In Vivo Effects of Clostridium perfringens Enteropathogenic Factors on the Rat Ileum

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An experimental model was established using the terminal ileum of the rat for characterizing and studying the effects of crude cell-free extract from Clostridium perfringens upon physiological and histological parameters involved in the transport process. Further work was done with the model system using purified enterotoxin (protein) from the cell extract. Using an in vivo perfusion technique it was found that crude extract induces a reversal of net transport, from absorption in controls to secretion, of water, sodium, and chloride. Glucose absorption was greatly inhibited, whereas potassium and bicarbonate transports were unaffected. Crude extract also caused histological damage to the villus epithelium by denuding the villus tips, thereby leaving the lamina propria exposed. Similar responses in transport of water, sodium, chloride, bicarbonate, and glucose were caused by purified toxin. Little or no histological damage resulted from the pure toxin activity. However, the toxin was shown to have the capacity to denude villus tips under the proper experimental conditions.

In recent years, food poisoning due to strains of Clostridium perfringens has come under more intensive study and consideration because of its increasing prevalence in the United States (1). Studies have shown that enteric responses similar to those found in C. perfringens food poisoning can be induced in rabbit ileal loops by challenge with viable cells (3) and cell-free preparations (4). Further study has shown that purified protein collected from a cell-free extract has biological activity similar in some respects to that of the crude preparations (5, 14). To date no physiological study has been reported that describes in detail the specific responses by the intact intestine to preparations of active enteropathogenic factors from sporulating cultures of C. perfringens. The results reported in this paper characterize and define in vivo transport and morphological responses by the perfused intact rat terminal ileum to active factors from sporulating cultures of C. perfringens.

MATERIALS AND METHODS

Crude cell-free extract was prepared essentially according to the method of Duncan and Strong (4). Cultures of C. perfringens NCTC 8239 (Hobbs type 3) were kindly supplied by Charles L. Duncan (Food Research Institute, University of Wisconsin). Cells were grown overnight in fluid thioglycollate medium followed by a 2.0% (vol/vol) inoculum into Duncan-Strong sporulation medium. These were incubated for 8 h at 37 C and harvested by centrifugation. Packed cells were sonically treated with a Branson S 110 sonifier until the cells were disrupted, and the sonic extract was then centrifuged at 27,000 x g for 10 min. The supernatant was pushed through a 0.45-μm membrane filter (Millipore Corp.) with prefilter by positive pressure. One-milliliter portions were lyophilized, sealed under nitrogen, and stored at -70 C.

Purified toxin from C. perfringens (NCTC 8239) was also kindly supplied by Charles L. Duncan. Samples were prepared in his laboratory according to the method already described (15), lyophilized, and delivered by mail. Potency of the toxin was determined in Duncan’s laboratory to be 5,000 erythematous (14) units per mg of protein as measured in guinea pigs. The lyophilized samples were suspended in normal glucose Ringer solution at sufficient dilution to cause 1 ml to contain the desired potency (erythemal units per ml) of toxin. One-milliliter portions were stored frozen at -70 C in 5-ml vials until used.

The technique of intestinal perfusion was that previously used in this laboratory (16) with minor modifications. Female Wistar strain rats weighing between 175 and 220 g were used in the crude-extract experiments, and males of the same strain weighing between 260 and 380 g were used for the pure toxin experiments.

After weighing, animals were anesthetized by intraperitoneal injection of a 5% solution of sodium pentobarbital at a dose of 32.5 mg/kg of body weight. Animals were then tied down and an incision was made along the abdominal midline. A portion of the terminal ileum was exposed and cannulated using polyethylene cannulas. The segment usually extended for 15 to 20 cm oral from the ileocecal junction. Care was taken to maintain the mesenteric blood supply

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intact. The exposed segment was kept moist at all times by regular periodic applications of warm glucose Ringer solution.

In the crude extract experiments, 1 ml of extract (2,000 erythemal units per ml) was incubated in the segment for 45 min prior to the beginning of perfusion. In pure toxin ion transport studies, 1 ml (160 erythemal units per ml) was incubated for 20 min, and in the pure toxin glucose transport experiments, 1 ml (250 erythemal units per ml) was incubated for 15 min.

Crude-extract controls and pure toxin glucose controls consisted of incubating 1 ml of glucose Ringer solution in the segment for 45 and 15 min, respectively. Pure toxin ion transport controls consisted of incubating heat-inactivated pure toxin (60 C for 10 min) for 20 min prior to perfusion.

After the incubation period, the cannulas were attached to a closed perfusion system with a reservoir containing perfusion solution with known amounts of ions, glucose, and polyethylene-1,2-

\[ ^{14} \text{C} \text{glycol} \] as a nonabsorbable marker. The toxin in the incubated segment mixed with and remained in the perfusate throughout the experiments. After the pump was started, it was run for 15 min to allow the perfusate to equilibrate with the segment contents. At the end of the equilibration period a sample was taken and designated as zero time. Small samples were withdrawn at succeeding 15 min intervals for 60 min (five samples total). The segments were perfused at a rate of 1.7 ml/min.

The composition of the glucose Ringer solution was as follows, in millimoles per liter: NaCl, 130; KCl, 5; CaCl₂, 1.3; NaHPO₄, 1.2; NaHCO₃, 10; and glucose, 20. Equilibration of the Ringer solution with CO₂ was accomplished by bubbling with gas mixture (95% oxygen-5% carbon dioxide). The final pH of the solution was approximately 7.0.

Perfusion solution was prepared like the glucose Ringer solution except for the addition of sufficient polyethylene-1,2-

\[ ^{14} \text{C} \text{glycol} \] (molecular weight 4,000) (New England Nuclear) to give a final concentration of approximately 0.15 μCi per ml of perfusion solution (PEGC₁⁴ specific activity, 0.60 μCi/mg).

Samples were analyzed as follows: fluid volume by changes in radioactive concentration of ¹⁴C-labeled polyethylene glycol (11) as determined with a Beckman LS-250 liquid scintillation system; Na⁺ and K⁺ with a Beckman DU-2 spectrophotometer with flame attachment; Cl⁻ with a Buchler-Cotlove chloridometer; glucose using the Glucostat enzyme reagents (Worthington Biochemical Corp.); and bicarbonate was calculated from continuously recorded pH and the barometric pressure by means of the Henderson-Hasselbalch equation (16).

Histological sections were taken from animals used in perfusion experiments and from animals used in specific histological experiments. The animals used in the histological experiments were treated in a manner similar to that used for animals in perfusion experiments, except that toxin preparations were incubated in the segment with no subsequent perfusion. Instead, at 10-min intervals, sections were removed from the segment with care taken to maintain an intact blood supply to the remaining segment. All sections taken for histological study were placed immediately in Bouin solution for 4 to 6 h and then rinsed and stored in 70% ethanol until embedded in paraffin. Thin sections were prepared from the paraffin mounts and stained with hematoxylin-eosin or carmine-mucin dyes.

RESULTS

Table 1 shows the results obtained from a series of transport experiments using crude cell-free extract to induce transport alterations. It can be seen that a consistent net absorption of water in controls was significantly reversed by the action of crude extract to net secretion throughout the 60-min period studied. Chloride transport was similarly altered by the action of the extract. Sodium transport was inconsistent in controls and experimental animals. However, a substantial rate of absorption at 15 and 45 min in controls was altered significantly to a large rate of luminal accumulation at comparable intervals in experimental animals. A small amount of secretion at 30 and 60 min in controls was contrasted by large secretory rates in animals exposed to the cell-free preparations. The difference was significant at 30 min but not at 60 min.

Crude extract appeared to have no significant effect upon potassium transport. Control and experimental animals displayed net secretion throughout the experiments, and the differences between groups were insignificant. The same observation was made for bicarbonate transport.

Glucose was absorbed by the normal rat terminal ileum at a declining rate with time. Whereas extract-treated animals also absorbed glucose on a net basis, the rate of absorption was significantly reduced throughout the experimental period, even to the point of a small net luminal gain of glucose at 30 min.

A typical histological alterations observed when a segment of the terminal ileum was exposed to crude cell-free extract for various lengths of time is shown in Fig. 1A–C. Figure 1A shows a control portion of the terminal ileum unexposed to extract. It is a typical picture of normal intact elongated villi seen in the rat terminal ileum. Figure 1B, after 20 minutes exposure to extract, shows widespread breakdown at the villus tips with relatively large portions of epithelial lining missing. Figure 1C at 50 minutes shows the progression to a point of severe desquamation of the villus epithelium that seems to have reached maximal severity between 20 and 50 min. Heat inactivation of the extract for 10 minutes at 80 C destroyed histopathological activity.
Net transport of water and solutes in control animals and animals treated with pure toxin is shown in Table 2. It can be seen that net absorption of water in controls was reversed to net secretion by the action of the pure toxin. The toxin had a similar effect upon net chloride transport. The consistent absorption of chloride in controls was inhibited significantly, even to the point of secretion at 15 and 60 min in experimental animals.

Sodium transport activity varied greatly in controls, and the transport response to the action of the toxin varied as well. Control animals absorbed sodium throughout the experiment, whereas toxin-treated animals experienced a net loss of sodium, except at 45 min. However, due to substantial variation among animals, the difference was significant at 60 min only, of borderline significance at 30 min and not significant at 15 and 45 min. Bicarbonate was secreted into the lumen in control as well as experimental animals. There was no significant difference between values obtained from the two groups. Net glucose uptake in controls was significantly reduced by the action of the pure toxin.

Figure 1D–E shows histological effects as a function of time of pure toxin on the tissue morphology of the rat terminal ileum. Compared to the control segment shown in Fig. 1A, 1D after 30 min exposure shows nearly the same normal villus morphology as 1A, except for an occasional small area of disruption in the epithelium at some villus tips. Figure 1E (60 min) shows the appearance of histological damage that has progressed beyond the minor disruption seen in Fig. 1D. The essentially intact epithelium (Fig. 1D) is typical of the condition seen in each section at up to 30 min of toxin exposure. The condition seems not to progress much beyond what occurs within 50 min. The first trace of response can be seen at between 10 and 30 min and begins to progress between 30 and 50 min, after which time it seems to remain unchanged.

Figure 1F shows a section from a rat at the conclusion of a perfusion experiment in which pure toxin was used. Slight epithelial disruption can be seen at the tips of several villi, but the epithelium is essentially intact. The animal from which the segment was taken responded to the pure toxin by significant net water secretion into the lumen and a significantly inhibited rate of glucose absorption.

Table 3 shows water transport rates observed in two experiments with crude extract and three with pure toxin.

**DISCUSSION**

In this study, diarrheal symptoms induced
EFFECTS OF C. PERFRINGENS ON RAT ILEUM during food poisoning in man and experimental animals due to an enterotoxin found in sporulating cells of C. perfringens have been reproduced in an experimental rat model. It is thought that the filtered sonic extract used is reasonably close in composition to active factors found in the gut during actual cases of C. perfringens food poisoning. This conclusion is based on the observation that enterotoxin is not released by the organism except upon lysis of sporulating cells (2). With the energy levels and times used in sonic treatment for production of extract, the process can be viewed as being an enhancement of the normal rate and process of lysis of the bacterial cells.

It was shown in Table 1 that crude cell-free

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**Fig. 1.** Effects on epithelial morphology of crude cell-free extract and pure toxin under various experimental conditions. (A) Control section from terminal ileum; (B) section from same animal after 20 min exposure to crude extract; (C) section after 50 min exposure to crude extract; (D) section exposed to pure toxin for 30 min; (E) section exposed to pure toxin for 50 min (same animal as in D); (F) section exposed to pure toxin during a perfusion experiment for 60 min (taken at the conclusion of the experiment). Magnification, ×50. Stain, hematoxylin and eosin. Toxin concentration: A-C, 2,000 erythemal units per ml; D-F, 250 erythemal units per ml.
extract has pronounced effects upon the normal function of the terminal ileum. Net water, sodium, and chloride transport reversals from absorption to secretion are rather common observations in diarrhea induced in other systems, including cholera toxin (8) and staphylococcal enterotoxin B (16). However, inhibition of glucose absorption is an important deviation from observations during cholera and staphylococcal diarrheal syndromes. In these disorders, glucose absorption is maintained at normal levels while fluid and electrolytes are being lost to the lumen.

Since intestinal transport normally consists of two simultaneous fluxes, serosal-mucosal (S-M) and mucosal-serosal (M-S), when water and electrolytes appear in the lumen on a net basis, it is important to understand whether the toxin is inducing an increase in the efflux (S-M) decrease in influx (M-S), or changes in both. Since glucose is absorbed at the same rate in both experimental and control animals in experimental cholera (13) and staphylococcal food poisoning (16), it appears that the absorptive mechanisms remain normal. C. perfringens crude extract inhibited net glucose absorption. A decreased rate of net glucose absorption implies decreased mucosal to serosal movement or a leakage back into the lumen at a rate sufficient to cause a decreased net inward movement. The histological changes seen with the crude extract support a leakage or decreased absorptive surface concept due to surface desquamation by action of the toxin. However, a similar decrease in glucose absorption was seen under the influence of the pure toxin (Table 2) in the apparent absence of severe histological damage (Fig. 1F).

Potassium and bicarbonate transport remained essentially unaffected by the action of the crude extract. Little work has been done with potassium transport, possibly because its movement into or out of the intestine seems to be passive and dependent almost entirely upon

### Table 2. Effects of pure toxin upon net transport in the terminal ileum

<table>
<thead>
<tr>
<th>Transport system</th>
<th>Preparation</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>Control</td>
<td>0.46 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.09 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Toxin</td>
<td>-1.38 ± 0.28</td>
<td>-0.93 ± 0.18</td>
<td>-0.77 ± 0.20</td>
<td>-1.21 ± 0.20</td>
</tr>
<tr>
<td>Cl</td>
<td>Control</td>
<td>181.4 ± 42.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>272.8 ± 44.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>219.6 ± 30.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>192.1 ± 22.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Toxin</td>
<td>-4.5 ± 34.4</td>
<td>53.5 ± 27.3</td>
<td>5.8 ± 30.3</td>
<td>-44.6 ± 26.9</td>
</tr>
<tr>
<td>Na</td>
<td>Control</td>
<td>125.5 ± 167.3d</td>
<td>364.8 ± 190.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47.5 ± 166.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>125.0 ± 87.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Toxin</td>
<td>-88.2 ± 76.1</td>
<td>47.7 ± 27.7</td>
<td>35.9 ± 103.3</td>
<td>-202.3 ± 84.9</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>Control</td>
<td>-66.4 ± 9.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-94.1 ± 15.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-84.2 ± 13.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-108.9 ± 11.4&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Toxin</td>
<td>-81.4 ± 11.6</td>
<td>-108.7 ± 22.0</td>
<td>-71.7 ± 12.4</td>
<td>-63.9 ± 18.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>Control</td>
<td>54.8 ± 5.6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>52.1 ± 5.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td>47.6 ± 9.6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>35.5 ± 4.3&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Toxin</td>
<td>18.4 ± 3.0</td>
<td>21.5 ± 5.8</td>
<td>16.3 ± 4.0</td>
<td>8.2 ± 2.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are mean ± standard error (n = 7 animals in all cases), mucosal to serosal being positive. Water movement is expressed as μl/cm per min; solute movement is expressed as nmol/cm per min.

<sup>b</sup> P < 0.001 (P-values as in Table 1).

<sup>c</sup> P < 0.01.

<sup>d</sup> Not significant.

<sup>e</sup> P = 0.054.

<sup>f</sup> P < 0.02.

### Table 3. Comparison of water transport rates during various experimental conditions

<table>
<thead>
<tr>
<th>Prepn</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract (2,000)</td>
<td>-2.12 ± 0.41</td>
<td>-1.67 ± 0.34</td>
<td>-1.35 ± 0.22</td>
<td>-0.53 ± 0.18</td>
</tr>
<tr>
<td>Extract (2,000)</td>
<td>-1.75 ± 0.23</td>
<td>-1.13 ± 0.41</td>
<td>-0.94 ± 0.14</td>
<td>-0.61 ± 0.36</td>
</tr>
<tr>
<td>Toxin (160)</td>
<td>-1.38 ± 0.28</td>
<td>-0.93 ± 0.18</td>
<td>-0.77 ± 0.20</td>
<td>-1.21 ± 0.20</td>
</tr>
<tr>
<td>Toxin (250)</td>
<td>-1.22 ± 0.06</td>
<td>-1.23 ± 0.16</td>
<td>-0.83 ± 0.14</td>
<td>-1.11 ± 0.15</td>
</tr>
<tr>
<td>Toxin (160)</td>
<td>-1.30 ± 0.34</td>
<td>-0.75 ± 0.33</td>
<td>-1.48 ± 0.32</td>
<td>-1.20 ± 0.20</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values in parenthesis indicate erythemal units per ml incubated in the intestine in the form of active factor (crude extract or pure toxin). Values are means ± standard error (n = 7 animals in all cases except toxin [250] where n = 9), mucosal to serosal being positive. Transport of water is expressed as μl/cm per min.
a concentration gradient (S. F. Phillips and W. H. J. Summerskill, J. Clin. Invest. 45:1056, 1966), and the interdependence between transport of potassium and other substances across the intestinal wall is obscure at this point.

According to Hubel (6, 7), bicarbonate is exchanged for chloride, which means as chloride is actively absorbed, bicarbonate ions are secreted into the lumen in exchange for the lost chloride ions. Some bicarbonate secretion persists even when chloride is secreted into the lumen (7). In this study, the rate of net bicarbonate secretion into the lumen is similar whether chloride is absorbed, as in controls, or secreted, as in extract-treated animals. These observations could cast some doubt on the significance of bicarbonate-chloride exchange. It should be noted though that change in pH was the sole indication of bicarbonate transport used. The possibility that factors other than HCO$_3$- transport may have contributed to the pH change has not been excluded. Another possible explanation for a difference in net chloride transport with no difference in bicarbonate transport is that influx of chloride may be the major source of bicarbonate exchange, and the influx of chloride may have been essentially the same in control and extract-treated animals. It may have been an increase in the efflux rate that caused net chloride loss in extract-treated animals.

The histological evidence obtained through these experiments shows that there are definite observable tissue responses to crude cell-free extract and purified toxin. Though histological lesions are observed in diarrheal syndromes due to bacterial infections of the gut (Salmonella enterocolitis, 10, 12; shiga dysentery, 9), they have been conspicuously absent in the toxin-induced diarrheas (cholera and staphylococcal syndromes). No report has been made of histological damage in experimental human C. perfringens-induced diarrhea. It seems unlikely that histological lesions equivalent in severity to those seen in this model would exist in clinical cases, since the symptoms subside in a day or two. This may be due to differences in species sensitivity and concentration of active factors in the gut.

Alterations in fluid and ion transport due to the action of pure toxin were similar to those seen using crude extract. Some histological damage can be induced with pure toxin when exposed to the intestine for sufficient periods of time and at sufficient concentrations. In perfusion experiments, pure toxin was exposed at full strength for 15 or 20 min and then diluted 1:8 by perfusate. In these cases, histological damage was either absent or minimal.

The implications of these observations are as follows: (i) the activity of the crude extract is due to the presence of the protein toxin in the extract rather than other extraneous factors present. This conclusion is based on the observation that pure toxin induced transport and histopathological responses in the rat terminal ileum similar to those induced by extract. Increased destruction of villus epithelium by crude extract could be due to the higher concentration of toxin (crude extract $[2,000$ erythemal units per ml] versus pure toxin $[160$ to 250 erythemal units per ml]), and could be augmented by enzymes present in the sonicate.

(ii) At the levels shown, there is little or no dependence of fluid transport response (the parameter primarily noted during apparent diarrhea) upon toxin concentration. Crude extract containing $2,000$ erythemal units per ml elicited a response close to that of pure toxin with $160$ to $250$ erythemal units per ml. Transport experiments (unpublished data) attempted in this lab with lower concentrations of pure toxin ($100$ to $135$ erythemal units per ml) indicated that animals did not experience consistent net secretion or absorption of water. Analysis of polyethylene-$[1,2^{-14}$C]glycol concentration indicated that fluid was being absorbed and secreted intermittently, fluctuating from slight net secretion to slight net absorption, and resulted ultimately in little or no net fluid transfer. Heat-inactivated material ($0$ erythemal units per ml) resulted in normal fluid absorption. Therefore, fluid absorption effectively counter-balanced fluid secretion with toxin concentrations less than $160$ erythemal units per ml, and comparable significant net secretion is induced by toxin concentrations of $160$, $250$, and $2,000$ erythemal units per ml. The water movements measured in five different groups of experiments using the three toxin concentrations listed did not vary much more than would be expected due to individual animal differences (Table 3). Certainly the variation seen is not proportional to the nearly 10-fold difference in toxin concentration. Between $160$ and $2,000$ erythemal units, little difference is seen in fluid transport alteration.

(iii) The toxin is different from other common enteropathogenic toxins in that it inhibits glucose absorption. This would tend to indicate the possibility that net luminal gain of solutes may be due to decreased absorption which tends to unmask pre-existing rates of loss to the lumen. However, it is the feeling of the author that such is not the case. Preliminary experiments currently being conducted with pure toxin indicate that normal mucosal-to-serosal sodium flux, for example, occurs during net

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luminal gain of water and sodium. A stronger likelihood is that glucose absorption inhibition is due, at least in part, to an energy involvement or malfunction of some sort that involves glucose absorption and possibly glucose utilization. Other preliminary studies indicate that the toxin causes metabolic disturbances in vitro using everted sacs of rat terminal ileum. Further study is needed to determine the mechanism by which the active factor induces the transport and histological alteration observed.

ACKNOWLEDGMENTS
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LITERATURE CITED