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Immunological cross-reactivity of enterotoxins of *Aeromonas hydrophila* and cholera toxin

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SUMMARY

Pre-incubation with anticholera toxin (ACT) significantly reduced intestinal secretion induced by cell-free broth preparations of heat-labile toxins (LT) of *Escherichia coli* and *Aeromonas hydrophila* in jejunal perfusion experiments in rats *in vivo*. Pre-incubation with ACT also prevented cytotoxicity by *E. coli* LT in the Y1 cell culture system. Pre-incubation had no effect on cytotoxicity in Y1 and L132 cell lines or on haemolytic activity with cell-free preparations of *A. hydrophila*. In another series of experiments rats were immunized with cholera toxin given as an intraperitoneal priming dose followed 12 days later by intraduodenal boosting. Immunization significantly protected against net intestinal fluid secretion induced by enterotoxigenic *E. coli* and *A. hydrophila* and by cholera toxin.

INTRODUCTION

Immunological cross-reactivity between cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) has been demonstrated in diverse test systems. These include measurement of skin permeability (Evans *et al.*, 1973), fluid secretion in ileal loops (Holmgren *et al.*, 1973), cytotoxicity in cell culture systems (Donta & Smith, 1974) and immunodiffusion techniques (Gyles, 1974). However, antiserum to either toxin more effectively neutralizes homologous than heterologous toxins (Gyles & Barnum, 1969).

Enterotoxins of other organisms may also cross-react with CT and LT. These include the heat-labile toxins of *Klebsiella pneumonae* and *E. cloacae* (Klipstein & Engert, 1977) and Salmonella toxin (Sandefur & Petersen, 1977) but there is conflicting evidence concerning cross-reactivity with Aeromonas enterotoxins (Donta & Haddow, 1978; Dobrescu, 1978).

The present study attempts to clarify the immunological cross-reactivity of CT with Aeromonas toxins using cell culture, suckling mouse and intestinal perfusion systems as well as haemolysin assay.

MATERIALS AND METHODS

Organisms. The strain of *A. hydrophila* used was isolated from faeces of an adult with diarrhoea and identified according to Schubert (1974). We had previously shown that this strain produced haemolysins and enterotoxin detectable in the suckling mouse and intestinal perfusion assays and cytotoxins affecting Vero and Y1 cell systems.

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The strain of *E. coli* used was isolated from faeces of an Australian Aboriginal child without diarrhoea. This organism produced LT detectable in intestinal perfusion, Y1 adrenal cells and enzyme-linked immunosorbent assay (ELISA), but no ST detectable in the suckling mouse assay.

Organisms were stored at room temperature in maintenance medium consisting of 5 g of agar, 5 g of sodium chloride, 2·5 g of Difco Bacto peptone 0118 (DIFCO Labs, Detroit, Michigan, USA), 2·5 g of Oxoid peptone L34 (Oxoid Ltd, Basingstoke, UK) in 200 ml of phosphate buffer (2·8 g Na_2HPO_4 in 134 ml of distilled water and 1·3 g of K_2HPO_4 in 66 ml of distilled water) and 800 ml of distilled water at pH 6·7.

Bacterial preparations. Trypticase soy broth with 0·6% yeast extract L21 (Oxoid Ltd, Basingstoke, UK) (TSB–YE) was inoculated with the organism to be tested and incubated at 37°C and 300 rev min^{-1} on an environmental incubator shaker (New Brunswick Scientific, Edison, New Jersey, USA) for 24 hr. Fifty millilitres of broth was added to 250-ml Erlenmeyer flasks or 5 ml of broth to 25-ml flasks depending on the volume required. Cell-free preparations were made by filtering through a Millipore type HA filter (pore size 0·45 μm) (Millipore Corp., Bedford, Massachusetts, USA) after centrifuging at 300 rev min^{-1} for 30 min. All tests were carried out within 2 days of preparing supernatants which were stored at 4°C until used.

ASSAYS FOR EXOTOXINS

Enterotoxin assays

Suckling mouse test. Suckling mice were used for the assay of Aeromonas enterotoxin as described previously (Burke *et al.*, 1981). In this method both intestinal weight: body weight ratio and the amount of diarrhoea are considered as indices of response.

Intestinal perfusion. For perfusion experiments in immunized rats, cell-free preparations were dialysed overnight against phosphate-buffered saline (PBS) consisting of 8 g of NaCl, 1 g of Na_2HPO_4 and 0·02 g of NaH_2PO_4 in 1 l of distilled water at pH 7·2 so that the osmolality of the test solution was 300 ± 10 mOsm. In neutralization experiments, cell-free preparations of *A. hydrophila* and *E. coli* were incubated with the appropriate volume of antiserum for 1 hr at 37°C before perfusion; 5 μg 100 ml^{-1} of phenol red was added as a non-absorbable marker. Rats were anaesthetized using intraperitoneal chloral hydrate and a segment of jejunum approximately 20 cm long was cannulated as described previously (Thelen, Burke & Gracey, 1978). Before perfusion of immunized rats, 5 ml of PBS + 3000 Kallikrein Inactivator Units ml^{-1} of trasylol (Bayer, Leverküsen, Germany), a proteinase inhibitor, was gently syringed through the cannulated jejunal segment, collected and stored at −20°C. These samples were used for measurement of secretory antitoxin. The segment was perfused with cell-free preparations of *A. hydrophila*, *E. coli* or cholera toxin, 2 μg ml^{-1} in PBS, at 10 ml hr^{-1} after rinsing with the same solution. After equilibration for 30 min, perfusate was collected for 1 hr; the segment was then drained and its length measured under the tension of a standard weight. There were at least eight rats in each experimental group.

Phenol red was determined spectrophotometrically at 560 nm using 0·5-ml volumes of perfusate alkalinized with 1 ml of 1 M NaOH.

Net water flux is given in μl of water transported cm^{-1} of intestine h^{-1} with negative values indicating net fluid secretion.

Haemolytic activity. Using microtitre trays (Linbro, Hamden, Connecticut, USA), 100-μl volumes of doubling dilutions of the test solution in PBS were added to an equal volume of a 1% suspension of rabbit erythrocytes. Haemolysis was recorded after incubation for 1 hr at 37°C then 1 hr at 5°C.

Cell culture methods. Cell-free supernatants were tested in monolayers of Vero and Y1 cell systems. Vero cells were maintained in Eagles medium with L-glutamine to which was added 10% of foetal calf serum (FCS), 35 mg ml^{-1} of $NaHCO_3$, 50 IU ml^{-1} of penicillin, 200 IU ml^{-1} of streptomycin and 100 μg ml^{-1} of neomycin with neutral red indicator. Cephamanidole, 50 μg ml^{-1}, and gentamicin, 10 μg ml^{-1}, replaced the other antibiotics for growth of Y1 cells.

For neutralization experiments, 20 μl of the test solution was added to 100 μl of cell monolayers in 96-well microculture trays (Linbro, Hamden, Connecticut, USA) and incubated for 18 hr in an

atmosphere of 95% oxygen and 5% carbon dioxide. Wells were then examined microscopically and disruption or rounding affecting more than half the cells recorded as positive.

Since rounding of Y1 cells with Aeromonas preparations may be seen only after heating to 56°C for 10 min has destroyed cytotoxicity (Wadstrom *et al.*, 1976), neutralization experiments were carried out before and after heating the cell-free preparations in this way.

Immunological methods

Production of antiserum. Purified cholera toxin, supplied by Dr H. Moon, was used in a concentration of 25 μg ml^{-1} of normal saline mixed with 1 ml of complete Freund's adjuvant (Difco Labs, Detroit, Michigan, USA) and injected subcutaneously into sheep and rabbits. Booster doses of 25 μg of toxin with 1 ml of incomplete Freund's adjuvant were given subcutaneously at intervals of 2–8 weeks.

Animals were bled 7–10 days after the third injection and anti-serum was stored at −20°C in aliquots of 5 ml until used.

Neutralization experiments. For cell culture and suckling mouse assays, cell-free supernatant from broth cultures of the *A. hydrophila* or *E. coli* strain were incubated for 1 hr at 37°C with antiserum to cholera toxin (ACT) in equal volumes and in dilutions of 1 in 5, 10, 25, 50, 100, 250 and 500 in PBS.

Immunization of rats. Male and female Sprague–Dawley rats weighing between 150 and 300 g were immunized with cholera toxin using the method of Pierce & Koster (1980). For primary immunization, 20 μg of purified cholera toxin diluted in PBS to a concentration of 100 μg ml^{-1} was emulsified with an equal volume of Freund's incomplete adjuvant (Commonwealth Serum Laboratories, Melbourne, Australia) and given intraperitoneally in a final volume of 0·4 ml. Twelve days later a booster immunization of 20 μg of CT in 0·1 ml of its original diluent was injected directly into the duodenum using a 26 gauge needle after the duodenum had been exposed by a small laparotomy. Animals were kept lightly anaesthetized using ether for all immunization procedures. None of the immunizing dosages were sufficient to cause diarrhoea in the experimental animals.

Determination of antitoxin titres. Immunized rats were bled from the tail vein 0, 4, 12, 16, 20 and 40 days after primary immunization. Serum was separated and stored at −20°C until tested. These samples and serum from immunized sheep or rabbits were assayed for antitoxin activity using a modification of ELISA. Secretory antitoxin in intestinal washings from immunized rats was measured in the same way. The method is similar to that described by Sack *et al.* (1980) but omits GM1 ganglioside for binding.

For titration, serum was diluted to allow determination within the range of the blocking assay; intestinal washings were not diluted.

Antitoxin titre was determined by measuring activity in ELISA after incubation for 1 hr at room temperature of 100 μl of CT with 100 μl of serial two-fold dilutions of intestinal washings or serum in PBS + 1% FCS. The amount of CT was chosen as four times that giving an optical density reading of 1·00 in ELISA.

Each ELISA plate included reference anti-serum against cholera toxin, Swiss Serum Institute Anticholera Serum (SSVI), and the following controls: PBS + 1% FCS; CT + PBS + 1% FCS; PBS + 1% FCS + serum or intestinal washings.

The equivalence point for each sample of serum was determined from the linear range of the data points, after logit transformation of the data and antitoxin units were estimated by reference to SSVI.

Measurement of cellular antitoxin. Mesenteric lymph nodes were removed from immunized and unimmunized rats after intestinal perfusion and placed in 3–5 ml of PBS at 4°C, then in a Petri dish with approximately 2 ml of PBS + 10% FCS. After dissecting off all fatty tissue, nodes were chopped into small pieces and pressed through a fine sieve to retain connective tissue which was discarded. This cell suspension was filtered through non-absorbent cotton wool and, after making up to 5 ml with PBS + 10% FCS at 4°C, centrifuged at 1,000 rev min^{-1} for 5 min at 4°C. The cell pellet was collected, washed twice in PBS + 10% FCS, centrifuged at 1,000 rev min^{-1} for 5 min at 4°C and finally suspended in 100% FCS, from which cell smears were prepared on washed glass slides. The smears were fixed in 96% ethanol for 30 min at 4°C and stored at 4°C in a desiccator.

Fluorescein staining of antibody-containing cells. Antibody-containing cells in mesenteric lymph nodes were counted by the method of Pierce & Gowans (1975). Prepared slides were washed for 30 min in approximately 1 l of cold PBS, dried, then sequentially incubated with 10 μl of purified cholera toxin (100 μg ml^{-1} in PBS) and 10 μl of rabbit anticholera toxin labelled with fluorescein isothiocyanate (FITC) diluted to a concentration of 120 μg ml^{-1} in PBS. Each reagent was allowed to react for 1 hr in a humid chamber at room temperature; for FITC-labelled antibody, incubation was carried out in a dark cupboard. Slides were washed in 1 l of PBS for 30 min after each application.

Stained smears were mounted in a semipermanent modified polyvinyl alcohol (PVA) medium described by Lennette (1977) and examined under dark ground illumination at a magnification of 950. The proportion of fluorescent cells was derived from a total count of about 1,000 lymphocytes. No fluorescent lymphocytes were seen in smears of mesenteric lymph nodes from immunized rats in which CT was omitted from the staining sequence. Mesenteric lymph node smears from immunized animals were included as controls.

Statistical methods. Student's *t*-test was used for comparison of means. Differences were considered significant at the 95% level. In data such as antitoxin titres, where variance increased with increase in mean, logarithmic transformation of data was used in statistical analysis.

RESULTS

Neutralization experiments

Intestinal perfusion. Incubation with anticholera toxin (ACT) for 1 hr at 37°C before jejunal perfusion significantly decreased intestinal secretion in the presence of cell-free broth preparations of enterotoxigenic *A. hydrophila* or *E. coli* which produced LT only. Figs 1 and 2 show the change in intestinal net water flux related to the concentration of ACT used. Regression analysis, using a model of the form $y = a + bx + cx^2$, showed the relationship to be significant ($P < 0{\cdot}05$) for both *A. hydrophila* and *E. coli*.

Cell culture systems. Cell death in Y1 and L132 cultures was unchanged by pre-incubation of cell-free broth preparations of *A. hydrophila* with ACT. Using similar preparations of the *E. coli* strain which produced LT only, changes in Y1 cultures were prevented by ACT at a concentration

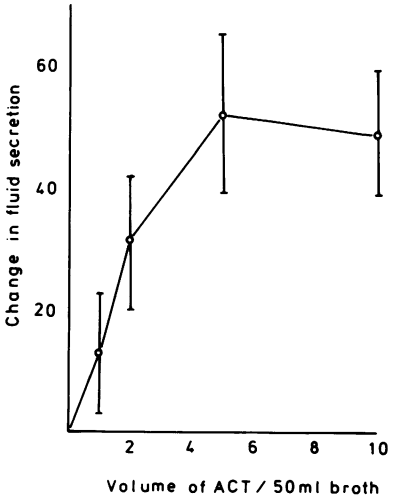

Fig. 1. Net intestinal fluid secretion (μl cm^{-1} hr^{-1}) in perfusion experiments *in vivo* with cell-free supernatants of *Aeromonas hydrophila*. Increasing concentrations of ACT in the broth supernatants caused a significant reduction in net fluid secretion across the intestine which is expressed as an increase in net fluid absorption ($n = 8$).

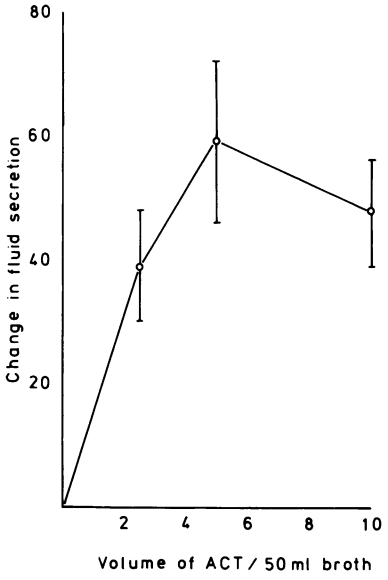

Fig. 2. Net intestinal fluid secretion (μl cm^{-1} hr^{-1}) in perfusion experiments *in vivo* with cell-free supernatants of *Escherichia coli*. Increasing concentrations of ACT in broth supernatants caused a significant reduction in net fluid secretion across the intestine which is expressed as an increase in net fluid absorption ($n=8$).

of 1:250. Controls using ACT showed no effect in Y1 or L132 cell cultures. Both Y1 and L132 cell systems were unaffected after heating of preparations of *A. hydrophila* to 56°C before testing.

Suckling mouse assay. Incubation of a cell-free preparation of *A. hydrophila* with ACT did not affect the response in suckling mouse tests even using up to 250 times the concentration of ACT found to neutralize *E. coli* LT in the Y1 cell system.

Haemolysin assay. Titres of haemolytic activity were unaffected by pre-incubation of broth filtrates for 1 hr with anticholera toxin in doubling dilutions from 1:2 to 1:128.

Immunization experiments

Humoral response in immunized rats. The humoral response to immunization was measured at 4-day intervals for 24 days and at 40 days after primary immunization. The time–response curve is shown in Fig. 3. The highest antitoxin titres occurred about days 12–20.

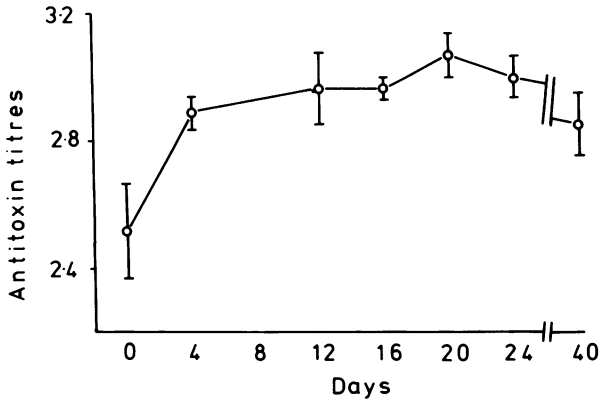

Fig. 3. Serum antitoxin titres after primary intraperitoneal immunization with cholera toxin. Values are expressed as mean $\pm$ s.e. after logarithmic transformation.

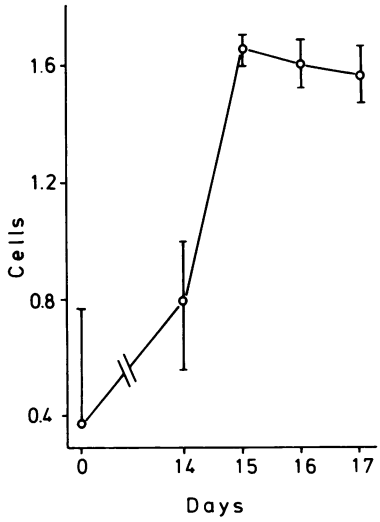

Fig. 4. Cholera-antitoxin-containing cells (ACC) in mesenteric lymph nodes of rats immunized intraperitoneally with cholera toxin and boosted 12 days later with intraduodenal injection of cholera toxin. Cell counts are expressed as $\log_{10}$ of ACC 10^{-3} ($\pm$s.e.m.) ($n=4$).

Cellular response in immunized rats. The proportion of cholera-antitoxin-containing cells (ACC) in the mesenteric lymph nodes of rats was determined for four consecutive days from the second day after intraduodenal booster immunization. Fig. 4 shows the response to immunization in relation to time with results expressed as $\log_{10}$ ACC per 1000 cells. The highest proportion of antitoxin-containing cells occurred 4 days after booster immunization with the maximal response about day 15.

Intestinal perfusion of immunized rats. In eight unimmunized rats, perfusion of CT produced net water secretion of 10·53 μl cm^{-1} hr^{-1} (s.e.m. = 0), while in eight rats perfused 16 days after immunization there was net absorption of 16·85 μl cm^{-1} hr^{-1} (s.e.m. = 9·9).

The change in net fluid secretion in relation to time after immunization for *A. hydrophila* and the *E. coli* producing LT only is shown in Fig. 5. Regression analysis showed the relationship to be significant ($P<0{\cdot}05$) for both organisms using a model of the form $y=a+bx+cx^2$. The maximum difference between control and immunized rats occurred between days 12 and 14 for *A. hydrophila* and between days 14 and 16 for *E. coli*.

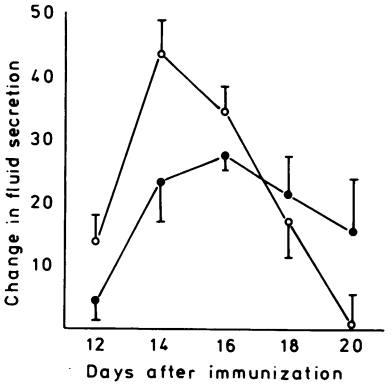

Fig. 5. Change in net fluid secretion after immunization with cholera toxin. Perfusion experiments *in vivo* with cell-free preparations of (o) *Aeromonas hydrophila* and (•) *E. coli* ($n=8$).

Table 1. Antitoxin titres* in the serum and jejunal washings† in relation to immunization of rats with cholera toxin

	n	Jejunal washings	*n*	Serum
Control	13	0·64 (0·7)	31	364·55 (54·88)
Day 12			23	1,509·73 (148·28)
Day 14	4	1·24 (0·315)‡	4	77·80 (99·72)
Day 16	10	0·73 (0·07)	3	1,597·16 (278·18)
Day 18	4	1·11 (0·14)‡	4	1,265·90 (244·88)
Day 20	4	1·26 (0·21)‡	4	1,452·78 (240·50)
Day 40	3	0·67 (0·02)	2	723·00 (156·31)

* Mean and (s.e.m.).

† Antitoxin units ml^{-1} of collected jejunal washings.

‡ Significantly different from control value ($P < 0·05$).

Demonstration of enterotoxins in cell-free preparations. The cell-free broth preparations used in perfusion experiments were assayed in other test systems to confirm that toxin was present. All Aeromonas preparations used were positive for enterotoxin when assayed in the suckling mouse system. *E. coli* preparations assayed using ELISA contained LT detectable at a dilution of 1 : 2 or more in all perfusates used.

Immune response in rats used for perfusion studies. Antitoxin titres in the serum at day 12 and at the time of perfusion demonstrated an increase over pre-immunization values in all animals immunized with CT and used in perfusion experiments (Table 1).

The approximate peak titres of antitoxin activity in jejunal washings occurred about day 14 and day 20 after primary immunization (Table 1).

DISCUSSION

Aeromonas produces several exotoxins (Trust & Chipman, 1979) which complicates interpretation of responses in different test systems. Donta & Haddow (1978), using Y1 and HeLa cell cultures, demonstrated cytotoxicity which was not neutralized by antisera to enterotoxins of *E. coli*, *V. cholerae*, *S. dysenteriae* or *C. perfringens* used in amounts sufficient to neutralize homologous toxin. However, enterotoxin was not detected using the rabbit ileal loop system and cytotoxicity cannot be equated with enterotoxicity in their study. Dobrescu (1978), using a strain of Aeromonas which produced enterotoxin detectable in the rabbit ileal loop assay, found that antiserum to LT neutralized the effects of Aeromonas toxin on Y1 cell cultures.

Wadstrom *et al.* (1976) reported partial inhibition of Aeromonas enterotoxin by antiserum to CT or LT in preliminary experiments but later failed to confirm this using rabbit ileal loops as the test system, although immunodiffusion studies showed a single precipitate against antisera to CT (Ljungh, Popoff & Wadstrom, 1977). Boulanger *et al.* (1977) were also unable to neutralize the effects of *A. sobria* enterotoxin with antiserum to LT using rabbit ileal loop or suckling mouse assays. However, amounts of antisera in excess of the volume required for neutralization of the effects of LT were not used in these studies.

Our study has shown immunological cross-reactivity of *A. hydrophila* enterotoxin with cholera toxin using in-vivo perfusion of rat jejunum. Significant differences in net fluid secretion were found in experiments after incubation of cell-free preparations of enterotoxigenic *A. hydrophila* with anti-cholera toxin. Perfusion experiments, using rats immunized with cholera toxin by intraperi-

toneal priming and intraduodenal boosting, also showed fluid secretion in the presence of Aeromonas enterotoxins to be significantly decreased in immunized animals.

Cross-reactivity between cholera toxin and *E. coli* LT is well established (Holmgren *et al.*, 1973). Our experiments confirmed this finding and showed that the decrease in fluid secretion during perfusion of *E. coli* LT in immunized rats is similar to the response with preparations of enterotoxigenic *A. hydrophila*. Intestinal fluid secretion was also decreased in immunized rats perfused with cholera toxin. These findings confirm the validity of the experimental technique chosen.

The maximal response to immunization occurred after 12–16 days as measured by the proportion of cholera-antitoxin-containing cells in mesenteric lymph nodes, antitoxin titres in jejunal washings and serum antitoxin levels. This peak corresponds to the time of the maximal effect on fluid secretion.

Pre-incubation of cell-free preparations of *A. hydrophila* with anticholera toxin did not neutralize the effects in the suckling mouse assay even with concentrations in excess of those which decrease intestinal fluid secretion in the rat perfusion system. Experiments in our laboratory suggest that there may be more than one Aeromonas enterotoxin, as suggested by Boulanger *et al.* (1977), analagous to production of ST and LT by strains of *E. coli* but unlike these toxins in their heat lability. We have found that heating cell-free preparations of *Aeromonas* sp. to 56°C for 10 min prevents the response in the suckling mouse assay. However, intestinal secretion is still demonstrable in the rat perfusion model using heated samples. Even after heating cell-free preparations to 100°C for 30 min, net fluid absorption was significantly less than normal in this system. It seems likely that *Aeromonas* sp. may produce a heat-labile toxin, detectable in the suckling mouse assay and which does not cross-react with cholera toxin, as well as a more heat-stable toxin which causes secretion during intestinal perfusion and cross-reacts with cholera toxin.

Our studies confirm that cytotoxicity of *A. hydrophila* is not neutralized by anticholera toxin. Wadstrom *et al.* (1976) suggested that Aeromonas enterotoxins could be detected in cell culture systems after heating to 56°C to inactivate proteases which may cause non-specific positive reactions. However, we found no activity detectable using Y1 or Vero cell cultures after heating cell-free preparations of the Aeromonas strain chosen to 56°C for 10 min.

Haemolysin titre is also unaffected by incubation with anticholera toxin which suggests that this toxin is also different from Aeromonas enterotoxin detectable using intestinal perfusion.

Conflicting results regarding cross-reactivity of Aeromonas exotoxins with cholera toxin probably reflect the range of test systems used. Results in cell culture, for example, apply to cytotoxins but not necessarily to enterotoxins of *Aeromonas* sp. Failure to neutralize enterotoxin activity in rabbit ileal loops (Boulanger *et al.*, 1977) may be explained by the use of anticholera toxin in amounts sufficient only to neutralize homologous toxin.

Our observations extend the range of known organisms capable of producing enterotoxins which cross-react with cholera toxin.

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