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Detection of Heat-Labile Escherichia coli Enterotoxin with the Use of Adrenal Cells in Tissue Culture

Abstract. *Cell-free culture filtrates of heat-labile enterotoxin-producing strains of* Escherichia coli *are capable of inducing morphological changes and steroidogenesis in monolayer cultures of adrenal cells. These tissue culture changes are similar to those induced by cholera enterotoxin and cannot be effected by culture filtrates of other enterotoxigenic or enteropathogenic types of bacteria. The results of the tissue culture studies correlated well with those done in the standard intestinal-loop systems and suggest that this tissue culture system could be used to significantly aid epidemiological and molecular studies with heat-labile* Escherichia coli *enterotoxin.*

The toxic manifestations of cholera enterotoxin and crude cell-free extracts of enteropathogenic strains of *Escherichia coli* have been shown to be associated with activation of intestinal mucosal adenyl cyclase and increased intracellular levels of cyclic adenosine monophosphate (AMP) (*1*). Several extraintestinal effects of cholera enterotoxin are also thought to be linked to stimulation of the adenyl cyclase–cyclic AMP system (*2*). Recently Donta *et al.* demonstrated that picogram quantities of the purified cholera enterotoxin were capable of inducing morphologic changes and steroidogenesis in monolayer cultures of adrenal cells in tissue culture (*3*). We report here that cell-free culture filtrates from enterotoxigenic strains of *E. coli*, but not those from nontoxigenic strains, are capable of inducing morphological changes and steroidogenesis similar to those induced by cholera enterotoxin.

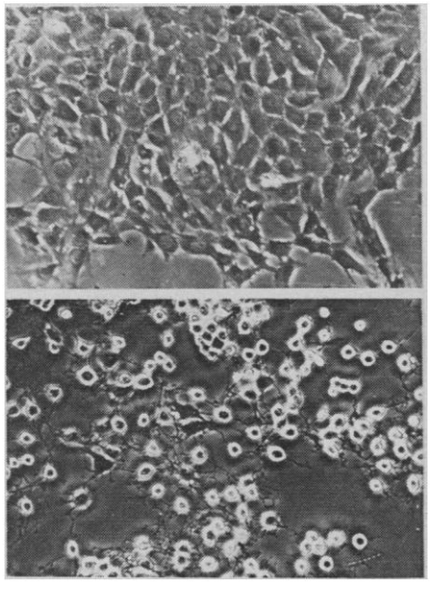

Fig. 1. Photomicrographs (phase-contrast, unstained, ×100) of monolayer cultures of Y1 cells. (Top) Untreated cells. (Bottom) Y1 cells 20 hours after addition of 0.1 ml of a culture filtrate of *E. coli* 0148 to the tissue culture medium.

A variety of *E. coli* strains isolated from American soldiers in Vietnam with diarrhea were provided by Dr. Samuel Formal of the Walter Reed Army Institute of Medical Research. These lyophilized strains were grown in trypticase–soy broth at 37°C in a shaking water bath for 24 hours. Then the culture was centrifuged to remove the bacteria and the supernatant was filtered through 0.22-μm filters for use in the tissue culture or ileal-loop studies. For the tissue culture studies, 0.2 ml of the sterile filtrate was added to 2.0 ml of the tissue culture medium (Ham's nutrient mixture F-10 supplemented with 15 percent horse and 2.5 percent fetal calf serum) which bathed late-logarithmically growing monolayer cultures of Y1 adrenal cells on 60- by 15-mm disposable plates (Falcon), and the plates were incubated at 37°C in a humidified atmosphere of 95 percent air, 5 percent CO_2. Quantitation of the Δ^4,3-ketosteroids that were secreted into the tissue culture medium was done by extracting the steroids from the medium with methylene chloride, separating the aqueous and organic phases, and evaporating the latter to dryness, then redissolving the residue in absolute alcohol and determining the solution's absorption at 242 nm. The spectrophotometric values were then converted to and expressed as nanomoles of steroid per plate of cells (or milligrams of cell protein) per incubation time interval. The jejunal-loop studies were performed in triplicate in 9-week-old rabbits, with aliquots of the same sterile filtrates used for the tissue culture studies. Two milliliters of the filtrate was used for each 10-cm loop, and after 18 hours the resultant fluid accumulation was expressed as milliliters of fluid per centimeter of gut (*4*).

Figure 1 depicts the morphological changes that are inducible by enterotoxigenic strains of *E. coli* and which closely resemble those effected by adrenocorticotropin (ACTH) and cholera enterotoxin. Whereas the changes inducible by ACTH begin a few min-

Table 1. Comparison of the responses of Y1 adrenal cells (morphological changes and steroidogenesis) and 9-week-old rabbit jejunal loops to cell-free culture filtrates of *E. coli*. The jejunal-loop activity is expressed as milliliters of liquid per centimeter of intestinal loop and represents the average value of three 10-cm loops. Steroidogenesis is expressed as nanomoles of steroid produced per plate of cells per 24 hours of incubation (*3*); morphological changes are graded as those strongly positive (about 90 percent or more of cells rounded) at 4 to 6 hours (++) or those moderately positive (about 25 to 75 percent of all cells rounded) at 6 to 8 hours (+).

E. coli strain	Morphological changes	Jejunal-loop index	Steroidogenesis
0148	++	3.07	20.44
M42-43*	0	0	0
121 V2B	0	0	0
301V-2MC-334a	0	0	0
201V-3MC-3342	0	0	0
111V-3MC-B2C	0	0	0
H-12808	0	0	0
V.N. 211VB	0	0	0
V.N. 161V1B	0	0	0
5 IV BB	+	1.80	13.94
5 IV AB	++	2.03	19.22
71 IV 1B	+	0.77	9.38
145 IV-4MC	0	0	0
76 IV 2M	0	0	0
78 IV 2MC	0	0	0
98 1 1MC	0	0	0
971 1MC	0	0	0
H-10407	++	3.70	17.68
171V1MC	0	0	0
29A1V1B	0	0	0
1461V-1B	0	0	0
TML-R66†	0	0	0
B2C	+	0.97	9.72
B7A	++	2.93	20.44

* This is actually a strain of *Shigella flexneri*, not *E. coli*. † Not *E. coli*, but a strain of *Salmonella typhimurium*.

utes after the exposure of cells to the pituitary hormone and become maximal in 20 to 40 minutes, those effected by culture filtrates of toxigenic *E. coli* do not begin for 3 to 4 hours following addition of the toxin to the medium. It is possible that higher concentrations of toxin could decrease this lag period, but in studies with cholera enterotoxin, this lag period could not be decreased to less than ½ hour, with maximal rounding occurring 1 to 2 hours later (*5*). Also in contrast to ACTH, once morphological changes are induced by the toxins, these changes appear to be irreversible, even after removal of the toxin, for periods of time up to 72 hours. Even in the continual presence of maximal stimulating amounts of ACTH, Y1 adrenal cells begin to revert to their normal flattened appearance by 16 to 20 hours and are completely back to normal by 24 to 30 hours.

Table 1 summarizes the results of the tissue culture and jejunal-loop studies with the 24 strains of bacteria employed. As can be seen, there was a 100 percent correlation of the jejunal-loop and tissue culture results, even to the extent that filtrates H10407, B7A, 0148, and 51VAB were the most potent and filtrates B2C and 71-IV-1B were the least potent of the active samples in both systems. A variety of other strains of bacteria known to be associated with intestinal diarrhea and isolated from patients with the disorder, including *Salmonella* sp., *Shigella* sp., and *Vibrio parahemolyticus*, as well as purified preparations of staphylococcal enterotoxins A and B (provided by M. Bergdoll) and *Clostridium perfringens* type A enterotoxin (provided by C. Duncan), did not induce any morphologic or steroidogenic changes in these tissue culture cells.

It has been shown that there are two types of *E. coli* enterotoxins: one that is heat-labile and has a delay in onset of action and maximal response in the ileal-loop similar to those of cholera, and the other a heat-stable toxin that causes its intestinal effects without delay, with a maximal response and termination of this response that occurs much earlier than that of the heat-labile enterotoxin (*6*). In order to evaluate the tissue culture system's specificity toward the *E. coli* enterotoxins, a variety of *E. coli* strains known to produce either heat-stable toxin (ST) alone or ST plus heat-labile toxin (LT) were grown overnight at 37°C in trypticase–soy broth in a shaking water bath, and the cell-free culture filtrates were tested for their morphologic altering ability. The results of these experiments are shown in Table 2 and demonstrate that only the LT-ST producers were capable of effecting the morphological changes. In separate experiments, only the unheated culture filtrates of the LT-ST producers were able to effect increases in steroid production by Y1 adrenal cells.

Table 2. Correlation of the type of *E. coli* enterotoxin produced by various strains and its effects on rounding by Y1 adrenal cells.

E. coli strain	Serotype	Enterotoxin produced*	Rounding†	
			BA	BAH
123	043:K−:H28	0	−	−
124	08:K(A):NM	0	−	−
253	013:K−:H11	0	−	−
263	08:K87,K88$_{ab}$:H19	ST-LT	+	−
431	0101:K(A):NM	ST	−	−
598	08:K87,K88$_{ab}$:H19	ST-LT	+	−
613	0101:K(A):NM	ST	−	−
637	064:K+:NM	ST	−	−
987	09:K(A):NM	ST	−	−
G1108E	0141:K85,K88:H4	ST-LT	+	−
2176E8	0138:K81	ST	−	−
1262	09:K+:NM	ST	−	−
A-1	0149:K91,K88	ST-LT	+	−
1291	0149:K91,K88	ST-LT	+	−
F11	?	0	−	−
F11(P155Ent$^+$)	?	ST-LT	+	−
Troyer	09:K35:NM	ST	−	−
123‡		0	−	
431‡		ST	−	
Broth‡		0	−	

* ST, heat-stable toxin; LT, heat-labile toxin; ST-LT, both toxins; 0, no toxin. The type of toxin or toxins produced was determined by the effects of BA and BAH cultures in the jejunal loops of 3-week-old pigs and 9-week-old rabbits 18 hours after injection of either 5 ml per 10-cm loop (pigs) or 1 ml per 10-cm loop (rabbits). † Determined at 20 hours of incubation; BA, trypticase–soy broth agitated cultures; BAH, heated at 100°C for 15 minutes. Plus (+) indicates positive reaction; minus (−), no reaction. ‡ These extracts were concentrated 25 times.

It would seem, therefore, that this tissue culture system is only activated by the heat-labile enterotoxins of *E. coli* and *Vibrio cholerae*, which would imply that their mechanisms of action are similar to each other and dissimilar to those of other enteropathogenic bacteria. These results also suggest that the intestinal changes effected by bacteria other than the cholera vibrio and heat-labile enterotoxigenic *E. coli* probably do not involve the adenyl cyclase–cyclic AMP system. It could be, however, that Y1 adrenal cells lack the appropriate receptor sites for these other toxins.

The detection of enterotoxigenic strains of *E. coli* has relied almost exclusively on the use of intestinal-loop systems. The serotyping of strains of *E. coli* obtained from outbreaks of travelers' or other types of diarrhea is not an accurate method for detecting enterotoxin-producing strains because the determination for enterotoxigenicity resides in a transferable episome (*7*), and not in a surface antigen. It would appear from our studies that the adrenal cell tissue culture system can be used as a specific test for heat-labile *E. coli* enterotoxin that will be more sensitive, simpler, and less expensive than the intestinal-loop system. The tissue culture system should be quite useful in epidemiologic studies of enteric disease in people and animals as well as serve as a model system with which the molecular mechanisms of action of *E. coli* and cholera enterotoxins can be easily studied. Although quantitation of steroidogenesis could be used for the epidemiological studies, it would be much simpler and more sensitive a method to use the occurrence of the morphological changes induced by the *E. coli* enterotoxin as the assay parameter for the presence of enterotoxin in stool or culture filtrates. Preliminary experiments using 4-day-old pigs infected with LT-producing *E. coli* strains have demonstrated the ability of the adrenal cell tissue culture system to detect heat-labile enterotoxin in the stools of these animals during the diarrheal phase of their illness.

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Electroencephalographic Evidence for Retention of Olfactory Cues in Homing Coho Salmon

Abstract. *Differences were observed in the magnitude of the evoked electroencephalographic response to 1 percent morpholine for homing coho salmon* (Oncorhynchus kisutch) *exposed to morpholine as fingerlings 1 month before smolting as compared to salmon not exposed to morpholine as fingerlings. These results indicate that olfactory information has been retained for 18 months, the period between smolting and the homeward migration.*

Since the studies of Hara *et al.* (*1*) suggested that the electroencephalographic (EEG) technique might be used as a bioassay for home stream recognition in migrating salmon, many physiological (*2*) and behavioral experiments (*3–6*) have been performed to interpret the significance of the EEG home stream responses. Much of this work has been summarized by Hara (*7*).

Despite early reports that the EEG technique was specific in that only the home stream water elicits a characteristic evoked potential (*1, 3*), more recent work (*4–6*) has failed to find such a specificity. In addition, analysis of the EEG technique is complicated because other factors such as *p*H, ionic strength, and nonspecific stimulatory products also affect the EEG response.

We used the EEG technique to determine whether coho salmon fingerlings can be imprinted by exposure to an artificial chemical, morpholine (*8*), and to see if they can retain the information from this chemical until the adult spawning migration 18 months later. Dizon *et al.* (*5*) showed a significant difference in the magnitude of the evoked potentials to morpholine for a group of coho salmon exposed to morpholine as fingerlings 9 months earlier as compared to an unexposed group. Since the salmon were held in a hatchery after chemical exposure and were not sexually mature when tested, we felt it necessary to see whether adult salmon also showed this response. A longer paper on this work appears elsewhere (*9*).

Two groups of 8,000 coho salmon fingerlings were held in large tanks at Oak Creek, South Milwaukee, Wisconsin, in April 1971 and supplied with water (*10*) pumped from Lake Michigan. Both groups were marked by different fin clips. One group was exposed to morpholine at 5×10^{-5} mg/liter; the other was not. The 5-week exposure period, starting 3 weeks before smolting and ending 2 weeks afterward, was chosen because it was viewed as adequate for imprinting (*11*). The salmon were then released at the mouth of Oak Creek. During the spawning migration in the fall of 1972, morpholine was released into Oak Creek at a stream concentration of about 1×10^{-4} mg/liter. It was hypothesized that if the salmon had imprinted to morpholine as the home stream chemical, then only the exposed group of fish would recognize Oak Creek as the home stream since it contained morpholine.

Salmon were captured in the mouth of Oak Creek upon their return as adults, paralyzed with Flaxedil (2 mg per kilogram of body weight), and restrained in a holding box. Their gills were flushed with city tap water saturated with oxygen. A portion of the skull over the forebrain was removed with a dental drill to permit the insertion of an electrode (*12*) into the olfactory bulb. The EEG recordings were made with a Grass Instruments polygraph equipped with a model 7P5 preamplifier, and the signals were integrated with a Grass model 7P10 integrator (which has an infinite time constant). Heartbeat monitored by electrocardiography (EKG) indicated the condition of the fish. Both EEG and EKG signals were recorded on magnetic tape with an FM tape recorder.

Fourteen chemical solutions and water samples were tested by introduction into the nares. Morpholine at 1, 0.1, and 0.01 percent was used to see if the fish had "remembered" the imprinting chemical. Three other morpholine solutions at these concentrations buffered with sodium bicarbonate (0.01*M*, *p*H 7.5) served to control for *p*H, which varies in the unbuffered solutions and is a factor in the EEG response (*5, 9*). Two buffer solutions were tried, one at *p*H 9.5 to control for *p*H and another at *p*H 7.5 to control for ionic strength. Phenethyl alcohol at 0.1 and 0.01 percent was used to determine whether an organic compound other than morpholine would elicit evoked potentials. Lake Michigan and Oak Creek waters were chosen to test for the influence of water that the fish had recently experienced and to determine the presence of nonspecific stimulatory products. Sodium chloride (0.06*M*) served as an internal standard. Finally, South Milwaukee city tap water was used to rinse the fishes' nares between samples and to prepare test solutions. Approximately 10 ml of each sample were delivered in a random sequence through a Pasteur pipette to the nares at an approximate rate of 1 ml/sec.

The experiment was standardized by dividing the integration of the reaction to each test solution by the integration of the NaCl record (*13*). Differences in means for all fish were evaluated for significance by the Mann-Whitney *U* test (*14*); responses separated by less than 0.03 were considered the same rank.

Eleven imprinted and nine nonimprinted fish were tested with the EEG technique. The responses of these two groups to 1 percent morpholine were significantly different ($U = 12$, $P \leq .01$) (Table 1). If only the salmon tested three or more times are compared (eight imprinted and six nonimprinted salmon), the differences between the two groups are also significant ($U = 3$, $P \leq .001$). No fish responded to concentrations of 0.1 and 0.01 percent morpholine. The magnitude of the evoked potentials to morpholine for the 11 imprinted fish was roughly correlated with the period in the migration at which they were tested. The fish at the start and the end of the season gave