INFECTION AND IMMUNITY, Aug. 1974, p. 320-327

Vol. 10, No. 2
Printed in U.S.A.

Cyclic Adenosine Monophosphate and Alteration of Chinese Hamster Ovary Cell Morphology: a Rapid, Sensitive In Vitro Assay for the Enterotoxins of *Vibrio cholerae* and *Escherichia coli*

RICHARD L. GUERRANT, LAURENCE L. BRUNTON, TERRY C. SCHNAITMAN, LIONEL I. REBHUN, AND ALFRED G. GILMAN

Departments of Internal Medicine, Pharmacology and Biology, University of Virginia, Charlottesville, Virginia 22901

Received for publication 1 April 1974

The major limitation to our understanding of the clinical importance of enterotoxigenic *Escherichia coli* in diarrheal illness has been the lack of a simple rapid assay for the enterotoxin produced by certain *E. coli*. On the basis of the activation of adenylate cyclase by heat-labile enterotoxin of *E. coli* (LT) and by cholera toxin (CT) in intestinal and other tissues, cultured Chinese hamster ovary (CHO) cells with known morphological responses to dibutyryl cyclic adenosine 5'-monophosphate (AMP) were exposed to these enterotoxins. Crude culture filtrates of LT-producing *E. coli* and CT stimulated cyclic AMP accumulation and cell elongation in CHO cells. The similarity of time course, concentration dependence, and potentiation by phosphodiesterase inhibitors suggested cyclic AMP mediation of the morphological change. Heat inactivated CT and LT in this system. Choleragenoid inhibited CT; antiserum against CT inhibited both enterotoxin effects. In contrast to culture filtrates of 16 strains of *E. coli* known to produce LT, culture filtrates from 13 *E. coli* that do not produce LT did not alter CHO cell morphology. The morphological change is a simple, specific assay for these enterotoxins and detect 3×10^{-17} mol of CT or a 1:250 dilution of crude culture filtrate of LT-producing *E. coli* 334.

Substantial evidence indicates that the lumenal accumulation of isotonic fluid in diarrhea caused by *Vibrio cholerae* and certain *Escherichia coli* results from the actions of enterotoxins produced by these organisms (7, 17). These toxins stimulate adenylate cyclase activity and elevate intracellular concentrations of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in intestinal mucosa (3, 27, 32, 33). The magnitude and time course of adenylate cyclase stimulation correlate well with changes in sodium and water fluxes (20, 21). Other agents that increase intracellular cyclic AMP (theophylline, vasopressin) and exogenous cyclic AMP itself mimic the effects of these toxins on short-circuited rabbit ileal mucosa (1, 13).

Noting that cholera enterotoxin can elevate cyclic AMP content in numerous tissues, Bourne and co-workers have suggested that this property of the toxin makes it a unique pharmacological tool for testing hypotheses involving cyclic AMP and cell function (2). *E. coli* and cholera enterotoxins stimulate cyclic AMP accumulation in thyroid slices (29), and cholera toxin induces cyclic AMP-mediated events in fat cells (35), platelets, liver (P. D. Zieve, N. F. Pierce, and W. B. Greenough III, Clin. Res., 1970, 18:690), and leukocytes (2).

Most studies of the actions and mechanisms of these enterotoxins have been carried out on intestinal loops or with a skin permeability model (4). Whereas functional relationships can be studied with these preparations, they represent heterogeneous cell populations in which circulatory factors cannot be ruled out. In addition, their use for routine assay purposes is cumbersome and expensive. Consequently, only a few studies have been made of the incidence of enterotoxin-producing *E. coli* as a cause of acute "undifferentiated" diarrhea (17, 18).

Hsie and Puck have reported that the clonal line CHO-K1, a Chinese hamster ovary (CHO) derivative, responds with distinct morphological and biochemical changes after treatment with dibutyryl cyclic AMP, testosterone, and certain prostaglandins (24, 25). Accordingly, we have studied the effects of cholera and *E. coli* enterotoxins on intracellular cyclic AMP con-

centrations of a variety of cultured cell lines, and we have correlated these changes in cyclic nucleotide metabolism with morphological changes in CHO cells. This characteristic morphological alteration appears to serve as a simple marker of elevated cyclic AMP concentration and thus forms the basis of an assay for these enterotoxins.

MATERIALS AND METHODS

Cyclic AMP determinations. Confluent cultures of a number of cell lines were obtained by plating 2×10^5 to 3×10^5 cells per 60-mm dish and growing for 4 to 6 days in the appropriate medium (Dulbecco's modified Eagle medium, with 5 or 10% fetal calf serum [FCS] for all lines but CHO) with medium changes every other day and the day prior to the experiment. CHO-K1 cells were obtained from the American Type Culture Collection, Rockville, Md. The sources and origins of other cells used here have been described elsewhere (16). For studies of cyclic AMP concentrations in CHO cells, stock cultures were maintained in F10 medium (containing 2 g of $NaHCO_3$ per liter) with 10% FCS in 10% CO_2 at 37 C. To determine cyclic AMP concentrations under density and growth conditions comparable to those used in the morphological studies, 10^6 to 1.5×10^6 CHO cells were plated per 60-mm dish and grown in F10 with 10% FCS for 8 to 12 h to allow cell attachment. Plates were then washed with F10 without FCS, and 2 ml of F10 with 1% FCS was added. Theophylline or 1-methyl-3-isobutylxanthine (MIX; Aldrich Chemical Company, Milwaukee, Wis.) was included as indicated. After a 30-min incubation, appropriate dilutions of enterotoxin were added, and plates were incubated at 37 C for the indicated times.

Incubations were terminated by aspiration of the medium and addition of 1 ml of cold 5% trichloroacetic acid to each plate. Samples were assayed for cyclic AMP by the procedure of Gilman (15) as slightly modified by Gilman and Murad (*in* J. G. Hardman and B. W. O'Malley (ed.), *Methods in Enzymology*, in press). Cellular protein precipitated by the trichloroacetic acid was dissolved in 0.2 N NaOH and determined by the method of Lowry et al. (28). Cyclic AMP concentrations are expressed as picomoles of cyclic AMP per milligram of cellular protein, and each point represents the average from two or more samples of cells, each assayed in duplicate. Sample values were within 5% of the average.

Morphological studies. Stock cultures of CHO cells were grown in F12 medium supplemented with 10% FCS in 6% CO_2 at 37 C and were passaged by trypsinization. For morphological studies, a suspension containing approximately 5×10^3 cells in 0.25 ml of F12 medium plus 1% FCS was added to each chamber of an eight-chamber culture slide (Lab-Tek Products, Naperville, Ill.). Enterotoxins were added immediately after plating. Phase-contrast microscopic examination was made at appropriate times to determine the percentage of cells that had become clearly bipolar, were elongated at least three times their width, and had lost their knoblike projections. Each morphological datum on all graphs presented represents the mean percentage of cells elongated from 400 cells counted in a total of four studies. These were done on at least two different days, with a maximal range of ± 6% of cells elongated in studies showing an effect.

Purified cholera toxin (lot 0572) and Swiss Serum Vaccine Institute antiserum against purified cholera toxin were provided by R. S. Northrup and C. E. Miller, National Institute of Allergy and Infectious Diseases, for whom the toxin is prepared by R. A. Finkelstein in Dallas (14). Choleragenoid, the naturally occurring toxoid of cholera toxin, was kindly provided by R. A. Finkelstein. Choleragenoid was incubated with cells 30 min prior to their exposure to cholera toxin. Antiserum against purified cholera toxin and nonimmune rabbit serum were preincubated with the two enterotoxins at 37 C for 30 min prior to cell challenge.

Well-studied, enterotoxin-producing and control *E. coli* strains (10, 11, 21, 22, 31) were grown in syncase medium by the procedure of Sack (31). Sterile filtrates of these cultures were prepared by centrifugation at 10,000 × *g* for 30 min at 5 C and passage through a 0.22-μm membrane filter (Millipore Corporation, Bedford, Mass.). When such filtrates were added to cell cultures, the concentrations were expressed as dilutions of the original filtrates.

RESULTS

Cholera toxin effects. Initial studies showed that cholera toxin (1 μg/ml) produced an elevation of intracellular cyclic AMP in each of 18 different mammalian cell lines. Figure 1 shows the development of prominent responses to cholera toxin in several cell lines and the variable but definite lag phase. The lag is a characteristic feature of the effect of cholera toxin on cyclic AMP in all cells studied (2, 20, 27, 29, 32).

Preliminary studies with CHO cells showed the morphological response was maximal at 24 h after cholera toxin exposure with cells grown in 1% FCS. Subsequent studies of cyclic AMP concentrations and morphology were made under these conditions, unless otherwise stated. The concentration dependence of the effect of cholera toxin on cyclic AMP content in the CHO cells is shown in Fig. 2. This effect is enhanced by the phosphodiesterase inhibitor, MIX, and is antagonized by equimolar or greater concentrations of choleragenoid. Incubation with 0.5 mM MIX causes a significant 38% increase in basal cyclic AMP content as well ($P < 0.01$, $n = 14$ pairs). The effect of 0.05 mM MIX, as shown in Fig. 2, is smaller, if present, upon basal cyclic AMP content. Heated toxin (100 C, 20 min) causes no response. Similar results are seen on the percentage of cells elongated with cholera toxin (Fig. 3), with morphological changes becoming maximal at a concentration of cholera toxin 10-fold lower

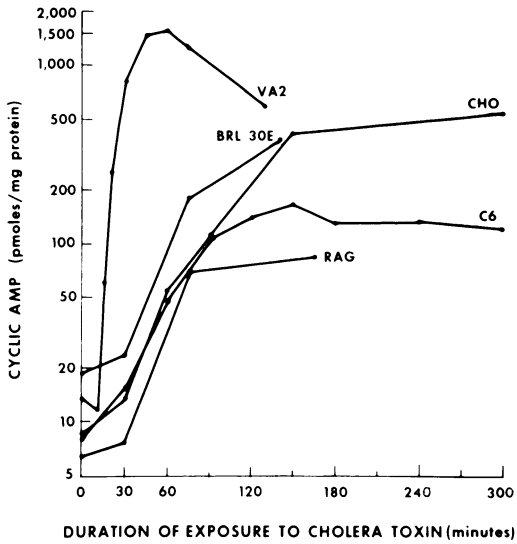

FIG. 1. *Time course of cyclic AMP accumulation due to cholera toxin. Confluent cultures were incubated with 1 mM theophylline, and after 30 min cholera toxin was added (1 μg/ml except 10 μg/ml for C6). At indicated times incubations were terminated as described in the text. Cell lines: VA2, SV40 transformed human fibroblast; C6, rat glioma; BRL30E, buffalo rat liver; RAG, mouse renal adenocarcinoma; CHO, Chinese hamster ovary.*

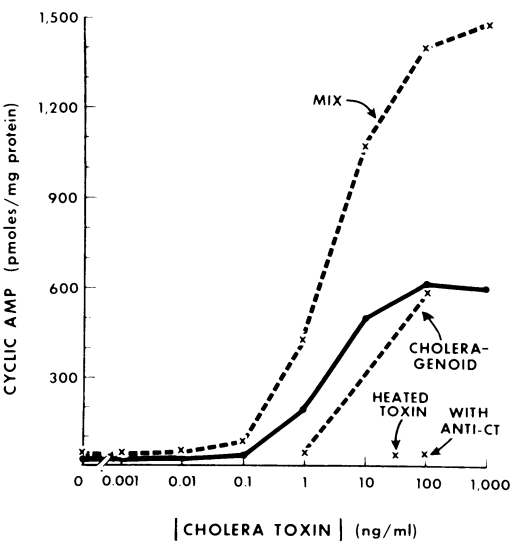

FIG. 2. *Cyclic AMP accumulation in CHO cells incubated for 24 h with cholera toxin in 1% FCS with (×) or without (●) 0.05 mM MIX. The 32% elevation in cyclic AMP concentration with 0.01 ng/ml of cholera toxin is significant ($P < 0.05$, $n = 5$ pairs) by Student's t test. Inhibition of this response by choleragenoid (100 ng/ml) and inactivation of cholera toxin by heating at 100 C for 20 min or by preincubation with antitoxin are also shown.*

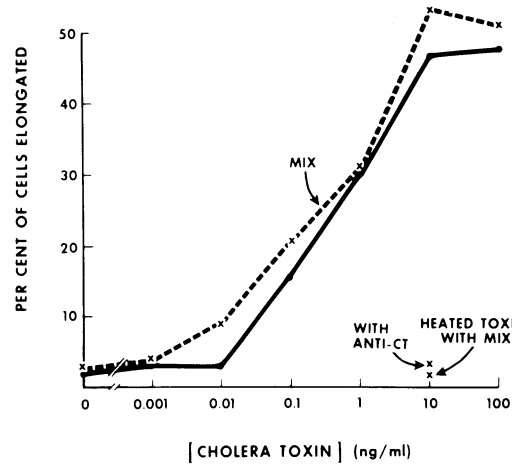

FIG. 3. *Morphological responses in CHO cells incubated for 24 h with cholera toxin in 1% FCS with (×) or without (●) 0.05 mM MIX. Heated toxin and toxin preincubated with antitoxin have no effect on morphology.*

than necessary for a maximal cyclic AMP response. MIX slightly enhances the toxin effect, and heating (as above) inactivates the toxin. Swiss Serum Vaccine Institute cholera antitoxin neutralizes both cyclic AMP and morphological effects of cholera toxin when 10 to 100 ng of cholera toxin per ml is preincubated with a 1:500 dilution of antiserum against purified cholera toxin (Fig. 2 and 3). Similar to results with cyclic AMP concentrations, morphological effects of cholera toxin are inhibited in a dose-related fashion by choleragenoid (Fig. 4). The morphological changes with cholera toxin are identical to those shown in Fig. 6b with the filtrate of enterotoxigenic *E. coli*.

E. coli enterotoxin effects. Similar concentration-dependent effects of crude culture filtrates of heat-labile enterotoxigenic *E. coli* strain 334 (ECT 334) on cyclic AMP concentrations are shown in Fig. 5. Heat-inactivated ECT 334 and the filtrate of a control nontoxigenic *E. coli*, strain 10405, are ineffective, and, as with cholera toxin, MIX enhances the effect of the active toxin. Since serum has been shown to inhibit cholera toxin effects on HeLa cells (6), we examined the effects of FCS upon ECT 334-stimulated CHO cells. There were 50 and 95% reductions in ECT 334-stimulated cyclic AMP levels by 1 and 10% FCS, respectively.

The elongation, polarization, and loss of knoblike projections of CHO cells are striking by 24 h after exposure to a 0.08 dilution of ECT 334, but not to the same dilution of control filtrate (Fig. 6). The effects of varying dilutions of these crude *E. coli* culture filtrates on mor-

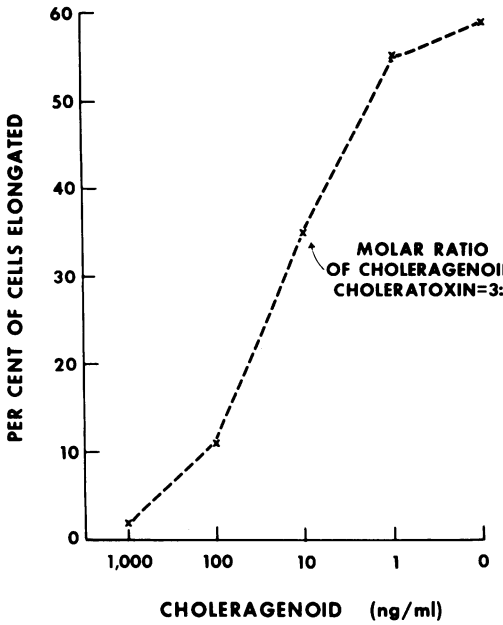

FIG. 4. *Antagonism by choleragenoid of the effect of cholera toxin on CHO cell morphology. Choleragenoid was added to the cells 30 min prior to the addition of cholera toxin (10 ng/ml), and cells were incubated for 24 h. Medium includes 0.05 mM MIX.*

phology are shown in Fig. 7. MIX enhances the ECT 334 effect on morphology, whereas FCS slightly inhibits this change, as it inhibits the effects on cyclic AMP concentrations. Heat-inactivated ECT 334 has no effect on morphology. Preincubation of a 1:25 dilution of ECT 334 with a 1:500 dilution of Swiss Serum Vaccine Institute cholera antitoxin neutralizes both the cyclic AMP and the morphological effects of the toxin (Fig. 5 and 7), whereas a similar dilution of non-immune serum had no effect when preincubated with either enterotoxin.

Figure 8 shows the time course of cyclic AMP accumulation due to cholera toxin (A and B) and to culture filtrates of *E. coli* 334 (C and D) and *E. coli* 10405 (E). The presence of MIX enhances the elevation of cyclic AMP due to either toxin. MIX does not alter the lag period characteristic of either toxin effect. The lag period is notably longer with *E. coli* culture filtrates than with cholera toxin. Immediate, reversible effects on cyclic AMP, such as those described previously in canine small bowel (21) on adenylate cyclase activity with *E. coli* toxin, are not seen in the CHO cells. Cells exposed for 15 min to cholera toxin and then washed several times to remove unbound toxin follow the same time course of cyclic AMP alteration.

The time course of the development of morphological change is more difficult to quantitate. Protruding knobs disappear as early as 2 h after the addition of cholera toxin and at 4 h with toxigenic *E. coli* filtrates. The elongation is maximal at 24 to 30 h, showing that the morphological effects of elevated cyclic AMP begin to be expressed fairly rapidly but are not complete for many hours. In contrast to the reversible morphological effect of dibutyryl cyclic AMP, the morphological alterations due to both cholera and *E. coli* enterotoxins persist for 96 h, despite their removal after 4 h. Over a 24-h time course, control *E. coli* filtrate at the 0.08 dilution had no significant effect upon the measured parameters.

To determine our ability to distinguish between toxigenic and nontoxigenic *E. coli*, 29 strains previously studied in animal models were examined. Strains 334 (O15:H11), 10407 (O78:H11), B2C (O6:H16), B7A (O148:H28), 408-3 (O78:H12), 19662, 9116, 17974, 19437, and 19094, kindly provided by R. B. Hornick, R. B. Sack, and D. J. Evans, were originally isolated in India, Bangladesh, Viet Nam, and Arizona (10, 11, 17, 31) from patients with watery

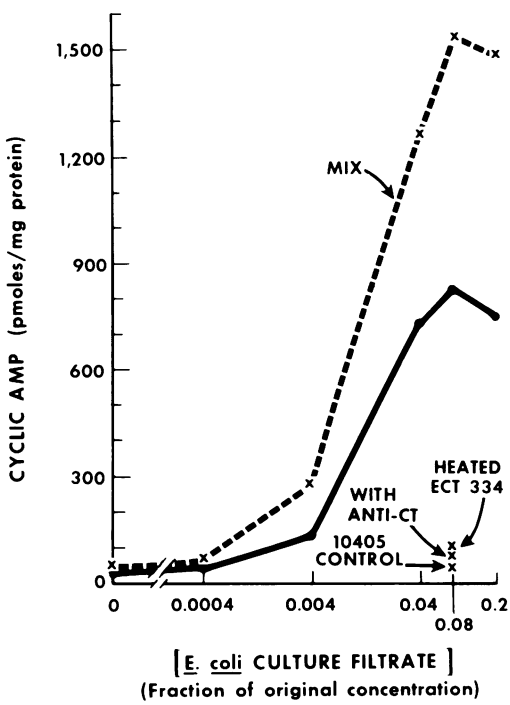

FIG. 5. *Cyclic AMP accumulation in CHO cells incubated for 24 h with E. coli 334 culture filtrate (ECT 334), with (×) or without (●) 0.05 mM MIX. Heated ECT 334, filtrate from control E. coli 10405 (10405 control), and ECT 334 premixed with antiserum against purified cholera toxin are inactive.*

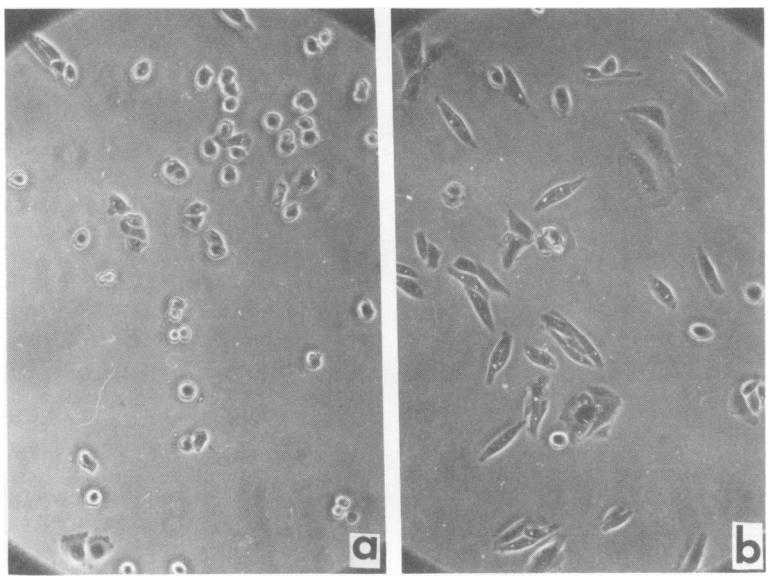

FIG. 6. *Cultures of CHO cells incubated for 24 h in 0.08 dilutions of crude culture filtrates of control E. coli 10405 (a) and toxigenic E. coli 334 (b). Magnification ×100, phase contrast.*

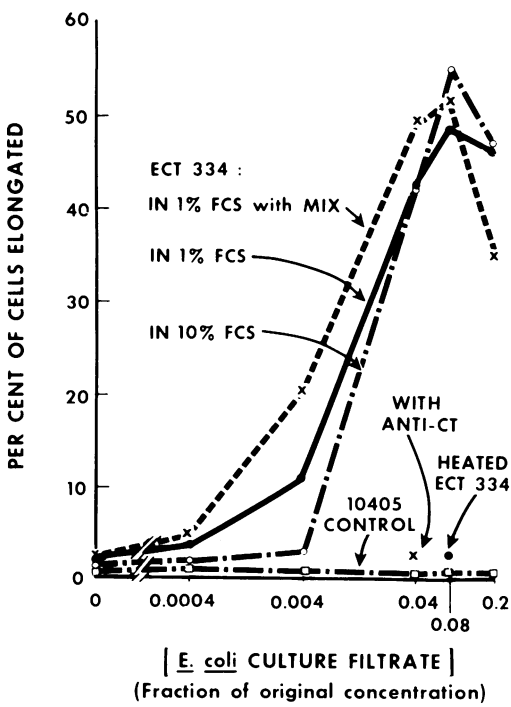

FIG. 7. *Morphological responses in CHO cells incubated for 24 h with ECT 334 under indicated conditions (MIX = 0.05 mM) or with 10405 control with 10% FCS. Heated ECT 334, 10405 control, and ECT 334 pre-mixed with antiserum against purified cholera toxin are inactive.*

diarrhea and are strongly positive by our morphological criteria (average difference from results with control human strains = 39% of cells elongated; $P < 0.001$ by Student's t test [Fig. 9]). Nontoxigenic human strains (all negative in the rabbit ileal loop test) included 10405, 408-4 (O78:H12), HS (non-typable), 1624 (O144:K?:H−), 1272 (O124:K72:H−), and 4608 (O143:K?:H−), the last three being invasive strains (10). These control strains were clearly negative in our CHO assay (Fig. 9). The six toxigenic human *E. coli* filtrates studied produced significant cyclic AMP elevations when compared with the effects of the control strains (average difference = 890 pmol per mg of protein; $P < 0.004$ by Student's t test).

Well-studied porcine strains of *E. coli* (22) were provided by H. W. Moon and C. L. Gyles. Those producing a heat-stable toxin only [711 (K12); 2176E8 (O138:K81); 987 (O9:K(A)); 1351 (O9:K35); (K12, 88ab); P16 (O9:K9); and E57 (O138:K81)] were negative in the CHO assay, whereas those producing heat-labile toxin as well [263 (O8:K87, 88); 1291 (O149:K91, 88); 1260 (O141:K85, 88:H4); P155 (O149:K91, 88ac); 711 with P155 plasmid; and (K12,88ac)] were clearly positive in this assay (Fig. 9).

DISCUSSION

Purified cholera toxin and crude culture filtrates of heat-labile, enterotoxin-producing *E. coli* produce similar elevations of cyclic AMP

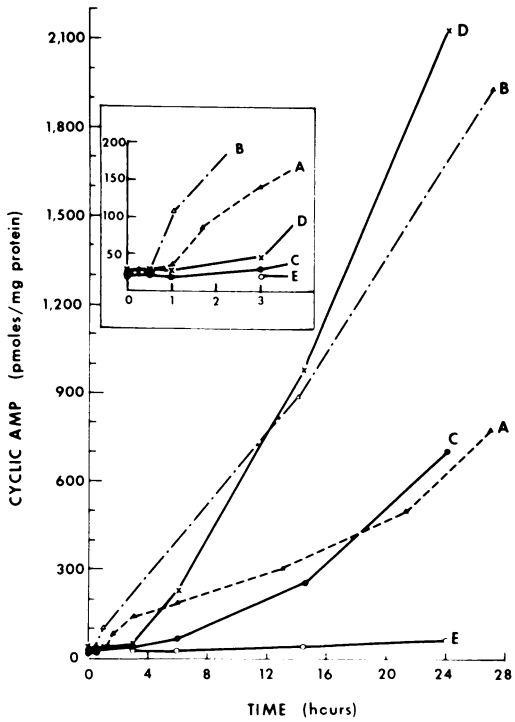

FIG. 8. *Time course of cyclic AMP elevation caused by the enterotoxins. Curves A and B represent effect of cholera toxin, 1 μg/ml, in the absence (A) and presence (B) of 0.1 mM MIX. Lines C and D show the time course of ECT 334, 0.08 of original filtrate concentration, with (D) and without (C) 0.5 mM MIX. Curve E shows the lack of effect of a 0.08 dilution of 10405 control in the presence of 0.5 mM MIX. Insert shows early time course.*

and parallel characteristic morphological changes in CHO cells. This demonstration of elevated cyclic AMP concentrations during elongation supports Puck's suggestion that cyclic AMP is involved in the alterations of morphology, agglutinability, collagen synthesis, contact inhibition, and microtubule polymerization in CHO cells (30). The similar enhancement of cyclic AMP and morphological effects of both enterotoxins by phosphodiesterase inhibition provides further evidence for the cyclic AMP mediation of the morphological response. In addition, FCS antagonized both cyclic AMP and morphological effects of enterotoxins on CHO cells. The specificity of this assay for the heat-labile enterotoxins of both *V. cholerae* and *E. coli* is further shown by the inhibition of either toxin effect by preincubation with antiserum against purified cholera toxin, as shown in animal loop studies (23).

The concentration of cholera toxin causing half-maximal cyclic AMP accumulation in CHO cells is 3 ng/ml (approximately 3×10^{-11} M), a result identical to that obtained in glioma line C6 (L. L. Brunton and A. G. Gilman, unpublished observations). This concentration is three orders of magnitude smaller than the binding constant of cholera toxin to rat thymocytes (J. M. Boyle, I. D. Goldfine, and J. D. Gardner, Clin. Res., 1973, **21**:509) and one to two orders of magnitude smaller than the binding constants of toxin-fat cell and toxin-liver membrane complexes (P. Cuatrecasas, J. Clin. Invest., 1973, **52**:22a) (5). Whereas quantitative comparisons of these different systems are difficult, this disparity could be a reflection of a number of "spare receptors" for cholera toxin stimulation of cyclic AMP accumulation. Thus, maximal accumulation of cyclic AMP could result when only a fraction of toxin receptors are occupied.

The insensitivity of serological screening for "enterophathogenic" *E. coli* has been demonstrated (17, 18). Enterotoxin-producing capability has recently been shown to be transferrable between strains of *E. coli* by a plasmid (34), thus rendering serological screening unreliable.

The CHO assay provides a morphological index of enterotoxin activity somewhat similar to those described by other workers using HeLa cells (26) and the adrenal tumor line Y1 (8, 9). The exquisite sensitivity of these assay systems

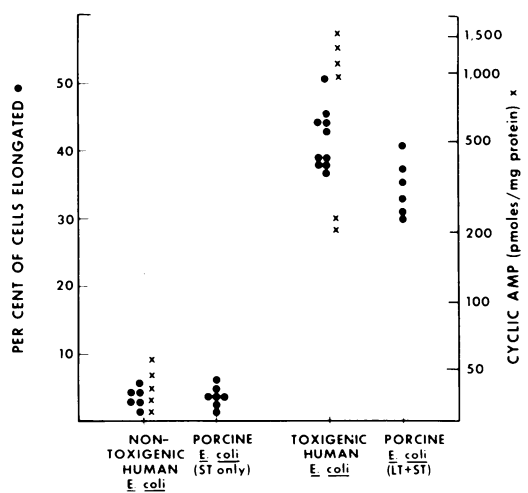

FIG. 9. *Elongation (●) and cyclic AMP accumulation (×) in response to toxigenic and nontoxigenic human E. coli strains, and morphological effect (●) of porcine E. coli strains that produce only heat-stable enterotoxin (ST only) and that produce heat-labile toxin as well (LT + ST). Incubation mixtures contain 1% FCS and 0.05 mM MIX.*

may prove adequate for assay of antitoxic immunity in the sera of patients with recent enterotoxic diarrhea. In contrast, however, to the responses of Y1 cells (S. T. Donta, 1973 Symposium on Cholera: U.S.-Japan Cooperative Medical Science Program, in press), the responses of CHO cells to cholera toxin are specifically blocked by the natural toxoid, choleragenoid. This suggests that the binding site for cholera toxin in the CHO cells is similar to that in intestinal tissue.

The cyclic AMP-related morphological changes described here serve as an extremely sensitive assay for the presence of these enterotoxins. The sensitivity of this assay for cholera toxin is 100 to 10,000 times more sensitive than the skin permeability, fat cell lipolysis, and ileal loop assays used previously (19) and is similar to the sensitivity of the adrenal tumor line Y1 (8). The CHO assay for heat-labile *E. coli* enterotoxin is 5 to 100 times more sensitive than the skin permeability and rabbit ileal loop assays used heretofore (12). We believe this assay will be clinically useful in detecting *E. coli* capable of producing heat-labile enterotoxin.

The morphological and cyclic AMP effects of enterotoxin activity are heat labile. Heat-labile enterotoxin alters cyclic AMP concentration in intestinal tissue, presumably accounting for the resulting diarrhea (21). In contrast, *E. coli* filtrates with heat-stable substances that cause fluid accumulation in animal intestinal loops have no effect on CHO cell morphology, suggesting that they may be acting through a different mechanism. Further studies on intestinal tissue are needed to clarify the mechanism by which these heat-stable toxins act.

In addition to the description of a specific, simple, sensitive assay of heat-labile enterotoxins, observations are presented that these enterotoxins elicit elevations in cyclic AMP concentrations in cells from many species, suggesting that they may be useful as pharmacological tools in producing cyclic AMP-mediated responses and in studying mechanisms of regulation of cyclic AMP synthesis.

ACKNOWLEDGMENTS

We thank H. Anderson for technical help and Merle A. Sande and Gerald L. Mandell for their assistance.

Public Health Service grants GM 02139-03 from the National Institute of General Medical Sciences and NS 10193 supported this work. This paper has been selected for the John Horsley Award on 10 May 1974.

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