INFECTION AND IMMUNITY, Apr. 1997, p. 1293–1298 Vol. 65, No. 4
0019-9567/97/$04.00+0

Potent Membrane-Permeabilizing and Cytocidal Action of *Vibrio cholerae* Cytolysin on Human Intestinal Cells

ALEXANDER ZITZER,* TRUDY M. WASSENAAR, IWAN WALEV, AND SUCHARIT BHAKDI

Institute for Medical Microbiology, Johannes Gutenberg University, 55101 Mainz, Germany

Received 22 October 1996/Returned for modification 5 December 1996/Accepted 28 January 1997

Many strains of *Vibrio cholerae* non-O1 and O1 El Tor that cause diarrhea do not harbor genes for a known secretogenic toxin. However, these strains usually elaborate a pore-forming toxin, hitherto characterized as a hemolysin and here designated *V. cholerae* cytolysin, whose action on intestinal cells has not yet been described. We report that *V. cholerae* cytolysin binds as a monomer to Intestine 407 cells and then assembles into detergent-stable oligomers that probably represent tetra- or pentamers. Oligomer formation is accompanied by generation of small transmembrane pores that allow rapid flux of K^+ but not influx of Ca^{2+} or propidium iodide. Pore formation is followed by irreversible ATP depletion and cell death. Binding of fewer than 10^4 toxin molecules per cell in vitro is lethal. The possibility is raised that production of this toxin by bacteria that are in close contact with intestinal cells is rapidly cytocidal in vivo, and death of intestinal cells may be a cause of diarrhea.

Enteropathogenic *Vibrio cholerae* can elaborate two basically different categories of exotoxins. The first is represented by classic cholera toxin (CT), zonula occludens toxin, and accessory cholera toxin (7, 25). The genes encoding the latter are located in the "virulence cassette region" and do not occur independently of the CT gene (6). A membrane-damaging toxin, hitherto referred to as a hemolysin (4, 9, 11, 12, 21, 30, 31) and here designated the *V. cholerae* cytolysin (VCC) to underline its capacity to damage nucleated cells, represents the second type of *Vibrio* exotoxin. Production of VCC is not coupled to the expression of CT.

V. cholerae O1 biotype El Tor, the cause of the present pandemic of cholera, was historically differentiated from classical *V. cholerae* O1 by its ability to produce VCC. Later, Richardson et al. demonstrated that classical *V. cholerae* O1 strains produce another (second) hemolysin. This second hemolysin remains to be more closely characterized, but it appears to be distinct from VCC (21). A pathogenetic role of VCC has not been clearly established, however, and a few conflicting reports exist in the literature. Thus, *V. cholerae* O1 mutants in which both the CT and VCC genes were deleted still caused diarrhea in volunteers (15). On the other hand, deletion of the gene for HlyU, the regulator of VCC gene expression, led to a 100-fold decrease in virulence in an animal model (28). Also, epidemiological studies have shown that gastroenteritis cases due to CT-negative, hemolytic *V. cholerae* O1 El Tor have occurred (10, 17). During a 3-year period from 1989 to 1991, 31 patients infected with *V. cholerae* O1 strains that were hemolytic but lacked the gene encoding CT were identified in Uzbekistan (34). Evidence thus persists that the hemolytic phenotype is, indeed, an indicator of virulence in CT-negative *V. cholerae* O1 strains. In the same context, *V. cholerae* non-O1 strains recognized as important causes of diarrheal diseases and isolated worldwide (3, 16) usually do not produce CT (29) but are hemolytic.

The genes encoding *V. cholerae* O1 El Tor and non-O1 hemolysin have been cloned and sequenced and found to be highly homologous to each other (4). The proteins, which we here collectively designate VCC, have been purified and partially characterized as pore-forming toxins (11, 30, 31). Their membrane-permeabilizing action has been demonstrated in erythrocytes, planar lipid bilayers, and, most recently, liposomes (14, 32, 33). The VCC has been shown to be cytotoxic to various cell lines (11, 32, 33). The VCC from *V. cholerae* non-O1 evokes fluid accumulation in the rabbit ileal loop and suckling mouse models (12), so an investigation into the effects of VCC on intestinal cells appeared warranted. We report on a remarkably potent cytocidal action of VCC on human intestinal cells and provide evidence that cell death is due to generation of pore-forming toxin oligomers on target membranes.

MATERIALS AND METHODS

Reagents. RPMI 1640, Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), glutamine, penicillin-streptomycin, phosphate-buffered saline (PBS), and Hanks' balanced salt solution (HBSS) were obtained from Biochrom (Berlin, Federal Republic of Germany [FRG]). Chemical reagents were purchased from Sigma (Deisenhofen, FRG). Carrier-free, $Na^{125}I$ was obtained from Amersham Corp. (Braunschweig, FRG). A polyclonal rabbit antiserum against purified VCC was produced as previously described (32). Staphylococcal alpha-toxin, *Escherichia coli* hemolysin, and streptolysin O (SLO) were prepared in our laboratory as previously described (2).

Bacterial strain and purification of VCC. *V. cholerae* O1 El Tor 8731 (9) was kindly supplied by Robert Hall of the U.S. Food and Drug Administration, Washington, D.C. VCC was purified from culture supernatants by ethanol precipitation (final concentration, 40%), preparative isoelectric focusing in a sucrose density gradient, and hydroxyapatite chromatography (35). Specific activity of VCC toward erythrocytes paralleled toxicity for intestinal cells during VCC purification.

Radioiodination of VCC. VCC (20 μg) and 500 μCi of ^{125}I (as NaI) were incubated in siliconized glass tubes coated with 2.5 μg of 1,3,4,6-tetrachloro-3α,6α,-diphenylglucouril (Iodo-Gen; Sigma) for 5 min at room temperature. The reaction was stopped by transferring the mixture to a fresh vial containing dithiothreitol final concentration, 10 mM). Bovine serum albumin was added to 0.1%, and the labelled protein was purified by gel filtration (PD-10 column; Pharmacia-Biotech, Uppsala, Sweden). The specific activity was approximately 700 Ci/mmol of iodinated VCC. Approximately 50% of the radiolabelled toxin exhibited the capacity to bind and lyse rabbit erythrocytes. The labelled VCC was stored in aliquots at −70°C.

Cell culture. The Intestine 407 cell line, which originated from the jejunum and ileum of a 2-month-old human embryo, was generously donated by B. A. M. van der Zeijst of the Department of Bacteriology, Institute of Infectious Diseases and Immunology, University of Utrecht, Utrecht, The Netherlands. The cell line was cultured in DMEM with 2 mM glutamine supplemented with 10% FCS, 100

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie, Johannes Gutenberg-Universität, Hochhaus am Augustusplatz, 55101 Mainz, Germany. Phone: 49 (6131) 17-2865 or 7341. Fax: 49 (6131) 39-2359. E-mail: makowiec@goofy.zdv.uni-mainz.de.