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Inhibition of the Secretory Activity of *Escherichia coli* Heat-Stable Enterotoxin by Indomethacin

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The effect of indomethacin on the net intestinal accumulation of fluid induced by *Escherichia coli* heat-stable (ST) enterotoxin in the infant mouse model was examined. Indomethacin, when administered with ST enterotoxin, caused a striking decrease in net intestinal fluid accumulation. This inhibition of ST activity was dose dependent with various concentrations of indomethacin ($P < 0.01$). A significant inhibition of toxicity was also observed when indomethacin was given before ($P < 0.01$) or after ($P < 0.02$) ST enterotoxin challenge. No significant differences in fluid accumulation were observed between control mice treated with buffer alone and those challenged with only indomethacin. These data indicate that indomethacin markedly decreases the net intestinal fluid accumulation induced by *E. coli* ST enterotoxin. Further studies on the potential use of indomethacin in both the prophylaxis and the therapy of diarrheal diseases appear warranted.

Transient contamination of the small intestine by strains of *Escherichia coli* that produce heat-labile (LT) or heat-stable (ST) forms of enterotoxin is a recognized cause of acute diarrheal disease. Both LT and ST enterotoxins induce net intestinal fluid accumulation in numerous animal systems (4, 7, 26) and are responsible for human diarrheal disease (5, 13, 23-25). Recent studies have shown that the bioactivities of the LT and ST forms of *E. coli* enterotoxin are inhibited by bismuth subsalicylate and phenylbutazone, respectively. Ericsson et al. (6) have shown that bismuth subsalicylate inhibits the secretory activity of *E. coli* and *Vibrio cholerae* enterotoxins in ligated rabbit ileal loops. Ohgke and Wagner (21) have demonstrated the inhibitory effect of phenylbutazone against *E. coli* ST enterotoxin in infant mice. Other studies have shown that indomethacin inhibits the diarrheal response induced by *V. cholerae* enterotoxin in rabbits (14) and rats (16). Indomethacin also inhibits intestinal fluid accumulation when administered before or after challenge with *Salmonella typhimurium* (11, 12) and *Shigella flexneri* (14). The present study extends these observations with indomethacin to the ST enterotoxin of *E. coli*. The effect of indomethacin on the toxic activity of ST enterotoxin was examined in the infant mouse model before, during, and after initiation of intestinal fluid accumulation.

MATERIALS AND METHODS

Microorganism. *E. coli* strain PB-122-B1 (serotype O80:H9) was obtained from Doyle Evans (University of Texas Medical School, Houston). This strain was originally isolated from a case of traveler's diarrhea in Mexico and has maintained stability of the ST enterotoxin plasmid in the laboratory (8). The organism was grown in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) and preserved by lyophilization.

Toxin preparation. A lyophilized culture of the microorganism was reconstituted and inoculated onto a Trypticase soy slant (Difco). After 18 h of incubation at 37°C, the slant was harvested with 1.0 ml of synthetic medium, and 0.1 ml was used to inoculate a preculture of 50 ml of synthetic medium in a 250-ml Erlenmeyer flask. The synthetic medium was prepared by the method of Alderete and Robertson (1). The preculture was then incubated for 18 h at 37°C and used as a seed culture for toxin production. Erlenmeyer flasks (1 liter) containing 200 ml of synthetic medium (1) were inoculated with 25 ml of the seed culture, resulting in an initial optical density at 540 nm of 0.05. The organism was grown for 8 h in a platform shaker (160 rpm) at 37°C, at which time the culture was centrifuged (16,100 × *g*) for 1 h and the supernatant was filter sterilized (0.45 μm; Millipore Corp., Bedford, Mass.). The sterile culture filtrate was concentrated 10-fold and washed on an Amicon UM-2 ultrafiltration membrane (Amicon Corp., Lexington, Mass.). The retentate was filter sterilized and assayed for toxic activity in infant mice.

Infant mouse assay. The infant mouse model of Dean et al. (4) was routinely used to assay for toxic activity. The specificity of this model for *E. coli* ST enterotoxin has been shown (9). Briefly, infant mice (2 to 4 days) were randomly separated into groups of four, injected with a 0.1-ml sample containing 0.02% Niagara Sky Blue, and incubated at room temperature for 3 h. The animals were then sacrificed by decapitation, and the entire intestinal tract was removed and

weighed. The ratio of intestinal to remaining body weight was used as a measure of the amount of toxic activity as described elsewhere (4). If dye was not present in the stomach at sacrifice, the infant was discarded.

A dose-response titration curve was constructed from dilutions of the concentrated UM-2 retentate. A dilution giving a toxic response in the linear portion of the titration curve was routinely used. In all subsequent drug studies this was referred to as a standard dilution of toxin. The standard toxin dilution gave a ratio of approximately 0.100 when mixed with an equal volume of 0.134 M potassium phosphate buffer (PPB) at pH 7.0.

Simultaneous treatment with indomethacin. A stock solution of indomethacin (Merck Sharp and Dohme, West Point, Pa.) was suspended in PPB (pH 7.0) to a final concentration of 800 μg/ml; a fresh stock was prepared daily. Standard serial dilutions of the stock solution were made in PPB (pH 7.0) to yield final concentrations ranging from 20 to 400 μg/ml. An equal volume of the standard toxin dilution was then added to each of the drug concentrations, and the solution was blended in a Vortex mixer before injection. The assay for toxic activity was performed as previously described.

Pre- and post-treatment with indomethacin. Indomethacin (10 μg in 50 μl of PPB) was administered percutaneously into the stomach of each infant mouse 30 min before or 30 min after toxin challenge. Control mice were injected with 50 μl of PPB 30 min before or 30 min after challenge with the same standard toxin dilution. All mice were sacrificed 3 h after toxin challenge, and the ratio of intestinal to body weight was determined as described above. The ability of ST enterotoxin to induce detectable intestinal fluid accumulation 30 min postchallenge was determined by sacrificing groups of mice 30 min after toxin injection.

RESULTS

The previously described method of *E. coli* ST enterotoxin assay (4, 10) as modified in this study has been used to construct a dose-response curve. The titration curve is shown in Fig. 1. As shown, each point on the graph represents a mean ratio obtained from eight animals challenged with various dilutions of UM-2 retentate. These results illustrate the general applicability of the toxin titration method. A response ratio in the linear portion of the titration curve was required for the assay of drug effect to allow titration of residual toxic activity (Fig. 1). Therefore, control animals received a standard toxin dilution resulting in a final ratio of approximately 0.100 when mixed with an equal volume of buffer. In drug-treated animals, a standard toxin dilution was mixed with an equal volume of buffer containing indomethacin and titrated for residual toxic activity.

The reproducibility of the toxin titration over the range of drug concentrations used was shown by repeated titrations of drug with the standard

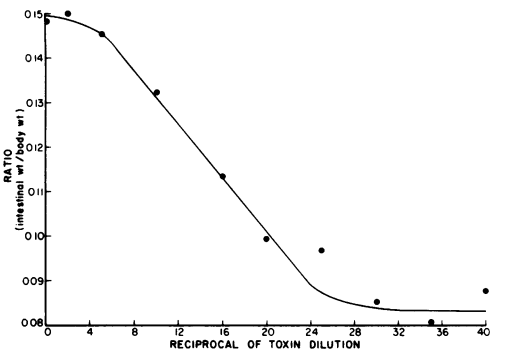

FIG. 1. *Toxin titration curve of the concentrated sterile culture filtrate of E. coli ST enterotoxin. Each point represents the mean value from eight infant mice.*

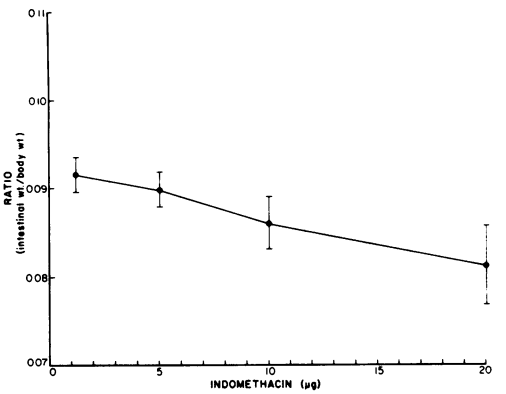

FIG. 2. *Effect of indomethacin on net intestinal fluid accumulation induced by E. coli ST enterotoxin. Each point represents the mean value ± standard deviation (n = three groups). Toxin alone gave a response ratio of 0.105 ± 0.005 where n = 11 groups (data not shown).*

dilution of toxin (Fig. 2). As shown, the simultaneous injection of infant mice with various concentrations of indomethacin and the standard toxin dilution resulted in a significant inhibition of net intestinal fluid accumulation. In the absence of indomethacin, titration of the standard toxin dilution gave a response of 0.105 ± 0.005 (data not shown). This response, in the presence of various drug concentrations, was significantly diminished and dose dependent. The difference between toxin alone and each drug concentration tested was significant ($P < 0.01$). Indomethacin alone (40 μg) caused no significant accumulation of intestinal fluid (see below).

Both pre- and post-treatment of infant mice with 10 μg of indomethacin resulted in the inhibition of net intestinal fluid accumulation. When 10 μg of indomethacin was injected 30 min before

the administration of a standard dilution of ST enterotoxin, there was a significant ($P < 0.01$) decrease in fluid accumulation when compared with control groups of mice receiving buffer 30 min before or 30 min after challenge with the same toxin dilution (Table 1).

To test the ability of indomethacin to inhibit net fluid accumulation induced by ST enterotoxin after initiation of the response, it was necessary to determine whether ST enterotoxin had an appreciable effect within 30 min. Groups of mice were injected with the standard toxin dilution and sacrificed 30 min later, and the ratio of intestinal to body weight was determined. As shown in Table 1, a significant amount of fluid had already begun to accumulate ($P < 0.01$) as compared with the buffer control. Animals injected with either buffer alone or indomethacin alone (40 μg) showed no significant difference in the ratio of intestinal to body weight. Next, mice were injected with the standard toxin dilution followed by an injection of indomethacin (10 μg) 30 min later. There was a significant decrease ($P < 0.02$) in fluid accumulation in these mice as compared with the toxin control (Table 1).

DISCUSSION

This study demonstrates the ability of indomethacin to significantly inhibit the net intestinal fluid accumulation induced by *E. coli* ST enterotoxin. This inhibition was dose dependent from 1 to 20 μg when the drug was given simultaneously with toxin. Treatment of mice 30 min before toxin challenge resulted in a significant inhibition of net fluid accumulation, as did drug treatment after initiation of the response. Results from mice sacrificed 30 min after toxin challenge indicated that a significant amount of fluid had already accumulated by the time of drug treatment. Other studies have shown a significant accumulation of intestinal fluid as early as 60 min after toxin challenge (10).

TABLE 1. *Effect of pre- and post-treatment of infant mice with indomethacin 30 min before or 30 min after toxin challenge*

Procedure	Drug concn (μg)	Ratio[a]
Pretreatment	10	0.068 ± 0.007
Post-treatment	10	0.074 ± 0.009
Toxin control	None	0.097 ± 0.006
Indomethacin control	40	0.058 ± 0.002
Toxin (30 min)	None	0.080 ± 0.003[b]
Buffer control	None	0.062 ± 0.001

[a] Ratios represent the mean intestinal weight/body weight ± standard deviation. All P values were determined by the Student t test. Differences between pre- or post-treatment and toxin control (see text for details) were significant at $P < 0.01$ and $P < 0.02$ respectively; no significant difference was observed between the indomethacin control and buffer alone.

[b] This value was significantly ($P < 0.01$) higher than buffer alone.

Previous studies in infant mice have dealt primarily with qualitative determinations of toxic activity, whereas the present study was concerned with a quantitative decrease in fluid accumulation. Therefore, a dose-response titration curve was required. When the concentrated UM-2 retentate was used, the response was approximately linear from a ratio of 0.140 to 0.090. The lower limit of significant toxicity is in close agreement with earlier reports which range from 0.090 (4) to 0.083 (10). These results emphasize the need to use initial concentrations of toxin which yield ratios of 0.100 or greater.

The mechanism of inhibition of intestinal fluid accumulation by indomethacin is presently unknown. Although indomethacin is a potent inhibitor of prostaglandin synthesis, the role of these substances in diarrheal syndromes is a matter of speculation. Prostaglandins are involved in the profuse watery diarrhea characteristic of pancreatic cholera (17), induce fluid secretion in perfused loops of dogs and isolated ileal loops (22; Q. Al-Awqati, M. Field, N. F. Pierce, and W. B. Greenough III, J. Clin. Invest. **49**:2a, 1970), and are known to affect cyclic AMP (cAMP) levels in several tissues (2, 3, 20). Because cholera enterotoxin induces fluid accumulation by stimulation of adenylate cyclase and thus elevation of intracellular cAMP levels, it has been suggested that prostaglandins serve as intermediates for the stimulation of adenylate cyclase. In support of this concept, Jacoby and Marshall (16) have demonstrated the ability of indomethacin to inhibit fluid accumulation in intestinal loops of rats after challenge with cholera enterotoxin.

Additional studies have failed to confirm the association between inhibition of prostaglandin synthesis and decreased cAMP levels. Kimberg et al. (19) found that cAMP levels after challenge with cholera toxin were not influenced by addition of indomethacin, although intestinal fluid accumulation was decreased. Wald et al. (27) have shown that indomethacin inhibits secretion and unidirectional sodium flux in control and toxin (cholera)-challenged jejunal loops. Because indomethacin had no effect on cAMP levels, these authors suggest that the drug may be affecting a prostaglandin-mediated reaction beyond the stimulation of cAMP.

E. coli LT induces fluid accumulation in a manner similar to cholera enterotoxin (18). Although the mechanism of action of *E. coli* ST is not clearly defined, recent studies have sug-

gested that ST acts by elevating intracellular cGMP levels (9, 15). Giannella et al. (11, 12) and Gots et al. (14) have demonstrated the ability of indomethacin to inhibit fluid accumulation induced by *Shigella* and *Salmonella*. In contrast to cholera-mediated secretion, the increase in cAMP caused by *Salmonella* infection is inhibited by indomethacin (11). Inhibition of fluid secretion, after it is well established, has been shown in rhesus monkeys infected with *Salmonella* and then treated with indomethacin (12). However, indomethacin was shown to stimulate absorption of water and sodium in the ileum of control animals. Recently, Ohgke and Wagner (21) have shown that phenylbutazone inhibits the secretory activity of *E. coli* ST when given subcutaneously 30 min before toxin challenge. Although further investigations will be required to define the inhibitory mechanism of indomethacin, it would appear that a prostaglandin-mediated step is involved in a wide range of diarrheal syndromes.

Further studies may indicate the therapeutic use of indomethacin in the treatment of bacterial diarrhea. Indomethacin has been used to successfully treat pancreatic cholera (17). Recent studies by Ericsson et al. (6) have shown that Pepto-Bismol inhibits the activity of cholera and *E. coli* LT enterotoxins in tissue culture and ligated ileal loops. However, the preparation was ineffective once toxin was bound to intestinal mucosa. Results of the present study and other investigations (12) demonstrate that indomethacin is effective after initiation of fluid accumulation and may therefore prove to be effective in the treatment of diarrheal symptoms. In light of the drug's significant inhibition of intestinal fluid accumulation induced by a variety of microorganisms, it would seem appropriate to conduct clinical trials.

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