Aeromonas cytotoxic enterotoxin cross reactive with cholera toxin

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Summary. Isolation by affinity chromatography from crude culture filtrate of *Aeromonas sobria* of protein that cross reacted with cholera toxin (CT) revealed a toxin that produced fluid accumulation in rat ileal loops and in infant mice and caused rounding of Y1 adrenal cells. All these activities were neutralised by antiserum to CT. There was no haemolytic or cytotoxic activity associated with this CT-cross reactive cytotoxic enterotoxin. CT-cross reactive material detected in enzyme linked immunosorbent assay (ELISA) was produced by 25% of *Aeromonas* isolates from faeces of children with or without diarrhoea—26% of *A. sobria*, 20·0% of *A. hydrophila* and 24% of *A. caviae* tested gave positive ELISA results. Most strains that produced this cytotoxic enterotoxin but no cytotoxic enterotoxin were isolated from children without diarrhoea. Toxin preparations from *Aeromonas* spp. that completely inhibited adenosine-5'-diphosphate-induced platelet aggregation, an effect related to elevation of intracellular cAMP, were, with one exception, cross reactive with CT in ELISA.

Introduction

Immunological cross-reactivity between cholera toxin (CT) and the heat labile toxin (LT) of *Escherichia coli* is well recognised (Clements and Finkelstein, 1978). The investigation of other bacteria for toxins cross reactive with CT suggests that there is a family of CT-like enterotoxins that may be produced by *Klebsiella pneumoniae* and *Enterobacter cloacae* (Klipstein and Engert, 1977), *Salmonella* spp. (Sandefur and Peterson, 1977) and *Campylobacter jejuni* (Ruiz-Palacios *et al.*, 1983).

Reports of CT-like activity of *Aeromonas* spp. have been inconsistent. Wadstrom *et al.* (1976) initially found that antiserum to CT or LT partially inhibited aeromonas enterotoxin and that there was a single precipitin line with antisera to CT in immunodiffusion studies with aeromonas enterotoxin (Ljungh *et al.*, 1977). However, they were unable to confirm their findings. Boulanger *et al.* (1977) failed to neutralise the effects of aeromonas enterotoxin in rabbit ileal loop or suckling mouse assays but Dobrescu (1978), James *et al.* (1982) and Jiwa (1983) demonstrated cross reactions between exotoxins of *Aeromonas* spp. and LT or CT. This

apparent conflict probably reflects the multiplicity of extracellular products of *Aeromonas* spp. present in crude culture filtrates. It is now clear, for example, that both cytotoxic (Asao *et al.*, 1984) and cytotoxic (Chakraborty *et al.*, 1984) enterotoxins of *Aeromonas* spp. are capable of producing fluid accumulation in suckling mice, so that the use of such a model would not allow detection of neutralisation of one toxin in the presence of the other.

More recently immunologically-based assays have demonstrated CT-like activity produced by strains of *Aeromonas* spp. (Shimada *et al.*, 1984; Campbell and Houston, 1985). Shimada *et al.* (1984) did not report biological activity related to this CT-reactive product and, although Campbell and Houston (1985) showed fluid accumulation in rabbit ileal loops with culture filtrates that cross reacted with CT, they failed to demonstrate that this enterotoxicity was associated with the CT-reactive fraction and not with cytotoxic enterotoxicity. In the present study we have used enzyme linked immunosorbent assay (ELISA) to detect CT-like activity and affinity chromatography to isolate a CT-cross reactive protein produced by faecal isolates of *Aeromonas* spp., and we have related the results to species, production of cytotoxic enterotoxin and the presence of diarrhoea.

Materials and methods

Bacteria

Four strains of *Aeromonas* spp. known to produce CT-like activity were kindly provided by Dr R. Sakazaki of the Department of Bacteriology, National Institute of Health, Kamiosaki, Tokyo. Three were *A. sobria* and one *A. hydrophila* (Popoff, 1984). All other strains were isolated from faeces of children with or without diarrhoea during 1980–1981 in a study reported elsewhere (Gracey *et al.*, 1982). All strains isolated by primary culture on blood agar, as well as those isolated only after enrichment in nutrient broth or alkaline peptone water, were included in this study. Of the 205 strains isolated, 143 were *A. sobria*, 25 *A. hydrophila* and 37 *A. caviae* when classified according to Popoff (1984).

Strains were stored in 3-ml volumes of maintenance medium consisting of agar 5 g, NaCl 5 g, peptone 0118 (Difco) 2.5 g, peptone L32 (Oxoid) 2.5 g in 200 ml of PBS (containing Na₂HPO₄ 2.8 g in 134 ml of distilled water and KH₂PO₄ 1.3 g in 66 ml of distilled water), and 800 ml of distilled water, pH 6.7.

Bacterial preparations

For assay of biological activity and for production of crude CT-like toxin, *Aeromonas* spp. were grown in Tryptone Soya Broth (Oxoid) supplemented with yeast extract (Oxoid) 0.6% shaken at 300 rpm in an environmental incubator shaker (New Brunswick Scientific, Edison, NJ, USA) at 37°C for 22 h. Sterile broth filtrates for the biological tests were obtained by centrifugation at 2000 g for 10 min at 4°C and filtration through 0.45-µm membrane filters (Millipore) and were stored at 4°C until tested, within 24 h of preparation.

Biological assays

Suckling-mouse tests were performed as described by Burke *et al.* (1981); an intestinal weight/body weight (IW/BW) ratio of ≥ 0.08 was the criterion of a positive result. For routine testing of *Aeromonas* spp., three mice were used for each test with two tests per sample. With the cytotoxic enterotoxin isolated by affinity chromatography, we used six mice for each test with two tests per sample. As fluid secretion in infant mice challenged with CT does not occur as quickly as the response to *E. coli* heat stable toxin (STa) (Baselski *et al.*, 1977), groups of mice were held for the usual period of 3 h or for 5 h.

The rat ileal loop test was performed as described by Pierce (1977) with at least two rats for each test. Results were expressed as ml of fluid/cm of intestine; values greater than 0.2 ml/cm were considered positive.

Cytotoxicity and cytotoxicity tests. Y1 cells were used for assay of cytotoxicity and L132 cells for assay of cytotoxicity (Donta *et al.*, 1974). For titration, 25 µl of the toxin diluted with PBS were added to 100 µl of cell monolayers in 96-well microculture plates (Linbro, Flow Laboratories, Irvine, Ayrshire) and incubated for 18 h in CO₂ 5% at 37°C. The response was considered to be

cytotoxic if there was rounding of >50% of cells or cytotoxic if there was death of >50% of cells.

Haemolytic activity was assayed as described previously (Burke *et al.*, 1984b). Lysis of at least 50% of the erythrocytes was considered a positive result.

Definition of enterotoxin activity

Production of cytotoxic enterotoxin was defined by cytotoxicity in cell culture, a positive haemolysis assay and IW/BW ratio >0.08 in the suckling-mouse test. Cytotoxic enterotoxin was recognised by cross reactivity with CT in ELISA and a positive suckling-mouse test. Cytotoxic effects in Y1 cells could be recognised in the presence of cytotoxic enterotoxin by heating cell-free supernates to 56° for 20 min before addition to cell cultures (Chakraborty *et al.*, 1984). Results in Y1 cells were concordant with results in ELISA so that, in subsequent discussion, this cytotoxic enterotoxin is considered to be the material detected in ELISA.

Assay for cross reactivity with CT

Immunisation procedures. Antisera to CT were produced by repeated inoculations of purified toxin (Calbiochem-Behring, La Jolla, CA, USA) into a sheep and a rabbit. Animals were given primary immunisation subcutaneously with Freund's complete adjuvant and two subcutaneous booster immunisations at monthly intervals with Freund's incomplete adjuvant. The doses of CT for primary and booster immunisations in the sheep were 60, 40 and 40 µg. In the rabbit the respective doses were 10, 5 and 5 µg of CT.

Enzyme-linked immunosorbent assay (ELISA). The assay was performed as described by Yolken *et al.* (1977). The optimal dilutions of reagents were first determined by chessboard titration. All samples were tested in triplicate, working volumes were 100 µl, and all dilutions of antigens and antisera were made with phosphate-buffered saline-Tween-0.1% bovine serum albumin buffer. This buffer contained NaCl 8g, KH₂PO₄ 0.2 g, Na₂HPO₄ 1.14 g, KCl 0.2 g, NaN₃ 0.2 g and Tween 20 0.5 ml/L of distilled water, pH 7.4. Bovine serum albumin was added to give a final concentration of 0.1%. Sheep anti-cholera toxin serum was used as the solid phase, diluted rabbit antiserum to cholera toxin as the second antibody, and alkaline phosphate-conjugated goat anti-rabbit IgG antiserum (Miles Laboratories, Rehovot, Israel) was used as the conjugate. Coated plates were stored at -20°C until use to maintain optimal sensitivity of the assay. Optical densities at 405 nm were determined with Titertek Multiscan automatic spectrophotometer. Values greater than twice the optical density of the control were considered positive.

Platelet aggregation test (PAT)

Twenty strains were used for comparison of the PAT with results of ELISA: *A. sobria* 11, *A. hydrophila* 7 and *A. caviae* 2.

Platelet aggregation is influenced by compounds that affect intracellular levels of AMP (Salzman, 1972). Enterotoxins which increase intracellular cAMP inhibit platelet aggregation. This inhibition of ADP-induced platelet aggregation is the basis for the use of the PAT for assay of cAMP-mediated enterotoxins (Fumarola and Miragliotta, 1981).

Bacterial preparation. *Aeromonas* strains were grown in screw-cap tubes containing 5 ml of Trypticase Soy Broth (BBL 11767) with yeast extract (Difco 0127) 0.6% for 48 h at 37°C on a roller drum (60 rpm) with the tubes nearly horizontal. Cultures were centrifuged and the supernates were filtered through 0.45-µm membrane filters (Millipore). Filtrates were endotoxin free as shown by the Limulus gelation assay.

Preparation of platelets. Normal donors who had received no medication such as aspirin in the 10 days preceding venepuncture provided the platelets. Trisodium citrate (3.8%) in a ratio of 10:1 v:v was used as the anticoagulant. Citrated platelet-rich plasma was separated by centrifugation at 100g for 20 min at room temperature and platelet-deficient plasma was obtained by sedimentation of platelets from samples of the platelet rich preparation at 250g for 20 min. The platelet count in the platelet-rich plasma was adjusted to 300 000/µl by dilution with platelet-deficient plasma.

Platelet aggregation. The photometric technique of Born (1962) with an Elvi 840 Aggregometer (Elvi Logos, Milan, Italy) was used to measure platelet aggregation at 37°C. Platelet rich plasma from each donor was incubated in 0.3-ml volumes with 0.1 ml of each bacterial filtrate. Saline and cholera toxin were, respectively, the negative and positive controls. After incubation for 15 min, samples were challenged with 1.8–6.4 µmol of adenosine-5' diphosphate, disodium salt (ADP Sigma, St Louis, MO, USA) as the aggregating agent. Failure to aggregate platelets, as seen with CT, produced no increase in the percentage transmission measured photometrically.

Preparation of cytotoxic enterotoxin

A strain of *A. sobria* isolated from faeces of a patient with diarrhoea was used as the source of the CT-cross reactive enterotoxin. Solid ammonium sulphate was added to the cell-free supernate to 60% of saturation at 4°C. The precipitate was left at 4°C overnight and then was collected by centrifugation at 13 500g for 20 min, dissolved in and dialysed against phosphate-buffered saline (PBS) pH 7.3 and kept frozen at –70°C until use. This material is referred to as crude enterotoxin.

Protein determination. Protein content was determined as described by Read and Northcote (1981) with bovine serum albumin as a standard.

Immunoabsorption. The γ globulin of sheep anti-serum to CT, precipitated at room temperature with 40% saturated ammonium sulphate, was dissolved in PBS, dialysed against 10 mM Tris-HCl buffer, pH 8.0, (starting buffer) at 4°C overnight and loaded onto the DEAE Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) column equilibrated with the same buffer. The column

was eluted with linear gradient of 0–300 mM NaCl in starting buffer. Fractions containing IgG were pooled, concentrated by ultrafiltration and equilibrated against 0.1M sodium bicarbonate, pH 10.0. A portion containing 225 mg of protein was mixed with 10 ml of 1,1'-carbonyldiimidazole activated agarose (Reacti-Gel, Pierce Chemical Company, Rockford, IL, USA) and tumbled overnight at 4°C. Active groups were blocked by the addition of 1.0M ethanolamine at pH 9.0 for 3 h at room temperature. After washing with water, 1M NaCl, water and PBS, the coupled gel was poured into a column to which crude enterotoxin was added. After washing with PBS the column was eluted with 3M KSCN, pH 7.2. The eluted protein was dialysed against PBS, concentrated by ultrafiltration and stored at –70°C. The column was regenerated by washing with PBS.

SDS-PAGE on gradient gels. The eluted protein was analysed by electrophoresis on slabs of acrylamide 2.5–27% gradient (Gradient Laboratories, Pyrmont, Australia) in the presence of sodium dodecyl sulphate (SDS-PAGE) (Lambin, 1978). The gel was stained with Coomassie brilliant blue R250 (Bio-Rad Laboratories, Richmond, CA, USA) and then destained. Mol.-wt markers (Pharmacia, Uppsala, Sweden) were included.

Neutralisation tests

Equal volumes of the purified enterotoxin (20 µg/ml) and anti-CT serum were mixed, incubated for 60 min at 37°C, and assayed in ileal loops, suckling mice and Y1 cells.

Results

Isolation of the CT-cross reactive product

Before the application of crude aeromonas enterotoxin, the ability of the column to bind CT was assessed. The capacity of the column, as indicated by depletion of applied CT and its recovery from the column, was at least 150 µg of CT.

When the crude enterotoxin of *A. sobria* was applied to the column, the protein reacting in ELISA with antiserum to CT was retained. Only 5% of the adsorbed protein was eluted with 0.1M glycine-HCl buffer, pH 2.5. The rest of the bound material was eluted with 3M KSCN and subsequently only this material was used in the study.

This eluted product had about 10% of the biological activity of the crude enterotoxin. No activity was detectable in the suckling-mouse test, rat ileal loop test or in Y1 cells at a concentration of 2 µg/ml but at a concentration of 10 µg/ml this product showed activity in all of these test systems.

In the suckling-mouse test, the IW/BW ratio was 0.82 after incubation for 3 h with the eluted material

in a concentration of 10 µg/ml. Pre-incubation with antiserum to CT produced an IW/BW ratio of 0.055. With samples incubated in suckling mice for 5 h, the eluted material produced an IW/BW ratio of 0.12 and after pre-incubation with cholera antitoxin the IW/BW ratio was 0.07.

In the rat ileal-loop test, the eluted product in a concentration of 10 µg/ml, resulted in a mean fluid accumulation of 0.27 ml/cm (SEM 0.01). In loops containing this product pre-incubated with cholera antiserum, mean fluid accumulation was 0.11 ml/cm (SEM 0.02).

The addition of the product to Y1 cells produced rounding (fig. 1a) which was neutralised by cholera antitoxin (fig. 1b). No cytotoxic or haemolytic activity was demonstrated.

In SDS-PAGE without reducing agent, the material eluted with 3M KSCN showed three major protein bands (fig. 2) with apparent mol. wts (10^3) 43.5, 29.5 and 27.0. A protein of high mol. wt, beyond the range of the markers used, was also detected. By extrapolation its mol. wt was estimated at 150×10^3 . Treatment with 2-mercaptoethanol did not change the electrophoretic pattern of the eluted material.

Screening for CT-cross reactive cytotoxic enterotoxin

Of the 205 faecal isolates of *Aeromonas* spp. tested, toxin preparations from 51 (25%) cross reacted with CT in ELISA. The distribution of species (table I) was not significantly different between ELISA-negative and ELISA-positive strains (χ^2 test, $p > 0.05$). Strains positive in ELISA were 26% of the *A. sobria* strains, 20% of *A. hydrophila* and 24% of *A. caviae* tested.

The relationship between production of cytotoxic and cytotoxic enterotoxin is shown in table II. All but 11 strains (2 *A. hydrophila* and 9 *A. caviae*) that produced cytotoxic enterotoxin also produced cytotoxic enterotoxin. Results were positive in the suckling-mouse assay if strains produced either or both enterotoxins. All positive results were detected

Table I. Cytotoxic enterotoxin detected by ELISA in relation to species

ELISA result	Number of isolates (%) that gave the indicated result		
	<i>A. sobria</i> (n = 143)	<i>A. hydrophila</i> (n = 25)	<i>A. caviae</i> (n = 37)
Positive (n = 51)	37 (26)	5 (20)	9 (24)
Negative (n = 154)	106 (74)	20 (80)	28 (76)

Fig. 1a. Rounding of Y1 cells produced by the toxin isolated by affinity chromatography ($\times 100$).

Fig. 1b. Neutralisation of cytotoxic effects on Y1 cells by pre-incubation of the toxin with antiserum to CT ($\times 100$).

after incubation for 3 h and, although the IW/BW ratio increased after incubation for 5 h, no strains gave negative results at 3 h and positive results after 5 h.

Most of the strains that were positive in ELISA were associated with diarrhoea (table III) but all except 11 of these strains also produced cytotoxic enterotoxin (table II). There was a similar association between diarrhoea and production of cytotoxic enterotoxin amongst strains that did not produce cytotoxic enterotoxin, i.e., epidemiological data did not suggest that diarrhoea occurred more frequently when strains produced both enterotoxins. Of the 11

Fig. 2. Sodium dodecyl sulphate-polyacrylamide gradient gel electrophoresis (SDS-PAGE) of the isolated enterotoxin (10 µg). Lane 1, mol. wt reference proteins: from the top of the gel these proteins were phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and lactalbumin (14 400); lane 2, sample heated at 100°C for 5 min in the absence of mercaptoethanol; lane 3, sample incubated at 37°C for 3 h in the presence of mercaptoethanol 5%; lane 4, sample heated at 100°C for 5 min in the presence of mercaptoethanol 5%.

strains that produced cytotoxic but not cytotoxic enterotoxin, only one *A. caviae* strain was associated with diarrhoea, in a patient from whom a salmonella was isolated from the same faecal specimen.

Platelet aggregation test

Eight strains inhibited platelet aggregation completely. Only one of these strains gave negative results in ELISA, but the optical density attained with this strain was consistently just below twice

Table II. Cytotoxic enterotoxin detected by ELISA in relation to suckling-mouse tests, production of cytotoxic enterotoxin, and species

ELISA result	Positive result in suckling-mouse test	Cytotoxic enterotoxin test					
		Positive			Negative		
		S	H	C	S	H	C
Positive (n = 51)	51	37	3	0	0	2	9
Negative (n = 154)	123	105	18	0	1	2	28

S = *A. sobria*, H = *A. hydrophila*, C = *A. caviae*.

Table III. Cytotoxic enterotoxin detected by ELISA related to the presence of diarrhoea

ELISA result	Number of strains in which cytotoxic enterotoxin was detected					
	<i>A. sobria</i>		<i>A. hydrophila</i>		<i>A. caviae</i>	
	D ⁺	D ⁻	D ⁺	D ⁻	D ⁺	D ⁻
Positive (n = 51)	28(2)*	9(7)	3	2	1	8(1)
Negative (n = 154)	74(5)	32(28)	12(2)	8(6)	6(1)	22(2)

D⁺ diarrhoea, D⁻ no diarrhoea.

*Numbers in parenthesis are numbers of strains isolated after enrichment but not on primary culture.

the control value, the criterion chosen for a positive result in ELISA. Five strains partially inhibited platelet aggregation but gave negative results in ELISA. The remaining seven strains tested in PAT did not inhibit platelet aggregation and showed no CT-like activity in ELISA (table IV).

The effect on platelet aggregation did not correlate with production of cytotoxic enterotoxin. Of the eight strains that completely inhibited platelet aggregation, two produced only cytotoxic enterotoxin and six produced both cytotoxic and cytotoxic enterotoxin. Of the seven strains that caused no inhibition of platelet aggregation, four produced cytotoxic enterotoxin in high titre.

Discussion

About one-quarter of *Aeromonas* spp. isolated from faeces produced cytotoxic enterotoxin that cross reacted with CT in ELISA. Production of cytotoxic enterotoxin was not associated with a particular biotype. Shimada *et al.* (1984) found that

Table IV. Comparison of results in platelet aggregation tests and ELISA

Platelet aggregation test result	ELISA result	
	Positive	Negative
Inhibition	7 (5)*	1† (1)
Partial inhibition	0	5 (3)
No inhibition	0	7 (4)

*Numbers in parenthesis are numbers of producers of cytotoxic enterotoxin.

†This strain consistently produced optical-density readings just below the critical value for classification as positive in ELISA.

all their CT-positive strains were "*A. hydrophila*", a group that would include both *A. hydrophila* and *A. sobria* if strains were classified according to Popoff (1984). They found no CT-positive *A. caviae* but, in our study, 24% of *A. caviae* strains were CT-positive. We have previously found that *A. caviae* rarely show potential virulence factors such as production of cytotoxic enterotoxin, fucose-resistant haemagglutination (Burke *et al.*, 1984a) or invasiveness (Watson *et al.*, 1985), consistent with the reported low virulence of this species (Schubert, 1967). Production of cytotoxic enterotoxin appears to be more common than these other potential virulence factors in *A. caviae*. However, only one of the *A. caviae* strains that gave positive results in ELISA was associated with diarrhoea, and in that patient a salmonella was isolated from the same faecal sample.

It may be that diarrhoea is associated with cytotoxic enterotoxin only if the *Aeromonas* strain also possesses the required colonisation factors, analogous to the situation with enterotoxigenic *E. coli* (Satterwhite *et al.*, 1978). None of the *A. caviae* strains that gave positive results in ELISA showed fucose-resistant haemagglutination, the pattern, in our experience, most commonly produced by strains isolated from patients with *Aeromonas*-associated diarrhoea (Burke *et al.*, 1984a).

Diarrhoea was associated with the production of cytotoxic enterotoxin, both in strains that produced cytotoxic enterotoxin and those that did not. Because we found so few strains that produced only cytotoxic enterotoxin, it has not been possible to assess the possible contribution of the cytotoxic enterotoxin as a virulence factor in *Aeromonas* spp. However, data from the 11 non-cytotoxic, cytotoxic enterotoxin-producing strains suggest that the cytotoxic enterotoxin alone is not a virulence factor for aeromonads.

ADP induced platelet aggregation is known to be inhibited by substances that increase levels of intracellular cAMP in platelets (Salzman, 1972). CT, LT and salmonella CT-like enterotoxins, but not heat stable toxins of *E. coli*, *Klebsiella* or *Yersinia enterocolitica*, inhibit platelet aggregation (Fumarola and Miragliotta, 1983). Results in the platelet aggregation test with *Aeromonas* spp. are consistent with the suggestion that *Aeromonas* enterotoxins may act by increasing intracellular cAMP (Dubey *et al.*, 1981; Fumarola and Miragliotta, 1984).

The ability to inhibit platelet aggregation was distinct from production of cytotoxic enterotoxin but there was good correlation between strains that gave positive results in ELISA and the ability to completely inhibit platelet aggregation. The single

strain that completely inhibited platelet aggregation but was classified as negative in ELISA, consistently just failed to attain the critical value for a positive test, suggesting that the cytotoxic enterotoxin was present in low concentrations. This observation, and the failure of strains causing partial inhibition in the PAT to be positive in ELISA, suggests that PAT is a more sensitive assay than ELISA for the cytotoxic enterotoxin. However, the usefulness of PAT as a routine assay is limited by the difficulty of acquiring constant fresh supplies of human platelets from donors not exposed to drugs such as aspirin.

Shimada *et al.* (1984) presented evidence of immunological cross reactivity between CT and some strains of *Aeromonas* but did not establish that this CT-like toxin had biological activity. The enterotoxicity demonstrated in CT-cross reactive culture filtrates of *A. hydrophila* (Campbell and Houston, 1985) was not distinguished from the possible effect of cytotoxic enterotoxin that may have been present in the same preparation. By isolation of the CT-cross reactive material of *A. sobria* by affinity chromatography we have shown that this protein has the properties of a cytotoxic enterotoxin, neutralised by antiserum to CT. There was no cytotoxicity or haemolytic activity associated with this cytotoxic enterotoxin. We have shown previously that this same strain of *A. sobria* also produces a cytotoxic enterotoxin that causes fluid accumulation in rat ileal loops and in infant mice and is cytotoxic and haemolytic but none of these activities is neutralised by antiserum to CT. Thus, we have evidence to support the view that *Aeromonas* spp. may produce a cytotoxic enterotoxin that cross reacts with CT and a cytotoxic enterotoxin that does not cross react with CT. Both toxins are detectable in rat ileal loops and in infant mice (Asao *et al.*, 1984; Chakraborty *et al.*, 1984) but the cytotoxic enterotoxin is easily distinguished by its haemolytic or cytotoxic activity.

The relationship of the CT-cross reactive cytotoxic enterotoxin we have isolated to the aeromonas cytotoxic enterotoxins previously described is not clear. The enterotoxin isolated by Ljungh *et al.* (1981) does not cross react with CT, does not cause fluid accumulation in suckling mice and has a mol. wt of only 15 000 but the cytotoxic enterotoxin we isolated cross reacts with CT and causes fluid accumulation in suckling mice.

Chakraborty *et al.* (1984) have cloned an aeromonas cytotoxic enterotoxin without haemolytic or cytotoxic activity that, like the cytotoxic enterotoxin we have isolated, causes fluid accumulation in suckling mice but its mol. wt and ability to cross

react with CT have not been reported. Genetic probing failed to show homology with *E. coli* heat labile toxin (LT) but CT was not investigated (Chakraborty *et al.*, 1984).

It may be that apparent differences between these cytotoxic enterotoxins of *Aeromonas* spp. reflect technical differences between laboratories. However, we used cross reactivity with CT as the basis for purification of this cytotoxic enterotoxin and we cannot exclude the possibility of other cytotoxic enterotoxins of *Aeromonas* spp. that do not react with CT.

The need to use 3M KSCN for elution of the bound protein from the column resulted in a considerable decrease in biological activity, in agreement with findings reported for affinity purification of LT (Dafni *et al.*, 1978). Incubation of a crude LT preparation with 3M KSCN decreased biological activity by 10–90% (Dafni *et al.*, 1978).

SDS-PAGE identified four bands in the eluate. Dafni and Robbins (1976) and Dafni *et al.* (1978) also found multiple bands on SDS-PAGE analysis

of LT-including components with apparent mol. wt similar to the A (mol. wt 27 000) and B (mol. wt 58 000) subunits of CT. The multiple bands found with the cytotoxic enterotoxin of *A. sobria* are consistent with an analogous separation into subunits. Unlike the high mol.-wt band described by Dafni *et al.* (1978) the fraction of high mol. wt found on SDS-PAGE of *Aeromonas* cytotoxic enterotoxin is probably not immunoglobulin because it was unaffected by the presence of reducing agent. It is not known whether this band represents undissociated enterotoxin.

At present, the relative importance of cytotoxic and cytotoxic enterotoxins as virulence factors for *Aeromonas* spp. is not known and clarification awaits the development of an experimental model for *Aeromonas*-associated diarrhoea.

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