Use of Indomethacin to Demonstrate Enterotoxic Activity in Extracts of Entamoeba histolytica Trophozoites

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The present study was designed to develop and characterize animal models for the assay of enterotoxic activity in extracts of Entamoeba histolytica trophozoites. Marked water and electrolyte secretion occurred in both in vivo rabbit ileal loops and rat colon loops exposed to clarified sonic fluids of E. histolytica strain HM-1 trophozoites (106/ml) when the animals were first administered indomethacin (0.1 mg/kg). No effect on intestinal absorption was observed in animals exposed to Entamoeba extracts alone or after administration of a lower (0.01 mg/kg) dose of indomethacin. Higher doses (≥1 mg/kg) of indomethacin inhibited extract-induced secretion. No enterotoxic activity was detected with or without indomethacin, using extracts from the nonpathogenic E. histolytica-like Laredo strain, even at 10-fold-higher cell concentrations. The HM-1 enterotoxic activity was heat labile. Prior exposure of the loop lumen to fetuin (100 µg/ml) blocked the secretory response to subsequent HM-1 extract exposure, but postexposure of the loop to fetuin did not block secretion that had already been established by the amoeba extract. No histological changes were seen associated with the amoeba extract-induced secretion. The data suggest that E. histolytica HM-1 strain elaborates an enterotoxic activity capable of causing consistent secretion in the mammalian intestine that has had its mucosal cytoprotection impaired by indomethacin.

Entamoeba histolytica trophozoites invade the intestinal mucosa to produce a wide range of clinical signs and symptoms. Intestinal ulceration, dysentery, and watery diarrhea have each been reported with varying incidences (3). Pathogenic strains of E. histolytica have been shown to be capable of killing cultured mammalian cells on contact (2, 5, 7−9, 17, 18). The complexity of the invasive process can be appreciated from the study of Takeuchi and Phillips (22). These workers observed that when trophozoites were placed in contact with guinea pig large intestine, they damaged the enterocyte brush border. This allowed the amoeba to make closer contact with the mucosal surface. This contact was followed by detachment of the affected epithelial cells from their neighbors and the basement membrane, with the eventual penetration of the amoeba through the separated epithelial cells.

The contact killing by E. histolytica has implicated the trophozoite surface in the pathogenicity of amebiasis. The amoebae contain a surface lectin which appears to be involved in their ability to agglutinate erythrocytes (10), and a surface concanavalin A receptor that exhibits capping has also been reported (10, 23). However, neither the membrane lectin content nor the concanavalin A receptor capping could be correlated with amoeba strain virulence. Putative amoeba surface lysosomes also could not be correlated with virulence (5, 9); however, a correlation has been reported between the ability of a strain to phagocytize erythrocytes and its pathogenicity (24).

Early attempts to demonstrate a trophozoite cell-free toxin were unsuccessful, apparently because of the use of serum in the culture medium (1, 9, 13). When fractions of axenically cultured E. histolytica trophozoites were tested for cytotoxicity, using mammalian cells suspended in serum-free medium, rounding and detachment of the target cells was observed (1, 13). This cytopathic effect was inhibited by several serum factors including fetuin (16), alpha-2-macroglobulin (14, 18), and alpha-1-antiprotease (14). The E. histolytica cytotoxin was also reported to have enterotoxic activity when tested in the rabbit ileal loop (13). A correlation has been reported between Entamoeba strain virulence and the content of this cytotoxin/enterotoxin (15). Furthermore, a hemolytic activity detected in E. histolytica homogenates was found to be in higher concentrations in more virulent strains (12).

This study was undertaken to develop and
characterize an animal model to further investigate the enterotoxic activity of pathogenic strains of *E. histolytica*. Infeasibly high concentrations of trophozoite extracts were required to demonstrate the enterotoxic activity described by Lushbaugh and associates (13). This necessitated devising a method of increasing the sensitivity of the animal model to the enterotoxin.

Robert (19, 20) has reported that prostaglandins are involved in gastrointestinal mucosal cytoprotection, and that the prostaglandin synthesis inhibitor indomethacin impairs this cytoprotection. We therefore used indomethacin in this study to reduce mucosal cytoprotection and permit the full expression of any cytotropic/enterotoxic activity present in our amoeba extracts. Both the in vivo rabbit ileum and rat colon were used as models, using a dose of indomethacin that is presumed to impair cytoprotection but is not per se anti diarrheal (21).

**MATERIALS AND METHODS**

*Amoeba cultures.* *E. histolytica* strain HM-1 and the *E. histolytica*-like Laredo strain were cultivated axenically in Diamond TP-S-1 medium (4). Trophozoites were harvested from late-growth-phase cultures by chilling and centrifugation (500 × g). They were then washed twice in potassium phosphate (10 mM, pH 7.2)-buffered saline (400 mM). The washed cells were finally suspended in phosphate-buffered saline (PBS) at 300 mM at cell concentrations of 105, 106, and 107/mL.

These preparations were frozen and stored at −20°C until required. Within 72 h the frozen material was thawed in an ice-water bath and disrupted by sonicating at 20 W (Cell Disruptor 185, Branson Instruments Co., Danbury, Conn.) for 3 min at 4°C. The material was centrifuged at 1,000 × g for 30 min at 4°C. The supernatant suspension from this centrifugation, hereafter referred to as trophozoite extract, was tested for enterotoxic activity. The factor in the extract that produced intestinal secretion is hereafter referred to as enterotoxin.

*Rabbit model.* Adult New Zealand albino rabbits of either sex were starved for 24 h before use, but were given water ad libitum. Animals were anesthetized with intravenous pentobarbital, and procaine was injected at the site of a laparotomy. Two 30-cm-long distal ileum loops were prepared, and a polyethylene catheter was secured at the distal end of each loop. The proximal loop was used as a control and was inoculated with 12 mL of PBS (300 mM); the distal loop was used as the experimental loop and was inoculated with 12 mL of trophozoite extract. At the time of inoculation, 40 μL was taken from each loop for measurement of initial Na+ and K+ concentrations by flame photometry. Three hours later the fluid was withdrawn from each loop, its volume was measured, and a sample was again taken for the measurement of Na+ and K+ concentrations. The fluid taken from each loop was reconstituted after restoring its volume to 12 mL with PBS if absorption had taken place. This procedure was repeated for a second test period of 2 h. Net water, Na+, and K+ fluxes were then calculated for the two test periods and normalized to loop length. Only animals that showed continuous absorption in the control loop were included in this study.

Indomethacin was administered subcutaneously at a dose of 0.1 mg/kg 10 min before inoculation of the loop with trophozoite extract. This dose of indomethacin was one-half that required to inhibit cholerenterotonic- and mucus secretion while not inhibiting cholera enterotoxin-induced water secretion (G. J. Leitch, O. O. Njoku, and M. H. George, Physiologist 23:9, 1980). When fetuin was used, 12 mL of a 100-μg/mL solution of fetuin (Fetuin-Spiro method; GIBCO Laboratories, Grand Island, N.Y.) in PBS (300 mM) was inoculated into the test and control loops for 10 min. The fetuin solution was then removed, and the loops were inoculated with PBS or trophozoite extract as above.

*Rat model.* Adult male Sprague-Dawley rats were allowed food ad libidum. Animals were anesthetized intraperitoneally with pentobarbital. One proximal colonic loop was prepared in each animal by inserting a catheter 7 cm from the cecal-colonic junction, flushing the colonic contents back into the cecum, and securing the junction with a ligature. A control colonic loop was not used because, unlike the rabbit ileum, no case of spontaneous colonic secretion was ever observed in our rat population.

Net water fluxes were measured hourly for 4 h, using 0.5 mL of fresh test solution at the beginning of each hour. In the most commonly used protocol, the test solution in the first period was trophozoite extract, and in the subsequent three periods PBS was used. When fetuin was used in this model, the loop was inoculated with 0.5 mL of the fetuin solution for 10 min before inoculation with trophozoite extract.

Statistical analyses were performed by the Student *t* test. A value of *P* < 0.05 was considered significant.

A morphological study of the tissues of the rat model was undertaken, using at least two examples of each protocol. The experiments were terminated at the end of the 1st hour, and a section was taken from the middle of the loop and fixed in neutral Formalin. Paraffin-embedded sections were stained with hema-toxylin and eosin or with periodic acid-Schiff. The slides were coded and examined by an independent observer.

**RESULTS**

*Rabbit model.* When rabbit ileum loops were inoculated with either PBS or amoeba extract equivalent to 106 trophozoites/mL, there was a net absorption of water, Na+, and K+ (Table 1, 2, and 3). If indomethacin was administered before inoculation of trophozoite extract, net secretion of water, Na+, and K+ occurred in the experimental but not the control loop. This secretion persisted into the second collection period. Heating the HM-1 extract at 60°C for 10 min destroyed its enterotoxic activity. Similarly prepared extracts of the *E. histolytica*-like Laredo strain did not produce secretion in the rabbit ileum regardless of whether indomethacin was administered. In some preliminary experiments, exposing the loops of indomethacin-treated ani-
mals to fatten for 10 min before inoculating the HM-1 trophozoite extract completely inhibited the secretion otherwise seen in the experimental loop.

**Rat model.** In the initial experiments using the rat colon model, the loops of indomethinc-treated or untreated animals were exposed to trophozoite extract for 1 h and then to PBS for the following 3 h. Figure 1 illustrates the net water flux averaged over 4 h. In animals not treated with indomethacin, net water flux was always in the direction of absorption, although this absorption was significantly less in loops exposed to extract equivalent to $10^7$ HM-1 trophozoites/ml than in loops exposed to extract equivalent to $10^6$ trophozoites/ml or to PBS alone ($P < 0.05$). In animals treated with indomethacin (0.1 mg/kg), mucosal exposure to extract equivalent to $10^6$ and $10^7$ HM-1 trophozoites/ml caused secretion, both during the 1st hour when the trophozoite supernatant was in the lumen and during successive periods when PBS was in the lumen.

Figure 2 illustrates the results of an experiment designed to examine the effect of the duration of exposure to amoeba extract on the secretory response of the loop. All animals were administered 0.1 mg of indomethacin per kg and exposed to extract of $10^6$ HM-1 trophozoites/ml. Mucosal exposure to amoeba extract had to occur for at least 10 min before a secretory response was observed in the 1st hour and for 30 min before this secretion was sustained beyond the period when the supernatant was actually in the loop. Repeated loop inoculations with fresh extract at hourly intervals for 4 h did not increase the secretory response beyond that resulting from a 1-h extract exposure.

**Indomethacin dose-response curves were generated for both PBS-exposed loops and loops exposed to HM-1 trophozoite extract ($10^6$ cells/ml) for 1 h, followed by PBS for the subsequent 3 h. Figure 3 illustrates the average 4-h net water flux data and the time course of such fluxes in these experiments. In PBS-inoculated loops, the two higher doses of indomethacin tested (1 and 10 mg/kg) appeared to reduce the absorption averaged over 4 h (Fig. 3a). This effect was marginally significant with the 1-mg/kg dose ($P = 0.05$) but was not significant with the 10-mg/kg dose. In trophozoite extract-exposed loops, indomethacin produced a secretory response when the dose was 0.1 or 1 mg/kg, although in the latter case the response lasted for only 2 h.

### Table 1: Net water fluxes in rabbit ileal loops exposed to *E. histolytica* extract ($10^6$ HM-1 trophozoites/ml) with or without indomethacin (0.1 mg/kg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Control loop</th>
<th>Experimental loop</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-3 h</td>
<td>3-5 h</td>
</tr>
<tr>
<td>PBS</td>
<td>5</td>
<td>-1.43 ± 0.71</td>
<td>-0.61 ± 0.25</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>5</td>
<td>-1.14 ± 0.27</td>
<td>-0.11 ± 0.08</td>
</tr>
<tr>
<td>HM-1</td>
<td>5</td>
<td>-1.59 ± 0.28</td>
<td>-0.30 ± 0.11</td>
</tr>
<tr>
<td>HM-1 + indomethacin</td>
<td>6</td>
<td>-1.50 ± 0.40</td>
<td>-0.64 ± 0.25</td>
</tr>
<tr>
<td>HM-1 (heated) + indomethacin</td>
<td>5</td>
<td>-0.92 ± 0.23</td>
<td>-0.27 ± 0.13</td>
</tr>
<tr>
<td>Laredo</td>
<td>4</td>
<td>-0.86 ± 0.34</td>
<td>-0.52 ± 0.23</td>
</tr>
<tr>
<td>Laredo + indomethacin</td>
<td>5</td>
<td>-1.00 ± 0.28</td>
<td>-0.49 ± 0.22</td>
</tr>
</tbody>
</table>

* Negative values indicate absorption, and positive values indicate secretion.

### Table 2: Net K⁺ fluxes in rabbit ileal loops exposed to *E. histolytica* extract ($10^6$ HM-1 trophozoites/ml) with or without indomethacin (0.1 mg/kg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Control loop</th>
<th>Experimental loop</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-3 h</td>
<td>3-5 h</td>
</tr>
<tr>
<td>PBS</td>
<td>5</td>
<td>-8.8 ± 4.2</td>
<td>-6.8 ± 2.9</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>5</td>
<td>-9.4 ± 0.9</td>
<td>-7.8 ± 3.2</td>
</tr>
<tr>
<td>HM-1</td>
<td>5</td>
<td>-14.6 ± 2.0</td>
<td>-3.9 ± 1.7</td>
</tr>
<tr>
<td>HM-1 + indomethacin</td>
<td>6</td>
<td>-9.7 ± 1.9</td>
<td>-5.8 ± 2.4</td>
</tr>
<tr>
<td>HM-1 (heated) + indomethacin</td>
<td>5</td>
<td>-5.7 ± 2.0</td>
<td>-6.6 ± 1.6</td>
</tr>
<tr>
<td>Laredo</td>
<td>4</td>
<td>-8.0 ± 4.1</td>
<td>-10.8 ± 3.4</td>
</tr>
<tr>
<td>Laredo + indomethacin</td>
<td>5</td>
<td>-9.3 ± 3.1</td>
<td>-6.6 ± 2.5</td>
</tr>
</tbody>
</table>

* See Table 1.
TABLE 3. Net Na⁺ fluxes in rabbit ileal loops exposed to E. histolytica extract (10⁶ HM-1 trophozoites/ml) with or without indomethacin (0.1 mg/kg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Control loop (μeq/10 cm, mean ± SEM)</th>
<th>Experimental loop (μeq/10 cm, mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–3 h</td>
<td>3–5 h</td>
</tr>
<tr>
<td>PBS</td>
<td>5</td>
<td>-235 ± 121</td>
<td>-130 ± 85</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>5</td>
<td>-234 ± 111</td>
<td>-134 ± 84</td>
</tr>
<tr>
<td>HM-1</td>
<td>5</td>
<td>-167 ± 46</td>
<td>-90 ± 18</td>
</tr>
<tr>
<td>HM-1 + indomethacin</td>
<td>6</td>
<td>-221 ± 65</td>
<td>-140 ± 45</td>
</tr>
<tr>
<td>HM-1 (heated) + indomethacin</td>
<td>5</td>
<td>-149 ± 36</td>
<td>-84 ± 30</td>
</tr>
<tr>
<td>Laredo</td>
<td>4</td>
<td>-82 ± 37</td>
<td>-127 ± 30</td>
</tr>
<tr>
<td>Laredo + indomethacin</td>
<td>5</td>
<td>-120 ± 59</td>
<td>-55 ± 38</td>
</tr>
</tbody>
</table>

* See Table 1.

(Fig. 3b). The dose which resulted in a maximal secretory response was 0.1 mg/kg. At the highest indomethacin dose used, 10 mg/kg, there was no secretion even during the period of exposure to extract.

The enterotoxic activity of E. histolytica HM-1 extracts was heat labile when tested in the rat colon, as it was in the rabbit model. There was no colonic secretion if the indomethacin was administered at the beginning of the 3rd hour of the experiment rather than before the HM-1 extract exposure. No enterotoxin activity was detected in extracts of the nonpathogenic E. histolytica-like Laredo strain, even at concentrations equivalent to 10⁷ trophozoites/ml.

Fetuin (100 μg/ml in PBS) was placed in the colonic lumen of indomethacin-treated rats for 10 min. This solution was then removed and replaced with trophozoite extract for 1 h followed by PBS as above. Prior exposure of the mucosa to fetuin completely inhibited the secretory response otherwise observed with this dose of extract (Fig. 4). Replacement of the trophozoite extract with fresh extract at hourly intervals over a 4-h period failed to reverse the fetuin-induced block of secretion. On the other hand, if the 10-min mucosal fetuin exposure took place after rather than before the first 1-h exposure to trophozoite extract, the fetuin treatment failed to block the ongoing secretion.

No relationship was found between toxin-induced secretion and histological appearance in the rat model. Stained sections of mucosae from PBS-treated, HM-1 extract-treated, and Laredo strain extract-treated animals, all administered indomethacin, were indistinguishable from each other when observed by light microscopy. Fig-

![Fig. 1](https://iai.asm.org/)

**FIG. 1.** Four-hour net water fluxes in rat colon loops exposed to E. histolytica strain HM-1 extracts prepared at different cell concentrations. Loops were exposed to extracts for 1 h, followed by exposure to PBS for 3 h. Positive values indicate secretion, and negative values indicate absorption. Indomethacin was administered subcutaneously 10 min before extract inoculation of the loop. Values are mean ± standard error of the mean.

![Fig. 2](https://iai.asm.org/)

**FIG. 2.** Hourly net water fluxes in rat colon loops exposed to extract equivalent to 10⁶ trophozoites/ml for 0, 5, 10, 30, 60, and 240 min followed by PBS for the remainder of the 4-h experiment. All animals were administered 0.1 mg of indomethacin per kg before extract inoculation.
FIG. 3. (a) Four-hour net water fluxes in rat colon loops exposed to PBS or to extracts of \(10^6\) HM-1 trophozoites/ml for 1 h and PBS for 3 h, plotted as a function of the dose of indomethacin administered subcutaneously 10 min before inoculation of the loops. (b) Hourly net water fluxes in loops exposed to HM-1 extract in animals administered 0.01, 0.1, 1, and 10 mg of indomethacin per kg subcutaneously.

ure 5 illustrates sections of colon from indomethacin-treated animals in which the colon was exposed to PBS or HM-1 extract.

**DISCUSSION**

A cytotoxic/enterotoxic activity has been reported to occur in cell-free preparations of *E. histolytica* strain HM-1 (1, 13). This toxin has been partially purified, and it appears to be a protein with a molecular weight ratio of approximately 30,000 (13, 15). The role of this toxin in amebiasis has yet to be elucidated.

Our work was based on the assumption that reducing the cytotoxic effects of endogenous prostaglandins with the prostaglandin synthesis inhibitor indomethacin (19, 20) would exacerbate the in vivo effect of any toxin produced by *E. histolytica*. We did not attempt to determine whether the intestinal secretory response was due to one or several toxic components elaborated by the amoebae. Exposure to cell-free extracts from the pathogenic HM-1 strain of *E. histolytica* after injection of indomethacin resulted in a secretion of water and electrolytes by both rabbit ileum and rat colon (Tables 1, 2, and 3; Fig. 1), whereas extracts from the same or larger numbers of trophozoites of the nonpathogenic *E. histolytica*-like Laredo strain had no effect on these animal models under the same conditions (Tables 1, 2, and 3).

If the trophozoite extracts used in these experiments were cytotoxic, the degree of tissue damage they produced was slight. In the rabbit ileum, the K⁺/Na⁺ flux ratio was higher than that usually seen in a secretory diarrhea such as

FIG. 4. Four-hour net water fluxes in colon loops of rats exposed to HM-1 extract (\(10^6\) trophozoites/ml) and administered 0.1 mg of indomethacin per kg subcutaneously. Loops were inoculated with a fetuin solution for a 10-min period immediately before or after a 1-h inoculation with amoeba extract. In one fetuin-treated group, loops were inoculated with amoeba extract hourly for 4 h.
secretion to occur in response to an amoeba cytotoxin/enterotoxin, provided the toxin is used at a high enough concentration (13). In our study, an extract equivalent to 10^8 HM-1 trophozoites/ml caused a statistically significant reduction in the magnitude of normal colonic absorption in the absence of indomethacin (Fig. 1). Higher concentrations of the effective factor(s) may have produced net secretion.

Indomethacin has been reported to inhibit the intestinal secretion produced in response to a variety of secretagogues (21). The dose of 0.1 mg/kg (Fig. 1 and 3) appeared to be a dose that disrupted cytoprotection without actually inhibiting secretion. We interpret the observation that indomethacin could not induce secretion in animals previously inoculated with amoeba extract as indicating that this drug did not act to unmask some suppressed enterotoxin-stimulated secretion, but rather that it reduced cytoprotection during the time when the mucosa was exposed to extract.

In the presence of indomethacin, a secretory response was maintained after removal of the trophozoite extract from the loop (Fig. 1 and 2). This was a function of the mucosal exposure to extract and was not due to the time required for the indomethacin to take effect, since administering indomethacin 30 min before inoculation of the extract was as effective as administering the drug 10 min in advance of the extract, as was the general case.

It has been suggested that fetuin attaches to cells and prevents the action of amoeba cytotoxin/enterotoxin (16). This suggestion is confirmed in the present work, as exposing the luminal surface to fetuin before exposing it to the enterotoxin in indomethacin-treated animals blocked secretion in response to HM-1 trophozoite extract for 4 h. The fetuin was apparently avidly bound to the mucosa, since flushing three times with PBS did not affect the blocking of extract-induced secretion, nor did filling the lumen with fresh amoeba extract at hourly intervals (Fig. 4). The fetuin was not antisecretory per se, as it had no effect on already established secretion (Fig. 4).

The above data are consistent with the following hypothesis. One or more toxic factors elaborated by E. histolytica strain HM-1 attach to mucosal sites that have a high affinity for fetuin. If the toxin(s) is in high enough concentration, or if mucosal prostaglandin-mediated cytoprotection is impaired, secretion will occur.

ACKNOWLEDGMENTS

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An HM-1 strain of Entamoeba histolytica, designated HM-1:HL-3A, was obtained from Dr. William B. Lushbaugh. The

FIG. 5. Hematoxylin and eosin-stained, paraffin-embedded sections of rat colon loops exposed to PBS for 1 h (a) and to HM-1 extract (10^6 trophozoites/ml) for 1 h (b). Animals were administered indomethacin (0.1 mg/kg) subcutaneously 10 min before inoculation of the loops.

experimental cholera (11). The K^+ secretion was also higher than would be expected if the fluid appearing in the lumen were simply leaked interstitial fluid unaltered by intestinal absorption (i.e., the K^+/Na^+ net flux ratio would have been 1/30 instead of 1/10 as seen in the 0- to 3-h period in E. histolytica extract-treated loops). In the 3- to 5-h period this ratio decreased. One interpretation of these flux data could be that the K^+ secretion was the result of tissue damage occurring at the time the mucosa was exposed to amoeba extract. In the rat model, a histological study of hematoxylin and eosiin and of periodic acid-Schiff-stained, paraffin-embedded sections failed to reveal any morphological evidence of frank tissue damage demonstrable by light microscopy. Frank tissue damage was not expected, as even in isolated cell preparations amoeba extracts appeared to show transient cytopathic activity rather than cytotoxic activity (1, 14, 16). No attempt was made to measure K^+ net fluxes in the rat colon and use such fluxes as an indicator of tissue damage because the rat colon secretes K^+ under normal conditions (6).

Indomethacin is apparently not essential for...
authors wish to thank Dr. Marjorie M. Smith for her evaluation of histological sections.

LITERATURE CITED