

## Effects of Heat-Stable Enterotoxin of *Yersinia enterocolitica* on Ion Transport and Cyclic Guanosine 3',5'-Monophosphate Metabolism in Rabbit Ileum

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Strains of *Yersinia enterocolitica* produce a heat-stable enterotoxin which is positive in the suckling mouse bioassay. Partial purification by a procedure previously worked out for heat-stable *Escherichia coli* enterotoxin yielded a substance which increases particulate guanylate cyclase activity and short-circuit current and inhibits active Cl<sup>-</sup> absorption in rabbit ileal mucosa. These effects of *Y. enterocolitica* enterotoxin are similar to those of heat-stable *E. coli* enterotoxin, suggesting a common mechanism of action.

Culture supernatants of several strains of *Yersinia enterocolitica*, including ones isolated from children with gastroenteritis, possess enterotoxigenic activity in the infant mouse bioassay that is heat stable and that does not produce a response in the Y-1 adrenal cell assay (2, 12). These characteristics are the same as those of heat-stable enterotoxin (ST) from *Escherichia coli*, which has also been found to increase guanylate cyclase activity and cyclic guanosine 3',5'-monophosphate (cGMP) concentration in the mammalian small intestine (3, 6, 9).

We report here that *Y. enterocolitica* ST, partially purified by the same procedure we previously used for *E. coli* ST, stimulates guanylate cyclase activity, increases cGMP concentration, increases short-circuit current, and inhibits active Cl<sup>-</sup> absorption when added to rabbit ileum in vitro. Very recently Robins-Browne et al. (13) also reported stimulation of guanylate cyclase in homogenates of infant mouse small intestines by a partially purified *Y. enterocolitica* ST.

### MATERIALS AND METHODS

**Preparation and bioassay of *Y. enterocolitica* ST.** The details of the procedure for ST preparation will be published elsewhere (W. J. Laird, unpublished data). Briefly, *Y. enterocolitica* grown in broth culture (pH 7.0) for 36 h at 25°C was sedimented, and the supernatant was chromatographed on Amberlite XAD-2. The column was washed with 10% methanol, and the toxin was eluted with 100% methanol. The

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eluate was concentrated 40-fold by air drying and then extracted with chloroform. The aqueous layer was further concentrated to 2 ml and gel filtered on Sephadex G-10 pre-equilibrated with 50% methanol. The toxin activity in the infant mouse assay appeared as a broad peak immediately after the void volume. The eluate was concentrated by air drying and gel filtration on Sephadex LH-20 pre-equilibrated with 50% methanol. The toxic activity again appeared as a fairly broad peak after the void volume. The eluate was evaporated to dryness and stored in 50% methanol. The toxin was assayed by the suckling mouse bioassay as previously described for *E. coli* ST (6). A mouse unit was defined as the amount of ST producing a half-maximal increase in the ratio of total intestinal weight to total body weight (6).

**Electrical and Cl<sup>-</sup> flux measurements.** As previously described (5), pieces of distal ileum from New Zealand white male rabbits (2 to 3 kg) were stripped of muscle and mounted in Ussing chambers. The bathing medium was HCO<sub>3</sub>-buffered Ringer solution in which half the Cl<sup>-</sup> was replaced with equimolar amounts of SO<sub>4</sub><sup>2-</sup> and mannitol (31.25 mM each). The medium was bubbled with 5% CO<sub>2</sub> in O<sub>2</sub> and maintained at 37°C. Electric potential difference (PD), conductance (G<sub>t</sub>), short-circuit current (I<sub>sc</sub>; the amount of current needed to nullify the spontaneous transmural PD) and unidirectional fluxes from mucosa-to-serosa (m-to-s) and s-to-m were determined as previously described (5).

**cGMP measurements.** After measuring electrical responses in Ussing chambers, we rapidly (<20 s) transferred tissues from the chambers into 3 ml of 5% cold trichloroacetic acid containing 0.02 pmol of [<sup>3</sup>H]cGMP (21 Ci/mmol; New England Nuclear Corp., Boston, Mass.) as a recovery marker. Further processing of the samples was as described earlier (7). The trichloroacetic acid was extracted with ether, and the samples were evaporated to dryness and reconstituted in 0.1 M sodium acetate buffer, pH 6.2. The cGMP was determined by radioimmunoassay of the acetyla-

ted derivative (8), using [ $^{125}$ I]cGMP purchased from Becton, Dickinson & Co., Orangeburg, N.Y. and cGMP antibody kindly provided by A. L. Steiner (University of North Carolina, Chapel Hill).

**Guanylate cyclase assay.** Membranes were prepared and the assay was performed as previously described (6) with a few modifications. Instead of being homogenized, intestinal cells were sonicated for 75 s (15 s  $\times$  5) at 50 W (Sonifier cell disruptor, model W185 Heat Systems-Ultrasonics Inc., Plainsview, N.Y.). The modified assay mixture (45  $\mu$ l) contained [ $\alpha$ - $^{32}$ P]GTP (1  $\mu$ Ci; 0.4 mM), 5 mM MgCl<sub>2</sub>, 0.1 mM MnCl<sub>2</sub>, 50 mM tris(hydroxymethyl)aminomethane-(Tris)hydrochloride, 10 mM phosphocreatine, 2 U of creatine phosphokinase per tube, and 50  $\mu$ M cGMP. The reaction was initiated by a mixing of the individual additions of 10  $\mu$ l of toxin (in 50 mM Tris or buffer alone), 45  $\mu$ l of assay mixture, and 15  $\mu$ l (1 to 2  $\mu$ g of protein per  $\mu$ l) of membrane suspension. Assays were carried out for 10 min at 37°C, and the reaction was terminated by adding 0.5 ml of cold 50 mM Tris-hydrochloride (pH 7.4) containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.03  $\mu$ Ci of [ $^3$ H]-cGMP and then boiling for 3 min. A 500- $\mu$ l amount of 0.1 N HCl was also added to each tube. The [ $\alpha$ - $^{32}$ P]-cGMP formed was measured by a modification of the procedure of Birnbaumer et al. (1). The Dowex column was equilibrated with 0.1 M Imidazole-hydrochloride (pH 7.0) before loading the samples. After loading, the columns were washed with 4 ml of 50 mM HCl followed by 1 ml of 0.1 M Imidazole-hydrochloride. The cGMP fraction was eluted directly onto alumina columns with 2 ml of 0.1 M Imidazole-hydrochloride. This eluate from the alumina columns was discarded, the columns were washed with 3 ml of 0.1 M Imidazole-hydrochloride (pH 7.4) and the cGMP was eluted directly into 12 ml of Bray solution with an additional 3 ml of 0.1 M Imidazole-hydrochloride. Proteins were measured by the method of Lowry et al. (11). All reagents were obtained from previously listed sources (7).

## RESULTS

**Effects of *Y. enterocolitica* ST on ion transport.** The characteristics of the electrical

response to *Y. enterocolitica* ST are similar to those previously reported for *E. coli* ST (6). Upon addition to the luminal side, the enterotoxin produced a rapid (within 30 s) and persistent increase in PD, without causing a significant change in tissue resistance. *Y. enterocolitica* ST, like *E. coli* ST, increased I<sub>sc</sub> over a broad range of toxin concentrations (Fig. 1). The effects of *Y. enterocolitica* ST on unidirectional and net Cl<sup>-</sup> fluxes across short-circuited ileal mucosa are shown in Table 1. The enterotoxin, added at the end of period I, caused a decrease in J<sub>m $\rightarrow$ s</sub> and an increase in J<sub>s $\rightarrow$ m</sub>, resulting in a net decrease in Cl<sup>-</sup> absorption. Under similar experimental conditions, no significant changes developed in control tissues between the two periods. No changes in conductance were seen either in enterotoxin-treated or control tissues. Addition of toxin did not alter the I<sub>sc</sub> response to luminal addition of 10 mM glucose (data not shown).

**Effects of *Y. enterocolitica* ST on cGMP concentration and guanylate cyclase activity.** At the toxin concentration (5 mouse units per ml) which caused the changes in Cl<sup>-</sup> transport shown in Table 1, *Y. enterocolitica* ST caused a three-fold increase in tissue cGMP concentration (0.461  $\pm$  0.16 pmol of cGMP in treated versus 0.1516  $\pm$  0.05 pmol of cGMP in control tissues). The enterotoxin stimulated guanylate cyclase activity when added to ileal membranes (Fig. 1). As in the case of *E. coli* ST, higher concentrations of *Y. enterocolitica* ST were required to stimulate guanylate cyclase activity than to elicit electrical responses or changes in cGMP concentration. The highest concentrations of toxin tested appeared to inhibit enzyme activity, possibly due to an impurity.

## DISCUSSION

The present study demonstrates that a partially purified preparation of a ST elaborated by

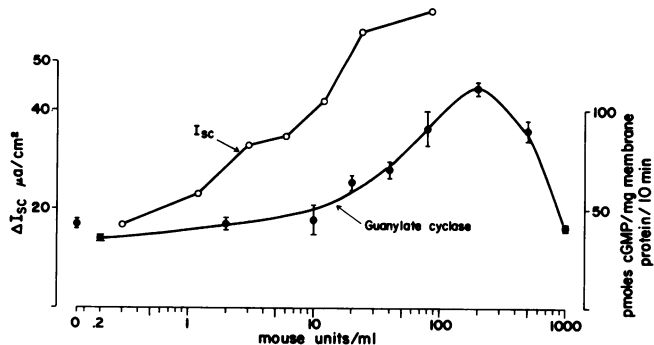


FIG. 1. Effects of various *Y. enterocolitica* ST concentrations on guanylate cyclase activity (●) and I<sub>sc</sub> (○). For guanylate cyclase activity the points represent the mean  $\pm$  standard error of the mean of four determinations, each carried out in duplicate. † represents basal guanylate cyclase activity. For I<sub>sc</sub> the points are representative values for one of four experiments. I<sub>sc</sub> responses were elicited by sequential additions (5 to 10 min apart) of ST to the same tissues.

TABLE 1. Effect of *Y. enterocolitica* ST on Cl<sup>-</sup> transport<sup>a</sup>

Tissues	J <sub>ms</sub> <sup>Cl</sup>	J <sub>sm</sub> <sup>Cl</sup>	J <sub>net</sub> <sup>Cl</sup>	Isc	G <sub>i</sub>
ST treated (8)					
Period I	8.71 ± 1.25	6.29 ± 1.67	2.42 ± 0.8	0.82 ± 0.47	28.96 ± 1.94
Period II	7.56 ± 0.85	7.37 ± 1.68	0.19 ± 0.84	2.60 ± 0.33	26.57 ± 1.52
Δ	-1.15 ± 0.31	+1.08 ± 0.12	-2.23 ± 0.61	+1.78 ± 0.55	-2.39 ± 2.88
P	<0.05	<0.01	<0.05	<0.05	NS
Control (4)					
Period I	7.61 ± 0.62	5.24 ± 0.5	2.37 ± 0.28	0.92 ± 0.18	25.23 ± 1.66
Period II	7.64 ± 0.50	5.19 ± 0.57	2.53 ± 0.24	1.28 ± 0.15	25.09 ± 1.77
Δ	0.03 ± 0.3	-0.05 ± 0.18	0.16 ± 0.52	±0.36 ± 0.2	-0.14 ± 0.36
P	NS	NS	NS	NS	NS

<sup>a</sup> Means ± 1 standard error of the mean for (*n*) experiments. Period I fluxes were measured 50 to 70 min after the tissues were mounted and before the addition of ST. Therefore, period I values are the Cl<sup>-</sup> fluxes for two groups of untreated tissues. ST (5 mouse units/ml) was added to the mucosal medium immediately after period I measurements. Period II fluxes were measured 20 to 50 min after period I in control tissues or after ST addition in experimental tissues. No additions between periods occurred in the control.  $J_{net}^{Cl} = J_{ms}^{Cl} - J_{sm}^{Cl}$ . Fluxes and Isc are in  $\mu\text{Eq}/\text{h} \cdot \text{cm}^2$ ; conductance (G<sub>i</sub>) is in  $\text{ms}/\text{cm}^2$ . NS, Not significant.

strains of *Y. enterocolitica* is closely related in its characteristics and mechanism of action to the ST of *E. coli*. Like *E. coli* ST, *Y. enterocolitica* ST stimulates particulate guanylate cyclase activity, increases cGMP concentration, causes an increase in PD and Isc, and inhibits active Cl<sup>-</sup> absorption in rabbit ileal mucosa. It is not known why higher concentrations of both *E. coli* and *Y. enterocolitica* ST preparations are required to stimulate guanylate cyclase activity in isolated membranes than to stimulate Isc in intact epithelium. However, in studies of hormone-adenylate cyclase interactions, losses of hormonal sensitivity after membrane isolation procedures are not uncommon.

As in the case of *E. coli* ST, cGMP is the probable intracellular mediator of *Y. enterocolitica* ST action. This hypothesis awaits confirmation, however, with fully purified ST preparations. It is worth noting that *E. coli* ST increases only cGMP and not cyclic adenosine 3',5'-monophosphate (cAMP) concentrations in intestinal mucosa (3, 6, 9). cAMP-related agonists (theophylline, cAMP analogs, and cholera toxin) appear to elicit net secretion both by inhibiting Na-coupled Cl absorption in villus cells and by stimulating active anion secretion, probably in crypt cells (4). Only the latter effect appears to alter Isc. Measurements of initial rates of uptake of Cl across the brush border indicate that *E. coli* ST, 8-bromo-cGMP, and theophylline all inhibit Na-dependent Cl uptake to the same extent (S. Guandalini, P. L. Smith, M. Rao, and M. Field, unpublished data). All these agents and *Y. enterocolitica* ST alter Isc and net transmural Cl<sup>-</sup> flux, indicating that they also affect the electrogenic component of the secretory process that probably originates in crypt cells.

Although comparison of the structural characteristics of *E. coli* and *Y. enterocolitica* ST

preparations would be highly speculative at this stage, preliminary evidence based on gel filtration studies suggest that these two STs differ in molecular weight (W. J. Laird, unpublished data). Recently, based on significant differences in heat stability and amino acid composition, the presence of two different *E. coli* STs isolated from a bovine and a porcine strain has been reported (10). Therefore, there may be a family of ST molecules, including *Y. enterocolitica* ST, which are similar in their biological activity and mechanism of action, but which differ somewhat in structure.

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