signal transmitted to ZO-1 protein (not a target for HA/P) on the inner membrane surface, through which the organization and structure of F-actin cytoskeleton was modified, this eventuating in tj rupture. However, the site in which occludin was hydrolyzed under the effect of HA/P was not identified up to the present time. According to the tj structure model [7] (Fig. 3), occludin was a transmembrane protein with 2 extracellular (B, D) and 3 intracellular (A, D, E) domains. The C-terminal domain E was bound to ZO-1. Although the authors [13] thought that HA/P did not penetrate inside the eukaryotic cell and cleaved occludin in extracellular domains B or D, they found the participants in intracellular domain E in both products of occludin degradation by HA/P. Hence, occluding cleavage site can be located in domain E. In that case, tj could be retained, while ZO-1 lost connections with the plasma membrane, this leading to reorganization of the actin cytoskeleton and cell rounding. In addition, further studies showed that claudins (proteins with similar structure) were also involved in tj formation [12]; their capacity to serve as the targets for HA/P was not proven.

The present findings confirmed the previous data obtained *in vivo* and the conclusions from these studies, according to which *V. cholerae* HA/P caused significant injuries in cell structure presumably because of wide spectrum proteolytic activity [4,6]. Degradation of protein components, in turn, led to metabolism disorders in eukaryotic cells. Hence, HA/P can be regarded as a virulence factor involved in the pathogenesis of cholera, particularly that caused by nontoxigenic strains of the agent.

The ultrastructural changes caused by HA/P in cultured cells were not described, but the results of electron microscopy of Int407 (intestinal cells) and HeLa treated with NMDCY (non-membrane-damaging cytotoxin) cytotoxic factor [5], a new independent toxin of *V. cholerae* [11], were published. It was hypothesized from published data that this factor was actually HA/P [3]. This was partially confirmed by HA/P capacity (detected in our study) to induce (similarly as NMDCY) reversible rounding of CHO and other cultured cells, not inhibited by monosugars [2]. Ultrastructural changes in cell cultures observed in our study virtually completely coincided with previous data on NMDCY [11], which was one more evidence in favor of identity of these factors.

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