Cholera Enterotoxin-Induced Mucus Secretion and Increase in the Mucus Blanket of the Rabbit Ileum In Vivo

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The in vivo rabbit ileum was used to study the relationship of cholera enterotoxin-induced water and electrolyte secretion and mucus secretion and to determine whether the enterotoxin influenced the intestinal mucus blanket. In experiments in which luminal fluid viscosity was used to assess mucus secretion, it was found that while cholera enterotoxin induced a sustained secretion of water and electrolytes, the toxin-induced mucus hypersecretion was short lived (3 to 5 h) and subsequent exposure of the mucosa to cholera enterotoxin or prostaglandin E_2 did not stimulate mucus secretion further. Dibutyryl cyclic AMP and theophylline caused a modest mucus secretion in ileal loops which differed from that of cholera enterotoxin in both magnitude and in the fact that the mucus secretion occurred 2 to 3 h after the onset of water and electrolyte secretion. An oral replacement solution was used in the ileum to reduce the enterotoxin-induced loss of water and electrolytes into the lumen. While such a solution slowed the appearance of acidic glycoprotein in the intestinal lumen, it did not change the amount of glycoprotein secreted over a 7-h period, suggesting that toxin-induced mucus secretion was not simply due to a flushing action of the experimentally caused diarrhea. To assess mucus blanket thickness, neutral glycoprotein was recovered from the blanket of rabbit ileal loops 7 h after exposure to cholera enterotoxin and the thickness of the mucus blanket was measured directly 4 and 18 h after toxin exposure. Both methods indicated that even though cholera enterotoxin-induced mucus hypersecretion had subsided and there was histological evidence of goblet cell mucin depletion, there was a sustained increase in mucus blanket thickness that was detectable for at least 18 h after mucosal enterotoxin exposure.

Cholera is characterized by a copious diarrhea, known as rice-water stool. Often mucus fragments may be the only formed material visible in such stool. In experimental cholera, the hypersecretion of electrolytes and water into the small intestinal lumen is associated with a hypersecretion of mucus (6, 7, 9, 16, 18, 19) and goblet cells in the area exposed to cholera enterotoxin are depleted of their content (22). The hypersecretion of water and electrolytes in cholera enterotoxin-treated small intestine is apparently sustained for as long as the mucosal epithelial cell adenylate cyclase is stimulated and tissue cyclic AMP (cAMP) levels are elevated (4, 10, 11), but there is some question as to whether or not the mucus hypersecretion of experimental cholera is cAMP mediated (15, 18). In rabbit ileal loops in vivo, the hypersecretion of electrolytes and water is sustained over several hours, while the hypersecretion of mucus peaks and then subsides (9, 16), suggesting that the mucus-secreting capacity of the mucosa has been overextended or exhausted. The intestinal mucus blanket represents an important defense system in the gastrointestinal tract (6). Compromising the mucus blanket during cholera, either as a result of goblet cell exhaustion or because of a flushing action of the mucosal secretion (16), would be expected to affect the course of the disease. The present study was designed to determine whether the mucus blanket integrity is maintained in experimental cholera and to further define the relationship between net water movement and mucus secretion in the small intestine.

MATERIALS AND METHODS

Measurement of mucus secretion and mucus blanket. Mucus secretion was assessed in two ways. In preliminary experiments, luminal fluid was harvested and the volume was adjusted to 1 ml/cm of rabbit ileum loop length. The apparent viscosity of the fluid was then measured at a shear rate of 1.84 s^{-1} at 25°C (16). In another experiment, mucus secretion was assessed chemically. Harvested luminal fluid was dialyzed exhaustively against 10 mM Tris hydrochloride (pH 7.0) at 4°C, and the neutral glycoprotein concentration was measured by a periodic acid-Schiff method (14) with mucin (type I; Sigma Chemical Co., St. Louis, Mo.) as a standard. Acidic glycoprotein secretion was measured by an alcin blue dye binding method (17), and the secretion of such glycoprotein is expressed as the milligrams of dye adsorbed. All glycoprotein secretion was normalized to ileum loop length.

The mucosal mucus blanket was assessed chemically and by direct observation. In the chemical method used, the in vivo ileal loop was filled with saline containing cetlypyridinium bromide (1 mg/ml) for 5 min (12). The luminal fluid was then harvested and pooled with two subsequent saline washes. The mucus blanket samples so obtained were then dialyzed and analyzed for neutral glycoprotein (14) and DNA (2). The physical method used to study the mucus blanket involved directly visualizing the blanket with a slit lamp and measuring the thickness with a pachymeter as described by Bickel and Kaufman (1). A section of rabbit ileum was everted, rinsed free of excess mucus in saline, and mounted on a test tube. The slit lamp was housed in a plastic chamber lined with damp sponges to maintain the relative humidity at or above 90% to prevent the sample from drying out. In each experiment, 10 measurements were made of the blanket thickness at randomly chosen sites on the villus tips and at intervillus areas. The values so obtained were averaged for each animal. All measurements were performed at room temperature.

Animal model preparation. Experimental cholera was produced with cholera enterotoxin (Sigma) in adult male New Zealand rabbits. All animals were starved overnight prior to the experiments. In preliminary experiments, the animals...
FIG. 1. Hourly net fluxes of water and mucus secretion in in vivo rabbit ileal loops exposed to cholera enterotoxin (CT). Negative net water flux \( J_{H_2O} \) values indicate absorption, and positive values indicate secretion. Mucus secretion was assessed by measuring the apparent viscosity in centipoise (cp) of the luminal fluid after normalizing fluid volume to ileal loop length. Mean ± standard error of the mean (SEM) (bars) \((n = 5)\).

were anesthetized with pentobarbital and a 20- to 25-cm-long loop of lower ileum was prepared in each (16). A catheter was secured in the distal end of the loop, which was then filled with 10 ml of bicarbonate-saline \((25 \text{ mm } \text{HCO}_3, 290 \text{ mosM})\) containing cholera enterotoxin. At hourly intervals, the loop was completely emptied, the content volume was measured, and the loop was again filled with 10 ml of bicarbonate-saline. In these experiments, the animals were maintained under general anesthetic for 9 h. In another group of animals, two loops were prepared and one was filled with bicarbonate-saline at hourly intervals, while the other was inoculated with dibutryl cAMP and theophylline in bicarbonate-saline.

In a third group of experiments, the animals were anesthetized with ether and a laparotomy was performed, using procaine as a local anesthetic at the incision site. A 20- to 25-cm-long loop of distal ileum was prepared with a catheter at each end. These catheters were led out through the abdominal wall and secured by purse-string sutures. The laparotomy was closed, and the animal was placed in a restraining harness and allowed to recover from the anesthetic. In these experiments, the loops were filled and emptied at 0.5-h intervals and the two 30-min samples were pooled. The loops were emptied by gravity and by peristalsis, followed by the injection of 20 ml of air into the catheter. Two different test solutions were used in these experiments, the bicarbonate-saline solution referred to above and the World Health Organization (WHO) oral rehydration solution (21) to which water was added to bring the osmolarity to 290 mosM. Animals were maintained in the restraining harnesses for 7 h, while procaine was administered at the laparotomy and purse-string suture sites at hourly intervals. At the end of the 7-h collection period, the loop was filled with cetylpyridinium bromide-saline \((1 \text{ mg/ml})\) while the animal was being anesthetized with pentobarbital prior to sacrifice.

In a fourth experimental protocol, the animals were anesthetized with pentobarbital and a 20- to 25-cm-long lower ileal loop was made with temporary ligatures. The loop was then inoculated with saline or saline containing cholera enterotoxin. Fifteen minutes later, the loop was emptied with a syringe, the ligatures were removed, and the laparotomy was closed. The animals were sacrificed 4 or 18 h later by an overdose of anesthetic. Tissue samples were taken for visual assessment of the mucus blanket, and samples were also fixed in neutral Formalin for subsequent histological study. Adjacent paraffin-embedded sections were stained for neutral glycoprotein by periodic acid stain and for acidic glycoproteins by alcian blue dye stain at pH 2.0.

**Statistical analyses.** In experiments involving more than two groups, the significance of differences between groups was measured by a one-way analysis of variance, followed by post hoc least-significant-difference tests to determine the significance of differences between pairs of means. Student’s \( t \) tests were used to determine the significance of differences between means in experiments involving two groups.

**RESULTS**

In preliminary experiments, animals were maintained under a general anesthetic for the duration of the experiment, allowing complete evacuation of the ileal loop at the end of each collection period. Such complete evacuation was not possible in subsequent experiments involving conscious animals. In the preliminary experiments, mucus secretion was assessed by measuring the apparent viscosity of the luminal fluid after normalizing the fluid volume to loop length. Increased apparent viscosity of the luminal fluid has been shown to correlate with increased nondialyzable hexose concentration in the luminal fluid (16). Figure 1 shows the results of such an experiment in which ileal loops were exposed to a supramaximal concentration of cholera enterotoxin \((50 \mu g)\) for 1 h. Water secretion gradually increased to a maximal level and remained at that level for the duration of the experiment, while mucus secretion into the luminal fluid exhibited a peak value and declined thereafter. A second period of mucosal exposure to the high dose of toxin did not affect the ongoing water secretion and did not initiate another detectable pulse of mucus secretion into the lumen.
When the initial enterotoxin dose was submaximal (0.5 μg), a similar pattern of reduced water and mucus secretion was observed. When the ileal mucosa was then exposed to 50 μg of enterotoxin during hour 7, a slight increase in water and mucus secretion was observed. Again, the increase in mucus secretion was transient, while the increase in water secretion was sustained. When these experiments were repeated with the supramaximal dose of cholera enterotoxin in hour 1 and another known secretagogue, prostaglandin E1 (10⁻⁵ M), in the luminal fluid during hour 7 (16), no second pulse of mucus secretion was observed and the plateau in water secretion was unaffected.

When dibutyryl cAMP and theophylline were substituted for cholera enterotoxin in ileal loops (Table 1), a significant (P < 0.01) net water secretion was observed in hour 1 and this secretion did not significantly change with time. A significant (P < 0.01) mucus secretion was also observed in cAMP-treated loops which was time dependent (P < 0.05). Post hoc tests indicated that the luminal fluid viscosity was only significantly increased in hour 4. No significant differences were detected histologically in goblet cell mucin content.

In another group of experiments with conscious animals, the ileal mucosa was exposed to saline or saline containing 100 μg of cholera enterotoxin for 1 h and the net fluxes of water and the secretion of acidic and neutral glycoproteins into the luminal fluid were measured. Figure 2 shows the results of these experiments, and Table 2 summarizes the total net water fluxes and the 1- to 7-h acidic glycoprotein secretion. The neutral glycoprotein secretion data are not shown because even after exhaustive dialysis, the glucose in the WHO solution interfered with the periodic acid-Schiff assay. The secretion of neutral glycoprotein into bicarbonate-saline-filled loops was 60% greater than that into control loops (P > 0.05), while the acidic glycoprotein secretion increased 112% with enterotoxin (P < 0.01). The evacuation of the loops at each collection period was less complete in these experiments than in those experiments discussed above with anesthetized animals. Nevertheless, a peak was seen in the secretion of acidic glycoproteins into bicarbonate-saline-filled enterotoxin-treated loops in the fourth hourly collection period, while such secretion increased more gradually in the WHO solution-filled enterotoxin-treated loops. Although there was a significant difference between the mean total 1- to 7-h net water flux in bicarbonate-saline-filled toxin-treated loops and in WHO solution-filled toxin-treated loops, there was no difference in the mean acidic glycoprotein secretion values of these groups.

Attempts were made at the end of these experiments to evaluate the mucosal mucus blanket to determine whether it had been compromised by the enterotoxin-induced mucus secretion into the lumen. Cetylpyridinium bromide was used to precipitate the blanket. Such treatment interfered with the alcan blue dye binding assay for acidic glycoproteins, and only the neutral glycoprotein content of the blanket was assessed. These data are expressed as a ratio of recovered neutral glycoprotein to DNA on the assumption that an increase in this ratio would signal an increase in glycoprotein content of the mucus blanket, even if the cetylpyridinium bromide treatment was responsible for some mucosal sloughing yielding epithelial cell glycoproteins. Cholera enterotoxin increased the blanket neutral glycoprotein/DNA ratio (Table 2). This increase was significant (P < 0.05) for the bicarbonate-saline-filled toxin-treated loops, but not for the WHO solution-filled toxin-treated loops.

The mucus blanket was also assessed in areas of intact

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### TABLE 1. Net water flux values and apparent viscosity values of luminal fluid in rabbit ileal loops in vivo inoculated at hourly intervals with bicarbonate-saline (control) or dibutyryl cAMP and theophylline in bicarbonate-saline (experimental)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Exptl treatment (mM)</th>
<th>Net water flux (ml/10 cm per h)*</th>
<th>Luminal fluid apparent viscosity (centipoise)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>Dibutyryl cAMP (5) + theophylline (5)</td>
<td>-2.89 ± 0.75</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>1-2</td>
<td>Dibutyryl cAMP (2.5) + theophylline (10)</td>
<td>-2.41 ± 0.94</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>2-3</td>
<td>Dibutyryl cAMP (2.5) + theophylline (10)</td>
<td>-0.80 ± 0.90</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>3-4</td>
<td>Dibutyryl cAMP (2.5) + theophylline (10)</td>
<td>-0.60 ± 1.02</td>
<td>2.6 ± 0.5</td>
</tr>
</tbody>
</table>

* Mean ± SEM, n = 5. Negative net water flux values indicate absorption, and positive values indicate secretion.

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ileum that had been exposed to 100 μg of cholera enterotoxin for 15 min or 18 h previously. Table 3 summarizes these data and shows that prior mucosal exposure to cholera enterotoxin significantly increased mucus blanket thickness at both villus tips and inter villus regions. Histological sections of tissue from these experiments were scored for goblet cell acidic and neutral glycoprotein content. Mucosal exposure to cholera enterotoxin 4 or 18 h previously resulted in a significant reduction in both acidic and neutral glycoprotein content of goblet cells.

**DISCUSSION**

Cholera enterotoxin has been shown to be a potent stimulant of both intestinal mucus secretion (6, 7, 9, 16, 19) and mucin synthesis (6, 7), and there is evidence that cholera enterotoxin preferentially stimulates the secretion of the most recently synthesized mucin (6). Previous studies (9, 16) and the present study showed that cholera enterotoxin-induced mucin secretion into the lumen was short lived, while toxin-induced water and electrolyte secretion was sustained for many hours. The fact that a second exposure to cholera enterotoxin or another secretagogue failed to produce a second mucous secretion response could be explained by one or more of three explanations. (i) The turnover of the small intestinal mucin pool that is responsive to cholera enterotoxin may have become exhausted or refractory to toxin. (ii) The initial bolus of mucus that appeared in the lumen may have represented mucus blanket that was flushed from the mucosal surface by the intestinal secretion of water and electrolytes. (iii) The mucus that initially appeared in the lumen was the result of a rapid evacuation of goblet cell content into the lumen, while subsequent secretion of newly synthesized mucin was slower and contributed to the gradual buildup of the mucus blanket.

It is unlikely that intestinal goblet cells became completely exhausted in 4 to 6 h as there was still a significant although reduced acidic and neutral glycoprotein content in enterotoxin-stimulated goblet cells for up to 18 h and the mucus blanket thickness remained significantly increased over the same period, suggesting that both mucin synthesis and secretion remained elevated for that period. The fact that goblet cell mucin content was reduced following mucosal exposure to cholera enterotoxin in both short-term (4-h) and long-term (18-h) experiments suggests that mucin synthesis lagged behind secretion in these cells. The possibility that some desensitization of the mucin synthesis and secretion system occurred cannot be excluded. Mouse intestinal mucosal adenylate cyclase is desensitized by repeated exposure to cholera enterotoxin (13), although adenylate cyclase stimulation by a single exposure to cholera enterotoxin has been shown to last for 48 h (11).

It is unlikely that the mucus appearing in the intestinal lumen was simply the result of a flushing action of the toxin-induced secretion of water and electrolytes as oral fluid replacement significantly reduced the net flux of water into the lumen while have no effect on the total acidic glycoprotein secreted into the lumen in a 7-h period. Similarly, luminal cAMP- and theophylline-induced water secretion preceded the appearance of mucus in the lumen by 2 to 3 h.

Cholera enterotoxin is a ubiquitous stimulator of adenylate cyclase activity (10). It has been generally assumed that all the small intestinal effects of this toxin are cAMP mediated. This may be true for the water and electrolyte flux effects of enterotoxin (4, 10, 11), but it seems less likely an explanation for the mucous secretion effects. It has not been possible to demonstrate a cAMP-theophylline stimulation of mucous secretion in the small intestine (15). In the present study, luminal cAMP and theophylline caused a weak stimulation of mucous secretion that was not seen in all animals and that lagged behind the water and electrolyte secretory response by 2 to 3 h. Thus, the mucus secretory response to cholera enterotoxin was not mimicked by exposure to exogenous cAMP. This stimulation of mucous secretion that was observed here but was not detected in shorter term in vitro experiments (15) may have been the result of a cAMP-mediated stimulation of synthesis (5) which eventually manifested itself in a modest mucus secretion.

**TABLE 2.** Cumulative net water fluxes and acidic glycoprotein secretion in rabbit ileum in vivo during the 1 to 7 h test periods and the neutral glycoprotein/DNA ratios of mucus blankets recovered at the end of 7 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Net water flux (mI/10 cm)a</th>
<th>Secreted acidic glycoprotein (mg of dye adsorbed/10 cm)a</th>
<th>Mucus blanket neutral glycoprotein/DNA (mg/mg)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (bicarbonate-saline)</td>
<td>−2.70 ± 1.13</td>
<td>7.68 ± 1.53</td>
<td>6.21 ± 0.89</td>
</tr>
<tr>
<td>Cholera enterotoxin (100 μg) WHO solution</td>
<td>+6.79 ± 2.90b</td>
<td>16.53 ± 1.96b</td>
<td>9.32 ± 2.37</td>
</tr>
<tr>
<td>Bicarbonate-saline</td>
<td>+18.89 ± 3.97b,c,e</td>
<td>16.26 ± 1.21b</td>
<td>18.65 ± 5.48b</td>
</tr>
</tbody>
</table>

* Mean ± SEM, n = 5 to 7. Negative net water flux values indicate absorption, and positive values indicate secretion.
* Different from control (P < 0.01).
* Different from WHO solution (P < 0.01).

**TABLE 3.** Mucus blanket thickness and neutral and acidic glycoprotein content of rabbit ileum goblet cells 4 and 18 h after exposure to saline or cholera enterotoxin (100 μg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blanket thickness (μm)</th>
<th>Goblet cell glycoprotein contentb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Villus tip</td>
<td>Intervillus region</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>18 h</td>
</tr>
<tr>
<td>Control</td>
<td>68 ± 19</td>
<td>83 ± 22</td>
</tr>
<tr>
<td>Enterotoxin</td>
<td>239 ± 27</td>
<td>263 ± 27</td>
</tr>
</tbody>
</table>

* Mean ± SEM, n = 5. P < 0.01 in all cases.
* Goblet cell glycoprotein content was scored subjectively with a 0 to 4 range.
The intestinal mucus blanket is believed to protect the underlying mucosa from mechanical and chemical damage and to influence the course of intestinal infections (6). The hypersecretion of mucus and mucus blanket thickening seen in experimental cholera may represent an attempt by the host to protect itself against *Vibrio cholerae* infection. The mucus blanket constitutes a physical barrier to *V. cholerae*, and only motile strains exhibiting chemotaxis can penetrate the mucus gel to adhere to the mucosa (8). Mucin is also known to bind cholera enterotoxin (20), while *V. cholerae* elaborates a metalloproteinase that degrades mucin and enables the enterotoxin to gain access to intestinal epithelial cell receptor sites (3).

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**LITERATURE CITED**


