Purification of cytotoxic enterotoxin of *Aeromonas sobria* by use of monoclonal antibodies

J. POTOMSKI, VALERIE BURKE, I. WATSON and M. GRACEY*

Gastroenterology and Nutrition Research Unit, Princess Margaret Children's Medical Research Foundation, and
*Department of Child Health, University of Western Australia, Perth, Australia.

Summary. Cytotoxic enterotoxin of *Aeromonas sobria* was purified by affinity chromatography with monoclonal antibodies. The purified enterotoxin gave a single protein band in polyacrylamide gradient gel electrophoresis and its mol. wt estimated by this technique was 63 000; it had a pI of 6.2. The purified enterotoxin caused fluid accumulation in rat ileal loops and in infant mice, was cytotoxic to cultured cells, was haemolytic to human erythrocytes, and was lethal to mice after intravenous injection. The relative concentrations of enterotoxin, cytotoxic and haemolytic activities were approximately the same in a culture filtrate and in purified, electrophoretically homogeneous enterotoxin. The three activities were also inactivated to the same extent after incubation for 10 min at 56°C. There was no immunological cross-reactivity with cholera toxin (CT) nor did antiserum to CT neutralise the biological effects of the toxin.

Introduction

Interest in *Aeromonas* spp. as enteric pathogens has led to the investigation of possible virulence factors concerned in the pathogenesis of diarrhoea. There have been several reports of haemolytic, cytotoxic and enterotoxic properties of *Aeromonas* spp. and of correlation between these exotoxin activities (Cumberbatch *et al.*, 1979; Burke *et al.*, 1981; Johnson and Lior, 1981). In contrast, haemolysins of *Aeromonas* spp. were reported to have no enterotoxic activity and to be distinct from an extracellular product of *Aeromonas* spp. with the properties of a cytotoxic enterotoxin (Ljungh *et al.*, 1981). More recently, purification of an aeromonas haemolysin suggests that haemolysis, cytotoxicity and enterotoxicity are properties of a single protein (Hostacka *et al.*, 1982; Asao *et al.*, 1984). The cloning of aeromonas enterotoxin without haemolytic or cytotoxic activity (Chakraborty *et al.*, 1984) appears to conflict with these reports.

In this study we report the purification of a cytotoxic enterotoxin of *A. sobria* and its biological, physicochemical and immunological properties.

Materials and methods

Bacterial strains and growth conditions

One strain of *A. sobria* (B33) and one strain of *A. hydrophila* (B337), identified by the criteria of Popoff

(1984), were used in the study. The *A. sobria* strain was isolated from faeces of a patient with gastro-enteritis and gave positive results in the rat intestinal loop test (RIL), the suckling mouse test, the cytotoxicity test and the haemolytic assay. The *A. hydrophila* strain was obtained from H. M. Atkinson (South Australian Institute of Technology, Adelaide, Australia) and gave negative results in all these tests. Bacteria were grown in Tryptone Soya Broth (Oxoid) supplemented with yeast extract (Oxoid) 0.6% at 37°C and 300 rpm in an environmental incubator shaker (New Brunswick Scientific, Edison, NJ, USA) for 22 h. Cell-free supernates for the biological tests were made by centrifuging cultures at 2000 *g* for 10 min at 4°C and filtering through 0.45 µm membrane filters (Millipore Corp., Bedford, MA, USA).

Preparation of crude antigen

The bacterial preparation was centrifuged at 4000 *g* for 30 min and solid ammonium sulphate was added to the supernate to 60% of saturation at 4°C. The precipitate was left at 4°C overnight and then was collected by centrifugation at 13 500 *g* for 20 min, and dialysed against phosphate buffered saline (PBS), pH 7.3.

Production of monoclonal antibodies

Female Balb/c mice (Murdoch University, Perth, Australia), 6–8 weeks old, were immunised twice with the *A. hydrophila* or *A. sobria* crude antigen. Mice were given 0.1 ml of crude antigen (1 mg/ml), emulsified with an equal volume of Freund's complete adjuvant, subcutaneously. The treatment was repeated 3 weeks later when crude antigen was emulsified with Freund's

incomplete adjuvant. After 3 months, mice were given booster injections of 5 µg of crude antigen in PBS intravenously 3 days before the hybridisation. Spleen cells (10^8) from hyperimmunised mice were fused with 10^7 NS-1 myeloma cells according to the method of Galfe and Milstein (1981). Culture supernates were assayed for cross-reactivity with crude antigen by enzyme-linked immunosorbent assay (ELISA). Hybridomas of interest were cloned and recloned by the limiting dilution method with mouse spleen cells as feeder layers. Ascites tumours were induced in pristane (2, 6, 10, 14-tetramethylpentadecane; Sigma Chemical Co., St Louis, Mo, USA) treated Balb/c mice by intraperitoneal injection of 3×10^7 cloned cells. Immunosuppression was achieved with an injection of cyclophosphamide c. 0.5 mg/20 g animal weight 24 h before cell transplantation.

ELISA

The hybridoma supernatant fluids were assayed for cross-reactivity with crude antigen ELISA. Microtitration plates (Linbro; Flow Laboratories) were coated with 100 µl of *A. sobria* or *A. hydrophila* crude antigen (200 µg/ml) in coating buffer (0.01M carbonate-bicarbonate buffer, pH 9.6) and incubated for 3 h at 37°C followed by at least 20 h at 4°C. The plates were washed three times with PBS-Tween 20 and 100 µl of hybridoma supernate diluted in PBS-Tween with fetal calf serum (FCS) 1% as added. After incubation for 2 h at 37°C, the plates were washed again and 100 µl of alkaline phosphatase-labelled sheep anti-mouse immunoglobulin diluted 1 in 2000 in PBS-Tween-1% FCS was added. The plates were allowed to incubate at 37°C for 2 h, washed and 100 µl of p-nitrophenylphosphate 1 mg/ml in diethanolamine buffer (pH 9.8) was added. The absorbance at 405 nm was read in a Multiscan reader (Flow Laboratories) after incubation for 1 h at 37°C; optical densities 0.2 above background were considered positive results.

Immunoabsorption

The γ globulin of mouse ascites fluid (10 ml) was precipitated by adding an equal volume of cold, saturated ammonium sulphate at 4°C. After 30 min at 4°C this preparation was centrifuged at 10 000 g for 30 min. The pellet was washed with 50% saturated ammonium sulphate, dissolved in one tenth of the original volume of coupling buffer (0.1M NaHCO₃, pH 8.5, containing 0.5M NaCl) and dialysed against coupling buffer for 24 h at 4°C. A sample containing 100 mg of protein was coupled to 5 g of CNBr Sepharose 4B according to manufacturer's instructions (Pharmacia Fine Chemicals, Uppsala, Sweden) and transferred to a column (C16/20, Pharmacia); *A. sobria* crude antigen (25 mg of protein in 12 ml of PBS) was then run through the column. Unbound material was removed by washing with PBS. Non-specifically-bound proteins were removed by washing with 0.1M borate

buffer (pH 9.0) containing 1M NaCl. The column was eluted with 3M KSCN (pH 7.4). All the bound material eluted in a single peak. Fractions constituting this peak were pooled, dialysed against PBS and stored at -70°C. Antigens purified by immunoabsorption were designated by the number of their specific monoclonal antibody.

Biological assays

The *suckling-mouse test* was done as described by Burke *et al.* (1981); the intestinal weight/body weight (IW/BW) ratio was the sole assay criterion. All samples were diluted with PBS and an IW/BW ratio of ≥ 0.08 was considered a positive result. Six mice were used for each test and two tests were performed on each sample. One mouse unit was defined as the minimum amount of protein that caused IW/BW ratio ≥ 0.08 .

The *rat ileal loop test* was carried out as described by Pierce (1977). In rats under ether anaesthesia, four segments in each rat were isolated by ties with additional ties separating consecutive loops. At least three rats were used for each test. Test solutions of PBS were injected via a 26-gauge needle in 0.25-ml volumes in alternate loops so that the lower loop contained solution or PBS in alternate animals. The abdomen was sutured and the animals were killed with ether 18 h later. Loops were excised and weighed, then drained to measure the volume of fluid. The length of the segment, with a standard weight attached, was measured against a vertical rule. Results were expressed as ml of fluid per cm of intestine; values > 0.2 ml/cm were considered positive. One loop unit was the minimum amount of protein that produced fluid accumulation of at least 0.2 ml/cm.

The *cytotoxic activity* of the cell-free supernate and the purified toxin was assayed in Y1 and CHO-K1 cells. For titration, 25 µl of doubling dilutions of the test samples of PBS were added to 100 µl of cell monolayers in 96-well microculture plates (Linbro; Flow Laboratories) and incubated for 18 h in CO₂ 5% at 37°C. Visible cytotoxicity was confirmed by trypan blue dye exclusion test. Death of $> 50\%$ of Y1 or CHO-K1 cells was considered a positive result. One cytotoxic unit was the minimum amount of toxin that produced a positive result in this test.

The *haemolytic activity* of toxin preparations was assayed with human group-O erythrocytes in 96-well microtitration plates; 100 µl of doubling dilutions of the toxin solution in PBS containing bovine serum albumin 0.1% were added to 100 µl of human red blood cells 1% v/v in PBS. Haemolysis was recorded after incubation for 1 h at 37°C then 1 h at 4°C. Lysis of at least 50% of the erythrocytes was considered a positive result. One haemolytic unit was the minimum amount of toxin that gave a positive result in this test.

Mouse lethal toxin activity was determined by the intravenous injection of 100 µl of toxin diluted in PBS into two adult Balb/c mice for each dilution and the amount of toxin required to kill 50% of the animals (LD50) was estimated.

Polyacrylamide gradient gel electrophoresis

The toxin was analysed by electrophoresis on slabs of 2.5–27% concave gradient acrylamide (Gradient Laboratories, Pyrmont, Australia) in Tris-borate buffer pH 9.6 (Margolis and Kenrick, 1968). Gels were run in constant voltage conditions for at least 2000 v.h. Proteins, including mol.-wt markers (Pharmacia), were stained with 0.1% Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, CA, USA) 0.1%, and destained in methanol 25% and acetic acid 10%.

Estimation of isoelectric point (pI)

Isoelectric focussing of the pure toxin (15 µg) was performed with a LKB Ultraphor electrofocussing unit (LKB, Bromma, Sweden) and a LKB Ampholine PAG plate, pH 3.5–9.5 according to manufacturer's instructions. After focussing, the pH gradient was measured by means of a semi-micro surface pH electrode (Orion Research Inc., Cambridge, MA, USA). Gels were stained with Coomassie brilliant blue R-250 0.12% to locate protein bands.

Immunoglobulin class, subclass and light chain

Monoclonal antibodies in growth medium from stable cell lines were concentrated 25-fold (Minicon B15, Amicon Corp., Danvers, MA, USA) and used in double immunodiffusion against specific rabbit antisera to mouse immunoglobulin M (IgM), IgG (7S), IgG1, IgG2a, IgG2b, IgG3, κ and λ light chain (Bionetics Laboratory Products, Kensington, MD, USA).

Double immunodiffusion

Double immunodiffusion was performed in agarose 0.9% in borate saline buffer pH 8.5 (ionic strength 0.175) containing polyethylene glycol 3% and thiomersal 0.01%.

Protein estimation

Protein was estimated by the method of Read and Northcote (1981) with bovine serum albumin (Sigma) as the standard.

Preparation of antisera

Antiserum to cholera toxin (CT) (Calbiochem-Behring, La Jolla, CA, USA), and antiserum to *A. sobria* cytotoxic enterotoxin were produced by repeated inoculations of purified toxins into rabbits. 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 cholera toxin for primary and booster immunisations were 10, 5 and 5 µg; the respective doses of *A. sobria* cytotoxic enterotoxin were 20, 10 and 10 µg. The animals were bled 10 days after the last injection.

Results

Production of monoclonal antibodies

The fusion of NS-1 myeloma cells and *A. sobria* crude antigen-immunised mouse spleen cells resulted in hybrid colonies appearing in all the 96 wells dispensed. When tested in ELISA, 44 supernates reacted with *A. sobria* crude antigen but not with *A. hydrophila* crude antigen. The two hybrid colonies that gave the highest binding in ELISA with *A. sobria* were selected for cloning by limiting dilution. After cloning and recloning, two stable cell lines, designated 1/20 C11 G9 and 4/5A8F5, producing monoclonal antibodies that reacted with *A. sobria* crude antigen, were obtained. These two monoclonal antibodies were found to be of the IgG1 and IgG2b subclasses with λ light chains as determined by double immunodiffusion.

Isolation of aeromonas cytotoxic enterotoxin

To prepare sufficient IgG for immunoadsorbent columns, 1/20 C11 G9 and 4/5 A8F5 cells were injected intraperitoneally into Balb/c mice and, when tumours had developed, ascitic fluids were collected and pooled. IgG1 and IgG2b were purified by ammonium sulphate precipitation and coupled to CNBr-activated Sepharose 4B., *A. sobria* crude enterotoxin was passed through these two columns and adsorbed material was eluted with 3M KSCN. Eluates derived from immunoadsorption columns were dialysed against PBS and concentrated by ultrafiltration. Ouchterlony immunodiffusion analysis showed lines of identity between 1/20 C11 G9 and 4/5 A8F5 eluates with antiserum to *A. sobria* crude antigen (fig. 1). Further electrophoretic and biological analysis confirmed their identity. When eluates from immunoadsorption columns were analysed by polyacrylamide gradient gel electrophoresis only one protein band was demonstrated (fig. 2)). This band had a faster migration rate than the standard of lowest mol. wt (67 000). An estimation of its size by regression analysis was 63 000. The pI of the toxin was 6.2 (5.4–7.1).

Biological activities of toxin

The protein purified by immunoadsorption produced a positive reaction in the rat intestinal loop test, the suckling-mouse test, the cytotoxicity test and the haemolytic assay (table). The dose-response curves of the toxin in these four tests are illustrated in figs. 3, 4 and 5.

In the suckling-mouse test, IW/BW ratio was

Table. Enterotoxic, cytotoxic and haemolytic activity in culture supernate and purified *Aeromonas* toxin

Toxin assay	Units/mg (SEM)* in		Activity ratio (pure toxin/ supernate)
	culture supernate	pure toxin	
Rat intestinal loop test	160 (0)	7 530 (190)	47
Suckling-mouse test	160 (0)	7 700 (101)	48
Y1 cytotoxicity test	2048 (0)	65 000 (8280)	32
Haemolytic assay	1024 (0)	39 700 (7823)	39

* Mean values and standard error from two to four experiments. See *Methods* for definition of units.

proportional to the amount of the toxin in the range 62.5–500 ng. The minimum amount of the enterotoxin detected was 130 ng and the maximum reaction was observed at 2 μ g/mouse; 16 μ g of the toxin was not lethal to the infant mice and IW/BW ratio (0.115 ± 0.005) was almost the same as for 500 ng (0.114 ± 0.007). The minimum amount of enterotoxin detected in the rat intestinal loop test was 109 ng. Higher doses of the toxin (500 ng) caused inflammation and haemorrhage.

The enterotoxin was cytotoxic to Y1 adrenal cells (65 000 units/mg) and CHO-K1 cells (fig. 6). The smallest amount of pure toxin that gave a positive response in the CHO-K1 assay was 10 pg. The toxin was also haemolytic to human erythrocytes (39 700 units/mg).

The relative concentrations of these activities were approximately the same in bacterial culture supernate and in purified, electrophoretically homogeneous, enterotoxin (table). Heating affected enterotoxicity, cytotoxicity and haemolysis similarly. After incubation at 56°C for 10 min, about 99% of its cytotoxic and haemolytic activities were

Fig. 1. Ouchterlony immunodiffusion analysis of the relationship of 1/20 C11G9 (1) to 4/5A8F5 (2) eluates with antiserum to *A. sobria* crude antigen (3).

lost and 8 loop and mouse units did not give positive results.

The enterotoxin was also lethal to mice (LD50 11 μ g/kg) after intravenous injection. A dose-related period of survival was observed; thus the animals that received 4 μ g of the toxin died within

Fig. 2. Gradient gel electrophoresis of the purified toxin. A. Mol.-wt reference proteins—thyroglobulin (669 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000) and albumin (67 000). B. Purified toxin (11 μ g). **Fig. 3.** Rat intestinal loop test: accumulation of fluid in response to amount of enterotoxin protein. Each point shows mean $\pm$ SD for 8 loops.

Fig. 4. Suckling-mouse assay: intestinal weight to remaining body weight ratio (IW/BW) in response to amount of enterotoxin protein. Each point represents mean $\pm$ SD for 18 mice.

Fig. 5. Haemolysin (○) and cytotoxin (●) titres related to the amount of enterotoxin protein. Cytotoxin and haemolysin titres are means, with one SD of 4 (haemolysin) or 5 (cytotoxin) experiments.

1 h, whereas those that received 0.25 μ g of the toxin survived for about 7 h. The lethal activity was also inactivated by heating for 10 min at 56°C.

The toxin did not react with antiserum to CT in ELISA. Ouchterlony immunodiffusion analysis

showed lines of non-identity between CT and *A. sobria* cytotoxic enterotoxin with antisera to both toxins (fig. 7). None of the biological activities of the purified enterotoxin was neutralised by antiserum to CT.

Discussion

The purification and characterisation of toxins of *Aeromonas* spp. has been difficult because of the complexity of the extracellular protein profile of this species and instability of these toxins during classical techniques of biochemical purification (Ljungh and Wadstrom, 1982). Affinity chromatography with monoclonal antibodies is being used increasingly for the rapid and efficient purification of individual components from complex biological mixtures. With this technique we have purified an exotoxin of *A. sobria* and found it to be haemolytic, cytotoxic and enterotoxic, apparently properties of a single protein, in agreement with earlier reports (Hostacka *et al.*, 1982; Asao *et al.*, 1984).

The enterotoxin had an apparent mol. wt of c. 63 000, a pI of 6.2 and was homogeneous as determined by polyacrylamide gradient gel electrophoresis. Co-purification of enterotoxic, cytotoxic and haemolytic activities and the loss of these activities after incubation for 10 min at 56°C supported the view that the toxin was homogeneous. Immunological homogeneity was indicated by double immunodiffusion.

Biological activities in rat ileal loops, suckling mice, cell culture and haemolysin assay were similar to the properties of a haemolysin previously purified from a strain of *A. hydrophila* (Asao *et al.*, 1984). Hostacka *et al.* (1982) also reported enterotoxic, cytotoxic and haemolytic activity from *Aeromonas* spp. in a fraction with mol. wt c. 60 000.

Similar to cytotoxic enterotoxin of *Shigella dysenteriae* and toxin A of *Clostridium difficile* (Gemmell, 1984), *A. sobria* cytotoxic enterotoxin causes fluid accumulation with damage to intestinal mucosa, induction of an inflammatory response and haemorrhage. Recently Thompson *et al.* (1984) reported that *Bacillus cereus* enterotoxin induces fluid accumulation in rabbit intestinal loops and is cytotoxic to Vero cells, haemolytic to rabbit erythrocytes and lethal to mice. Thus, haemolytic activity associated with enterotoxicity is not unique.

Although the suckling-mouse test is a technique routinely used for the detection of heat stable enterotoxins (STa's) produced by *Escherichia coli*, *Klebsiella pneumoniae* and *Yersinia enterocolitica*, we found, in agreement with other reports (Janossy and Tarjan, 1980; Burke *et al.*, 1981; Johnson and

Fig. 6. Photomicrographs (phase contrast, unstained) of monolayer cultures of CHO cells. (A) Cells treated with toxin. (B) Untreated control cells (PBS).

Lior, 1981; Olivier *et al.*, 1981; Turnbull *et al.*, 1984), that *A. sobria* cytotoxic enterotoxin detected in the suckling-mouse test is heat labile. The *A. sobria* enterotoxin is not as potent as STa's; the minimum effective dosage of aeromonas cytotoxic enterotoxin in the suckling-mouse test was 130 ng in comparison with 4.0 ng, 5.9 ng and 25 ng of *E.*

Fig. 7. Ouchterlony immunodiffusion analysis of the relationship of *A. hydrophila* toxin (1) to cholera toxin (2) with antisera to both toxins (3).

coli, *K. pneumoniae*, *Y. enterocolitica* STa's, respectively (Klipstein *et al.*, 1983; Inoue *et al.*, 1983).

In our study the cytotoxic enterotoxin produced by the *A. sobria* strain did not cross-react with CT. There have been reports of immunological cross reactivity between cholera toxin (CT) or *E. coli* heat labile toxin (LT) and aeromonas enterotoxin (Dobrescu, 1978; James *et al.*, 1982; Jiwa, 1983), not confirmed by other laboratories (Ljungh and Wadstrom, 1982). CT-like activity, detected in ELISA, has now been demonstrated to be produced by some strains of *Aeromonas* spp. (Shimada *et al.*, 1984; Campbell and Houston, 1985).

Our results indicate that cross-reactivity with CT is not a characteristic of the purified cytotoxic enterotoxin of *A. sobria* but we have found that crude cell-free broth preparations of the same strain of *A. sobria* do cross react with CT in ELISA. These findings would be explained if this strain of *A. sobria* produced a CT-like enterotoxin in addition to the cytotoxic enterotoxin. We have now isolated this CT-cross reactive enterotoxin by affinity chromatography.

REFERENCES

- Asao T, Kinoshita Y, Kozaki S, Uemura T, Sakaguchi G 1984 Purification and some properties of *Aeromonas hydrophila* hemolysin. *Infection and Immunity* **46**: 122-127.
- Burke V, Robinson J, Atkinson H M, Dibley M, Berry R J, Gracey M 1981 Exotoxins of *Aeromonas hydrophila*. *Australian Journal of Experimental Biology and Medical Science* **59**: 753-761.
- Burke V, Robinson J, Berry R J, Gracey M 1981 Detection of enterotoxins of *Aeromonas hydrophila* with suckling-mouse test. *Journal of Medical Microbiology* **14**: 401-408.
- Campbell J D, Houston C W 1985 Effect of cultural conditions on the presence of a cholera-toxin cross-reactive factor in

- culture filtrates of *Aeromonas hydrophila*. *Current Microbiology* **12**: 101–106.
- Chakraborty T, Montenegro M. A, Sanyal S C, Helmuth R, Bulling E, Timmis K N 1984 Cloning of enterotoxin gene from *Aeromonas hydrophila* provides conclusive evidence of production of a cytotoxic enterotoxin. *Infection and Immunity* **46**: 435–441.
- Cumberbatch N, Gurwith M J, Langston C, Sack R B, Brunton J L 1979 Cytotoxic enterotoxin produced by *Aeromonas hydrophila*: relationship of toxigenic isolates to diarrheal disease. *Infection and Immunity* **23**: 829–837.
- Dobrescu L 1978 Enterotoxigenic *Aeromonas hydrophila* from a case of piglet diarrhoea. *Zentralblatt für Veterinärmedizin B* **25**: 713–718.
- Galfre G, Milstein C 1981 Preparation of monoclonal antibodies: strategies and procedures. *Methods in Enzymology* **73**: 3–46.
- Gemmell C G 1984 Comparative study of the nature and biological activities of bacterial enterotoxins. *Journal of Medical Microbiology* **17**: 217–235.
- Hostacka A, Ciznar I, Korych B, Karolcek J 1982 Toxic factors of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. 1 Abteilung Originale A* **252**: 525–534.
- Inoue T, Okamoto K, Moriyama T, Takahashi T, Shimizu K, Miyama A 1983 Effect of *Yersinia enterocolitica* ST on cyclic guanosine 3'5' monophosphate levels in mouse intestines and cultured cells. *Microbiology and Immunology* **27**: 159–166.
- James C, Dibley M, Burke V, Robinson J, Gracey M 1982 Immunological cross-reactivity of enterotoxins of *Aeromonas hydrophila* and cholera toxin. *Clinical and Experimental Immunology* **47**: 34–42.
- Janossay G, Tarjan V 1980 Enterotoxigenicity of *Aeromonas* strains in suckling mice. *Acta Microbiologica Academiae Scientiarum Hungaricae* **27**: 63–69.
- Jiwa S F H 1983 Enterotoxigenicity, hemagglutination and cell surface hydrophobicity in *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas salmonicida*. *Veterinary Microbiology* **8**: 17–34.
- Johnson W M, Lior H 1981 Cytotoxicity and suckling mouse reactivity of *Aeromonas hydrophila* isolated from human sources. *Canadian Journal of Microbiology* **27**: 1019–1027.
- Klipstein F A, Engert R F, Houghten R A 1983 Immunological properties of purified *Klebsiella pneumoniae* heat-stable enterotoxin. *Infection and Immunity* **42**: 838–841.
- Ljungh A, Wadstrom T 1982 *Aeromonas* toxins. *Pharmacology and Therapeutics* **15**: 339–354.
- Ljungh A, Wretling B, Möllby R 1981 Separation and characterization of enterotoxin and two haemolysins from *Aeromonas hydrophila*. *Acta Pathologica et Microbiologica Scandinavica. Section B* **89**: 387–397.
- Margolis J, Kenrick K G 1968 Polyacrylamide gel electrophoresis in a continuous molecular sieve gradient. *Analytical Biochemistry* **25**: 347–362.
- Okamoto K, Inoue T, Moriyama T, Miyama A 1983 Heat-stable enterotoxin produced by *Yersinia enterocolitica*. *Toxicon* **3**: 325–328.
- Olivier G, Lallier R, Larivière S 1981 A toxigenic profile of *Aeromonas hydrophila* and *Aeromonas sobria* isolated from fish. *Canadian Journal of Microbiology* **27**: 330–333.
- Pierce N F 1977 Protection against challenge with *Escherichia coli* heat-labile enterotoxin by immunization of rats with cholera toxin/toxoid. *Infection and Immunity* **18**: 338–341.
- Popoff M 1984 *Aeromonas* Kluyver and Van Niel 1936 In: Krieg N R, Holt J G (eds) *Bergey's Manual of systematic bacteriology* vol. 1, 9th edn. Williams and Wilkins Co., Baltimore, pp 545–548.
- Read S M, Northcote D H 1981 Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Analytical Biochemistry* **116**: 53–64.
- Shimada T, Sakazaki R, Horigome K, Uesaka Y, Niwano K 1984 Production of cholera-like enterotoxin by *Aeromonas hydrophila*. *Japanese Journal of Medical Science and Biology* **37**: 141–144.
- Thompson N E, Ketterhagen M J, Bergdoll M S, Schantz E J 1984 Isolation and some properties of an enterotoxin produced by *Bacillus cereus*. *Infection and Immunity* **43**: 887–894.
- Turnbull P C B *et al.* 1984 Enterotoxin production in relation to taxonomic grouping and source of isolation of *Aeromonas* species. *Journal of Clinical Microbiology* **19**: 175–180.