Stimulation of Steroidogenesis in Tissue Culture by Enterotoxigenic *Escherichia coli* and Its Neutralization by Specific Antiserum

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Y1 adrenal cells in monolayer tissue culture react to culture filtrates from enterotoxigenic strains of *Escherichia coli*, but not to those from nontoxigenic strains, by undergoing morphological changes similar to those inducible by cholera enterotoxin and increasing their production of steroids. Heating destroys the ability of crude preparations of the *E. coli* enterotoxin to effect these tissue culture changes. None of a number of culture filtrates of other enteric or enteropathogenic bacteria were capable of inducing either the morphological changes or steroidogenesis. The maximal degree of steroidogenesis achievable with *E. coli* enterotoxin was comparable to that of cholera enterotoxin and could not be further increased by combination of the two toxins. As with cholera enterotoxin, removal of the *E. coli* toxin from the tissue culture medium after an initial brief exposure of the cells to the toxin was accompanied by some decrease in maximal steroidogenesis and no changes in either the onset or the permanency of the morphological changes. Antiserum raised to one of the crude *E. coli* enterotoxins was capable of completely neutralizing the steroidogenic effects and preventing the morphological changes secondary to a variety of different enterotoxigenic strains of *E. coli*. At the concentrations tested, this antiserum was not effective in preventing these same tissue culture changes inducible by cholera enterotoxin. The results of these and other related experiments suggest that cholera and heat-labile *E. coli* enterotoxins have similar mechanisms of action which are dissimilar to those of the other enterotoxins thus far described and tested.

One of the major obstacles to assessing the importance of enterotoxigenic strains of *Escherichia coli* in enteric disease of children and adults has been the absence of a simple, reliable, and inexpensive assay system for the enterotoxin. Current assay systems employ either serological methods that depend on the presence of certain capsular antigens or testing for the presence of enterotoxigenic activity in ileal loops with the use of a variety of animal models. The results of serological investigations are not entirely accurate in assessing the enteropathogenicity of *E. coli* because the production of enterotoxin by *E. coli* is not determined by a capsular antigen but rather by a transferable episome. The ileal loop method, although the currently recognized standard assay technique, is time consuming, relatively cumbersome and expensive, and perhaps not specific for enterotoxin.

Recently, we reported that adrenal cells in

tissue culture respond to picogram quantities of cholera enterotoxin by undergoing morphological changes and increasing their production of $\Delta^4,3$ -ketosteroids (3). This response led us to evaluate a number of culture filtrates of other enteropathogenic bacteria or their enterotoxins in this tissue culture model. The findings indicated that, in addition to cholera enterotoxin, only the heat-labile, cell-free culture filtrates from enterotoxigenic strains of *E. coli* were capable of effecting the similar morphological and steroidogenic changes seen with cholera (4). Thus, it would seem that this tissue culture system might find much use in epidemiological studies concerned with the importance of enterotoxigenic *E. coli* in enteric disease as well as with evaluation of serological responses to any vaccines that may be made with the toxin. A number of studies were therefore conducted to detail the morphological and steroidogenic effects of crude preparations of *E. coli* enterotoxin

and an inhibition of these effects by specific antisera. The results of these experiments are the subject of this report.

MATERIALS AND METHODS

Enterotoxins and antisera. Stock cultures of *E. coli* known to produce a positive ileal loop test as well as negative-reacting strains were provided by R. Finkelstein (12). Lyophilized preparations of culture filtrates of *E. coli* strains 408-3 and 408-4 as well as rabbit antisera to the crude material were obtained from R. B. Sack (13). Purified cholera enterotoxin was also provided by R. Finkelstein (6). The purified cholera toxin and the lyophilized *E. coli* filtrates were reconstituted in a 0.05 M tris(hydroxymethyl)amino-methane, .001 M ethylenediaminetetraacetic acid buffer system (pH 7.5), and dilutions were made with this buffer. Dilutions of the antisera were made in a phosphate-buffered saline solution (pH 7.2). Five strains of *Shigella dysenteriae* and *Shigella flexnerii* were obtained from P. Gemski, as was a strain of *Salmonella typhimurium*. Other *Salmonella* sp. were obtained from R. T. Howell (*S. enteritidis* agona), C. Muchnick (*S. newport*), and F. Koontz (*S. paratyphi*). F. Koontz also provided six strains of *Shigella sonnei* isolated from various outbreaks of human shigellosis in Iowa. Twelve strains of *Vibrio parahaemolyticus* were provided by J. Molenda. Thirty strains of various *Enterobacteriaceae* sp. isolated from patients in the clinical laboratory were provided by E. Berglund. Purified preparations of staphylococcal enterotoxins A and B and *Clostridium perfringens* A enterotoxin were obtained from M. Bergdoll and C. Duncan (14), respectively.

Preparations of crude enterotoxin were made from 16- to 24-h (unless otherwise specified) cultures that were incubated in Trypticase soy broth at 37 C in a shaking water bath. The cultures were centrifuged at $12,000 \times g$ for 10 min, and the supernatant was filtered through membrane filters (0.22 μ m pore size; Millipore Corp.) before use in the tissue culture experiments.

Tissue culture methodology. Y1 adrenal cells, derived originally from a radiation-induced adrenocortical tumor, were grown as monolayer cultures at 37 C in a humidified atmosphere of 95% air, 5% CO₂, and maintained in Ham nutrient mixture F-10 supplemented with 15% horse serum and 2.5% fetal calf serum as previously described (3). For each experiment, equal numbers of cells (usually $\sim 10^5$) were distributed into plates (60 by 15 mm; Falcon) and allowed to grow to near stationary phase growth (~ 1 week) before use in studies of steroidogenesis. The tissue culture medium was changed every 3 or 4 days and at the onset of each experiment.

Measurement of the $\Delta^4, 3$ -ketosteroids produced by Y1 cells was accomplished by extraction of the steroids from the culture medium with methylene chloride followed by separation and evaporation of the organic phase and then redissolving the steroid-containing residue in absolute alcohol and determining this solution's absorbance at 242 nm. Ultraviolet spectra of such extracts from toxin-stimulated cells confirmed the presence of a peak at 242 nm in all of

these samples. Steroid production was expressed as nanomoles per plate of cells per the incubation time period (3). Some of the variation seen in the basal amounts of steroid produced between experiments was due to either the total incubation time or the cell density; in addition, some subclonal lines of Y1 cells have higher basal rates of steroid production than do others.

For experiments in which antisera were used, the toxin and antiserum were preincubated at 37 C for 1 h before addition to the tissue culture medium at 1:10 dilution. Concentrations of toxin and antisera shown in the tables represent their final concentrations in the media.

RESULTS

When a variety of *E. coli* strains whose ileal loop response in the infant mouse had already been detailed (12) were tested in the tissue culture system, cell-free culture filtrates from only those with positive ileal loop responses were capable of effecting the characteristic morphological changes and steroidogenesis. Figure 1 depicts the morphological differences between Y1 adrenal cells exposed to toxin-negative and toxin-positive preparations. Table 1 details the steroidogenic effects of these same preparations. It was found that the increased ultraviolet absorbance at 242 nm present in extracts of medium containing the crude culture filtrates could be almost completely eliminated by prior dialysis for 24 h. Another acceptable alternative was to use dilutions (in phosphate-buffered saline) of the crude material. With this latter method, the background absorbance could be reduced or eliminated to a proportionately greater extent than was steroidogenesis. There has been no similar absorbance problem when partially purified (by diethylaminoethyl-cellulose) enterotoxin preparations have been used to induce steroidogenesis. No studies have been done to determine whether the background absorbance could be reduced by using different microbiological media or culture conditions.

A comparison of the amounts of enterotoxin produced in Trypticase soy broth cultures at 8, 24, and 48 h in either an incubator or a shaking water bath at 37 C reveals that, similar to the observations of others (10), maximal production occurred at 24 h in the shaking bath (Table 2). Based on the finding that 5 pg of pure cholera toxin is the smallest amount capable of effecting the morphological changes in this tissue culture system (3), if the dose-response curve of *E. coli* enterotoxin is similar on a weight basis to that of cholera, it can be estimated that approximately 5 to 10 ng of *E. coli* enterotoxin per ml is being produced under these culture conditions.

FIG. 1. Morphological effects of *E. coli* culture filtrates on Y1 adrenal cells 20 h after the addition of the filtrates to the tissue culture medium. The numbers shown correspond to those indicated in Table 1. (Phase contrast, unstained photomicrographs; magnification 150X.)

A number of other enteric pathogens and nonpathogens were examined to see whether their culture filtrates could either effect any morphological changes in Y1 cells or induce steroidogenesis. These included *Salmonella* sp. isolated from various outbreaks of human salmonellosis, *V. parahaemolyticus* strains from patients with gastroenteritis, *Shigella* sp. from both outbreaks of human shigellosis and those known to contain *Shigella* enterotoxin (7), purified preparations of staphylococcal enterotoxins A and B, and a purified *C. perfringens* A

enterotoxin, as well as a number of enteric bacteria such as *Enterobacter* sp., *Pseudomonas* sp., *Serratia* sp., *Proteus* sp., and *Klebsiella* sp. from the clinical laboratory. None of these culture filtrates or preparations were capable of effecting either morphological changes or steroidogenesis. Examination of 100 *E. coli* strains from healthy adult humans and 50 from healthy cows and hogs also failed to reveal any enterotoxin-producing strains. Similarly, culture filtrates from 25 strains of *E. coli* isolated from patients with urinary tract infections were without morphogenic or steroidogenic effects.

By using a lyophilized preparation of a dialyzed culture filtrate of *E. coli* shown to be very active in the rabbit ileal loop (13), concentration-dependent effects with respect to both morphological and steroidogenic changes in tissue culture could be demonstrated (Table 3). Similar preparations of a closely related strain (*E. coli* 408-4) that were ileal loop negative (13) were unable to effect any morphological or steroidogenic changes in Y1 adrenal cells. Comparison of the dose-response curves of cholera and the crude *E. coli* enterotoxins showed them to be parallel to each other.

A comparison of the maximal responses to the *E. coli* enterotoxin preparation and purified cholera enterotoxin showed no differences and no increased steroidogenic effects when a combination of maximal steroid-inducing concentrations of both of the toxins was used (Table 4). Also shown in the table is the absence of any morphogenic or steroidogenic effects of a heat-inactivated preparation of *E. coli* 408-3. Additive steroidogenic effects could be noted when

TABLE 1. Steroidogenic effects of *E. coli* culture filtrates^a

<i>E. coli</i> strain	Morphological response	Steroid production ^b
Broth control	—	4.4 ± 0.1
K-12	—	3.4 ± 0.6
Stanley	—	5.5 ± 0.3
015:H11 (339t _a)	+	9.6 ± 0.9
078:H12 (H10407)	+	15.5 ± 0.1
A-1 (Animal-Barnum)	+	16.8 ± 0.1
0148:NM (B7A)	+	12.2 ± 1.4
06:H16 (B2C)	+	8.0 ± 0.6

^a Cultures of *E. coli* were grown in Trypticase soy broth for 20 h in a shaking water bath at 37 C and filtered through 0.2-μm filters for use in the tissue culture experiments.

^b Steroid production is expressed as nanomoles per plate of cells per 20-h incubation, and the values shown represent the mean ± S.D. of four plates.

TABLE 2. Differences in the production of enterotoxin between stationary and agitated cultures of *E. coli*^a

Dilution of filtrate	5 h		24 h		48 h	
	Bath	Incubator	Bath	Incubator	Bath	Incubator
1:5	+++	+++	++++	++++	++++	+++
1:20	+	+	++++	++++	++++	+
1:60	-	-	++	+	++	-
1:80	-	-	++	-	-	-
1:100	-	-	+	-	-	-
1:160	-	-	+	-	-	-

^a Cultures of *E. coli* 078:H12 (H10407) (20 ml) were grown in 250-ml Erlenmeyer flasks at 37 C in a shaking water bath or stationary in an incubator. The cultures were filtered through 0.2- μ m filters before dilutions were made for use in tissue culture. The morphological changes were graded as - (no cells rounded), + (10 to 25% of cells rounded), ++ (25 to 50% of cells rounded), +++ (75% of cells rounded), ++++ (more than 90 to 95% of cells rounded).

TABLE 3. Steroidogenic effects of *E. coli* 408-3 on Y1 cells

Culture	Amt (μ g/ml)	Steroid production ^a	Morphological response
Control		1.18 $\pm$ 0.35	-
<i>E. coli</i> 408-3	100	9.19 $\pm$ 1.46	+
	10	6.76 $\pm$ 2.03	+
	1	3.98 $\pm$ 0.37	+
	0.1	1.76 $\pm$ 0.94	+
	0.01	1.31 $\pm$ 0.22	-

^a Expressed as nanomoles of steroid per plate of cells per 18-h incubation; mean of six plates $\pm$ S.D.

TABLE 4. Comparison of the steroidogenic effects of *E. coli* and cholera enterotoxins

Culture	Steroid production ^a	Cell rounding
Control	2.71 $\pm$ 0.91	-
<i>E. coli</i> 408-3 (100 μ g/ml)	10.79 $\pm$ 1.61	+
<i>E. coli</i> 408-3 (100 μ g/ml, boiled) ^b	3.93 $\pm$ 1.52	-
CT ^c (5 ng/ml)	13.23 $\pm$ 1.89	+
CT + <i>E. coli</i> 408-3	13.86 $\pm$ 0.89	+

^a Expressed as nanomoles of steroid per plate of cells per 19-h incubation; mean of eight plates $\pm$ S.D.

^b Heated at 100 C for 5 min.

^c CT, Cholera enterotoxin.

submaximal concentrations of cholera and *E. coli* enterotoxins were used.

When Y1 adrenal cells are exposed to cholera or *E. coli* enterotoxins, there is a delay of from 1 to 4 h before the onset of the morphological changes and subsequent steroidogenesis, especially when compared to ACTH (3). Once steroidogenesis is induced by either toxin, it proceeds at a linear rate for at least 24 h (Fig. 2). As with cholera enterotoxin but again in contrast to adrenocorticotropin (ACTH) (1), when

Y1 cells are exposed to the *E. coli* enterotoxin for short periods of time and then the toxin is removed, the morphological changes still appear and steroidogenesis is induced (Table 5). Once the morphological changes have occurred in response to either cholera or *E. coli* enterotoxins, these changes do not appear to revert back to normal, even up to periods of time of 72 h and with medium changes. ACTH- and cyclic adenosine monophosphate (AMP)-induced changes do, however, revert back to normal after 20 to 24 h, even in the continued presence of these inducing agents.

When preincubated with enterotoxin, an antiserum prepared against *E. coli* 408-3 was effective in both preventing the morphological

FIG. 2. Steroidogenic effects of cholera and *E. coli* enterotoxins. Cholera enterotoxin (O) (2 ng/ml) or an *E. coli* A-1 (Δ) culture filtrate was added to plates of Y1 adrenal cells at zero time, and then the steroid content (nanomoles/plate) of the medium was measured at various time intervals later. The media from four different plates were used for each time point. ●, Control plates, no toxin added to medium.

TABLE 5. Effect of removal of *E. coli* enterotoxin from the tissue culture medium on subsequent steroidogenesis^a

Treatment	Steroid production	Cell rounding
Control (10 min), wash, control	1.2 ± 0.6	—
Control (10 min), wash, ACTH	6.1 ± 0.6	±
ACTH (10 min), wash, control	1.7 ± 0.5	—
Control (10 min), wash, ECT	10.1 ± 0.8	+
ECT (1 min), wash, control	4.0 ± 0.9	+
ECT (10 min), wash, control	4.2 ± 0.9	+

^a After incubation of Y1 cells with medium ± ACTH (10 mU/ml) or *E. coli* 078 enterotoxin (ECT), the medium was removed and the monolayer cultures of cells were washed two times with phosphate-buffered saline and then fresh medium ± ACTH ± ECT were added for 20 h of incubation. At the end of this time, the morphological changes were recorded and steroid content of the medium was determined (nanomoles per plate; average of four plates ± S.D.)

response to the toxin and neutralizing its steroidogenic effects. An antiserum to *E. coli* 408-4 was unable to prevent or neutralize these responses. When similar experiments were performed with anti-408-3 serum and a variety of other *E. coli* enterotoxin-containing preparations, a similar complete neutralization of their steroidogenic effects was possible. In contrast to the findings that an antiserum to cholera enterotoxin was able to partially neutralize the steroidogenic response of Y1 adrenal cells to *E. coli* enterotoxins (2), the anti-408-3 serum was unable to inhibit cholera enterotoxin's morphogenic or steroidogenic effects (Table 6), even when used at antibody concentrations 10 times that required for neutralization of the activity of *E. coli* 408-3. Higher antibody concentrations were not tested.

DISCUSSION

Similar to our previous observations on the effects of cholera enterotoxin on the morphology and steroidogenesis of adrenal cells in tissue culture (3), we have found *E. coli* enterotoxin-containing preparations to be capable of these same effects. None of a number of preparations of other enteropathogenic or enteric bacteria tested were able to induce either the cellular morphological or steroidogenic changes. It has been shown that there are at least two types of

E. coli enterotoxin, one heat labile and not dialyzable, the other heat stable and dialyzable (8). Both the intestinal and extraintestinal effects of cholera enterotoxin have been linked to involvement of the adenylyl cyclase-cyclic AMP system (11), and it has been reported that *E. coli* enterotoxin's effects also are mediated through this same system (5). Although not proven, the effects of cholera and *E. coli* enterotoxins on Y1 adrenal cells are also probably mediated through their effects on adenylyl cyclase and intracellular cyclic AMP levels. That other enteropathogens, notably staphylococci, *C. perfringens*, *V. parahaemolyticus*, *Salmonella* sp., and *Shigella dysenteriae*, including some strains of *Shigella* known to produce an enterotoxin, were unable to effect any morphological or steroidogenic changes suggests that their enteric effects may be mediated by mechanisms not directly involving adenylyl cyclase and cyclic AMP. Although the effects of the heat-stable toxin of *E. coli* have been reported to be associated with increased intestinal adenylyl cyclase activity (5), we have found that heat-stable *E. coli* enterotoxin is not capable of affecting the morphology or steroid production of Y1 adrenal cells (4). It may be, however, that Y1 adrenal cells lack the appropriate receptors for heat-stable *E. coli* and possibly other enterotoxins that are present in normal intestine.

Even though there are two types of *E. coli* enterotoxin, the importance of each to human or animal enteric disease has not been well established. Many *E. coli* strains are known to produce both types of toxin, which can be distinguished by either their temporal responses in ileal loops or the use of different animal models to detect each type of toxin (9).

TABLE 6. Effect of *E. coli* 408-3 antiserum on steroidogenic effects of cholera and *E. coli* enterotoxins

Culture	Steroid production ^a	Cell rounding
Control	3.8 ± 0.4	—
Anti-408-3 (1:400)	4.0 ± 0.1	—
408-3 (5 µg/ml)	12.6 ± 1.4	+
408-3 + anti-408-3	4.4 ± 1.1	—
0148 ^b	13.8 ± 1.2	+
0148 ^b + anti-408-3	4.5 ± 0.5	—
CT ^c (2 ng/ml)	13.7 ± 1.4	+
CT + anti-408-3	13.4 ± 1.1	+

^a Expressed as nanomoles/plate/20-h incubation; mean ± S.D. of four plates.

^b Undiluted culture filtrate of *E. coli* 0148:NM (B7A).

^c CT, Cholera enterotoxin.

The tissue culture system of adrenal cells used in our studies that responds to only the heat-labile type of *E. coli* enterotoxin should prove to be valuable in epidemiological studies designed to assess the importance of such toxin-producing strains in enteric disease of humans and animals. We have recently found that we can detect *E. coli* enterotoxin in the stools of infected piglets even before the onset of the diarrheal phase of their illness (S. T. Donta, H. W. Moon, and S. C. Whipp, submitted for publication), and it may be possible that stool specimens alone may suffice for the detection of toxin in studies of the role of *E. coli* in diarrheal disease.

The ability of antisera prepared against *E. coli* enterotoxin to neutralize the morphogenic and steroidogenic effects of the toxin offers a potentially valuable method for assaying the effectiveness of the serological responses to any vaccines that may be made with the toxins. A standard curve similar to that developed for the neutralization of the effects of cholera enterotoxin-induced steroidogenesis by specific antitoxin (2) could also be developed for the *E. coli* enterotoxin-antitoxin system, and the amount of *E. coli*-specific antitoxin present in sera could be quantitated.

Although the molecular mechanisms through which the cholera and *E. coli* enterotoxins exert their effects on Y1 adrenal cells have yet to be investigated, it would seem that, based on the similar temporal relationship between exposure of the cells to the two toxins and the onset of the cellular morphological changes, their similar maximal steroidogenic effects, and their similar, apparently irreversible morphological effects, their mechanisms of action would be quite similar to each other and dissimilar in some ways to the effects of ACTH on this same system. Whether the actual receptor sites or subsequent loci of action differ between the two toxins and ACTH is currently under investigation.

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