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# Cytotoxic Activity of *Aeromonas hydrophila*

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Most strains of *Aeromonas hydrophila* tested demonstrated cytotoxic activity on several tissue-cultured cell lines. The cytotoxin is heat-labile, non-dialyzable, and immunologically distinct from that of *Shigella dysenteriae* and *Clostridium perfringens*. None of the aeromonas isolates was found to be enterotoxigenic by either tissue culture or rabbit ileal loop assays.

*Aeromonas hydrophila* is a pathogen of several vertebrate species, including man, and has been associated with a variety of clinical illnesses ranging from skin and wound infections to septicemia (9, 16–18, 20–22). Properties of *A. hydrophila* that have been reported to be associated with virulence of the organism include hemolytic, proteolytic, lecithinase, cytotoxic, and enterotoxic activities (1, 2, 24; S. T. Donta, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 17th, New York, N.Y., Abstr. no. 397, 1977).

Because one of the criteria for enterotoxicity used by one group of investigators was the ability of culture supernatants to effect morphological changes in Y1 adrenal tissue-cultured cells (13, 23, 24) and because the results of previous studies had indicated that only strains of *Vibrio cholerae* or *Escherichia coli* could effect such changes in Y1 cells (5, 9), it was of considerable interest to attempt to confirm the finding of enterotoxigenic strains of *A. hydrophila* and to compare its toxic properties in tissue culture with those of *V. cholerae* and *E. coli*.

## MATERIALS AND METHODS

**Organisms.** Enterotoxigenic strains of *A. hydrophila* were obtained from T. Wadström (23); nine additional isolates of *A. hydrophila* from a variety of clinical sources were obtained from J. Washington III (Mayo Clinic), and three more isolates were furnished by W. Sommers (Northwestern University). All of the strains obtained from T. Wadström were confirmed to be *A. hydrophila* with standard biochemical tests done in the clinical microbiology laboratory (courtesy of E. Berglund). The bacteria were stored on agar slants and inoculated into Trypticase soy broth for growth (usually at 37°C in a shaking water bath) and further testing.

**Tissue culture methods.** Monolayer cultures of Y1, HeLa, $C_6$, and HTC (hepatoma) cells were similar to those used previously (3, 4, 10) and were propagated in Ham nutrient mixture F10 supplemented with 15% horse serum and 2.5% fetal calf serum on disposable tissue culture flasks or plates (Lux-Biolabs) at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. Cytotoxicity was assessed morphologically by the presence of destructive changes and by the failure to exclude trypan blue. Nondestructive morphological changes compatible with those elicited by the heat-labile enterotoxins of *V. cholerae* and *E. coli* were further assessed by the ability of Y1 cells to increase their production of cyclic AMP and steroids (7); quantitation of cyclic AMP was by radioimmunoassay (Schwarz/Mann), and that of steroids was by methylene chloride-extractable fluorescence (25).

**Toxins, antitoxins, and chemicals.** The preparations of *V. cholerae* and *E. coli* toxins and antitoxins were those used in previous studies (10). Antisera to *Shigella dysenteriae* toxin and to *Clostridium perfringens* enterotoxin were obtained from G. Keusch and C. Duncan, respectively (10). The trypsin and Pronase used in some of the experiments were both obtained from Sigma Chemical Co.

**Ileal loop studies.** Two-kilogram albino rabbits (local supplier) were used in the ileal loop studies, and ligated loops were tested for fluid accumulation as in previous studies (6). Incubation was continued for 18 h after injection of 1.0 ml of culture filtrates into the ileal loops, and a positive reaction was interpreted as the accumulation of 1.0 ml or more of fluid per cm of a 10-cm loop.

## RESULTS

**Morphological activity.** All 11 strains of *A. hydrophila* obtained from T. Wadström, 7 of 9 strains obtained from J. Washington, and 1 of 3 strains obtained from W. Sommers demonstrated cytotoxic activity on either Y1 adrenal or HeLa cells (Table 1). (The isolate from Northwestern University active on Y1 cells was also active on $C_6$ rat glial and rat hepatoma cells.) The onset of cytotoxicity was as early as 30 min postexposure of Y1 cells to cell-free culture filtrates (strain K141) or as late as 7 h (strains K144, K185), the differences correlating well with the concentrations of cytotoxin in the filtrates. The earliest morphological changes consisted of some rounding of the cells but, in general, were better characterized as consisting of degenerative changes that became more appar-

TABLE 1. *Cytotoxicity of A. hydrophila*

| Strain | Cytotoxicity to:[a] | |
|---|---|---|
| | Y1 cells | HeLa cells |
| Swedish strains | | |
| K118, K138, K144 | + | − |
| K137, K139, K140, K141, K185, K194 | ++ | ++ |
| K142 | ++ | + |
| K206 | − | + |
| Mayo isolates | | |
| No. 2, 3, 5, 7, 8, 9, 12 | ++ | ++ |
| No. 4, 10 | − | − |
| Northwestern isolates | | |
| 1088, 1089 | − | − |
| 1143 | + | − |

[a] Degree of cytotoxicity is expressed as: ++, most cells involved; +, some cells involved; and −, no cytotoxicity.

ent with time (Fig. 1). By 3 h, cytotoxicity, as determined by the failure to exclude trypan blue, was discernible with isolate K141; by 24 h most cytotoxic preparations resulted in the dislodgment of tissue culture cells from plate surfaces.

**Steroidogenic activity.** None of the seven isolates of *A. hydrophila* selected for varying degrees of cytotoxic activity was capable of inducing steroidogenic changes of Y1 cells, and none of two isolates was capable of activating adenylate cyclase, in contrast to *V. cholerae* and *E. coli* toxin controls (Tables 2 and 3). In fact, filtrates from cytotoxic strains of *A. hydrophila* elicited a depression of basal steroid production.

**Properties of the cytotoxin.** The *A. hydrophila* cytotoxin was found to be non-dialyzable (strains K144, K185) and resistant to proteolytic treatment with trypsin (0.1% for 60 min, strains K138, K144, K185) or Pronase (0.1% for 30 min, strains K137, K140, K141, K194). The cytotoxic activity was not neutralized by prior incubation of cell-free filtrates (strains K138, K142, K144, K185) with antisera to *E. coli, V. cholerae, S. dysenteriae,* and *C. perfringens* enterotoxins in amounts capable of complete neutralization of homologous toxin activity.

In contrast to the resistance of the cytotoxin to proteolytic activity, the toxin was very susceptible to heat exposure. All cytotoxic preparations were inactivated by temperatures of 100°C for 5 min, and four such preparations tested at 56°C (strains K138, K141, K144, K185) were also inactivated in 5 min. With one isolate (K141), heat lability could be demonstrated at 45°C (Table 4).

Attempts to demonstrate enterotoxicity of the 11 Wadström isolates in ligated rabbit ileal loops were unsuccessful; incubation of one of the isolates at 24°C (conditions employed by the Swedish investigators [24]) did not lead to a positive loop reaction. Examination of some of the aeromonas filtrate-treated loops disclosed some hemorrhagic necrosis, but none of the loops manifested fluid output as was noted in control enterotoxigenic *E. coli* filtrate-treated loops at the end of 18 h.

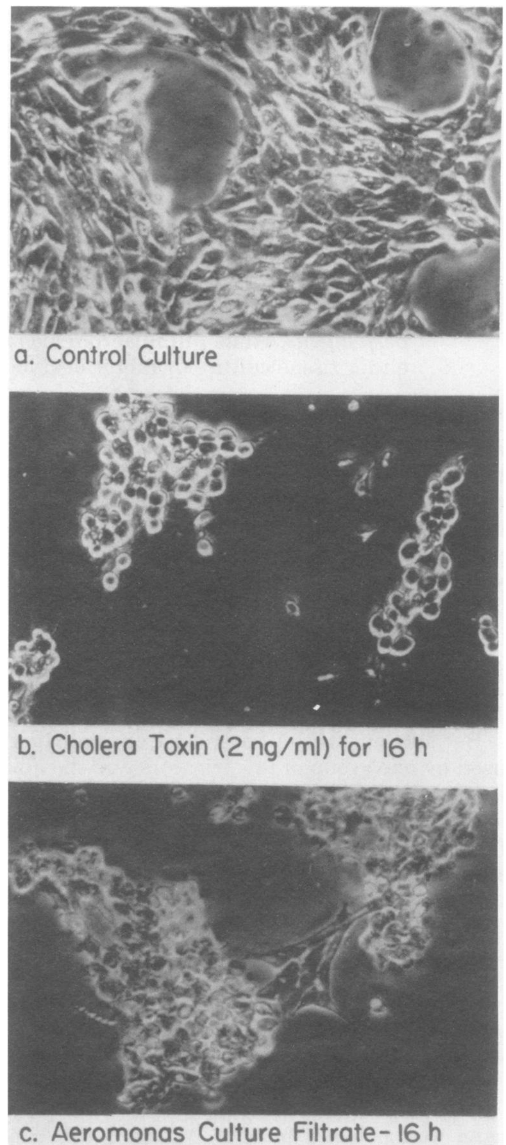


FIG. 1. *Phase-contrast photomicrographs of Y1 adrenal cells.*

## DISCUSSION

The cytotoxic activity of isolates of *A. hydrophila* has been noted previously. Wadström et

TABLE 2. *Effect of aeromonas filtrates on steroidogenesis*

| Filtrate | μg of steroids/plate[a] |
|---|---|
| Expt 1 | |
| Control | 23.4 ± 4.8 |
| ECT[b] (10 μg/ml) | 57.4 ± 5.5 |
| *Aeromonas* strain 2 | 8.8 ± 1.0 |
| *Aeromonas* strain 3 | 12.4 ± 3.6 |
| *Aeromonas* strain 4 | 17.1 ± 2.8 |
| Expt 2 | |
| Control | 16.4 ± 0.8 |
| CT[c] (2 ng/ml) | 23.8 ± 0.5 |
| *Aeromonas* strain K137 | 4.4 ± 0.9 |
| *Aeromonas* strain K140 | 3.8 ± 0.4 |
| *Aeromonas* strain K141 | 4.0 ± 0.4 |
| *Aeromonas* strain K194 | 5.3 ± 0.7 |

[a] Results are expressed as micrograms of steroids ± standard deviation per plate per 22 h (experiment 1) or per 16 h (experiment 2).
[b] ECT, *E. coli* enterotoxin (H10407).
[c] CT, Cholera enterotoxin.

TABLE 3. *Effect of aeromonas filtrates on adenylate cyclase activity*

| Filtrate | Adenylate cyclase activity (pmol of cyclic AMP ± standard deviation/plate per 7 h) |
|---|---|
| Control | 0.9 ± 0.1 |
| ECT[a] (10 μg/ml) | 23.5 ± 3.2 |
| *Aeromonas* strain 144 | 3.1 ± 0.2 |
| *Aeromonas* strain K185 | 3.4 ± 0.1 |

[a] ECT, *E. coli* enterotoxin (H10407).

TABLE 4. *Effect of heating on aeromonas cytotoxicity*

| Time (min) | Greatest dilution at which toxicity present at: | | |
|---|---|---|---|
| | 45°C | 48°C | 53°C |
| 10 | 1:160 | 1:80 | <1:10 |
| 30 | 1:160 | 1:10 | <1:10 |
| 60 | 1:80 | <1:10 | |
| 180 | <1:10 | | |

al. reported that they could denote both a cytotoxic and an enterotoxic component, using primarily Y1 adrenal cells in tissue culture, and that the cytotoxic component could be inactivated by heating the preparation for 10 min at 56°C, whereas the enterotoxic component required heating at 80°C for 15 min to be inactivated (13, 24). This group of investigators further reported as unpublished observations that the aeromonas enterotoxin causes a release of steroids from Y1 cells similar to that effected by cholera toxin (13).

There are conflicting reports in the literature regarding the enterotoxigenicity of isolates other than species of *V. cholerae, Vibrio parahaemolyticus, E. coli, S. dysenteriae,* and *C. perfringens.* Toxigenic strains of *V. cholerae* and *E. coli* elicit noncytotoxic morphological changes in several tissue culture systems, whereas the shigella and clostridial toxins produce a cytotoxic picture (10). Guerrant et al. reported that several strains of *Klebsiella* sp. were enterotoxigenic to Chinese hamster ovary cells (8), but enterotoxicity could not be confirmed by repeat testing in his laboratory or in ours; confirmation by rabbit ileal loop testing was also lacking. Wadström et al., primarily on the basis of Y1 adrenal cell and skin testing, reported that strains of a variety of *Enterobacteriaceae* were found to be enterotoxigenic, including *Klebsiella, Enterobacter, Proteus, Citrobacter, Serratia,* and *Aeromonas* species (23). This group of investigators felt that they were able to differentiate the cytotoxic from the enterotoxic reaction in tissue cultures by differential heating (24). Klipstein et al. have reported the isolation of enterotoxigenic strains of *Klebsiella* sp. and *Enterobacter* sp. from patients with sprue, using the perfused rat ileum (11, 12), yet representative isolates have not yielded positive reactions in either Chinese hamster ovary or Y1 cells or in standard rabbit ileal loop systems.

Although the reasons for these discrepancies have not been elucidated, some of the explanations offered include the relative sensitivities of the assay systems employed and the stability or expression of any toxin-coding plasmid. One of the major problems, however, may rest in the interpretation of the assay results. In the case of tissue culture system, a cytotoxic response could be construed as representing enterotoxicity if morphological changes alone are used; i.e., the cytotoxic process may involve rounding of cells before their dislodgment from tissue culture surfaces. Confirmation of enterotoxicity, however, requires concomitant specific biochemical alterations (e.g., adenylate cyclase activation or steroidogenesis) or correlation with intestinal assay systems. With intestinal loop systems, the inherent problems of positive loop responses in unstimulated preparations are well known, and a sufficient number of segments, separated by interloops, need to be employed in two or more animals before confidence can be placed in the data (14, 15). In addition, the use of sterile culture filtrates increases the probability that any fluid accumulation observed is due to an enterotoxic response; the use of preparations containing live organisms can lead to nonenterotoxic, inflammation-mediated loop reactions. In the reports of enteropathogenicity of strains of *A. hydrophila,* Sanyal et al. used predominantly live cultures and obtained positive loop responses; when culture filtrates were employed, the maximum results achieved were 1.1 ml of fluid per cm of gut, although it was stated that

a crude toxin preparation elicited fluid accumulation up to 1.7 ml per cm of gut (1, 19).

The details of the loop reactions found by the Wadström group, with one exception, were not reported (23, 24); the values reported with the one isolate ranged between 1.4 and 1.6 ml of fluid per cm of gut, but means and standard errors were not given (24). The results of our investigations did not confirm the presence of an enterotoxin as defined by either tissue culture or by standard ileal loop reactions; as regards the latter, a hemorrhagic type of necrotic reaction was noted in response to some of the aeromonas filtrates, but no fluid accumulation comparable to that of *E. coli* controls (>2.0 ml per cm of gut) was observed. It could be, however, that the enterotoxic properties of these strains were lost or suppressed upon transfer from one laboratory to the next. Recently, Boulanger et al. reported that some strains of *A. hydrophila* and *Aeromonas sobria* isolated from healthy and from moribund fish elicited positive rabbit loop and/or suckling mouse reactions; none of the details were given, but it was stated that all 8 strains of *A. sobria* gave both positive suckling mouse and rabbit loop tests, whereas 4 of 13 strains of *A. hydrophila* were positive in the rabbit loop test and 11 of the 13 were positive in the suckling mouse test (2).

The cytotoxin of *A. hydrophila* seems to be present in most strains, regardless of the clinical source. This high prevalance of cytotoxigenicity appears to be greater than that found in random sampling of isolates of *Enterobacteriaceae* (~1 to 3%). The nature and role for the cytotoxin(s) await further purification and characterization. Its sensitivity to heat suggests a protein nature; the resistance of the toxin to proteolytic treatment may be relatable to use of crude toxin material. The relationship of the cytotoxin to any leukocidic, hemolytic, or other toxic properties is under study currently as is the inducibility of the toxin under a variety of temperature and cultural conditions. With the availability of a purified cytotoxin, it should be possible to develop specific antisera that can be used to further assess the degree of toxin homogeneity among cytotoxigenic strains of *Aeromonas* spp. and to compare the aeromonas toxin with cytotoxins elaborated by other organisms.

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