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Purification and Characterization of an Extracellular Secretogenic Non-Membrane-Damaging Cytotoxin Produced by Clinical Strains of *Vibrio cholerae* Non-O1

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Some clinical strains of *Vibrio cholerae* non-O1 produce an extracellular factor that evokes a rapid and dramatic cytotoxic response which manifests as cell rounding of Chinese hamster ovary (CHO) and HeLa cells without accompanying membrane damage. This study was performed to establish the identity of the non-membrane-damaging cytotoxin (NMDCY), which was not inhibited by antitoxins against cholera toxin, heat-labile toxin of enterotoxigenic *Escherichia coli*, El Tor hemolysin, Shiga-like toxin I, and Shiga-like toxin II, indicating that NMDCY did not bear an apparent immunological relationship with the above toxins and hemolysin. Brain heart infusion broth and AKI medium supported the maximal production of NMDCY; culture supernatant of AKI medium was found to be free of hemolysin activity, whereas in brain heart infusion broth hemolysin was coproduced with NMDCY. Maximal production of NMDCY in AKI medium was observed at 37°C under shaking conditions with the pH of the medium adjusted to 8.5. NMDCY was purified to homogeneity by a three-step purification procedure which increased the specific activity of the cytotoxin by 1.7×10^5-fold. The denatured molecular weight of the purified toxin was 35,000, and the cytotoxin was heat labile and sensitive to trypsin. Purification of the cytotoxin revealed an enterotoxic activity as reflected by its ability to accumulate fluid in the rabbit ileal loop. Both the cytotoxic and enterotoxic activities of NMDCY could be inhibited or neutralized by antiserum raised against purified cytotoxin but not by preimmune serum. Immunodiffusion test between purified NMDCY and antiserum gave a single well-defined precipitin band which showed reactions of complete identity, while, in an immunoblot assay, a well-defined single band was observed in the 35-kDa region. Our results indicate that the cytotoxic and enterotoxic activities expressed by NMDCY appear to contribute to the pathogenesis of the disease associated with *V. cholerae* non-O1 strains which produce this cytotoxin.

For the past several years, we have been attempting to understand the pathogenic mechanism of the non-O1 serogroups of *Vibrio cholerae.* With the exception of the recently discovered serogroup O139 Bengal (31), the non-O1 serogroups of *V. cholerae* do not cause epidemics of cholera and are usually associated with sporadic cases of gastroenteritis and occasionally with extraintestinal infections (27). Among the epidemic-causing O1 and O139 serogroups of *V. cholerae*, cholera toxin (CT) has been identified as the major virulence factor (17, 30) which is primarily responsible for the clinical state of cholera. Studies conducted in our laboratory and elsewhere have shown that production of CT is an exception to the rule (29, 38) among the non-O1 non-O139 serogroups of *V. cholerae*. A variety of other putative virulence factors like CT-like enterotoxin (10, 52), El Tor hemolysin (7, 25, 51), Kanagawa hemolysin (13), Shiga-like toxin (34), and a heat-stable enterotoxin (NAG-ST [4]) have been proposed to explain the clinical manifestations of non-O1 gastroenteritis. A human volunteer study has demonstrated that, in the presence of adequate colonization factor(s), a NAG-ST-producing strain of *V. cholerae* non-O1 caused diarrhea of severity comparable to that seen with cholera (28). However, the frequency of occurrence of NAG-ST among clinical strains of *V. cholerae* non-O1 is low (36, 37), indicating that another, hitherto-unknown enterotoxigenic factor(s) may be involved in the causation of acute secretory diarrhea.

Active surveillance of cholera patients admitted to the Infectious Diseases Hospital, Calcutta, India, for the past several years has revealed that the prevalence of the non-O1 non-O139 serogroups is low among hospitalized cases (32, 39). However, in most instances, the clinical profile of patients infected with *V. cholerae* non-O1 non-O139 is virtually indistinguishable from that of patients with cholera (29). Thus, it became evident to us that the non-O1 non-O139 serogroups of *V. cholerae* are capable of causing a disease mimicking cholera in the absence of CT. This led us to hypothesize that a hitherto-unknown secretogenic factor(s) may be involved in inducing the cholera-like symptoms observed in patients infected with the non-O1 non-O139 serogroups. A search revealed that some clinical strains of *V. cholerae* non-O1 non-O139 produced a factor which evoked morphological changes of Chinese hamster ovary (CHO) and HeLa cells consistent with that of cell rounding or a cytotoxic response without accompanying membrane damage (29, 38). We performed this study to establish the identity of the non-membrane-damaging cytotoxin (NMDCY); to determine the best medium and appropriate cultural conditions for optimal production of the cytotoxin; to determine the immunological relationship of the cytotoxin with existing toxins produced by other enteric bacteria; and to purify, characterize, and ascertain the biological activity of this novel factor produced by some clinical strains of *V. cholerae* non-O1.

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MATERIALS AND METHODS

Bacterial strains. Three strains of *V. cholerae*, V59 (serogroup O5), V160 (O untypeable), and V249 (serogroup O26), isolated from hospitalized patients

admitted to the Infectious Diseases Hospital, Calcutta, between July 1989 and November 1991 with cholera-like diarrhea, were used in this study. These strains were biochemically characterized by the API 20E system (Bio Merieux Science, Montalieu-Vercieu, France) and serogrouped at the National Institute of Health, Tokyo, Japan (courtesy of Toshio Shimada). All three strains of *V. cholerae* non-O1 were previously documented to produce a cell-rounding effect without membrane damage on CHO, HeLa, and Vero cells (38). With specific DNA probes, the above strains were also found to be devoid of *ctx* (CT), *zot* (zonula occludens toxin), *ace* (accessory cholera enterotoxin), and *sto* (heat-stable enterotoxin) genes but were found to possess the structural gene (*hlyA*) for El Tor hemolysin (18, 22, 38, 47).

Optimization of growth conditions for production of the cytotoxin. (i) Medium. Different media such as brain heart infusion broth (BHIB), Casamino Acids yeast extract medium (CAYE) (2% Casamino Acids, 0.6% yeast extract, 0.871% K_2HPO_4, 0.25% NaCl, 0.25% glucose [pH 8.0 to 8.2] supplemented with 1 ml of filter-sterilized metal solution consisting of 5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$ per liter, dissolved in 0.001 N H_2SO_4), CAYE supplemented with lincomycin (CAYE-L) (lincomycin [Sigma Chemical Co., St. Louis, Mo.], 90 μg/ml), yeast extract-peptone water (YEP) (1.5% Bacto Peptone, 0.4% yeast extract, 0.5% NaCl), syncase medium (0.1% Casamino Acids, 0.5% Na_2HPO_4, 0.5% K_2HPO_4, 0.5% sucrose, 0.118% NH_4Cl, 0.0089% Na_2SO_4, and 1 ml of metal solution per liter), Mueller-Hinton broth, and AKI (14) medium (1.5% Bacto Peptone, 0.4% yeast extract, 0.5% NaCl, 0.3% filter-sterilized $NaHCO_3$ [pH 7.4]) were used to determine the most suitable medium for the production of NMDCY. T medium (0.58% NaCl, 0.37% KCl, 0.018% $CaCl_2 \cdot 2H_2O$, 0.01% $MgCl_2$, 0.0142% Na_2SO_4, 0.4% sucrose, 0.05% glucose) containing 500 μg of EDDA [ethylenediamine-di(*o*-hydroxyphenylacetic acid)] (Sigma) per ml as iron chelator was also used to examine whether the production of NMDCY was enhanced in an iron-depleted medium (45). All media and ingredients used for preparation of the media were from Difco Laboratories, Detroit, Mich.

(ii) Culture conditions. Three different culture conditions, namely, shaking, static, and both (6 h of static followed by 18 h of shaking [15]), were used to determine the optimal conditions for production of NMDCY. V249 was cultured in a test tube (preculture) for 4 h and subsequently cultured under different conditions in 50-ml conical flasks containing 5 ml of medium for 24 h. Shaking was performed in an orbital shaker (OSI-503; Firstek Scientific, Tokyo, Japan) at 120 rpm. For each culture condition, strains were incubated either at 30°C or at 37°C to determine the suitable incubation temperature for production of NMDCY.

(iii) pH. Upon determination of the suitable medium and optimal cultural conditions, the pH of the selected medium was adjusted along a range of 4.5 to 9.5 with 5 N HCl or 5 N NaOH. Strain V249 was examined at 37°C under shaking conditions to establish optimal pH supporting NMDCY production. Uninoculated medium was also concurrently introduced into the tissue culture assay as a control to assess whether the pH per se of the medium affected the cell line.

Assay for cytotoxic activity. The cytotoxic activity was assayed in 96-well flat-bottomed tissue culture plates (Nunc; Intermed, Roskilde, Denmark) with either CHO or HeLa cells. CHO cells were grown in Dulbecco modified Eagle medium (Gibco BRL, Grand Island, N.Y.) supplemented with 10% horse serum (Gibco) and penicillin G and streptomycin sulfate (Sigma) and incubated at 37°C in an atmosphere of humidified 5% CO_2–95% air (Haereus, Hanau, Germany). HeLa cells were grown under the same conditions but in Eagle's modified minimum essential medium (Gibco) containing 10% horse serum. After confluent growth of the cells in a 25-cm^2 flask, cells were washed twice with Hanks balanced salt solution (Gibco) and treated with 0.025% trypsin–EDTA (Sigma) solution. Approximately 10^3 trypsinized cells per well were dispensed in 96-well tissue culture plates and incubated for 24 h in a CO_2 incubator for confluent growth. Samples to be tested were filter sterilized (0.22-μm-pore-size filter; Millipore Corp., Bedford, Mass.) and were serially diluted in tissue culture medium containing 2% horse serum. After aspiration of medium from a 96-well plate, neat or diluted culture supernatant was added and incubated for 24 h in a CO_2 incubator and then examined with a phase-contrast inverted microscope (Hund, Wetzlar, Germany) to observe morphological changes. The titer of cytotoxic activity in a sample was defined as the reciprocal of the maximum dilution of the sample that rounded 100% of CHO or HeLa cells. The specific activity of a sample was defined as the ratio of the reciprocal titer of cytotoxic activity to the protein concentration. The viability of toxin-treated cells was determined by trypan blue dye exclusion technique.

Hemolysin assay. With 1% rabbit erythrocytes, hemolysin assay was performed as described previously (42). Culture supernatants of strain V249 grown in AKI medium and BHIB were serially diluted with phosphate-buffered saline (PBS) (pH 7.4), incubated with equal volumes of 1% rabbit erythrocytes and PBS at room temperature for 1 h, and then centrifuged at 800 × *g* for 10 min to remove the unlysed cells, and the released hemoglobin was assayed spectrophotometrically at 540 nm. Complete hemolysis (100%) was defined as the absorbance readings of the same number of erythrocytes lysed by water or detergent.

Secretion kinetics. Strain V249 was grown at pH 8.5 in AKI medium at 37°C under shaking conditions. At 2-h intervals, an aliquot of the growing culture was removed, filter sterilized (0.22-μm-pore-size filter; Millipore), and examined by tissue culture assay. Simultaneously, the bacterial pellet was washed twice with PBS containing 1 mM EDTA and 25 μm phenylmethylsulfonylfluoride (Sigma). Cells were then resuspended in 1 ml of the same buffer, and cell lysates were prepared by using an ultrasonic cell disrupter (Microson; Ultrasonics, Inc., Farmingdale, N.Y.). This was centrifuged, and the clear supernatant was filter sterilized and kept at −20°C. Concurrently, the growth curve of the strain was plotted by measuring the optical densities at $A_{560-600}$ by using a nephelometric flask in a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc., Long Island City, N.Y.).

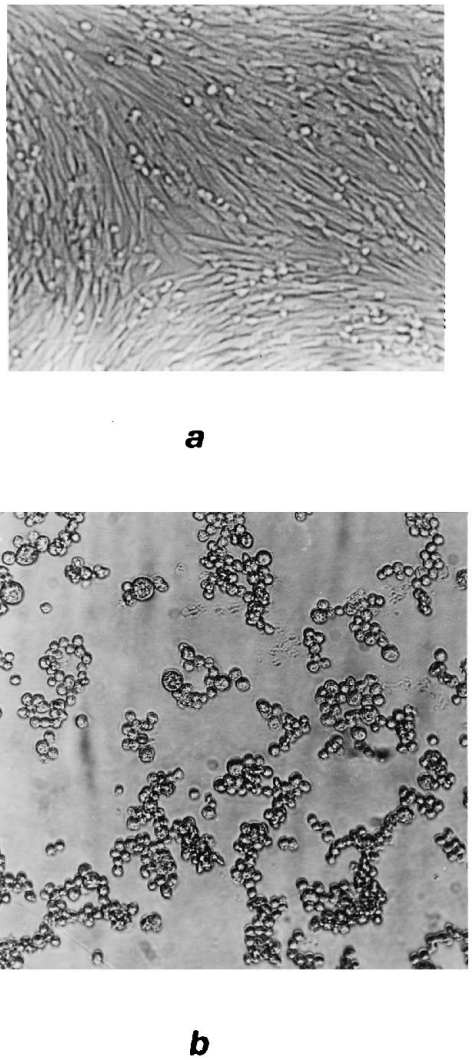

FIG. 1. Normal CHO cells (a) and cytotoxic effect which manifests as cell rounding without membrane damage evoked by culture supernatant of strain V249 (b).

Inhibition of cytotoxic activity with antitoxin antibodies. Antibodies against CT, labile toxin (LT) of enterotoxigenic *Escherichia coli*, El Tor hemolysin, Shiga-like toxin I (SLT-I), and Shiga-like toxin II (SLT-II) were used to determine if cytotoxic activity could be inhibited by the antitoxins. Antibodies against CT, LT, SLT-I, and SLT-II were made available by Yoshifumi Takeda, Research Institute, International Medical Center of Japan, Tokyo, Japan, while antibodies against El Tor hemolysin were made available by K. K. Banerjee, National Institute of Cholera and Enteric Diseases, Calcutta, India. All antibodies were previously determined by the donors to completely neutralize the activity of homologous toxin. Serially diluted culture supernatant was mixed with an equal volume of an excess amount of different antibodies separately and incubated for 2 h at 37°C. The incubated sample was then subjected to tissue culture assay to determine residual cytotoxic activity.

Inhibition assay with different monosaccharides. The inhibitory effect of different monosaccharides, namely, fucose, galactose, arabinose, rhamnose, mannose; *N*-acetylglucosamine, and *N*-acetylgalactosamine (Sigma) on cytotoxic activity of strain V249 was examined by the CHO cell assay as previously described (6). Filter-sterilized culture supernatants mixed with equal volumes of monosaccharides were serially diluted, incubated at 37°C for 3 h, and then introduced onto a confluent layer of CHO cells to measure the reciprocal titer. Sugar

TABLE 1. Evaluation of various media and cultural conditions for optimum production of the non-membrane-damaging cytotoxic factor produced by *V. cholerae* O26

Medium[a]	Reciprocal titer at temp and condition[b]:					
	37°C			30°C		
	Shaking	Stationary	Both[c]	Shaking	Stationary	Both[c]
BHIB	16	16	8	8	8	4
CAYE	0	0	0	0	0	0
CAYE-L	0	0	0	0	0	0
Syncase	0	0	0	0	0	0
AKI	16	4	4	8	4	4
YEP	4	4	8	4	2	2
MHB	8	0	4	4	2	2

[a] Media are defined in the text (Materials and Methods). MHB, Mueller-Hinton broth.

[b] The titer of NMDCY activity was defined as the reciprocal of the highest dilution which rounded 100% of CHO or HeLa cells; the titer was based on experiments repeated three times.

[c] This involved 6 h of incubation in a static condition followed by 18 h of shaking (15).

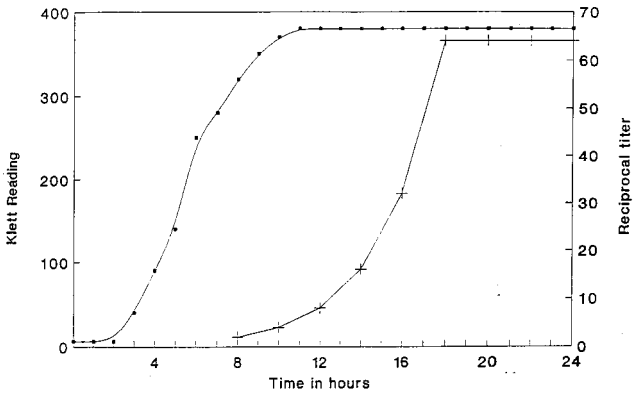

FIG. 2. The kinetics of cytotoxic activity manifested as cell rounding (+) by *V. cholerae* O26 (strain V249) and its growth curve (■) in AKI medium (pH 8.5) incubated at 37°C with shaking.

without culture supernatant was also added to CHO cells to determine if the sugar had any effect on the cell line.

Purification of NMDCY. Strain V249 was grown in AKI medium for 24 h at 37°C under agitation at 120 rpm in an orbital shaker (OSI-503; Firstek Scientific). Cells were harvested by centrifugation at 10,000 rpm for 20 min at 4°C with an RA-6 rotor (Kubota, Tokyo, Japan). The culture supernatant was concentrated 20-fold by ultrafiltration with a PM-10 membrane filter (Amicon, Inc., Beverly, Mass.). Concentrated supernatant was applied to an anion-exchange column (1.6 by 40 cm [Pharmacia LKB Biotech, Uppsala, Sweden]) of DE-52 (Whatman Laboratory, Maidstone, England) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.4) and then eluted at 4°C with the same buffer. Fractions exhibiting cell-rounding activity were pooled and concentrated by ultrafiltration with a PM-10 membrane. The concentrated DE-52 fractions were then subjected to hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (Sigma). The column (1.6 by 40 cm) was preequilibrated with 10 mM Tris-HCl buffer (pH 7.4) with 5% ammonium sulfate. The column was then washed with 2 bed volumes of the same buffer, and bound protein was eluted at 4°C with a linear gradient of 0 to 100% ethylene glycol at a flow rate of 10 ml/h. Fractions constituting the peak were pooled, dialyzed, concentrated, and then examined for cytotoxicity. The homogeneity of the active fractions obtained from hydrophobic interaction chromatography was analyzed by high-performance liquid chromatography (HPLC) on a reversed-phase column. The HPLC instrument used was a Beckman dual pump model 166 programmable solvent delivery module controlled externally by a computer (IBM PS/2 model 55SX) running System Gold software (Beckman Instruments, Inc., Fullerton, Calif.). The following method was programmed in this study: the silica-based hydrophobic C_{18} analytical column (Ultrasphere 5-μm spherical 80-Å [8 nm] pore, 4.6 by 250 mm [Beckman]) was equilibrated with 95% CH_3OH in 5% Tris-HCl buffer (pH 7.4), and then after injection of the sample, the column was developed at a flow rate of 1.0 ml/min for 20 min with 95% CH_3OH in 5% Tris-HCl buffer (pH 7.4) followed by a linear gradient of 20 to 80% CH_3OH in 10 mM Tris-HCl buffer (pH 7.4) for 60 min and finally allowed to run for another 40 min with 80% CH_3OH and 20% Tris-HCl, pH 7.4. At all steps of the purification and at other times, protein was estimated with the bicinchoninic acid protein assay kit at 37°C according to the manufacturer's instructions (Pierce, Rockford, Ill.). Bovine serum albumin was used as the standard.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (23) to analyze the purity of NMDCY. All chemicals used for this purpose were from Sigma Chemical Co. Samples in 10% glycerol–0.05% bromophenol blue–2% SDS–5% 2-mercaptoethanol–10 mM Tris-HCl buffer (pH 6.8) were run on a 12.5% polyacrylamide gel with a discontinuous buffer system at a constant voltage of 60 V for the stacking gel and 120 V for the resolving gel. Proteins were denatured by heating for 5 min in a boiling water bath. Proteins with known molecular weights (Pharmacia Biotech) were used as molecular weight markers and were fixed and stained with Coomassie blue.

Polyclonal antibody preparation. Antiserum to purified NMDCY was prepared by immunizing a New Zealand White rabbit by intramuscular and subcutaneous injections with 50 μg of NMDCY emulsified with an equal volume of Freund's complete adjuvant (Difco). This was followed by a booster injection with incomplete adjuvant (Difco) and two further boosters with NMDCY alone administered at 14-day intervals. Three days after the fourth injection, the animal was killed and bled. Serum was collected and stored at −20°C. The reactivity of the serum with purified NMDCY was examined by Ouchterlony immunodiffusion test.

Immunoblotting. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose membrane (0.45-μm pore size [Bio-Rad Laboratories, Richmond, Calif.]) electrophoretically (48) with a transblot apparatus (Bio-Rad) at 90 V for 1 h at 10°C in 20 mM Tris–150 mM glycine–20% (vol/vol) methanol, pH 8.3. Strips were then blocked with 3% nonfat dry milk (Bio-Rad) in Tris-buffered saline at 37°C for 2 h, washed, and incubated sequentially with polyclonal antibody against NMDCY (1:100 dilution [vol/vol]) and goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc.) conjugated to alkaline phosphatase (1:2,000 dilution [vol/vol]). Color was developed by 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (BCIP-nitroblue tetrazolium solution; Sigma).

Characterization of NMDCY. The following treatments were performed to further define NMDCY. (i) For trypsin treatment, 100 μg of the toxin per ml was incubated for 1 h at 37°C with trypsin (Sigma). Soybean trypsin inhibitor (Sigma) was added at a concentration of 5 μg/ml before performance of the tissue culture assay for NMDCY. The cytotoxic activity was then determined and compared with that of controls incubated with the same volume of medium or with trypsin that had previously been incubated at 37°C overnight with trypsin inhibitor. (ii) For heat treatment, samples of purified NMDCY were heated at 60, 70, 80, and 100°C for 10 min. The remaining cytotoxic activity was then determined as described above and compared with that of the unheated control. (iii) Inhibition studies were performed with the polyclonal anti-NMDCY antibody. Serial dilutions of NMDCY were inoculated in 96-well plates (50 μl per well). NMDCY was then preincubated for 4 h at 37°C with 50 μl of polyclonal antibody. After the preincubation, the mixtures were added to CHO cells and the assay was completed as described above. Inhibition of cytotoxic activity was determined by the ability of the antibody to abolish cell rounding compared with the control.

Ligated ileal loops in rabbits. The rabbit ligated intestinal loop assay was performed in young New Zealand White rabbits (about 2.0 kg) essentially as described by De and Chatterjee (11). Rabbits were subjected to fasting for 24 h prior to performance of the experiment. Animals were anesthetized with ketamine (Sigma). One-milliliter volumes of sample obtained at different stages of purification were injected into each 8-cm loop. Overnight culture of strain V249 in AKI medium was washed and resuspended in PBS (pH 7.4), and an inoculum of 1 ml containing approximately 10^6 organisms was introduced into the loop. AKI broth and PBS were used as the negative controls while commercially available pure CT (Sigma) was used as the positive control. Approximately 18 h later, the animals were sacrificed and the enterotoxic response was expressed as a volume-to-length ratio (fluid accumulation ratio) with a ratio greater than 1.0 indicating a strong positive response. A negative response was defined as loops with no fluid accumulation or a fluid accumulation ratio of less than 0.2. Neutralization of biological activity was performed by mixing undiluted anti-NMDCY serum and a solution (100 μg/ml) of purified NMDCY; the mixture was incubated for 60 min at 37°C, and then the rabbit ileal loop assay was performed as described above.

RESULTS

Description of tissue culture activity. Culture supernatants of all three strains of *V. cholerae* non-O1 exhibited a dramatic alteration in the morphology of CHO and HeLa cells consistent with that of cell rounding. There was complete disruption of the monolayer and dislodgement of tissue culture cells from

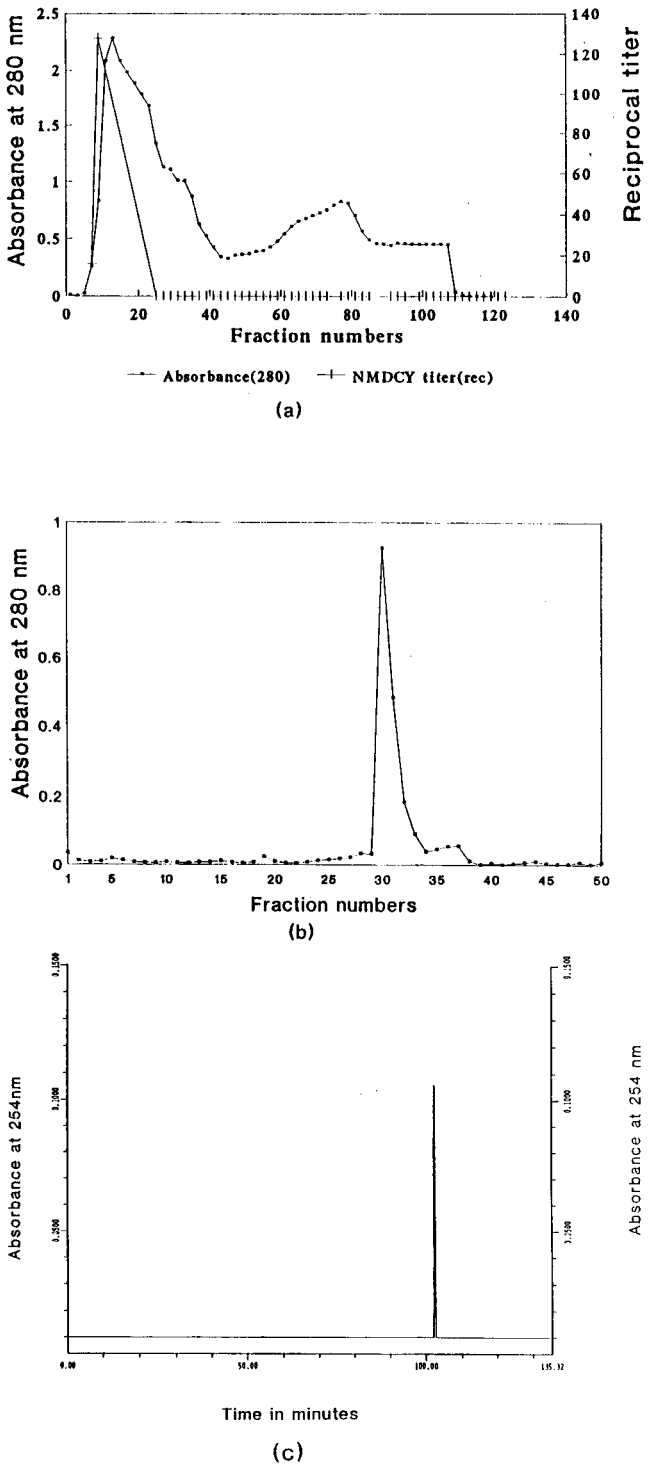

FIG. 3. Chromatographic profiles showing the purification of the *V. cholerae* O26 NMDCY from the culture supernatant of strain V249. (a) Anion-exchange chromatography of culture supernatant concentrated 20-fold by ultrafiltration with a PM-10 membrane on a DE-52 column; (b) hydrophobic interaction chromatography on phenyl-Sepharose CL-4B of pooled cytotoxic fractions from DE-52; (c) reversed-phase HPLC profile of NMDCY on a Beckman C_{18} silica-based analytical column.

the plate surface, but the change was not accompanied by membrane damage when examined by trypan blue dye. The onset of cytotoxicity was rapid (within 30 min to 1 h) but varied between strains and depended on the concentration of cytotoxin in the culture supernatant. The activity described above by all three strains of *V. cholerae* non-O1 could not be inhibited by antitoxins against CT, LT, SLT-I, SLT-II, and El Tor hemolysin, indicating that NMDCY did not bear apparent immunological relationships with the above toxins and hemolysin. Among the three strains, the culture supernatant of strain V249 exhibited the highest reciprocal cytotoxin titer and was therefore chosen for further investigation. The NMDCY activity exhibited by the culture supernatant of V249 could not be inhibited by simple monosaccharides examined in this study, indicating that the cytotoxin may not exhibit lectin-like activity. A representative photograph of the non-membrane-damaging cytotoxic effect evoked by the culture supernatant of V249 on CHO cells is shown in Fig. 1.

Optimum conditions for cytotoxin production. A variety of media and different cultural conditions including different incubation temperatures were assessed to determine which medium under what condition supported the optimum production of the cytotoxin when assayed in CHO cells. The amount of NMDCY produced was depicted as the reciprocal of the highest titer yielding 100% cell rounding of CHO cells. Strain V249 produced the highest titer of NMDCY in BHIB and AKI medium at 37°C (Table 1). In BHIB, the reciprocal titer of NMDCY was identical under both shaking and stationary conditions at 37°C, whereas in AKI medium, both at 30 and at 37°C, shaken culture yielded a higher titer of NMDCY. Significantly, the cytotoxin was not produced in CAYE, CAYE-L, and syncase medium both at 30 and at 37°C.

On the basis of the above, it was determined that both BHIB and AKI medium optimally supported the production of NMDCY. However, in BHIB a cytotoxic effect accompanied by membrane damage on CHO cells was observed in the lower dilutions, one which was not seen in AKI medium. Since strain V249 hybridized with a DNA probe specific for the structural gene (*hlyA*) of El Tor hemolysin (22), the membrane-damaging cytotoxic effect was attributed to the coproduction of the hemolysin. Consequently, the amount of hemolysin present in the culture supernatant of strain V249 grown in BHIB and AKI medium was determined. The culture supernatant of strain V249 grown in AKI medium was free of hemolytic activity as determined by its inability to lyse rabbit erythrocytes. In contrast, 10- and 40-fold dilutions of culture supernatant of strain V249 grown in BHIB yielded 100 and 20% hemolysis, respectively, of rabbit erythrocytes. It was, therefore, evident that BHIB also supported the production of hemolysin as well as cytotoxin. In this study, we were interested in characterization of the cytotoxin and therefore opted to grow strain V249 in AKI medium at 37°C under shaking conditions for optimum production of the cytotoxin. Under these conditions, it was clear that AKI medium with the pH adjusted to 8.5 optimally supported NMDCY production. NMDCY was not enhanced by being grown in T medium under iron depletion conditions but was expressed at a much lower titer compared with AKI medium.

Time course of cytotoxin production. The kinetics of the cytotoxin production by strain V249 in AKI medium were examined at 37°C under the shaking condition. Cytotoxic activity became discernible at the late logarithmic phase of growth (8 to 9 h) and continued to increase even into the stationary growth phase (till 17 h), after which it plateaued. Secretion of NMDCY, therefore, corresponded to growing and stationary growth phase cells as reflected by the growth curve

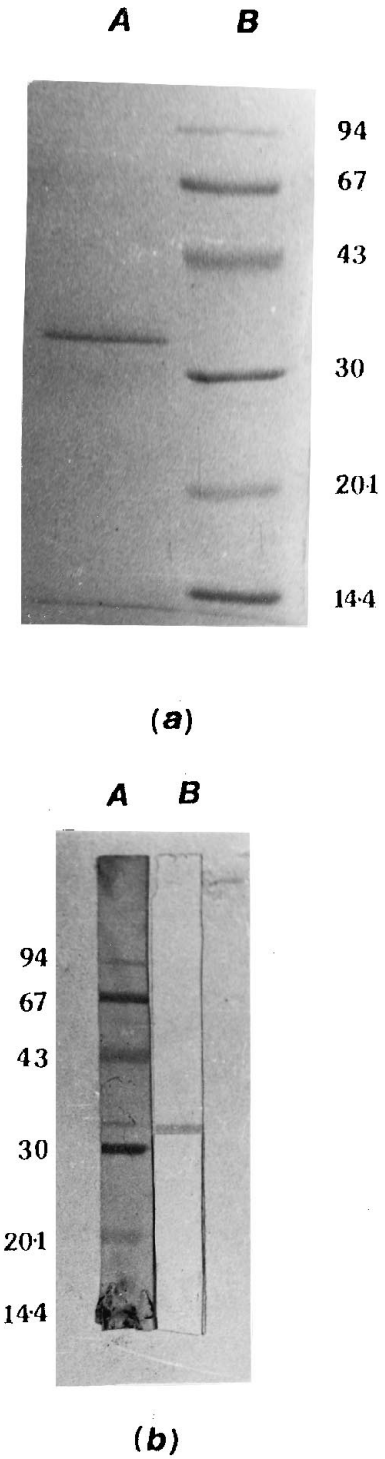

FIG. 4. (a) SDS-PAGE (12.5% acrylamide) of purified NMDCY obtained after hydrophobic interaction chromatography (lane A) and migration of marker proteins of known molecular mass (in kilodaltons) (lane B). (b) Immunoblot (Western transfer) of *V. cholerae* O26 cytotoxin. Purified cytotoxin after SDS-PAGE was transferred to nitrocellulose membrane electrophoretically with the Bio-Rad Trans-Blot electrophoretic transfer cell. The nitrocellulose paper was reacted with rabbit anti-NMDCY (lane B). Preimmune serum showed no reactivity. Lane A contains molecular mass markers (in kilodaltons).

(Fig. 2). The cell lysate of *V. cholerae* V249 prepared from cells harvested at different time periods did not evoke a cell-rounding effect on CHO cells.

Purification of cytotoxin. The cytotoxin in the supernatant of strain V249 was concentrated 20-fold by ultrafiltration and applied to an anion-exchange column. The elution pattern from this column is shown in Fig. 3a, from which it is clear that fractions exhibiting activity remained unabsorbed and eluted in the void volume while a substantial amount of contaminants eluted in the later fractions. Fractions exhibiting activity were pooled, again concentrated, and further subjected to hydrophobic interaction chromatography. Elution curves from this stage of purification are shown in Fig. 3b, from which it is clear that the cytotoxin eluted as a single peak at around 40 to 50% of ethylene glycol. At this stage, the specific activity of the toxin increased about 1.73×10^5-fold, and the final recovery rate was about 22%. Table 2 summarizes the typical data on the purification of NMDCY, from which it is clear that the specific activity of the cytotoxin gradually increased with the decline in the amount of protein, suggesting true purification of the factor. The homogeneity of 20 μl (0.2 μg) of the purified cytotoxin obtained from hydrophobic interaction chromatography was analyzed by reversed-phase HPLC, and from the profile, it was evident that the cytotoxin eluted as a sharp single peak (Fig. 3c). Analytical SDS-PAGE under denaturing conditions was performed to analyze the structural configuration and the purity of the cytotoxin. Samples of active fraction obtained after hydrophobic chromatography revealed that the cytotoxin yielded a single band in approximately the 35-kDa region (Fig. 4a).

Immunological characterization. Polyclonal antibody raised against purified cytotoxin in a rabbit showed a reciprocal titer of 32 in the immunodiffusion test. When the polyclonal antibody was used in an immunoblot assay with the cytotoxin antigen, a well-defined single band (Fig. 4b) was observed in the 35-kDa region. Preimmune rabbit serum used in the immunoblot experiment as the negative control showed no reactivity. Immunodiffusion test carried out between purified cytotoxin as antigen and the polyclonal antibody gave a single well-defined precipitin band against the toxin and showed reactions of complete identity (Fig. 5). The inhibition experiment revealed that the polyclonal antibody could completely inhibit the cytotoxic activity of crude culture supernatant of strain V249 and of purified cytotoxin on CHO cells. Further, polyclonal anti-NMDCY serum also inhibited the activity exhibited by the culture supernatants of the other two non-O1 strains (V59 and V160) on CHO cells.

Biological activity. The biological activity of the purified cytotoxin and the various fractions obtained during purification of NMDCY is shown in Table 3. In its crude state, the cytotoxin did not evoke fluid accumulation in the ligated loops of rabbits. However, upon purification the enterotoxic activity of the cytotoxin progressively increased but the fluid secretion was not hemorrhagic in nature. However, in comparison with CT, which was used as the positive control, 200 times more cytotoxin was required to evoke a positive fluid accumulation ratio. Interestingly, while the culture supernatant per se did not produce fluid accumulation, whole cultures of strain V249 when introduced into the loop evoked hemorrhagic fluid secretion. The secretory effect of purified NMDCY was completely blocked by prior incubation of the toxin for 60 min at 37°C with anti-NMDCY serum raised in a rabbit.

Physiochemical properties of purified NMDCY. The cytotoxic activity of the purified cytotoxin was unaffected on heating at 60°C for 10 min but did not retain activity on heating at 70°C and above for 10 min. Treatment of purified cytotoxin

TABLE 2. Summary of purification of NMDCY produced by *V. cholerae* O26 (strain V249)

Stage	Purification step	Vol (ml)	Protein		Cytotoxic activity in CRU[a]		Sp act	Recovery (%)	Fold purification
			mg/ml	Total mg	CRU/ml	Total CRU			
1	Crude culture supernatant	2,000	1.8	3,600	3.2×10^2	6.4×10^5	1.78×10^2	100	
2	Ultrafiltration with PM-10	100	5.6	560	4.3×10^3	4.32×10^5	7.71×10^2	67.5	4.3
3	Anion-exchange chromatography with DE-52	150	0.4	60	1.44×10^3	2.16×10^5	3.6×10^3	33.75	20.75
4	Hydrophobic interacting chromatography with phenyl-Sepharose CL-4B	8	0.1	0.8	1.73×10^4	1.4×10^5	1.73×10^5	21.6	972.04

[a] CRU, cell rounding unit, defined as the reciprocal of the highest dilution causing 100% rounding.

with trypsin resulted in complete loss of cytotoxic activity. The amount of toxin introduced into the cell line determines the reversibility of the effect of the cytotoxin. For example, 64-fold-diluted culture supernatant of strain V249 induced a cell-rounding effect after 24 h, which could be reversed within 4 h after removal of tissue culture medium. However, at a lower dilution (16-fold) the toxic activity on CHO cells was not reversible.

DISCUSSION

Considering the multiplicity of extracellular products produced by *V. cholerae* non-O1, it was important to initially determine that the cytotoxin being examined in this study was distinct from those already reported. To begin with, the morphological changes affected by the cytotoxin under study on CHO and HeLa cells were distinctly different from the cytotoxic activity exhibited by Shiga toxin on Vero cells (19, 21), the cytotoxic activity of hemolysins on tissue culture cell lines (5), the cell-rounding cytotonic effect of CT on Y1 adrenal cells (12), the cytolethal distending effect exhibited by a variety of enteropathogens (16), or the activity of the toxin which induces vacuolation of eukaryotic cells (9). The morphological changes on CHO and HeLa cells brought about by the *V. cholerae* non-O1 cytotoxin most closely resembled the cytopathic effect of toxins of *Clostridium difficile*, which does not involve cell damage when examined by trypan blue exclusion in in vitro studies and which manifests as rounding up of CHO and T-84 colonic carcinoma cells (24).

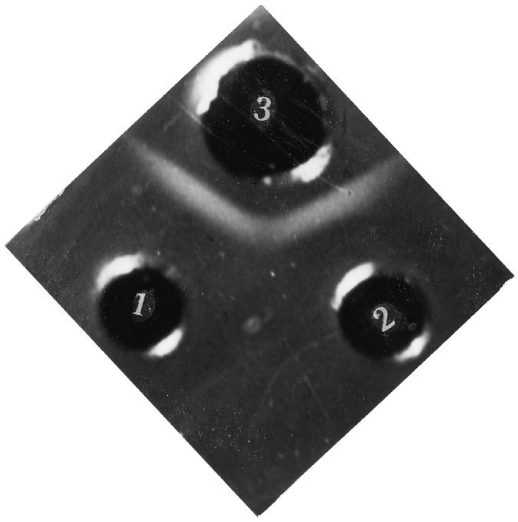

FIG. 5. Immunodiffusion test of NMDCY of *V. cholerae* O26 (strain V249). Well 1, culture supernatant of strain V249 grown in AKI medium; well 2, purified NMDCY; well 3, NMDCY antitoxin from an immunized rabbit.

Further evidence of the uniqueness of *V. cholerae* non-O1 cytotoxin came from the fact that none of the antitoxins against some of the currently known toxins and hemolysin inhibited the non-membrane-damaging activity elicited by the non-O1 cytotoxin. It was also clear from this study that medium influenced the production of NMDCY with media such as syncase, CAYE, and CAYE-L (all of which support CT production [15]) not supporting the production of NMDCY. This again would indicate that NMDCY is different from CT and CT-like toxins. That NMDCY was not in any way related to the El Tor hemolysin was also evident because AKI medium supported production only of NMDCY, not of the hemolysin. It was important to determine this, especially in view of the existing knowledge that classical strains of *V. cholerae* O1 produce a 27-kDa nonhemolytic but cytotoxic product which, in effect, is a truncated product of the El Tor hemolysin produced by the El Tor biotype of *V. cholerae* O1 (1, 2). It has been documented that some strains of *V. cholerae* non-O1 produce a bacterial cell-associated, heat-stable material that is cytotoxic for HeLa cells and that the cytotoxicity is completely neutralized by antibody to Shiga toxin (34). NMDCY was not related to this cytotoxin in that it is an extracellular product which was not neutralized by antibodies to both SLT-I and SLT-II.

Therefore, we decided to purify and characterize the non-O1 cytotoxin for the following reasons: (i) NMDCY of *V. cholerae* non-O1 was distinct from existing enteric toxins and hemolysin; (ii) NMDCY-producing *V. cholerae* non-O1 strains were the sole infecting agents in patients with cholera-like diarrhea; and (iii) the cytotoxic activity was rapid, dramatic and pronounced and appeared to be related to the pathogenesis. We have isolated and purified to homogeneity a 35,000-molecular-weight (denatured) cytotoxin from culture supernatant of strain V249 grown in AKI medium by a three-step purification procedure. The cytotoxic activity was determined to be heat labile, and the purified cytotoxin was quite unstable with a rapid decline in activity even when stored at 4°C. Further, the cytotoxin was sensitive to trypsin treatment, indicating that the active component is protein in nature. The cytotoxin had no subunits as determined by SDS-PAGE. Interestingly, progressive purification of the cytotoxin revealed an enterotoxic activity as reflected by its ability to accumulate fluid in the rabbit ileal loop. From the results of this study, the enterotoxic activity of NMDCY was 200-fold less than CT; in its crude state, NMDCY did not elicit any fluid accumulation. The enterotoxic activity of NMDCY was 100-fold less than the reported enterotoxic activity of Shiga toxin (35), 1.4-fold lower than the enterotoxic activity of a variant of SLT-IIv (26), and 10-fold less than the enterotoxic activity of toxin A of *C. difficile* (24). Immune rabbit serum to the isolated cytotoxic enterotoxin was

TABLE 3. Biological activity of the various fractions obtained during purification of NMDCY

Fraction no.	Sample	Amt of protein/no. of cells	Cytotoxic activity (CRU[a]/ml)	FA[b] ratio (mean ± SE; $n = 5$)
1	PBS (pH 7.4)			0
2	CT	0.5 μg		1.00 ± 0.07
3	V249 strain	10^7 organisms		1.3 ± 0.12
4	Culture supernatant of strain V249	1.8 mg/ml	3.2×10^2	0
5	NMDCY after anion-exchange chromatography	0.4 mg/ml	1.44×10^3	0.625 ± 0.04
6	Purified NMDCY	100 μg/ml	1.73×10^4	0.9 ± 0.08

[a] CRU, cell rounding unit; see footnote *a* in Table 2 for more information.
[b] FA, fluid accumulation.

used in immunoblots, and the antiserum was determined to be highly specific for the enterotoxic cytotoxin and was capable of inhibiting tissue culture activity and neutralizing biological activity in the rabbit ileal loop.

Enteric pathogens are known to produce both a cytotonic enterotoxin and a cytotoxic enterotoxin. CT is the prototype of a cytotonic enterotoxin while cytotoxins which elicit enterotoxic activity include Shiga toxin (21) and the cytotoxic enterotoxins of *Aeromonas* spp. (33, 40), *Salmonella* spp. (20), *C. difficile* (24), and *Bacteroides fragilis* (50). NMDCY appears to belong to the cytotoxic enterotoxin family and within this family to the non-membrane-damaging type like that of cytotoxic enterotoxins of *C. difficile* and *B. fragilis. C. difficile* toxin is known to exert a cytopathic effect, but the toxin-treated CHO or T84 cells remain viable even after 48 h (24). Likewise, concentrated bacterium-free culture supernatant of *B. fragilis* is known to exert a transient cytotoxic response on colon carcinoma cell line HT-29 which causes striking morphologic changes including loss of cell-to-cell attachments, rounding, swelling, and, in some cases, pyknosis (50). The *C. difficile* and *B. fragilis* toxins possess both cytotoxic and enterotoxic activities (46, 49, 50). It must be emphasized, however, that the similarity between NMDCY of *V. cholerae* O26 and the cytotoxic enterotoxins of *C. difficile* and *B. fragilis* is related only to the morphological changes that these toxins elicit on cell culture; the toxins differ substantially in their physical and basic molecular properties. The possibility of these cytotoxic enterotoxins which do not cause cell membrane damage belonging to a family needs to be investigated.

To our knowledge, this work represents the first purification to homogeneity of an NMDCY of *V. cholerae*. The *hlyA* gene is the most conserved genetic element among the virulence genes of *V. cholerae*, as has been documented recently (22), and it appears that production of El Tor hemolysin (or its truncated product), which has a membrane-damaging cytotoxic effect on different cell lines, obliterates or masks NMDCY. If grown in a suitable medium which inhibits production of the hemolysin, then it appears that a considerable percentage of clinical strains of *V. cholerae* non-O1 produce NMDCY (29). By using a sandwich monoclonal-polyclonal antibody-based enzyme-linked immunosorbent assay, it has been determined that 51.9% of the 368 strains of *V. cholerae* belonging to various serogroups examined produced NMDCY at varying concentrations; particularly significant was that 81.3% of the clinical strains of *V. cholerae* belonging to serogroup O1 produced this enterotoxic cytotoxin (43). A similar cell-rounding effect displayed by the non-O1 strains examined in this study has been observed in the extracellular products of environmental strains of *V. cholerae* non-O1 (3) and among fish strains of *Aeromonas* spp. (44) on cell lines of poikilothermic and homoiothermic species, and this has been reported as a cytotoxic effect with weak enterotoxic activity. Multifunctional cytotoxins exhibiting hemolytic and enterotoxic activity in addition to cytotoxicity have been purified from clinical strains of *Aeromonas* species. For instance, Chopra et al. (8) have identified a CT cross-reactive factor now referred to as cytolytic enterotoxin while Rose et al. (41) have purified a cytolytic enterotoxin that possesses an antigenic moiety that cross-reacts with CT.

Our data lead us to conclude that NMDCY, because of its enterotoxigenic potential, has a role in the pathogenesis of *V. cholerae* non-O1 infections. This, coupled with the proven antecedence of the association of cytotoxic enterotoxins with diarrhea, leads us to believe that NMDCY may constitute a covert virulence element in the whole cascade of events which enable the organism to precipitate the disease. At this point, however, we are unable to correlate the relevance of the cytotoxic activity with fluid accumulation in the ligated ileal loop. As proposed for the cytolytic enterotoxin of *Aeromonas hydrophila* (41), NMDCY appears to be a protein molecule with an activity that may manifest itself as cytotoxic or enterotoxic depending on the specificity of the target tissue, or this molecule may have one biological active site for cytotoxicity and another for enterotoxicity. We are currently developing an immunodetection assay to investigate the distribution of NMDCY among strains of *V. cholerae.*

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