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Characterization of non-membrane-damaging cytotoxin of non-toxigenic *Vibrio cholerae* O1 and its relevance to disease

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Abstract

The non-membrane-damaging cytotoxin which causes dramatic cell rounding of cultured HeLa cells was purified to homogeneity from a clinical strain (WO5) of non-toxigenic *Vibrio cholerae* O1 Inaba belonging to the El Tor biotype. The purified protein has a denatured molecular weight of 35 kDa and a native molecular weight of approximately 37 kDa indicating the monomeric nature of the protein. The 15 N-terminal amino acid sequence of non-membrane-damaging cytotoxin showed complete homology to the hemagglutinin protease previously purified and characterized from *V. cholerae* O1. Purified non-membrane-damaging cytotoxin from *V. cholerae* O1 was immunologically and biochemically identical to that previously purified from *V. cholerae* O26. Non-membrane-damaging cytotoxin was found to be enterotoxic in rabbit ileal loop assay inducing accumulation of non-hemorrhagic fluid at 100 μg and elicited a concentration dependent increase in short circuit current and tissue conductance of rabbit ileal mucosa mounted on Ussing chambers. A significant serum immunoglobulin G response against non-membrane-damaging cytotoxin was elicited by patients infected with *V. cholerae* O139 but not with *V. cholerae* O1. These properties make non-membrane-damaging cytotoxin a potential virulence factor of *V. cholerae* which should be taken into consideration while making live, attenuated recombinant vaccine strains against cholera. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Vibrio cholerae*; *El Tor biotype*; Non-membrane damaging cytotoxin; Cholera; Soluble hemagglutinin protease; Secretory activity; Diarrhoea

1. Introduction

Cholera, has plagued the world sporadically through recorded history. The disease has assumed pandemic proportions on several occasions since 1817 and is still a serious global threat. The O1 and O139 serogroups of *Vibrio cholerae* are associated with epidemic cholera while the other serogroups collectively known as non-O1 non-O139 serogroups are associated with sporadic cases of diarrhea and are ubiquitously found in the aquatic

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environment [1,2]. Toxigenic strains of *V. cholerae* O1 or O139 produce cholera toxin (CT), the activity of which largely accounts for the secretory diarrhea that is the hallmark of cholera [3]. A variety of other putative virulence factors, such as CT-like enterotoxin [4,5], El Tor hemolysin [6–8], Kanagawa hemolysin [9], shiga-like toxin [10] and heat-stable enterotoxin [11] are produced by some strains of *V. cholerae*, but their role in causing the disease is not adequately understood.

Despite the wealth of information currently available on the pathogenesis of *V. cholerae*, the mechanism by which non-toxigenic (NT) *V. cholerae* O1 and O139 (NT alludes to the absence of CT genes and therefore unable to produce CT) or clinical strains of *V. cholerae* non-O1 non-O139 devoid of CT genes are still capable of causing acute secretory diarrhea clinically indistinguishable from cholera caused by toxigenic *V. cholerae* O1 or O139 [12,13], continues to remain an enigma. The residual diarrhea caused by most of the recombinant oral cholera vaccine strains in human volunteer studies suggests the presence of as yet unknown toxin(s) capable of initiating a secretory response.

While investigating the cause of diarrhea due to NT *V. cholerae* O1 and O139 and due to clinical strains of non-O1 non-O139 *V. cholerae*, and the residual diarrhea caused by recombinant vaccine strains of *V. cholerae*, we found that certain clinical strains of *V. cholerae* non-O1 non-O139 produced a cytotoxic factor which manifested as rapid rounding of CHO and HeLa cells [12]. In subsequent studies, this cell-rounding factor was purified; the activity was due to a protein which, although 200-times less potent than CT, induced fluid accumulation in the rabbit ileal loop [14]. Interestingly, the cell-rounding factor designated as a non-membrane-damaging cytotoxin (NMDCY), was found to be widely distributed in strains of *V. cholerae*, irrespective of serogroup and also among strains of *V. parahaemolyticus*, *Aeromonas* spp. and *Shigella* spp. [15]. In the present study, we assessed the importance of NMDCY in *V. cholerae* belonging to the O1 serogroup. Our objective was to determine the biochemical and immunological relatedness between NMDCY purified from O1 and that previously purified from O26 [14], to compare the N-terminal homology of NMDCY from the O1 strain with known proteins and finally to confirm its enterotoxic property using in vivo and in vitro models.

2. Materials and methods

2.1. Bacterial strain and purification of the cell-rounding factor

An NT strain of *V. cholerae* O1 Inaba biotype El Tor (WO5) isolated during an outbreak and previously documented to produce a factor which caused rapid cell rounding of cultured HeLa and CHO cells was used in this study [13]. NMDCY from WO5 was purified using procedures identical to that used for purifying NMDCY from *V. cholerae* non-O1 non-O139 [14]. The native molecular weight was determined by loading purified protein (250 μg) onto a Sephacryl S200-HR (Sigma) column (1.6×40 cm Pharmacia) prequilibrated with Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl.

2.2. Immunoblot and inhibition of cell rounding by polyclonal antibody against NMDCY

Antiserum to the purified NMDCY from strain WO5 was prepared by immunizing a New Zealand white rabbit as described previously [14]. Inhibition of cytotoxic activity determined by the ability of the antibody to inhibit cell rounding compared with the control were performed with polyclonal anti-NMDCY prepared using NMDCY purified from *V. cholerae* O26 [14] and with polyclonal antibody raised against purified protein prepared in this study. Purified NMDCY from *V. cholerae* O26 incubated with immune and preimmune sera were used as positive and negative controls, respectively.

2.3. Amino acid sequencing

The N-terminal sequencing of the transblotted purified protein was performed using a precise Applied Biosystems 494 protein sequencer running a pulsed liquid blot sequencing program (Applied Biosystems Foster City, CA, USA) at the Sequencing Facility of MacQuarie University, Australia. The homology search was done with the SWISS PROT and NBRF-PIR protein sequence libraries.

2.4. Characterization of purified NMDCY

The following treatments were performed to further define the protein.

Trypsin treatment and chymotrypsin treatment were performed as described previously [14].

2.4.1. Hemagglutination (HA) assay

The technique for quantitation of HA was adapted from Jones et al. [7] using protein solution (100 μg ml^{-1}) two-fold serially diluted. Purified HA from a *V. cholerae* O2 strain was obtained from K. Banerjee, NICED, Calcutta and used as the positive control [16].

2.4.2. Measurement of protease activity

Casein was chosen as the substrate to assay proteolytic activity. The substrate–enzyme (Azocasein–protein) mixture was incubated at 37°C and the reaction terminated with 10% (w/v) trichloroacetic acid after 1 h. The precipitated protein was removed by centrifugation (12 000 × *g* for 4 min) and the supernatant transferred to a clean tube containing 525 mM NaOH. Absorbance was measured at 440 nm using a scanning spectrophotometer (Cecil Instruments Limited, Cambridge, London). Controls were substrate and buffer without enzyme and substrate and buffer with heat-inactivated enzyme. Inhibition study was performed by incubating the protein with Tris-HCl buffer containing 5 mM EGTA and EDTA, respectively, prior to the addition of the substrate.

2.5. Rabbit ileal loop assay

The ligated intestinal loop test was performed according to the method described by De and Chatterjee [17].

2.6. Ussing chambers experiment

The in vitro enterotoxic activity of NMDCY purified from *V. cholerae* O1 was tested by Ussing chambers experiment [18]. Purified protein at different concentrations was added to rabbit ileal tissue mounted on Ussing chambers. The change in I_{sc} (the amount of current needed to nullify the potential difference (PD)) and the PD (the difference in voltage measured on the mucosal side versus the serosal side of the tissue) were recorded at a 5-min interval for the first reading and then at 15-min intervals for up to 110 min [19]. At the end of every experiment, 200 μl of 0.5 M glucose was added to the mucosal side of each chamber. Only those tissues which showed an increase in I_{sc} in response to glucose indicating tissue viability were included in the analysis. From these measurements, G_t (tissue conductance) was calculated using Ohm's law: $I_{sc} = PD \times G_t$.

2.7. Serum antibody response

Acute and convalescent phase sera were collected from 16 cholera patients infected by either *V. cholerae* O1 or O139. Immunoglobulin G (IgG) specific

Table 1
Summary of purification of NMDCY produced by *V. cholerae* O1 strain WO5

State	Purification step	Volume (ml)	Protein conc. ($mg\ ml^{-1}$)	Total protein (mg)	Cytotoxic activity in CRU[a]		Specific activity	Recovery	Fold purification
					CRU/ml	Total CRU			
1	Crude culture supernatant	2000	1.7	3400	1.6×10^2	3.2×10^5	0.5×10^3	100	
2	60% Ammonium sulphate precipitation	80	5	400	1.28×10^3	1.02×10^5	0.25×10^3	31.85	5
3	Anion exchange chromatography with DE52	100	0.3	30	0.64×10^3	0.64×10^5	0.021×10^5	20	42
4	Hydrophobic interactive chromatography with phenyl Sepharose CL-4B	4	0.08	0.32	5.12×10^3	20.48×10^3	64.0×10^3	6.4	1280

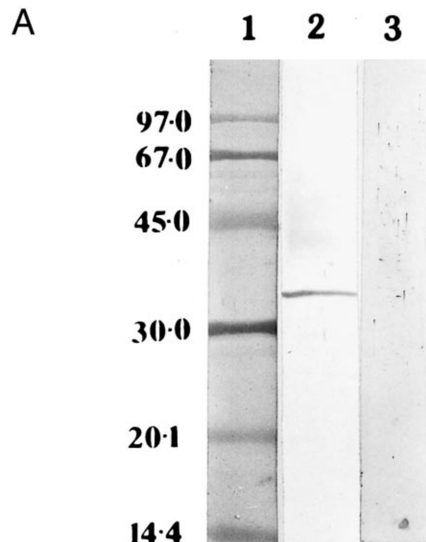
[a]CRU, cell-rounding unit, defined as the reciprocal of the highest dilution causing 100% rounding.

antibody responses in the paired sera were determined against purified NMDCY by ELISA in microtitration plates (NUNC, Rocksville, Denmark). Wells were coated with 100 μl of NMDCY at a concentration of 1 μg ml^{-1} in 10 mM PBS and 100 μl of purified CT (Sigma) at a concentration of 1 μg ml^{-1} in 10 mM PBS separately. Serial doubling dilutions of patients' sera in 1% BSA (100 μl per well) were added in triplicate for titration. Binding of the antibody to the antigen was visualized by adding 100 μl of *O*-phenylenediamine dihydrochloride (1 mg ml^{-1}; Sigma) as substrate per well. The plates were read spectrophotometrically at 492 nm using an microplate reader (Titertek; Flow Laboratories, Helsinki, Finland) and the titer was expressed as the reciprocal of the highest dilution of antiserum that showed an OD_{492} value of ≥0.2 in the assay. The root of the product of the titer showing the highest dilution was the geometric mean titer (GMT). A 3–4-fold increase in the antibody titer of the convalescent serum as compared to that of the acute phase serum was considered as a positive response.

3. Results and discussion

The NMDCY produced by *V. cholerae* O1 Inaba strain (WO5) El Tor biotype was purified to homogeneity by column chromatographies which were previously established for purification of NMDCY from *V. cholerae* O26 [14] (Table 1). NMDCY from O1 was found to be heat-labile, sensitive to trypsin and chymotrypsin and has a denatured molecular weight of 35 kDa. These properties are identical to that exhibited by NMDCY purified from O26 [14]. The native molecular mass of the purified protein determined by gel chromatography was approximately 37 kDa, suggesting the monomeric nature of the protein.

By Western blot analysis, the antiserum raised against NMDCY purified from WO5 recognized only a single band of 35 kDa and no other secreted *V. cholerae* constituent (Fig. 1A). Under the assay conditions used, a 1:64 dilution of the antiserum completely neutralized cell-rounding activity of the supernatant of *V. cholerae* WO5, whereas preimmune serum lacked the neutralizing activity. Furthermore, polyclonal antibody raised against O1



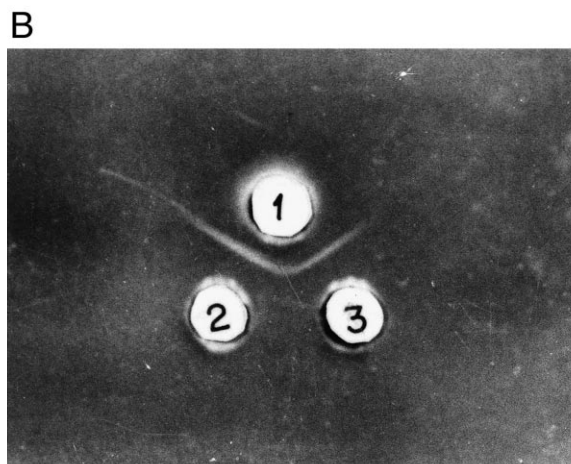


Fig. 1. (A) Immunoblot (Western transfer) of *V. cholerae* O1 NMDCY. Lane 1, molecular mass markers (in kDa); lane 2, purified NMDCY reacted with rabbit anti-NMDCY on a nitrocellulose paper; lane 3, total cell protein of strain WO5 reacted with rabbit anti-NMDCY on a nitrocellulose paper. (B) Immunodiffusion test of NMDCY of *V. cholerae* O2 and O26. Well 1, purified NMDCY from *V. cholerae* O1; well 2, *V. cholerae* O1 NMDCY anti-toxin from an immunized rabbit; well 3, *V. cholerae* O26 NMDCY antitoxin from an immunized rabbit.

NMDCY also inhibited the cell-rounding activity of both purified proteins, from *V. cholerae* O1 and O26. Immunologically, NMDCY from *V. cholerae* O1 cross-reacted with NMDCY purified from *V. cholerae* O26 as seen by the complete identity of

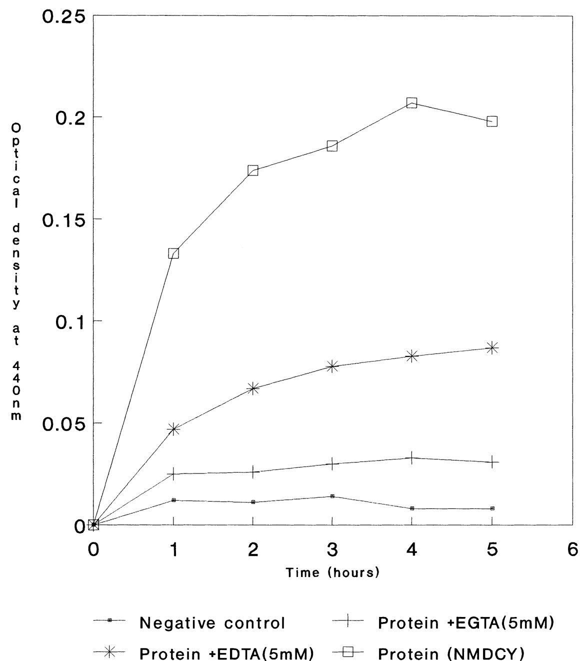


Fig. 2. Time course of protease activity. Protease activity was determined with azocasein as the substrate. The assay was performed exactly as described in Section 2. Each time point represents the mean of four determinations.

bands in Ouchterlony's double diffusion experiment (Fig. 1B). The cell-rounding effect exhibited by the purified protein was abolished by heating at 65°C for 10 min. NMDCY, therefore, belongs to the family of heat-labile toxins. The effect of protein inhibitors, such as chymotrypsin and trypsin, was also pronounced as incubation with trypsin and chymotrypsin for 1 h at 37°C resulted in the loss of cell-rounding activity.

The first 15 amino-terminal amino acids of the purified protein was determined as Ala Gln Ala Thr Gly Thr Gly Pro Gly Gly Asn Gln Lys Thr Gly. When compared with the SWISS PROT and NBRF-PIR protein sequence libraries, the N-terminal 15 amino acid residues of O1, NMDCY showed complete homology with the N-terminal 15 residues of the soluble hemagglutinin protease (HA/P) of *V. cholerae* O1 first purified by Finkelstein and Hanne [20,21]. However, unlike HA/P, NMDCY purified from strain WO5 did not show HA activity at a concentration of 10 μg using Balb/c mice and chicken erythrocytes, although the protein had significant protease activity which was inhibited by metal chelators, such as EGTA (5 mM) (Fig. 2). Extensive dialysis against EDTA (1 mM) and EGTA (1 mM) resulted in loss of protease activity, although the cell-rounding activity remained intact.

The secreted HA/P has been characterized as a zinc-dependent metalloprotease [22] with the ability to cleave several physiologically important substrates, including mucin, fibronectin, and lactoferrin [23]. It also nicks and activates CT and CT-related enterotoxins [24]. Besides the protease activity, HA/P also shows hemagglutinating activity [25,26] on erythrocytes of Balb/c mice, but this activity has not been consistently reproduced [27,28]. Our study showed that NMDCY has a protease activity which is inhibited by metal chelators, but has no hemagglutinating activity on Balb/c mice and chicken erythrocytes. The cell-rounding activity of NMDCY was, however, not inhibited by dialyzing the protein against EGTA and EDTA, indicating that the cell-rounding manifestation is not due to the protease activity. Although these data are very suggestive,

Table 2
Rabbit ileal loop response of NMDCY purified from *V. cholerae* O1 (strain WO5) at different concentrations

Sample	Volume (ml) of fluid accumulated	FA ratio[a] (mean ± S.D.; $n=4$)
NMDCY (50 μg)	−	−
NMDCY (75 μg)	3.5	0.5 ± 0.02
NMDCY (100 μg)	6.5	0.928 ± 0.05
Heat-inactivated NMDCY (100 μg)	−	−
CT (0.5 μg; positive control)4	7.6	1.085 ± 0.02
PBS (negative control)	−	−
AKI (medium control)	−	−
WO5 (10^8 cells)	7.1	1.01 ± 0.01

Length of the loop in all experiments was 7 cm. −, no fluid accumulation.
[a]FA, fluid accumulation.

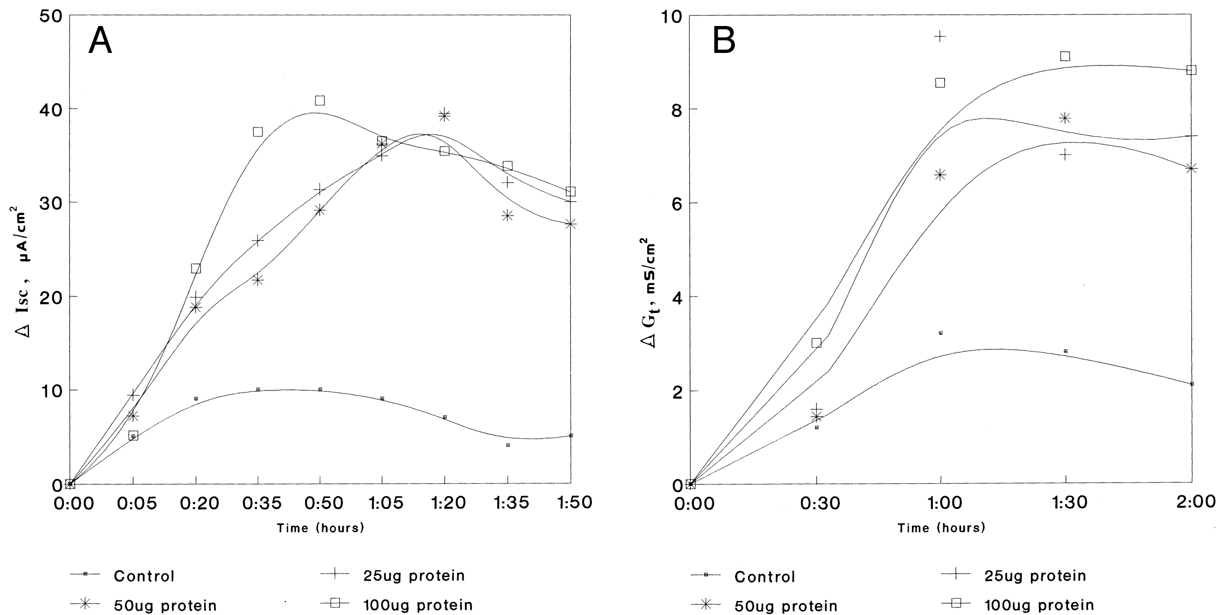


Fig. 3. (A) Time course of ΔI_{sc} of purified NMDCY from *V. cholerae* O1; each time point represents the mean of four determinations. Time 0 is the time at which the purified NMDCY was introduced into the arms of Lucite chambers. (B) Time course of ΔG_t of purified NMDCY from *V. cholerae* O1; each time point represents the mean of four determinations. Time 0 is the time at which the purified NMDCY was introduced into the arms of Lucite chambers.

further work needs to be done to determine if NMDCY and HA/P are indeed the same. There are a number of proteases now being characterized from various bacteria that have similar amino acid sequences and sizes yet turn out to be different proteins.

The in vivo enterotoxic activity was tested by introducing purified protein in rabbit ileal loop. At a protein concentration of 50 μg per loop and below, there was no fluid accumulation. At a protein concentration of 100 μg per loop, NMDCY elicited the accumulation of non-hemorrhagic fluid (Table 2). The non-toxigenic strain of *V. cholerae* (WO5) when introduced in the ileal loop also elicited fluid accumulation similar to the purified protein.

The in vivo secretory activity of NMDCY from *V. cholerae* O1 was further substantiated by an in vitro assay. Fig. 3A shows that at all protein concentrations the I_{sc} exhibited a gradual increase in rabbit ileal mucosa, with the peak being reached at different time intervals, depending on the protein concentration used, indicative of Cl^- secretion. At a protein concentration of 100 μg, the peak was attained at around 50 min which is also the concentration at which the protein elicited positive ileal loop response. A gradual increase in tissue conductance was also observed with an increase in the time interval which is a direct reflection of variation in short circuit current (Fig. 3B). This increase in tissue conductance may be due to alteration in the tight junction of the ileal tissue, which, in turn, could lead to fluid accumulation. The addition of glucose at the end of the experiment showed equivalent responses in treated and untreated tissues, indicating that glucose-Na^+ active cotransport function was unaffected (data not shown). The peak action of NMDCY is observed around 50–60 min after introduction of the toxin in Ussing chambers, which suggests that NMDCY is not a fast-acting toxin like heat-stable toxins [29], but the action is gradual. One point is clear that variation in tissue conductance and the change in short circuit current make NMDCY a factor similar to zonula occludens toxin (Zot) [30] of *V. cholerae*, *Clostridium difficile* toxin A and resembles accessory cholera enterotoxin (*Ace*), an enterotoxin of *V. cholerae* which increases short circuit

Table 3
Anti-NMDCY antitoxin IgG in the acute and convalescent sera of cholera patients

Antigen tested	Response rate (*x*/*n*)[a]		Geometric mean titer			
	O1 infected serum	O139 infected serum	O1 infected sera		O139 infected sera	
			Acute	Convalescent	Acute	Convalescent
Purified NMDCY	0/7	5/9	3200	3200	2300	7100
Cholera toxin	6/7	8/9	1983	6400	2625	8063

[a]Expressed as the number of sera showing positive response (*x*) per total number (*n*) tested. A positive response was considered to be a 3-fold or more rise in GMT value of convalescent phase serum as compared to that of corresponding acute phase serum.

current in Ussing chamber and causes fluid secretion in the ligated rabbit ileal loop [31].

Antitoxin antibody response in acute and convalescent sera of cholera patients was determined against purified CT and NMDCY by ELISA. A significant (≥3-fold) rise in the serum antitoxin antibody titer of IgG class was observed in the convalescent phase sera against CT in O1 and O139 infected patients (Table 3). Like CT, a significant (≥3-fold) rise in the serum antitoxin antibody titer of IgG class against NMDCY was also observed, although in only O139-infected patients. Five out of nine O139-infected patients showed a positive antibody response against NMDCY with a 3-fold rise in GMT value. However, no rise in IgG titer was observed against NMDCY in patients infected with the O1 serogroup.

The antitoxic IgG antibody response in serum has previously been shown to be a good correlate of the gut mucosal immune response to CT in cholera [32]. Although in *V. cholerae* infection, the organism does not come in contact with circulating blood, the serum antibody response induced by natural cholera infection is believed to be, in part, a systemic immune response and, in part, a spillover from the intestine into the circulation [33]. Our study indicated an interesting finding that NMDCY induces a strong antitoxin IgG response in patients with *V. cholerae* O139 infection, but not with *V. cholerae* O1 infection. The gradual evolution in the organism may have led to a stronger effect of NMDCY in *V. cholerae* O139 infection, the new etiologic agent of cholera.

This study, along with an earlier study conducted in our laboratory [14] for the first time, demonstrated the in vivo and in vitro secretogenic nature of NMDCY. The exact mode of action of the protein at the concentration of 100 μg in bringing about the intestinal ion flux is still under study. The concentration of NMDCY required for demonstration of enterotoxic activity is quite high and one might question the physiological relevance of this toxin. According to the purification data, the amount of protein necessary to produce a response in the ileal loop assay is the equivalent of the activity present in 60 ml of an overnight bacterial culture. A greater response was seen with only 10^8 bacterial cells which would indicate that NMDCY has only a minor contribution to the secretogenic potential of NT *V. cholerae*. Since a relatively large amount is required to induce diarrhea, its contribution to the disease depends upon the amount produced by the infecting strain.

Our previous studies have shown that the amount of NMDCY produced by strains of *V. cholerae* vary widely [15]. Therefore, it appears that strains of *V. cholerae* which produce NMDCY above a certain amount are capable of inducing diarrhea which might explain why certain strains of *V. cholerae* non-O1 non-O139 devoid of CT are associated with sporadic diarrhea [12,34]. One means to assess the contribution of NMDCY to the secretory response induced by bacterial cells would be to compare a mutant devoid of NMDCY and such a mutant is currently being constructed.

Acknowledgments

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