Purification and Characterization of a Cytotonic Protein Expressed In Vitro by the Live Cholera Vaccine Candidate CVD 103-HgR

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Cholera vaccines developed by the deletion of CTX genes from Vibrio cholerae induce a residual reactogenicity in up to 10% of vaccinees. A novel cytotoxic agent named secreted CHO cell elongating protein (S-CEP) was purified from culture supernatants of CVD 103-HgR (Levine et al., Lancet ii:467–470, 1988). Five fractionation steps yielded electrophoretically pure S-CEP with an M, of 79,000. A partially purified preparation caused fluid accumulation in a sealed infant mouse model. The amino terminus bore a unique sequence with strong homology to a cytotoxic toxin of El Tor V. cholerae.

Vibrio cholerae utilizes a complex array of structural and regulatory elements in the pathogenesis and immunogenicity of cholera. Although site-specific deletion mutagenesis of the genes encoding cholera toxin (11, 18) resulted in engendering significant protective immunity and the dramatic elimination of the symptoms of cholera gravis, clinical studies consistently reported a residual degree of diarrhea and malaise among vaccinees, indicating that a secretogenic activity remained (11, 16, 18, 21). The extent and intensity of symptoms among vaccinees appeared to be dependent on the dose, the study population, and the vaccine strain under investigation, although little is known of the bacterial factors or the host secretogenic mechanism involved (16, 21–25). Identifying and attenuating the cause of vaccine reactogenicity while preserving immunogenicity remains a major objective of oral vaccine development (19). Characterizing virulence determinants may also contribute to a better understanding of Vibrio pathogenesis and host responses.

Numerous model systems are available for the identification, assay, and characterization of secretogenic activities. Morphological studies on isolated mammalian tissue, such as the Chinese hamster ovary (CHO) cell assay, have provided useful information on nonlethal virulence factors from several bacterial pathogens because these tests do not require an endpoint of cell death (9). A wide range of whole animal systems, including the infant mouse model (13), has contributed to the identification and characterization of numerous virulence determinants from enteric pathogens.

In this study, we assayed culture supernatants from several ctxA-negative V. cholerae strains for cytotoxic activity on cultured CHO cells. A CHO elongation activity expressed by the cholera vaccine strain CVD 103-HgR was purified to electrophoretic homogeneity by using (NH₄)₂SO₄ fractionation and four chromatographic steps. Physical characteristics of the protein were determined, including the M, subunit structure, stability, and amino-terminal sequence. The purified protein was named secreted CHO cell elongating protein (S-CEP). A partially purified cytotoxic protein preparation induced fluid accumulation (FA) in the infant mouse model. The identification and description of a novel cytotoxic protein in V. cholerae raises questions about its expression and activity in the human intestine and its possible role in vaccine reactogenicity.

The following El Tor biotype strains of O serogroup 1 V. cholerae were studied: JBK 70 (a ΔctxAB mutant derivative of Inaba strain N16961 [11]) and three environmental isolates from Brazil, namely 8731, 1074, and 1196 (14). The classical biotype strains studied were CVD 103, CVD 103-HgR, and CVD 103-HgR2, all three of which are ΔctxA mutants of Inaba V. cholerae strain 569B (8). Overnight cultures in Luria broth (10 ml) were inoculated into 1-liter volumes of casamino-yeast extract broth (30 g of casamino acids [Difco; Becton Dickinson, Franklin Lakes, N.J.], 4 g of yeast extract [Difco], 0.5 g of K₂HPO₄ dissolved in 1 liter of H₂O, pH 6.8) in 2-liter flasks and incubated at 37°C in a rotary shaker for 18 h. Cultures were harvested by centrifugation (8,300 × g for 20 min at 4°C), and supernatants were sterilized by filtration (0.22-μm pore size) and stored at 0 to 4°C.

Both culture supernatants and chromatographic fractions (prepared as described below) were subjected to the CHO tissue culture assay conducted as reported elsewhere (9, 13). Briefly, CHO cells were cultured to confluence in Eagle minimal essential medium containing Hanks base salts (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% fetal bovine serum and 10% trypsin phosphate broth. After incubation at 37°C for 48 h in a humid atmosphere containing 5% CO₂, monolayers of CHO cells were released by treatment with 0.025% trypsin containing 1 μM EDTA. CHO cells were resuspended and seeded into fresh microtiter plates at a density of approximately 1,000 cells per well. The CHO cells were treated with a test or control sample (20 μl) and examined microscopically after 24 h of incubation. One CHO cell unit was defined as the reciprocal of the dilution that caused elongation of 50% of the cells in the well.

V. cholerae classical strain CVD 103-HgR was selected as the source of cytotoxic agent because of its relatively high level of activity compared with other organisms tested (data not shown), its role as a vaccine, and the extensive literature on this organism. An additional advantage in studying this strain is its deficiency in the expression of HlyA and engineered deletion of CtxA, which would otherwise exert, respectively, powerful

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cytolytic and cytotonic effects on CHO cells (5, 6, 8, 10, 15). The absence of cholera toxin and hemolysin activity removed a mask and thereby revealed a remaining CHO cell cytotonic activity which was purified in this study.

**Cytotonic toxin purification.** Culture supernatants of *V. cholerae* CVD 103-HgR were subjected to five steps of protein purification, (i) ammonium sulfate fractionation, (ii) anion exchange, (iii) cation exchange, (iv) hydrophobic interaction, and (v) gel filtration chromatography, to yield electrophoretically homogeneous protein bearing a single, unique amino-terminal sequence. Purification proceeded as follows.

In step 1, \((\text{NH}_4)_2\text{SO}_4\) was added to culture supernatants to 55% saturation (351 g/liter) and proteins precipitated overnight at 4°C. After centrifugation (8,300 \(\times g\) for 20 min at 4°C), precipitated proteins were resuspended, dialyzed, equilibrated against 20 mM Tris (pH 7.5) containing leupeptin (0.5 mg/liter), pepstatin (0.7 mg/liter) phenylmethylsulfonyl fluoride (1.0 mM), and EDTA (1.0 mM), and sterilized by filtration (0.2-\(\mu\)m pore size). Material obtained from 100 liters of culture was bulked.

In step 2, concentrated supernatant proteins were applied to Q-Sepharose anion-exchange resin in a prepacked fast protein liquid chromatography (FPLC) column (Amersham Pharmacia Biotech, Piscataway, N.J.), fractionated, and subjected to CHO cell assay. The S-CEP bound the anion-exchange chromatography column when loaded in low-salt buffer and eluted in the presence of NaCl in six fractions of 6 ml each.

Step 3 consisted of bulking the active fractions (numbers 7 to 12) from anion-exchange chromatography and applying the sample to a prepacked Mono-S cation-exchange FPLC column (Amersham Pharmacia Biotech). Pigment and other positively charged contaminating material in the bulked active fractions was removed during this step. The CHO cell assay identified the flowthrough (nonbinding) material as possessing the cytotonic activity, whereas a significant quantity of contaminating proteins and pigment bound to the matrix.

Step 4 comprised application of the bulked flowthrough activity to a prepacked hydrophobic interaction phenyl-Sepharose FPLC column (Amersham Pharmacia Biotech). The CHO cell-elongating activity fractionated in a decreasing concentration gradient from 0.3 M \((\text{NH}_4)_2\text{SO}_4\). Active fractions eluting from the phenyl-Superose FPLC column were concentrated by ultrafiltration centrifugation (Centricon 30; Amicon, Beverly, Mass.).

In step 5, the concentrate was applied to a prepacked Superose 12 gel filtration FPLC column (Amersham Pharmacia Biotech). The elution pattern of the fractions possessing CHO cell elongating activity was compared to that of known protein standards, indicating an active protein with an apparent molecular mass of 75,000 (Fig. 1).

**Protein analysis.** Protein concentrations were estimated either by using the Bio-Rad Protein Assay kit (Bio-Rad, Richmond, Calif.) (3) or by using visual estimations of sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels stained with Coomassie brilliant blue. The subunit composition of S-CEP was investigated by using PAGE (8 to 25% [wt/vol] total acrylamide) in the presence of SDS according to the manufacturer's instructions (PhastSystem; Amersham Pharmacia Biotech). Analysis of bulked active fractions from Superose-12 fractionation revealed an homogeneous, electrophoretically pure protein band migrating with an \(M_r\) of 79,000 (Fig. 2), with no visible evidence of copurifying subunit polypeptides. When compared to the elution pattern of the S-CEP activity directly from gel filtration chromatography (active moiety of \(M_r\) of 75,000), the estimated molecular masses
TABLE 1. Purification of S-CEP from *V. cholerae* CVD 103-HgR

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (10^6 U)</th>
<th>Recovery (% cumulative)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate ppt*</td>
<td>314.0</td>
<td>51.2</td>
<td>100</td>
<td>1.6 x 10^5</td>
<td>1.0</td>
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<tr>
<td>Anion exchange</td>
<td>21.3</td>
<td>30.1</td>
<td>58</td>
<td>1.4 x 10^6</td>
<td>9.0</td>
</tr>
<tr>
<td>Cation exchange</td>
<td>2.38</td>
<td>17.1</td>
<td>33</td>
<td>7.2 x 10^6</td>
<td>45.0</td>
</tr>
<tr>
<td>Hydrophobic interaction</td>
<td>0.093</td>
<td>8.3</td>
<td>16</td>
<td>8.9 x 10^7</td>
<td>556.0</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>0.033</td>
<td>6.4</td>
<td>12</td>
<td>19.4 x 10^7</td>
<td>1,212.0</td>
</tr>
</tbody>
</table>

* ppt, precipitation.

were sufficiently similar to support a model of S-CEP as a toxin active as a single major polypeptide chain. Previously described methods were used to determine the amino-terminal sequence of S-CEP (20). Samples were applied to multiple tracks of polyacrylamide gradient gels as described above. After electrophoresis, the minigels were separated from the plastic backing and electrophoretically transblotted onto polyvinylidene membranes (20) (Pro-Blott; PE Biosystems, Foster City, Calif.). Proteins were visualized with Coomassie brilliant blue, and bands corresponding to an Mr of 79,000 were excised and applied to the reaction cartridge of a PE Biosystems Model 477A automated amino-terminal sequencer. Twenty cycles of Edman degradation yielded a single amino-terminal protein sequence comprising NH3-Ser-Ser-Gly-Ala-Val-Val-Glu-Ser-Tyr-Ile-Gln-Gln-. This sequence was compared to the preliminary genome sequencing project reported by The Institute for Genome Research (TIGR) and was found to be identical to a chromosomally located protein sequence database, and the homology of the *V. cholerae* sequence with that of a putative *Aeromonas* virulence factor associated with gastroenteritis (4), suggest that similar sequences may be prevalent among a range of *Vibrio* and *Aeromonas* strains, and possibly beyond.

The El Tor sequence homologue with an additional 2 kb of flanking sequence was analyzed for open reading frames. The resulting extrapolated protein sequence encompassing the N-terminal amino acids was compared with sequences in the GenBank and SWISS-PROT databases. A close match was found with the phospholipase A1 of *A. hydrophila* (GenBank accession no. AAC64133.1). Lower scores were found with a family of DNA sequences from extracellular lipases from *A. hydrophila*. Significant homology was also found with a cell-associated protein with similar activity isolated from El Tor *V. cholerae* Inaba JBK 70, although the El Tor N-terminal sequence possessed six additional residues at the amino terminus (XGDETN-) (17). No homology was found with the recently described novel *V. cholerae* cytotoxin of Walia et al. (27).

The thermal stability of S-CEP was studied by incubating toxin for 15 min at 21, 56, and 100°C. The effect of pH was evaluated by incubation at 4°C for 24 h at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, followed by assay for CHO cell activity. The effects of the proteases trypsin (1.0 mg/ml), chymotrypsin (1.0 mg/ml), papain (1.0 mg/ml), subtilisin (0.1 mg/ml), and thermolysin (0.1 mg/ml) on the activity of the toxin were determined after digestion for 4 h at 37°C, except for thermolysin, which is optimally active at 45°C (1-h incubation). Residual activities were determined using the CHO cell elongation assay as described above. Incubation at 100°C abolished toxin activity. The toxin displayed stability from pH 5.0 to 10.0, with a reduction to 50% activity at pH 4.0. S-CEP was resistant to papain and thermolysin, but lost 50% activity after incubation with trypsin or chymotrypsin and 99% activity after incubation with subtilisin.

Partially purified toxin comprised bulked, active fractions from the third stage of purification (i.e., after cation-exchange chromatography) and was assayed in the sealed infant mouse model. By using previously described methods (1, 13), infant mice (3 to 5 days of age, approximately 3 to 4 g in weight, five mice per sample) were fed 50 μl (containing 53,250 U of activity) of protein purified through step 3 suspended in Evans’ Blue (0.01% [wt/vol]). After incubation for 6 h, the animals were sacrificed and the intestine plus stomach weight of each animal was measured. The FA ratio was expressed as 1,000 times the ratio of the weight of the stomach plus intestine to...
the remaining body weight. The FA ratios representing means ± standard deviations were compared to those of negative controls fed 0.01% Evans’ Blue in phosphate-buffered saline and positive controls fed cholera toxin. The following data indicate that significant FA was elicited. FA ratios induced by the toxin (74.0 ± 11.88) were significantly higher than those induced by buffer (57.0 ± 1.23; P = 0.012, paired t test). In comparison, FA ratios induced by 0.5 μg of cholera toxin (500,000 U) had values of 93.2 ± 2.8 (P ≤ 0.001; paired t test).

The complex regulatory and structural interactions between V. cholerae and the human host have yet to be fully described. Poorly understood aspects of cholera remain, for example, nontoxicigenic mutants present an unexplained reactogenicity in the adult volunteer model (15, 16, 22–25), and numerous putative virulence determinants have been identified without a clearly prescribed role in disease (2, 7, 10, 16, 26, 27). A range of model systems have identified several products of V. cholerae as potential reactogenic factors; however, evaluation of numerous cholera vaccine candidates bearing mutations in putative toxins has yet to identify a role for any of these virulence factors in reactogenicity in the adult volunteer model (12).

Recently, Silva et al. (21) suggested that a host inflammatory response might account for the symptoms observed in vaccinees. To the inventory of potentially bioactive macromolecules expressed and secreted by V. cholerae can now be added the S-CEP described in this report. A determination of the significance, if any, of S-CEP as a specific cause of vaccine reactogenicity may be clarified in the adult volunteer model.

REFERENCES