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Does Enteropathogenic *Escherichia coli* Produce Heat-Labile Enterotoxin, Heat-Stable Enterotoxins a or b, or Cholera Toxin A Subunits?

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Although most enteropathogenic *Escherichia coli* strains do not produce recognized enterotoxins, we wished to examine whether they produce any factors like heat-stable enterotoxin b or cholera toxin active subunits that might be missed by conventional assay methods. *E. coli* strains E851 (O142) and E2348 (O127) that had caused diarrhea in volunteers were negative for heat-labile enterotoxin and heat-stable enterotoxin a in Chinese hamster ovary cell and suckling mouse assays, failed to cause secretion in ligated small bowel loops from 6- to 8-week-old pigs after 4 to 5 h (used to show heat-stable enterotoxin b), and did not activate adenylate cyclase in pigeon erythrocyte lysates (used to demonstrate cholera toxin A subunit). We conclude that crude, unconcentrated culture filtrates and sonicates do not mimic heat-labile or heat-stable enterotoxins or cholera toxin or its A subunit and that enteropathogenic strains of *E. coli* probably have yet another mechanism or group of mechanisms by which they cause diarrhea.

The association of certain enteropathogenic *Escherichia coli* (EPEC) strains with diarrhea has been recognized since the work of Goldschmidt and Dulaney in the mid-1930s (7, 8, 17, 23). Two classic EPEC strains (O127 and O142) caused diarrhea in adult volunteers despite being negative in conventional assays for heat-labile enterotoxin (LT), heat-stable enterotoxin (STa), and invasiveness (26). In none of these studies of EPEC strains was the mechanism of diarrhea determined. In a recent report by Robins-Browne et al. (30), the EPEC strains that had caused diarrhea in adult volunteers were negative when tested in ligated rabbit ileal loops, rabbit skin vascular permeability tests, suckling mice, and Y1 adrenal cells, as well as being negative for guanylate cyclase activation of rat, rabbit, or infant mouse intestinal homogenates. They also found that these EPEC strains lacked detectable DNA for LT or STa. At present, EPEC strains include 13 serotypes recognized to cause diarrhea. Although these strains have usually been negative in tests for the conventionally recognized enterotoxins LT and STa, the possibility remained that an enterotoxin that is missed by conventional tissue culture, immunological, and biological assays might be present and contribute to the diarrhea caused by EPEC. Indeed, secretory enterotoxic products have been reported for EPEC strains in studies of perfused rat and dog intestinal segments (9). Our purpose was to confirm that the EPEC strains that recently caused disease in adult volunteers were negative in conventional tests for enterotoxins and to examine whether biological activities analogous to STb or cholera toxin active (A) subunits might be produced by these strains.

STb-producing *E. coli* strains P-3 and P-16 were obtained from Carlton Gyles. EPEC strains E851 (O142) and E2348 (O127), which had caused diarrhea in human volunteers (26), originated from outbreaks of infantile diarrhea in Glasgow and Taunton, United Kingdom. Strains 334 (LT) and C1-4 (an STa-producing isolate) were obtained from patients with diarrhea in Brazil. A nontoxigenic *E. coli* strain (10405) served as a control (20). EPEC and control strains were prepared as previously reported for testing for LT and STa (1, 15). To test for STb-like activity, the *E. coli* strains were incubated with shaking overnight at 37°C, centrifuged, sonicated in 3- to 10-s blasts, and heated at 65°C for 30 min. LT in crude culture filtrates was assayed in the Chinese hamster ovary (CHO) cell assay as previously described (19). STa was tested in 2- to 4-day-old suckling mice 3 h after 0.1 ml of crude culture filtrate was injected intragastrically (15). Fluid secretion induced by heated (65°C, 30 min), centrifuged *E. coli* sonicates in ligated small intestinal segments was assayed in 6- to 8-week-old weaned piglets and in 1.5- to 2-kg rabbits (10, 24). Porcine jejunal segments (ca. 6 cm long) were ligated and inoculated with 5 ml of test medium. Rabbit ileal loops (4 cm long) were inoculated with 1 ml of toxin control preparation. Data were expressed as the ratio of volume (in milliliters) to length (in centimeters) ± standard error of the mean.

Mucosal scrapings from *E. coli* toxin-treated animal segments were homogenized in saline as described by Forsyth et al. (13). Adenylate cyclase was measured by using [^{32}P]ATP conversion to cyclic [^{32}P]AMP by the method of Salomon et al. (33), except that the reaction mix consisted of 60 mM tricine containing 20 mM $MgCl_2$ (pH 8) and 1 mM ATP (10^6 cpm), and the reaction was stopped after 20 min by adding 100 μl of stopping solution containing 1% sodium dodecyl sulfate, 20 mM ATP, and 6.25 mM cyclic AMP (10^4 cpm of ^{3}H). The samples were chromatographed over Dowex and neutral alumina. NaF and isoproterenol were included as positive controls.

Activation of adenylate cyclase in pigeon erythrocyte lysates by *E. coli* toxins was tested by the method of Gill and King (16). Pigeon erythrocytes were lysed by freeze-thawing and incubated for 60 min with 50 μl of the test substance added. Cholera toxin (10 μg/ml) served as a positive control. The resulting toxin-lysate suspensions were incubated as described previously (16), except that the reaction was stopped after 30 min by first adding 0.1 ml of the standard

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stopping solution without sodium dodecyl sulfate and then boiling. Isoproterenol (5×10^{-5} M) or 10 mM NaF served as additional positive controls. The mixture was chromatographed as described above, and the results were expressed as percentages of the control values.

Culture filtrates of EPEC strains E851/71 (O142:H6) and E2348/69 (O127:H6) were confirmed to be negative in conventional tests for STa in the suckling mouse assay and for LT in the CHO cell assay.

In comparison with the effects of *E. coli* LT (secretion [± standard error of the mean], 0.71 ± 0.03 and 1.21 ± 0.04 ml/cm), STa (0.55 ± 0.11 and 0.30 ± 0.04 ml/cm), and cholera toxin (0.72 ± 0.03 and 1.2 ± 0.12 ml/cm) at 4 and 16 h, respectively, in three standard ligated rabbit ileal loop assays, EPEC strains E851 (0.24 ± 0.09 and 0.15 ± 0.04 ml/cm) and E2348 (0.16 ± 0.08 and 0.18 ± 0.04 ml/cm) caused no secretory responses and were comparable to the control *E. coli* strain 10405 (0.25 ± 0.07 and 0.20 ± 0.06 ml/cm) at both 4 and 16 h.

In contrast to the mean secretory response in duplicate weaned-pig intestinal segments after 4 to 5 h caused by both of the STb-producing *E. coli* strains, P3 (1.68 ml/cm) and P16 (1.53 ml/cm), EPEC strains E851 (0.20 ml/cm) and E2348 (0.11 ml/cm) gave responses that were not significantly different from those of the negative control strain, *E. coli* 10405 (0.39 ml/cm).

To examine for a cholera toxin or *E. coli* LT active subunit-like activity, the pigeon erythrocyte lysates incubated with EPEC culture sonicates were examined for adenylate cyclase activation. The effects of isoproterenol and cholera toxin on the lysates were striking in comparison to the effects of both EPEC strains and control *E. coli* 10405 culture filtrate material. The mean and standard deviation of determinations from three different experiments done in duplicate was 83.01 ± 22.8%, 284.4 ± 151.5%, and 7,049 ± 407.3% for strain 10405-treated, isoproterenol-stimulated, and cholera toxin-treated lysates, respectively; the latter two values are significantly different from that for the strain 10405-treated lysates ($P = 0.01$). Sodium fluoride stimulated adenylate cyclase activity 19-fold over that in untreated controls. Sodium fluoride also caused similar 15- to 18-fold stimulations over controls in the presence of culture filtrates from *E. coli* strains 10405, E851, E2348, P-3, and P-16 and with cholera toxin. EPEC strains E851 and E2348 consistently showed no adenylate cyclase activation analogous to that caused by active subunits of cholera toxin or LT or analogous to that caused by adenylate cyclase-stimulating agents. In additional studies done to exclude the possibility that an inhibitory effect of the crude culture filtrates might have masked an effect of EPEC products on pigeon erythrocyte lysate adenylate cyclase activity, the effect of cholera toxin was seen even when the crude culture filtrates from EPEC strains were added to cholera toxin in concentrations similar to those used in the test solutions.

The historical evidence that EPEC causes disease is strong (4, 17). The decline in interest in EPEC serotypes stemmed from (i) the development of our understanding of *E. coli* enterotoxins (11, 12, 21) and their importance in the etiology of diarrhea (3, 22, 27, 32), (ii) the apparent decline in the frequency of recognizable EPEC outbreaks, (iii) similarities in isolation rates of EPEC between cases and controls (14), and (iv) the discovery that enterotoxin production could be encoded on plasmids transmissible to any *E. coli* serotype (34). Nonenterotoxigenic and noninvasive EPEC strains have been associated with diarrhea despite their inability to produce conventionally recognized enterotoxins and lack of invasiveness (26). There are several potential mechanisms of action. Close adherence of bacteria to the small intestine brush border in a locally destructive fashion (5, 18, 29, 31, 35) may, in EPEC strains, be associated with a variable adherence factor encoded on a transmissible plasmid (2). An enterotoxic effect may impede intestinal absorption or elicit net fluid secretion (9, 25). A cytotoxic product detectable in Vero cells or a shigella-like enterocytotoxin may be elaborated. Although the original studies with these two strains were negative for Vero cytotoxin (26), O'Brien et al. (28) have reported the production of Shiga-like HeLa cell cytotoxin that is neutralized by antiserum to Shiga toxin from strain E851 that was grown on iron-depleted medium. As conflicting reports suggest that Shiga toxin may activate adenylate cyclase (6), the possibility that an adenylate cyclase-activating toxin is being produced by certain EPEC strains was considered. However, even with the higher ATP substrate concentrations, we still found no evidence for an adenylate cyclase-activating toxin in the same EPEC strain.

As reported by others, we too find no evidence for LT or STa in the CHO cell or suckling mouse assay. Furthermore, in contrast to positive LT and STa controls, the crude culture filtrates of the EPEC strains that we tested, which had caused diarrhea in volunteers, failed to cause significant secretion after 4 or 16 h in ligated adult rabbit ileal segments, nor did they produce STb-like activity or LT or cholera toxin A subunit-like activity in the porcine ligated segment or pigeon erythrocyte lysate adenylate cyclase assays compared with both positive and negative controls.

We therefore conclude that crude, unconcentrated culture filtrates and sonicates of EPEC strains that have been historically associated with infantile diarrhea and that have recently demonstrated their capacity to cause noninflammatory diarrhea in adult volunteers do not mimic *E. coli* LT, cholera toxin, *E. coli* STa, *E. coli* STb, or cholera toxin active subunits. These studies lend further support to the concept that EPEC causes diarrhea by yet another mechanism(s) besides the classically recognized enterotoxins and invasiveness. It appears that these strains closely adhere to the mucosa, and we cannot exclude the possibility that EPEC might deliver an enterotoxic or cytotoxic product locally to the cells on close adherence in vivo.

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