Ultrastructural Evidence of Invasive Activity of *Vibrio Cholerae*

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The development of experimental cholera in suckling rabbits is associated with typical cholerogenic syndrome: the presence of *Vibrio cholerae* in the blood, bile (in 60 and 70% cases, respectively), small and large intestine (in 100% cases). Simultaneously with enterocyte desquamation and increased permeability of the blood-enterocyte barrier, the vibrios are released into villous stroma and then into the microcirculatory bed. The zot toxin is involved in the mechanism of *Vibrio cholerae* invasion, the corresponding gene is present in the genome of the studied strain.

Key Words: *cholera; Vibrio cholerae invasion; small intestine; ultrastructure*

Virulent strains of *Vibrio cholerae* produce cholera toxin (CT) inducing (through messenger systems and bioactive substances) hypersecretion of water and electrolytes in the small intestine [13,15]. Diarrhea syndrome caused by CT postpones the role of other manifestations of cholera, for example, changes in the myocardium [7], lung [2], renal medulla and cortex [4,5,8].

The virulence of *V. cholerae* is determined by three key virulence genes in their chromosome: ctxAB responsible for the synthesis of toxic subunit A of CT, tcpA regulating production of structural unit of toxin-coregulated adhesion piles, and toxR global regulatory gene controlling the expression of ctxA and tcpA. The ctxAB genes are present in the bacteriophage CTXφ or CTX element, which contains these genes and genes of accessory cholera enterotoxin (ace) and zonula occludens toxin (zot) causing destruction of tight junctions (s. zonulae occludentes) [10].

We found that non-cholerogenic *V. cholerae* strains are capable of invasion, penetrate into the lamina propria, are located near the capillaries and often reach the circular (inner) layer of the lamina muscularis [3,6]. Classical and El Tor vibrios were isolated from the blood, liver, and spleen.

The aim of our study was to provide ultrastructural evidence of invasion of toxigenic *V. cholera* into the stroma and microcirculatory bed of suckling rabbits in experimental cholera, bacteriological studies of the blood, bile, small and large intestine, and molecular genetic characterization of the chosen strain.

MATERIALS AND METHODS

The experiments were carried out on 10-12-day-old rabbits (n=16) infected with cholera after 24-hour fasting. The rabbits received through a polyethylene gastric tube 1 ml 3% sodium carbonate for neutralization of gastric content, 1 ml 18-h culture of *Vibrio cholerae eltor* P-5879, and again 0.5 ml sodium bicarbonate. The infective dose was 10^5 microbial bodies/ml (by optical opacity standard). This dose led to the development of typical cholerogenic syndrome after 24 h, with accumulation of transparent or semi-transparent serous fluid in the intestine, containing vibrios in high concentrations (10^8-10^9 cell/ml). The duration of rabbit life after infection was 24-48 h. After 24 h the animals were narcotized with a lethal dose of Nembutal. Controls (n=4) received 1.5 ml sodium bicarbonate and

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1 m isotonic NaCl solution. Bacteriological studies of the blood, bile, small and large intestine were carried out, cholerogenic activity was evaluated, and inoculations from the viscera onto Marten agar plates were made. Fragments of the jejunum at a distance of 15 cm from the duodenum were examined under an electron microscope. The fragments 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, semithin sections were stained with toluidine blue. The sections

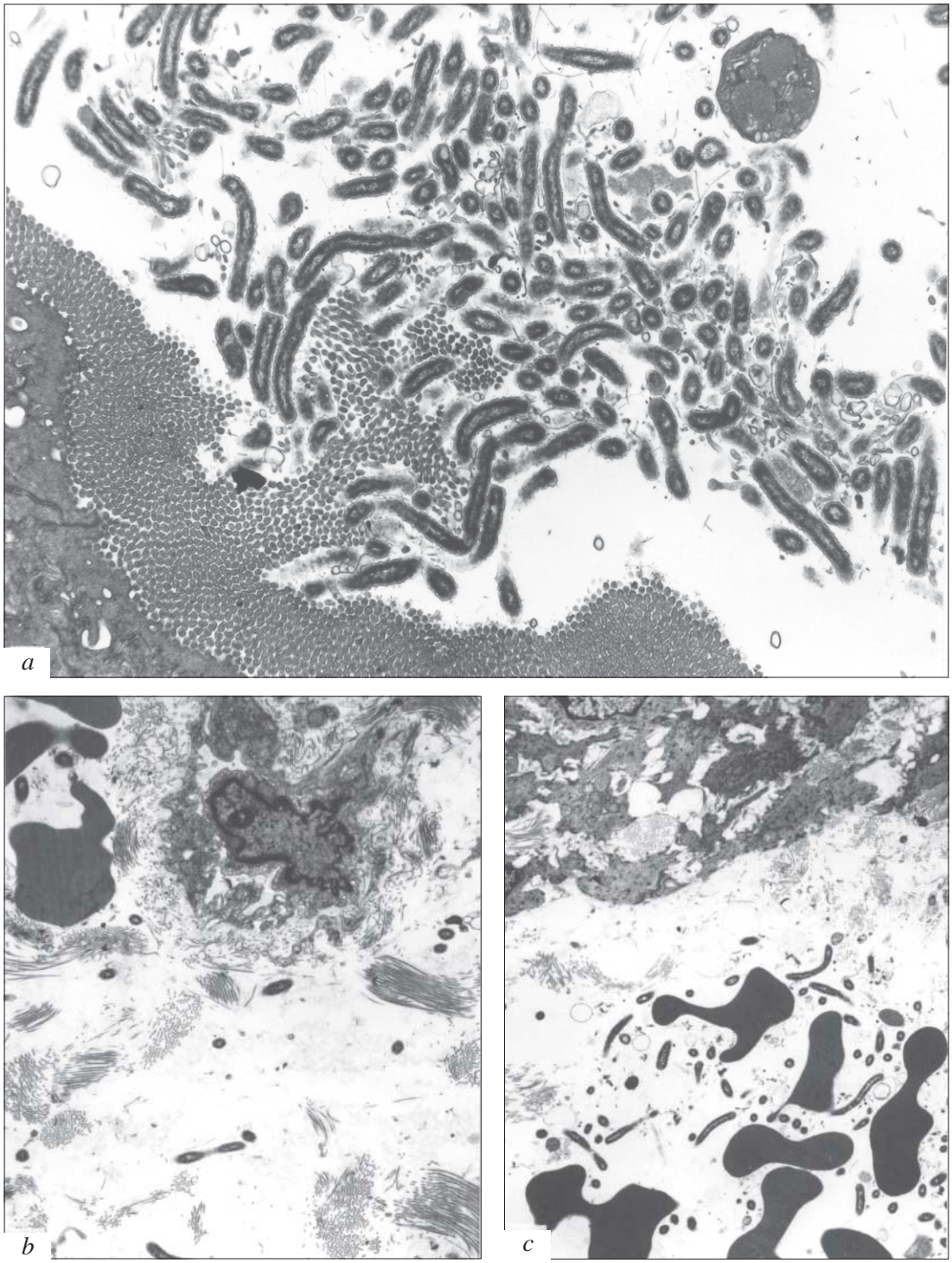

Fig. 1. Ultrastructural changes in the small intestine of suckling rabbits with experimental cholera. *a*) rejection of microvilli with *V. cholerae* adhered to them, ×4000; *b*) invasion of vibrios into the stroma; solitary erythrocytes near intact capillary, ×2000; *c*) numerous cholera vibrios and erythrocytes located in the stroma near smooth-muscle cells of circular muscles, ×2000.

prepared from the same blocks on an LKB-8800 ultramicrotome were then contrasted with uranyl acetate and lead citrate by the method of Reynolds and examined under a JEM-100 B electron microscope. The presence of genetic determinants of virulence in the genome of the studied *Vibrio cholerae eltor* P-5879 strain was determined by molecular probing and PCR.

RESULTS

The rabbits infected with virulent *Vibrio cholerae eltor* P-5879 strain develop choleric syndrome after 24 h. The syndrome is characterized by hyperemia and extensive accumulation of transparent fluid in the small intestine. In the large intestine distention and the presence of small floccular compact lumps in the liquid contents are observed. Bacteriological study showed the presence of cholera vibrios in the blood and bile in 60 and 70% cultures, respectively, and in 100% cultures inoculated with the material from the small and large intestine.

According to the data of molecular probing and PCR, *Vibrio cholerae eltor* P-5879 strain contains full-length tandem duplicated CTX element including the

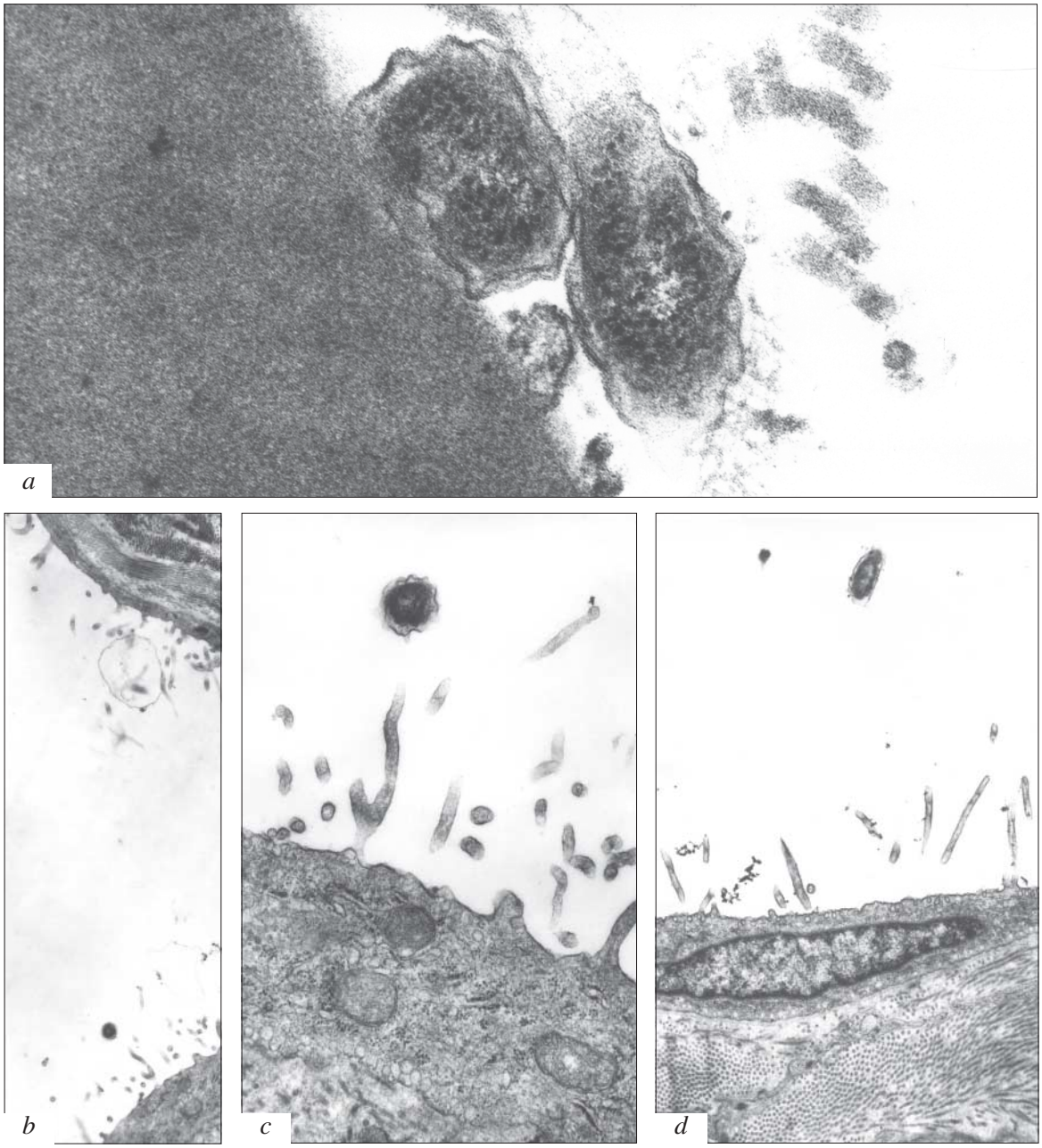

Fig. 2. Absence of hemolysis in *V. cholerae* contact with erythrocyte (*a*, ×50,000) and appearance of solitary *V. cholerae* in the lumen of collector veins (*b-d*) in experimental cholera, ×4000 (*b*), ×15,000 (*c*), and ×6000 (*d*).

ctxAB genes, accessory zot and ace toxins, and flanking RS sequences. Its genome also contains tcpA, toxR, and genes of cytotoxic RTX-complex rtxA and rtxC.

Electron microscopy of the jejunum from control suckling rabbits receiving isotonic saline revealed no lesions. In animals with cholera focal involvement of the villi and balloon degeneration of epitheliocytes were observed even on semithin sections. Ultrastructural analysis showed transformation of epithelial cells into fluid-filled giant vacuoles, drastic thinning of endothelial cells, and pronounced transendothelial micropinocytosis in villous capillaries [6].

Numerous cholera vibrios located in the lamina propria and even penetrating into the microcirculatory bed attract special interest. The process starts with rejection of enterocyte microvilli of the small intestine together with adhered cholera vibrios from the apical plasmalemma, and initial destruction of the epithelial layer (Fig. 1, *a*). Enterocyte desquamation is paralleled by a drastic increase in the permeability of the blood-enterocyte barrier: from intensification of transendothelial micropinocytosis to loosening of the endothelial cells and their desquamation into the vascular lumen [1]. In other words, this creates conditions for the appearance of vibrios in the villous stroma and then in capillaries (Fig. 1, *b*). Interestingly, extravasal erythrocytes which left the vessels by diapedesis were seen near intact capillaries. In cases with more severe capillary injuries *V. cholerae* were surrounded by red blood cells in the interstitial spaces (Fig. 1, *c*). The absence of hemolysis in vibrio-erythrocyte contact is worthy to note (Fig. 2, *a*). Cholera vibrios appear in the microcirculatory system: they were seen in the collector veins (>200 μ in diameter) (Fig. 2, *b-d*).

Apart from induction of typical cholerogenic syndrome and the relevant ultrastructural changes in the small intestine, *Vibrio cholerae eltor* P-5879 strain is capable of invasion. It seems that zot toxin is involved in invasion. The corresponding gene is present in the genome of the studied strain. The mechanism underlying the effect of zot (analog of zonuline, eukaryotic modulator of tight junctions) consists in reversible destruction of tight junctions [9]. Labilization of contacting surfaces near the apical surfaces of the neighboring epithelial cells loosens their contacts, thus creating conditions for vibrio release into the stroma.

We cannot rule out the involvement of one more factor complex recently detected in *V. cholerae* in the above-described processes. This complex is encoded by the so-called RTX gene cluster closely connected to the CTX element located 693 n. p. downstream its distal RS element, and includes 4 linked genes rtxA, rtxC, rtxB, rtxD [12]. Since strain P-5879 contains rtxA (toxin proper) and rtxC (its activator) genes, we can admit with high probability that this strain can produce bioactive rtxA. This high-molecular-weight toxin causes depolymerization of stress actin fibers and covalent binding of actin into dimers, trimers, and multimers of higher order, which leads to loosening of tight junctions [11].

Thus, despite the presence of soluble hemolysin hly gene characteristic of El Tor vibrios this gene is not expressed in the studied strain, which explains the absence of hemolysis and extravasal erythrocytes.

REFERENCES

1. E. A. Bardakhch'yan, Yu. M. Lomov, and I. A. Gusatinskii, *Arkh. Patol.*, **55**, No. 1, 29-34 (1993).
2. E. A. Bardakhch'yan, Yu. M. Lomov, and N. G. Kharlanova, *Byull. Eksp. Biol. Med.*, **120**, No. 8, 173-177 (1995).
3. E. A. Bardakhch'yan, Yu. M. Lomov, N. G. Kharlanova, *et al.*, *Arkh. Patol.*, **62**, No. 1, 24-29 (2000).
4. E. A. Bardakhch'yan, N. G. Kharlanova, Yu. M. Lomov, *et al.*, *Byull. Eksp. Biol. Med.*, **131**, No. 2, 223-227 (2001).
5. E. A. Bardakhch'yan, N. G. Kharlanova, Yu. M. Lomov, *et al.*, *Arkh. Patol.*, **63**, No. 6, 30-34 (2001).
6. V. P. Vlasov, N. G. Kharlanova, E. V. Monakhova, *et al.*, *Dokl. Rossiisk. Akad. Nauk*, **352**, No. 2, 278-280 (1997).
7. Yu. M. Lomov, N. G. Kharlanova, T. I. Tkacheva, and E. A. Bardakhch'yan, *Arkh. Patol.*, **57**, No. 6, 58-63 (1995).
8. N. G. Kharlanova, Yu. M. Lomov, T. I. Tkacheva, and E. A. Bardakhch'yan, *Morfologiya*, **109**, No. 3, 67-71 (1996).
9. R. El Asmar, P. Panigrahi, P. Bamford, *et al.*, *Gastroenterology*, **123**, 1607-1615 (2002).
10. A. Fusano, *Ann. NY Acad. Sci.*, No. 915, 214-222 (2000).
11. K. J. Fullner and J. J. Mekalanos, *EMBO J.*, **19**, No. 20, 5315-5323 (2000).
12. W. Lin, K. J. Fullner, R. Clayton, *et al.*, *Proc. Natl. Acad. Sci. USA*, **96**, No. 3, 1071-1076 (1999).
13. F. Qadri, R. Raqib, F. Ahmed, *et al.*, *Clin. Diagn. Lab. Immunol.*, **9**, No. 2, 221-229 (2002).
14. M. Trucksis, J. E. Galen, and J. Michalski, *Proc. Natl. Acad. Sci. USA*, **90**, No. 11, 5267-5271 (1993).
15. R. A. Wapnir and S. Teichberg, *J. Nutr. Biochem.*, **13**, No. 4, 190-199 (2002).