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Escherichia coli Enterotoxin-Induced Steroidogenesis in Cultured Adrenal Tumor Cells

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A heat-labile, Pronase-sensitive factor has been partially purified from cell-free culture filtrates of enterotoxigenic *Escherichia coli*. The partially purified factor contains both protein and carbohydrate moieties and appears to be *E. coli* enterotoxin (ECT). ECT binds to cultured adrenal tumor cells rapidly and irreversibly leads to adenosine 3',5'-cyclic monophosphate formation and steroidogenesis after a 60-min lag phase. Further studies indicate that it interacts with the cholera toxin receptor site on adrenal cells rather than the adrenocorticotropin receptor to activate adenyl cyclase. Mixed gangliosides block stimulation of steroidogenesis in response to both *E. coli* and cholera enterotoxin. In contrast to adrenocorticotropin, ECT has no additive effect on cholera toxin-induced steroidogenesis. The protein moiety of ECT is similar to cholera enterotoxin because horse serum anticholeragenoid prevented stimulation of steroidogenesis by either enterotoxin. Cultured adrenal cells provide a quantitative assay system that has facilitated the purification and characterization of *E. coli* enterotoxin.

Clonal cell lines of a mouse adrenal cortex tumor are capable of secreting steroids and of responding to adrenocorticotropin (ACTH) and adenosine 3',5'-cyclic monophosphate (cAMP) with increased steroidogenesis (2, 23). Several laboratories have shown that cholera enterotoxin (CT) stimulates steroidogenesis in adrenal tumor cells (5, 25; C. N. Kwan and R. M. Wishnow. Exp. Cell Res., in press; R. M. Wishnow and P. Feist, J. Infect. Dis., in press). Recently, *Escherichia coli* enterotoxin (ECT) induction of steroidogenesis was reported (6). In that report, results of the adrenal tumor cell assay for ECT paralleled results of the rabbit jejunal loop assay. This paper describes the partial purification of a heat-labile factor found only in filtrates from enterotoxigenic *E. coli* which stimulates steroidogenesis in cultured adrenal cells and appears to be a heat-labile enterotoxin. The present communication also demonstrates that ECT increases cAMP after a 60-min lag phase, leading to increased steroidogenesis in cultured adrenal tumor cells. In rabbit intestinal loops, it appears that ECT and CT interact with separate receptor sites (13, 20). In contrast, our studies in adrenal tumor cells suggest that both enterotoxins bind to the same membrane receptor. This tissue culture system can be used for screening enterotoxin-producing *E. coli* strains and for following the purification of ECT.

MATERIALS AND METHODS

E. coli enterotoxin purification. *E. coli* strains were kindly provided by S. Gorbach, V.A. Hospital, Sepulveda, Calif. Strain H-10407 was used for purification of ECT. The growth conditions and culture medium have been described by Evans and Evans (7). The medium contained 2.0% Casamino Acids (Difco), 0.15% yeast extract (Difco), 0.25% NaCl, 0.65% tris-(hydroxymethyl)aminomethane base, 0.368% K_2HPO_4, 0.132% KH_2PO_4, 0.25% glucose, and 1 ml of a trace salt solution per liter. We inoculated 1,500-ml broth cultures, and after 18 h of incubation, cells were removed by centrifugation twice at 7,800 $\times$ *g* for 30 min. The supernatant liquid was concentrated through a TCF-10 ultrafiltration system with PM10 membrane filter (Amicon) and then passed through an XM-100A membrane filter (Amicon). The retentate was dialyzed against a 10-fold excess of 0.01 M phosphate buffer (pH 7.5), followed by centrifugation at 30,000 $\times$ *g* for 30 min. A 15-ml volume of the supernatant liquid was placed on a diethylaminoethyl-cellulose (Bio Rad) column (1.5 by 10 cm) and eluted with a linear gradient of 0 to 0.4 M NaCl in phosphate buffer. The optical density at 280 nm and steroidogenic ability were determined for each fraction. Carbohydrate content was assayed for each fraction by the method of Badin et al. (1).

To determine the effect of Pronase on ECT activity, 10 μg of the pooled DEAE peak fraction of ECT in 0.2

ml was incubated overnight at 37 C with and without Pronase and added to cells. ECT was adjusted to pH 4.0 in 0.05 M potassium phosphate buffer, and after an overnight incubation, the ECT was added to adrenal monolayers. To determine its thermal stability, 10 μg of ECT in 0.2 ml was heated in a 65 C water bath for 5, 15, or 30 min, and added to adrenal cells. The medium was removed and assayed for steroids after a 4-h incubation period at 37 C.

Cell culture procedures, cAMP, and steroid assays. The incubation procedures and fluorometric steroid assay for Y-1 adrenal tumor cells have been described previously (24). Adrenal monolayer morphology was observed under a phase-contrast microscope (Nikon MS). To measure cAMP, the medium was removed from monolayer cultures, 4.0 ml of cold redistilled ethanol was added, and the cells were mechanically removed from the plates. The cell suspension was heated to 100 C for three 1-min periods, and the pellet was removed by centrifugation at 400 × *g*. Ethanol was removed by evaporation to dryness and the cell extract was redissolved in 1.0 ml of 0.05 M sodium acetate (pH 4.0). cAMP in a 50-μliter portion was determined by the protein binding method of Gilman (11). The pellet was dissolved in 0.1 N NaOH and protein was determined by the method of Lowry et al. (19).

Incubation procedures. Confluent cultures were washed twice with 2.0 ml of phosphate-buffered saline. Then 2.0 ml of Eagle minimal essential medium without serum containing ECT or purified cholera enterotoxin (9) was added for a 3-h incubation period at 37 C in a humidified atmosphere of 5% CO_2 in air. In the time course experiment, 4 μg of protein per ml of the pooled DEAE peak of ECT was added to each of eight duplicate adrenal cultures and incubated at 37 C. At 0, 0.5, 1, 2, 3, 4, 5.5, and 24 h of incubation, the medium was removed and saved for the steroid assay.

In the inhibition experiments, CT (2 ng/ml) or ECT (4 μg/ml) was added to duplicate adrenal cultures preincubated with mixed gangliosides or horse serum anticholeragenoid (8) for 10 min and steroid production was determined at the end of a 3-h incubation period. To determine the effect of CT plus ACTH on steroidogenesis, increasing amounts of CT were added to six identical adrenal cultures in duplicate. Additional monolayers were treated with 25 ng of CT per ml plus 0.1, 0.25, 0.5, or 1.0 mU of ACTH per ml. Identical adrenal cultures were incubated with increasing amounts of CT or ECT to determine if enterotoxins produced an additive effect of steroidogenesis. Five additional cultures were incubated with 20 ng of CT per ml plus the same increasing amounts of ECT, and steroid levels were determined after 3 h of incubation. Cell cultures were washed twice with 0.9% saline and lysed with 2.0 ml of 0.1 N NaOH, and protein was determined by the method of Lowry et al. (19).

Reagents. The following reagents were used for the incubations and assays: porcine ACTH (Armour), cholera enterotoxin, and horse serum anticholeragenoid (8, 9, prepared under contract for NIAID by R. A. Finkelstein), choleragenoid (9, supplied by R. A. Finkelstein), gangliosides (type III, Sigma), lipopolysaccharide (*E. coli* O111:B4, *Salmonella typhosa*, and *Serratia marcescens*, Difco), Pronase (Calbiochem.), [^{3}H]cAMP (24 Ci/mmol) (New England Nuclear), and corticosterone (Mann).

RESULTS

Purification and properties of ECT. Cultured adrenal tumor cells provide a simple quantitative assay system that has facilitated the detection, purification, and characterization of ECT. When ECT was added to monolayer cultures of adrenal tumor cells, the cells changed from flattened, irregular appearing cells to spherically shaped cells in 60 min, and began to produce steroids (Fig. 1). ACTH, cAMP, and CT also cause rounding-up of adrenal cells (5, 17, 26; C. N. Kwan, and R. M. Wishnow, Exp. Cell Res., in press; R. M. Wishnow and P. Feist, J. Infect. Dis., in press). We have correlated this morphological response with intracellular cAMP concentration rather

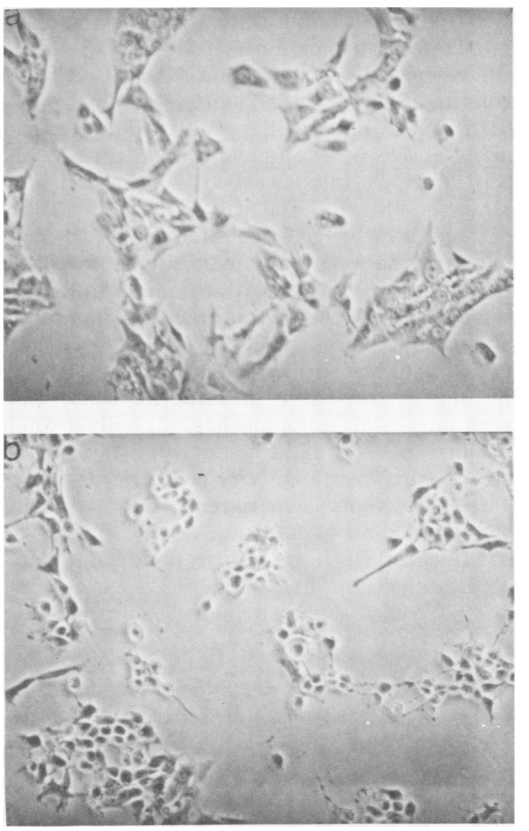

FIG. 1. *Photomicrographs of monolayer cultures of adrenal tumor cells under phase contrast (magnification ×100). (a) Control; (b) 60 min after addition of purified ECT.*

than steroidogenesis (Kwan and Wishnow, In Vitro, in press).

Cell-free culture filtrates from *E. coli* strains, H-10407, 410G, and 334, contain both heat-stable and heat-labile enterotoxin and were positive in our assay system. Nine *E. coli* strains, including 10405, known to be negative in the standard ileal loop assay system, did not stimulate steroidogenesis or alter adrenal morphology (personal communication, S. Gorbach). In addition, lipopolysaccharides (5 μg/ml) from *E. coli*, *S. typhosa*, and *S. marcescens* did not stimulate steroidogenesis during an 18-h incubation period.

Culture filtrates from *E. coli* strain H-10407 were highly active in stimulating steroidogenesis and were used for the purification of ECT (Table 1). At each step of the purification, all fractions were assayed for their steroid-producing activities. Cell-free filtrates from 1,500-ml broth cultures were concentrated 30-fold through a TCF-10 ultrafiltration system. A PM 10 membrane filter, was first used and then an XM-100A membrane filter was used, resulting in a 50-fold purification. After dialysis, 15 ml of the retentate was placed on a DEAE-cellulose column and eluted with a linear gradient of 0 to 0.4 M NaCl in phosphate buffer. The steroidogenic active peak eluted between 0.12 M and 0.16 M NaCl and was about 200-fold purified. However, this material still contains two-thirds carbohydrate, suggesting that the toxin molecule may be a complex molecule as previously indicated (15, 18). The purified enterotoxin is sensitive to mild acid treatment, is heat labile, and is inactivated by Pronase (Table 2), indicating that the active moiety of ECT is protein in nature. In Table 2, Experiment 1, ECT stimulation of steroidogenesis is only twofold because an overnight incubation at 37 C destroys most of the activity of heat-labile ECT. Three additional experiments showed similar steroidogenic responses.

Effect of ECT on cultured adrenal cells. The addition of heat-labile ECT to cultured adrenal cells results in increased intracellular cAMP and steroidogenesis after a 60-min lag phase (Fig. 2). Steroid production and cAMP were still elevated 24 h after adding ECT. The same results were obtained with three different adrenal tumor sublines although steroid production varied among the sublines. The slow steroidogenic response to ECT compared to ACTH could be due to delayed attachment of ECT to its membrane receptor. However, when cells were exposed to ECT for 10 min, and then washed thoroughly with phosphate-buffered saline, steroidogenesis during a 3-h incubation period was the same as in cells continuously exposed to ECT. This experiment suggested that ECT is rapidly and irreversibly bound to its adrenal membrane receptor.

Comparison of ECT and CT. Further experiments suggest that ECT and CT have the same binding sites on adrenal cells and have immunochemically similar active protein moieties. Mixed gangliosides are effective inhibitors of both CT- and ECT-induced steroidogenesis (Fig. 3a). When 50 ng of gangliosides per ml was

TABLE 2. *Effects of Pronase, low pH, and heating on ECT activity*

Expt	Assay conditions	Steroid (μg/ml of protein/h)
1[a]	Control	0.025
	+ ECT, 5 μg/ml	0.059
	+ Pronase, 0.05 μg/ml	0.027
	+ ECT + Pronase	0.029
	+ pH 4 treated ECT, 5 μg	0.040
2[b]	Control	0.027
	+ ECT, 5 μg/ml	0.260
	+ Heated ECT (5 min, 65 C)	0.070
	+ Heated ECT (15 min, 65 C)	0.055
	+ Heated ECT (30 min, 65 C)	0.030

[a] Expt 1, The effect of overnight Pronase treatment and acidification on ECT stimulation of steroidogenesis. In this experiment heat-labile ECT itself was preincubated at 37 C overnight before being added to adrenal monolayers.

[b] Expt 2, The effect of heating ECT to 65 C on steroidogenesis.

TABLE 1. *Purification of E. coli enterotoxin*

Fraction	Vol (ml)	Protein (mg/ml)	Carbohydrate (mg/ml)	Sp act[a]	Fold purified
Crude supernatant	1,600	2.20	0.035	0.091	1.0
PM 10 retentate	180	1.85	0.160	0.945	10.4
XM-100A retentate	50	0.65	0.200	4.440	48.6
Pooled active DEAE peak fraction	15	0.04	0.090	20.000	220.0

[a] Micrograms of steroid per milligram of cell culture protein per hour per milligram of toxin protein.

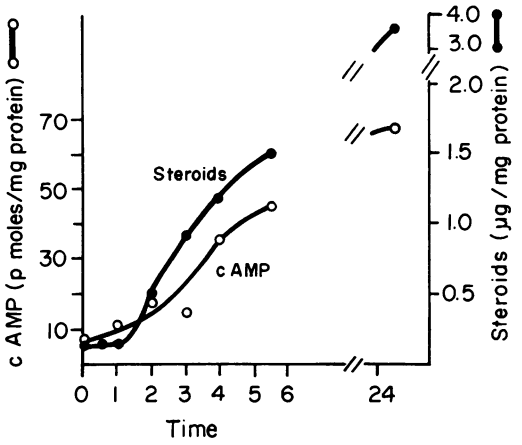

FIG. 2. *Time course of ECT stimulation of cAMP and steroidogenesis. Each point represents the mean of duplicate determinations.*

added to adrenal cells together with ECT (8.0 µg/ml) or CT (2.0 ng/ml), toxin-induced steroidogenesis was reduced by more than 50%. On the basis of this experiment, it appears that ECT receptors involve gangliosides and may be identical to CT receptors. In addition, ECT is inactivated by horse serum anticholeragenoid, but at 10 times the amount required to inactivate CT, suggesting similar active protein moieties (Fig. 3b). Experiments with other adrenal sublines also showed inhibition of enterotoxin-induced steroidogenesis by gangliosides and horse serum anticholeragenoid. Unlike in the ileal loop assay (13, 20), preincubation of adrenal cells with choleragenoid, the naturally occurring toxoid of *Vibrio cholerae*, did not inhibit stimulation of steroidogenesis by CT or ECT. In fact, 500 ng of choleragenoid per ml itself stimulated adrenal steroidogenesis.

Figure 4a shows that if increasing doses of ACTH are added to 25 ng of CT per ml, additional steroidogenesis occurs. In contrast, ECT has no additive effect when added to saturating amounts of CT for a 3-h incubation period. This suggests competition between the enterotoxins for the same membrane receptor (Fig. 4b).

DISCUSSION

Cell-free culture filtrates of enterotoxin-producing strains of *E. coli* stimulate cAMP formation and steroidogenesis in cultured adrenal tumor cells. Nontoxigenic strains of *E. coli* do not affect adrenal tumor monolayers. These results agree with the report of Donta et al. (6). However, another group has reported lipopolysaccharide stimulation of steroidogenesis in cultured adrenal cells which we were unable to confirm (25).

This new tissue culture assay system using a morphological marker and steroidogenesis has enabled us to purify ECT 200-fold by using an Amicon ultrafiltration system and DEAE ion-exchange chromatography. In agreement with other investigators, partially purified ECT is heat labile, acid labile, and Pronase sensitive (15). This material contains both protein and carbohydrate moieties. Other workers, using the ileal loop assay, have been unable to separate heat-labile ECT from endotoxin or polysaccharides (15, 18).

A 60-min lag phase was noted before partially purified ECT increased intracellular cAMP, stimulated steroidogenesis, and altered the morphological appearance of cultured adrenal cells. In contrast, Donta et al. observed a 3- to 4-h lag before a crude culture filtrate of ECT affected adrenal morphology (6). The shorter lag we observed could be due to the fact that our enterotoxin preparation was purified more than 200-fold, or to differences in the adrenal sublines.

ACTH, cAMP, dibutyryl cAMP, CT, and ECT cause cultured adrenal cells to change from a flattened to spherical morphology (5, 6, 17, 26, Wishnow and Feist, J. Infect. Dis., in press). This specific morphological response correlates with increased intracellular cAMP concentration, since both aminoglutethimide and cycloheximide blocked steroidogenesis without affecting toxin-induced rounding-up of adrenal monolayers (Kwan and Wishnow, In Vitro, in press). Cultured adrenal cells pro-

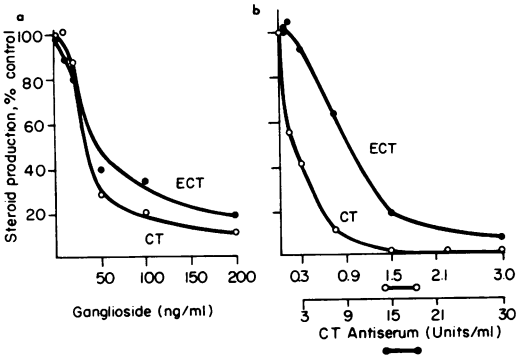

FIG. 3. *Inhibition of CT- and ECT-stimulated steroidogenesis by gangliosides and horse serum anticholeragenoid. (a) CT or ECT was added to cells preincubated with mixed gangliosides, and steroidogenesis was measured. (b) CT or ECT was added to cells preincubated with horse serum anticholeragenoid and steroidogenesis was determined at the end of 3 h.*

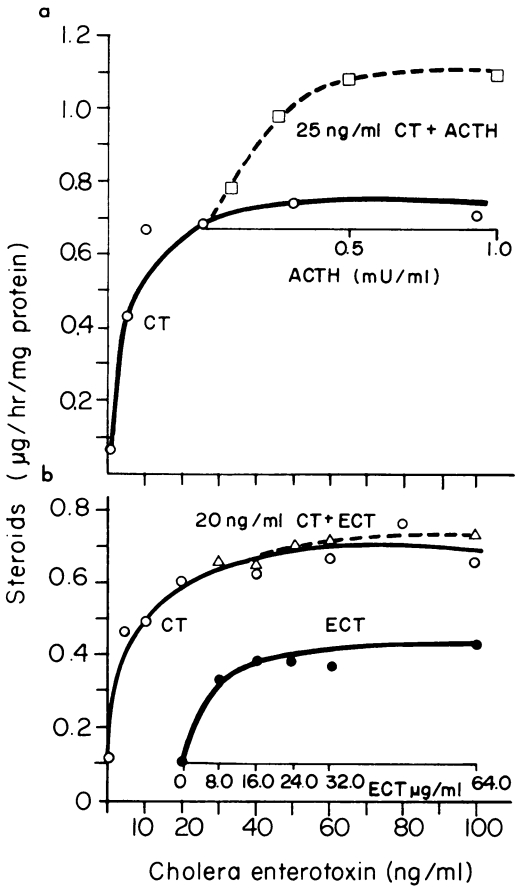

FIG. 4. *The effect of CT plus ACTH or CT plus ECT on steroidogenesis. (a) Increasing amounts of CT were added to identical adrenal cultures and additional cultures were treated with 25 ng of CT per ml plus increasing amounts of ACTH. (b) Identical adrenal cultures were incubated with increasing amounts of CT or ECT. Additional cultures were incubated with 20 ng of CT per ml plus the same increasing amounts of ECT. Each point represents the mean of two separate steroid determinations.*

vide a sensitive assay system that can be used to follow the further purification of ECT. This system can also be utilized to screen for heat-labile, enterotoxin-producing strains of *E. coli* as serotyping cannot predict all enterotoxin-producing strains (12). Donta et al. have reported that heat-stable *E. coli* enterotoxin will not stimulate steroidogenesis in cultured adrenal cells (6). In agreement with that report, crude filtrates from *E. coli* strains containing both heat-stable and heat-labile enterotoxins lost steroidogenic activity after being incubated at 65 C.

ECT, like CT, has a long lag phase and a prolonged stimulatory effect upon cultured adrenal tumor cells (5, 6, 25; Kwan and Wishnow, Exp. Cell Res., in press, R. M. Wishnow and P. Feist, J. Infect. Dis., in press). In contrast, the addition of ACTH leads to a rapid increase in cAMP and steroidogenesis (Kwan and Wishnow, Exp. Cell Res., in press). Neither cholera nor ECT can be removed by washing adrenal cells 10 min after toxin exposure, suggesting that both toxins bind rapidly to adrenal membrane receptors. Cuatrecasas has demonstrated rapid binding of ^{125}I-labeled cholera toxin to fat cells (3). The delayed onset of CT action has been observed in a variety of cell types and may be due to a slow membrane rearrangement whereby the toxin-receptor complex relocates to the adenyl cyclase activation site (4). The GM_1 brain ganglioside appears to be the natural membrane receptor for CT since it binds CT at a 1:1 molar ratio (14, 16). A mixed ganglioside preparation blocked *E. coli* and cholera enterotoxin induction of steroidogenesis equally. Therefore, it is likely that gangliosides are part of the ECT receptor and possibly ECT and CT have the same receptor site.

Choleragenoid blocks CT-induced fluid accumulation in the intestinal loop assay (13, 20). However, in adrenal cells, choleragenoid did not inhibit CT stimulation of steroidogenesis and 500 ng of the toxoid per ml stimulated steroidogenesis. The absence of a choleragenoid inhibitory effect could be due to the greater sensitivity of the adrenal system to CT, or to differences in the specificity of membrane receptor sites. The stimulatory effect of the toxoid could be explained by contamination of choleragenoid with less than 5% CT which would not be detected by low pH gel electrophoresis (S. van Heyningen, personal communication).

Previously, we have shown that CT does not interact with the ACTH membrane receptor because a mutant adrenal line unresponsive to ACTH was stimulated by CT (Wishnow and Feist, J. Infect. Dis., in press). The present report demonstrates that CT has an additive effect on ACTH stimulation of steroidogenesis. However, ECT does not increase CT-induced steroidogenesis, suggesting that both enterotoxins compete for the same membrane receptor sites. In contrast, ileal loop studies suggest that the toxins interact with separate intestinal receptors (13, 20). However, in those studies heat-stable enterotoxin was not separated from heat-labile ECT and could cloud the results.

Immunological cross-reactivity between heat-labile ECT and pure CT has been demonstrated previously (22). Our partially purified ECT can also be inactivated by horse serum anti-choleragenoid, indicating that the active pro-

tein moiety is similar or identical to CT. The similarities between the properties and mechanism of action of both enterotoxins suggest that they are structurally related and compete for the same adrenal membrane receptors. Recent studies raise the intriguing possibility that ECT is determined by a plasmid that was originally transmitted from a *V. cholerae* or other vibrio species (10, 21).

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