THE USE OF INDOMETHACIN TO DEMONSTRATE AN
ENTEROTOXIC ACTIVITY IN EXTRACTS OF
ENTAMOEBA HISTOLYTICA TROPHozoITES

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Axenically cultured *Entamoeba histolytica* trophozoites of the pathogenic strain HM-1 were harvested in phosphate buffered saline (PBS). The cells were disrupted with ultrasonic energy, centrifuged, and the supernatant fluid tested for enterotoxic activity in the *in vivo* rabbit ileum loop and the rat proximal colon loop. This HM-1 extract, equivalent to $10^6$ trophozoites/ml, had no effect on intestinal absorption in either animal model, but secretion resulted in both preparations when indomethacin (0.1 mg/kg) was administered subcutaneously to reduce cytoprotection. In the rat colon a lower dose of indomethacin was ineffective in allowing the ameba preparation to cause frank secretion, while higher doses, 1 and 10 mg/kg, directly inhibited this secretory response. In the absence of indomethacin, a preparation equivalent to $10^7$ trophozoites/ml reduced colonic absorption without producing secretion, while a preparation of $10^7$ trophozoites/ml of the non-pathogenic *E. histolytica*-like Laredo strain was ineffective even in the presence of 0.1 mg/kg indomethacin. 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 enterotoxin exposure in indomethacin-treated animals. Fetuin inhibition of secretion could not be reversed by inoculating the loop with fresh trophozoite extract at hourly intervals for 4 hours.
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A special thanks is given to my family for their financial supports and prayers; also to Mrs. Ifeyinwa Udezulu for her encouragements and to God for making it all possible.
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INTRODUCTION

Amebic dysentry is one manifestation of amebiasis in which infestation of the gastrointestinal tract occurs with a species of ameba, *Entamoeba histolytica*. This amebic infection often results in a copious diarrhea, which is evidenced by the evacuation of 6 to 8 mucoid, blood-stained stools a day (Hashim and Pittman, 1970; Craig and Faust, 1977). The pathogenic effects of this microorganism are seen world-wide, but the mode of its invasion is still not well understood.

Lushbaugh and colleagues (1979) partially purified a heat labile cytotoxin/enterotoxin from this organism which elicited secretion in the in vivo rabbit ligated loop. Due to the large number of trophozoites required to repeat the experiment of Lushbaugh and colleagues, we designed this project to reduce the volume of required trophozoite extract. This was achieved by increasing intestinal mucosal sensitivity to the trophozoite extract. The prostaglandin synthesis inhibitor, indomethacin, was employed to impair the mucosal cytoprotection (Robert 1977). This enhanced the ameba extract intestinal secretory response. The rabbit ileal and rat colonic loop models were used for this enterotoxic assay.

When the rabbit ileum and rat colon were inoculated with a cell free extract of the pathogenic *E. histolytica* strain HM-1:HL-3 following indomethacin administration there was a secretion of water and electrolytes into the intestinal loops. Extracts of the non-pathogenic *E. histolytica*-like Laredo strain did not produce secretion under similar conditions. Prior luminal exposure to fetuin inhibited this HM-1 extract induced secretion,
indicating that fetuin and *E. histolytica* enterotoxic activity share the same receptors on the mucosal surface, with fetuin either having higher affinity for the receptor or acting like a lectin by binding both ameba toxin and receptor.
SECRETORY DIARRHEA


diarrhea caused by *E. coli* heat stable enterotoxin appears to be mediated by increased tissue cyclic GMP levels (Sack, 1975; Hughes et al., 1978; Field et al., 1978). Calcium is necessary for all excitation-secretion coupling (Berridge, 1979), and the Ca++ ionophore A23187 induces *in vitro* rabbit ileum and colon to secrete electrolytes (Frizzel, 1977; Bolton and Field, 1977).

**Normal Mechanisms of Water and Electrolyte Transport in the Intestine**

Much of the understanding of intestinal electrolyte transport has been gained from studies of rabbit small intestinal preparations. Absorption is presently believed to be associated with mature villus enterocytes and secretion with cells of the crypts of Lieberkuhn (Hendrix, 1977; Elliot et al., 1979). In rabbit ileal models there are three major absorptive systems for electrolytes: (1) active electrogenic Na⁺ absorption; (2) Na⁺ absorption coupled to Cl⁻; (3) Na⁺ absorption coupled to nonelectrolytes. A fourth system that involved both a secretory and absorptive component is the Cl⁻-HCO₃⁻ exchange pump.

**Na⁺ Absorption** - In the intestine there appears to be a carrier mediated electrogenic transport of Na⁺ occurring in the absence of luminal non-electrolytes (Schultz and Frizzel, 1972; Schultz et al., 1974). At the luminal surface Na⁺ is driven into the enterocytes by the electrochemical gradient produced by the low intracellular Na⁺ concentration and the cell electronegativity (Koopman and Schultz, 1969; Schultz et al., 1966). This process was not affected by the Na⁺-K⁺ ATPase inhibitor, ouabain (Chez et al., 1967). The extrusion of Na⁺ from the cell into the serosal solution is an active transport process. Sodium is driven from the cell to the serosal surface by an energy requiring carrier system. This carrier system is an integral part of the Na⁺-K⁺ ATPase pump mechanism present in all animal cells that transport Na⁺ (Skou, 1957). Both the ATPase and the ion translocase process have been
shown to be oubain sensitive (Schultz and Zalusky, 1964; Charney and Donowitz, 1978). The ATPase is located in the basolateral membrane of epithelial cells (Fujita et al, 1971: Quigley and Gotterer, 1969: Fujita et al, 1972; Sterling, 1972). The extrusion of Na+ to the serosa has been shown to result in electrical positivity and increased osmolarity on the serosa which draws water through the tight junctions together with Cl− (Diamond and Bossert, 1967; Haljamae et al, 1973).

**Na-nonelectrolyte Absorption** – Another group of carrier mediated systems involving Na+ coupled to nonelectrolytes, such as glucose, amino-acids and bile salts has been demonstrated (Crane, 1965; Schultz and Curran, 1970; Schultz, 1977). These carrier systems are powered by the Na+ influx into the enterocytes down a favorable electrochemical gradient, while the non-electrolytes can move against an unfavorable chemical gradient. The resulting increase in Na+ pumping also contributes to the electrical positivity and high osmolarity of the serosa which aids in the reabsorption of water and Cl− (Haljamae et al, 1973).

**Na+-Cl− Absorption/Secretion** – A third carrier system involves Na+ and another electrolyte, Cl−. This have been designated an electrically neutral NaCl coupled pump (Frizzel et al, 1976: Frizzel et al, 1979). This pump appears responsible for the absorption of much of the Na+ and Cl− in the small intestine. Sodium entry in this model is down a favorable electrochemical potential gradient (Schultz et al, 1966: Koopman and Schultz, 1969). It was unaffected by ouabain (Chez et al, 1967). This Na+ entry was also found to be facilitated through a membrane carrier molecule with binding sites for both Na+ and Cl−, and binding of Na+ to the carrier could be inhibited by K+ in the mucosa (Frizzel and Schultz, 1972). Chloride is driven into the cell from the mucosa by the energy generated by the favorable electrochemical gradient of Na+, much as was the...
case in the Na\(^+\)-nonelectrolyte coupled system (Crane, 1965; Frizzel et al., 1975). The exit of Cl\(^-\) from the cell to the serosa is the passive diffusion down a favorable electrochemical gradient (Frizzel and Schultz, 1979). Sodium is believed to be moved from the enterocytes to the serosa through the Na\(^+\)-K\(^+\) ATPase powered pump.

The neutral NaCl pump can be specifically inhibited by elevated intracellular cyclic AMP levels (Layssac et al., 1974; Merten et al., 1974; Frizzel et al., 1975). It has been proposed that this inhibition of the NaCl neutral pump occurs on the mucosal surface, while on the serosal surface a passive diffusion of Na\(^+\) into the enterocyte continues, followed by Cl\(^-\). As Cl\(^-\) accumulates in the enterocyte the electrochemical gradient will favour diffusion out of the cell. An increase in mucosal membrane permeability has been postulated to explain the Cl\(^-\) secretion at the mucosa (Kimberg, 1974). This also results in the movement of water and Na\(^+\) to the mucosa through the tight junctions, resulting in a secretion of water and electrolytes (Kimberg, 1974).

\(-\text{HCO}_3^-\) Secretion/Absorption

The mammalian ileum and colon have been shown to contain another carrier system that shows absorption of Cl\(^-\) and secretion of HCO\(_3^-\) (Hubel, 1967; Swallow and Code, 1967; Hubel, 1969). The chloride is believed absorbed through a Cl\(^-\)-HCO\(_3^-\) exchange pump (Dietz and Field, 1973; Sheeren and Field, 1975) and HCO\(_3^-\) excreted against an electrochemical gradient (Turnburg et al., 1970). It has been proposed that the intracellular HCO\(_3^-\) is generated by the endogenous carbonic anhydrase activity in the cell using metabolic CO\(_2\) (Turnburg et al., 1970; Carter and Parson, 1971).
In the colon, Na\(^+\) absorption occurs against a steeper transmucosal electrochemical gradient than in the ileum, but it is also dependent on the basolateral membrane Na\(^+\)-K\(^+\) ATPase pump system (Cooperstein and Hogben, 1959; Frizzel et al., 1976).

**Pathophysiology of Electrolyte Transport**

Cholera is the most widely studied of the secretory diarrheal diseases. *Vibrio cholerae* is a microorganism capable of producing a protein enterotoxin. The enterotoxin has been purified and found to be a protein of a molecular weight of 84,000 daltons (Finkelstein and Lospalluto, 1969; 1970). This enterotoxin can induce the secretion of isotonic fluid by the intestinal mucosa. The fluid secretion is believed to be confined to the small intestine (Carpenter and Greenough, 1968) and there are variations of the secretory responses from one animal species to another (Banwell et al., 1970). For example, the human jejunum has been shown to produce more fluid than the ileum during *Vibrio cholerae* infections.

Choleragen, the cholera enterotoxin, has two component fragments, A and B. The A component was found to be capable of stimulating adenylate cyclase activity (Gill and King, 1975; Cassell and Selinger, 1977; Cassell and Pfeurrer, 1978). This process occurred by reducing the disulfide bond in the A component which then dissociated into A\(_1\) and A\(_2\). The A\(_1\) component catalyzed the cleavage of NAD to nicotinamide and ADP-ribose which was transferred to the regulatory component of adenylate cyclase. The regulatory component is a GTP binding protein that activates adenylate cyclase. It was found that the GTP hydrolysis was blocked by ADP-ribose and this resulted in constant activation of adenylate cyclase (Cassell and Selinger, 1977; Gill and Meren, 1978). The B component has been implicated as the protein which binds to the cell membrane receptor
sites, GM₁, gangliosides (Finkelstein, 1973; Van Heyningen et al., 1971; Cuatrecasus, 1973; Fishman et al., 1977). The increase in the adenylate cyclase activity with increased CAMP levels has been shown to result in the active secretion of water and electrolytes (Field, 1971).

The effects of cholera toxin induced diarrhea do not seem to damage the epithelial cells (Gangarosa et al., 1960) and no inhibition of sugar and amino acid absorption has been detected (Phillips, 1964), so that these two non-electrolyte pumps are presently used in oral rehydration therapy for cholera diarrhea.

Our present project was designed to study the in vivo effects of *Entamoeba histolytica* on certain intestinal animal models. This microorganism causes amebic dysentery (Losch, 1875; Councilman and Lefèvre, 1891; Walker and Sellards, 1913). *Entamoeba histolytica* is a protozoa classified under the genus *Entamoeba*. Biologically this organism has a complicated life cycle but the cyst and the trophozoites are the only stages of clinical importance in amebic infection. Amebic infection is acquired by ingestion of the cysts which under favourable conditions excyst to a mobile trophozoite. These conditions include adequate pH range, an anaerobic environment, as well as bacterial and nutritional factors necessary for trophozoite growth (Maegraith, 1955).

In amebic dysentery there is an evacuation of six to eight mucoid, blood-stained feces a day (Hashim and Pittman, 1970; Craig and Faust, 1977). The dysentery is often associated with a copious diarrhea. The mucus and blood stained feces seen during an ameba infection are due to lesions produced by ameba trophozoites located mostly in the mucosa of the large intestine (Jimenez, 1981). These ulcerative lesions have been found to be generally confined to the mucosal epithelium during the primary stage, but in advanced
cases they might extend to the lamina propria and muscularis and may result in extra intestinal infection of the liver (Brandt and Tamayo, 1970).

The invasive effects of amebic infection are seen as ulcerative lesions on the intestinal mucosa. The pathogenesis of this invasion was summerized by the work of Takeuchi and Phillips (1975) in which they demonstrated in a germ free guinea pig model that ameba trophozoites damage mucosal cells by first altering their attachment to adjacent cells. This enabled the trophozoites to move between the enterocytes towards the submucosa. This study suggested that ameba invasion required an ameba-cell surface interaction.

Attempts have been made to correlate ameba strain virulence with some factors assumed to be involved in ameba pathogenesis. One of these factors was an ameba lectin-like activity. *Entamoeba* has a surface lectin-like receptor which is capable of capping and agglutination of red blood cells (Trissel et al., 1977; Mattern et al., 1980; Kobiter and Mirelman, 1980). Another factor that has been studied is an ameba surface active lysosome (Eaton et al., 1969; Eaton et al., 1970; Proctor and Gregory, 1972). However, the correlation between *Entamoeba* strain virulence and these factors is poor.

When ameba trophozoites were placed in mammalian cell suspensions the presence of cytotoxic or cytopathic factors was suggested by the fact that rounding and detachment of cells occurred (Bos and Van de Gried, 1977; Lushbaugh et al., 1979). This has been confirmed by Ravdin and colleague (1980) in a study in which they demonstrated the ameba cytotoxic effects on monolayer of Chinese hamster ovary cells. This cytotoxic rounding effect of the cells was reversed when the cells were placed in fresh medium. This cytotoxic effect has been reported to be inhibited by fetuin (Mattern et al., 1980) and alpha-2-macroglobulin (Ravdin et al., 1980; Lushbaugh et al., 1981).
The occurrence of copious diarrhea in certain cases of amebic dysentry seems to indicate the presence of an enterotoxin. In 1979 Lushbaugh and colleagues were able to isolate a heat labile enterotoxin with an approximate molecular weight of 30,000 daltons, capable of inducing water and electrolyte secretion in the \textit{in vivo} rabbit ileum. Based on these findings we designed our studies with the aim of further characterising this enterotoxin and developing another more reliable \textit{in vivo} animal model for its assay. Due to the high trophozoite concentration required by Lushbaugh and associates (8 mg of partially purified protein) to produce ameba extracts with detectable enterotoxin activity, we first needed a method of increasing intestinal mucosal sensitivity to enterotoxin. This was done by employing the prostaglandin synthesis inhibitor, indomethacin.

Prostaglandins are derived metabolically from arachidonic acid (Nugteren and Heazelhof, 1973; Hemberg and Samuelsson, 1974). They have been reported to be involved in gastrointestinal mucosal cytoprotection (Robert et al, 1978; Chandburg and Robert, 1978). Such cytoprotection is the ability to protect the gastrointestinal mucosa from lesions induced by several nonsteroidal anti-inflammatory compounds (NOSAC). This property has been demonstrated by all groups of prostaglandins studied. In 1968, Robert and colleagues demonstrated that exogenous protaglandin E1 was able to protect rat stomach against lesion induced by gastric acid secretion. This prostaglandin treatment was also effective in individuals with certain gastrointestinal tract diseases such as peptic ulcer disease (Levy, 1974; Robert, 1974). We used indomethacin to inhibit this protaglandin mediated mucosal cytoprotection in an attempt to enhance the ameba enterotoxic effect of our extracts. We employed the rabbit ileum at first, but due to the high trophozoite extract volume required, we also used a smaller animal model, the rat colon, for our \textit{in vivo} assays of enterotoxic activity.
MATERIAL AND METHODS

Materials

Chemicals

Diamond's TPS-1 broth was purchased from North American Biologicals (Miami, Florida). Inactivated horse serum was purchased from Gibco Laboratories (Grand Island, New York) and Diamond's vitamin mix was purchased from North American Biologicals (Miami, Florida). Indomethacin was purchased from Sigma Chemical Company (St. Louis, Missouri). Fetuin (Spiro method) was purchased from Gibco Laboratories (Grand Island, New York). All other chemicals used were reagent grade.

Equipment and Supplies

Biological Hood - A Contamination Control Inc. hood (Landsdale, Pennsylvania) was used for sterile transfer of Entamoeba histolytica.

Sonicator - A Branson Sonifier cell disruptor 185 (Branson Sonic Power Company, Danbury, Connecticut) was used to lyse Entamoeba histolytica trophozoites.

Osmometer - An Osmette automatic osmometer from Precision Systems, Inc. (Newton, Massachusetts) was used to measure the osmolarity of all solutions.

Flame Photometer - A flame photometer, model 443 (Instrumentation Laboratories, Inc., Lexington, Massachusetts) was used to measure the concentrations of sodium and potassium in luminal fluid. Unopettes (Becton Dickson, Rutherford, New Jersey) were used to collect samples and dilute them for flame photometry.

Thermometer - A tele-thermometer Y.S.I. model 44 (Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio) was used to check the abdominal temperature of animals. Plastic hot water bottles were used to maintain the
animals' body temperature.

**Animals** - Adult New Zealand white rabbits of either sex and adult male Sprague-Dawley rats were used. Both species were purchased locally from Davies Small Animal Farm (Stockbridge, Georgia).

**Methods**

**Amebic Cultures**

*Entamoeba histolytica* strain HM-1:HL-3 and the *E. histolytica*-like Laredo strain trophozoites were maintained in Diamond's TPS-1 medium (Diamond, 1968). Trophozoites were grown in capped 15 ml test tubes. *Entamoeba histolytica* were recultured by splitting the trophozoites into 2 portions after 48 hours of logarithmic growth. *Entamoeba histolytica*-like Laredo strain trophozoites were recultured by splitting the trophozoites into 2 portions after 7 days of logarithmic growth. Ameba trophozoites were harvested from the culture tubes during the late growth phase by chilling in ice water for 5 minutes. The chilled culture tubes were centrifuged for 2 1/2 minutes at room temperature at 500 x g to separate the trophozoites from the supernatant. The supernatant was removed from the culture tubes by suction. The trophozoites were then washed twice in potassium phosphate (10 mm, pH 7.4) buffered saline (400 mOsm). The washed cells were finally suspended in phosphate buffered saline (PBS) of 300 mOsm in cell concentrations of $10^5$, $10^6$, and $10^7$ trophozoites/ml. This material was then stored at -30°C until needed.

**Trophozoite Extract**

The trophozoites were thawed in ice water at 4°C. They were disrupted by ultrasonic disruption at 4°C for three periods of three minutes each. The sonicate was centrifuge for thirty minutes at 7000 x g in a refrigerated
centrifuge at 4°C. The supernatant was poured off into 4 tubes of 3 mls each and these samples were maintained at 4°C for not more than two hours prior to use.

**Surgical Procedures**

New Zealand white rabbits were starved overnight but were allowed water *ad lib* before surgery. They were anaesthetized using intravenous pentobarbital as a general anaesthetic. Procaine was also used as a local anaesthetic at the incision site. A laparotomy was performed and the ileum was washed thoroughly with warm saline. Two 30 cm loops were made in the distal portion of the ileum. The animal was injected subcutaneously with 0.1 mg/kg of indomethacin in 10 mM phosphate buffer, pH 7.2. Fifteen minutes later the lower loop was inoculated with 13 mls of the ameba extract and the upper loop was used as a control and was injected with the same volume of 300 mOsm PBS. Initially two 20 μl samples of luminal fluid were taken for the measurement of sodium and potassium concentrations. At the end of the 3 hours both loops were drained using a syringe and needle. The volumes of the fluid were measured and samples were again taken for the measurement of electrolyte concentrations. The fluid volumes of both loops were readjusted to 13 mls with PBS if absorption had occurred, and the loops reinoculated with the same solution. Two hours later the procedures for the measurement of fluid volume and electrolyte concentrations were repeated. The net fluxes of water and electrolytes were then calculated as shown in equations 1 and 2. Only those animals that showed continuous absorption in the control loop were in these studies.
Equation 1

**Net water flux**

\[ J_{\text{net} \, H_2O} = \frac{V_f - V_i}{\text{length of loop (multiple of 10 cm)}} \]

- \( V_f \) = final vol (ml)
- \( V_i \) = initial vol (ml)

Equation 2

**Net ion flux**

\[ J_{\text{net \, ion}} = \frac{C_f \cdot V_f - C_i \cdot V_i}{\text{length of loop (multiple of 10 cm)}} \]

- \( C_f \) = final concentration (\( \mu \)Eq/ml)
- \( C_i \) = initial concentration (\( \mu \)Eq/ml)

When the rat model was employed the surgical procedure was repeated except that only one proximal colon loop was prepared. A loop 7.0 cm in length was made in the colon after first flushing the contents back into the cecum. A catheter was inserted in the distal portion of the loop. The loop was then exposed to 0.5 ml of ameba extract or 300 mOsm PBS for one hour. These solutions were replaced with 0.5 ml PBS at hourly intervals. The volume of fluid was measured hourly for 4 hours. Indomethacin, 0.1 mg/kg was injected subcutaneously 10 minutes prior to inoculating the loop with ameba extract. This protocol differed from that of the rabbit experiments in that fresh solutions were instilled at hourly intervals and electrolyte concentrations were not measured. Statistical analyses were performed using the student t test. A value of \( p < 0.05 \) was considered significant.

**Protocols**

**Protocol for Experiments Using Different Trophozoite Concentration**

Rat colon loops were exposed to ameba extract equivalent to \( 10^5 \), \( 10^6 \), or \( 10^7 \) HM-1 trophozoites/ml for an hour with and without subcutaneous injection.
of 0.1 mg/kg indomethacin. Hourly net water fluxes were measured as above and 4 hour net water fluxes were calculated.

**Protocol for Time of Exposure Experiment**

Rat colon loops were inoculated with extracts of $10^6$ HM-1 trophozoites/ml for 5, 10, 30, 60 and 240 minutes following subcutaneous injection of 0.1 mg/kg indomethacin. During the remainder of the four experiments the loops were exposed to PBS. Hourly net water fluxes were measured for 4 hours.

**Protocol for Indomethacin Dose Response Curve Experiment**

Rats were subcutaneously injected with 0.01, 0.1, 1 and 10 mg/kg of indomethacin 10 minutes prior to inoculating colon loops with extracts of $10^6$ HM-1 trophozoites/ml for 1 hour followed by inoculating the loops with PBS for 3 hours. Animals were also exposed to 0.01, 1 and 10 mg/kg indomethacin 10 minutes before inoculating loops with PBS for all four hour test periods.

**Protocol for Fetuin Experiment**

Rat colonic loops were exposed to 0.1 mg/ml fetuin for 10 minutes prior to 1 hour mucosal exposure to ameba extract ($10^6$ HM-1 trophozoites/ml). PBS was used as the test solution in the 3 hours subsequent test periods except in one experiment where loops were exposed to fetuin for 10 minutes followed by exposure to ameba extract at hourly intervals for 4 hours, and in another experiment where fetuin exposure took place immediately after ameba extract exposure. In these experiments all animals were administered 0.1 mg/kg indomethacin subcutaneously 10 minutes prior to the administration of extract.
RESULTS

Rabbit Model - When the rabbit model was used there was either absorption of secretion in the experimental loop and absorption in the control loop. Inoculation of both the experimental and control loops with 13 ml s PBS alone resulted in absorption of water, Na⁺ and K⁺ in both untreated animals and animals administered 0.1 mg/kg indomethacin. Ileal loops exposed to ameba extracts of $10^6$ HM-1 trophozoites/ml also showed absorption of water, Na⁺ and K⁺. On the other hand, ileal loops of indomethacin treated animals inoculated with this amebic extract showed secretion of water, Na⁺ and K⁺. Heating the HM-1 trophozoite extract at 60°C for 10 minutes destroyed the enterotoxic activity. Extracts of the non-pathogenic *E. histolytica*-like Laredo strain ($10^6$ trophozoites/ml) did not produce ileal secretion of water, Na⁺ and K⁺, whether the animals had been treated with indomethacin or not. (Tables I, II and III).

Rat Model - The colonic loops of indomethacin treated and untreated animals were exposed to 0.5 ml trophozoite extract for 1 hour and sequentially exposed to 0.5 ml of PBS for 3 hours (Figure I). When measured for the entire 4 hour period there was a colonic secretion of water in animals treated with indomethacin and inoculated intraluminally with the HM-1 extract equivalent to $10^6$ and $10^7$ trophozoites/ml. Indomethacin treated animals showed no luminal secretion with colonic exposure to an extract equivalent to $10^5$ HM-1 trophozoites/ml. Net absorption of water also took place in loops of animals not administered indomethacin but inoculated with extracts equivalent to $10^5$, $10^6$ or $10^7$ HM-1 trophozoites/ml. Using the 4 hour net flux data this absorption was significantly less in loops exposed to extract equivalent to $10^7$ HM-1 trophozoites/ml when compared to that of loops exposed to extract
Table 1. Net water fluxes (ml/10cm) in rabbit ileal loops exposed to trophozoite supernatant (10^6/ml) with or without indomethacin (0.1 mg/kg).

<table>
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<tr>
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<th>(N)</th>
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<td>Indomethacin</td>
<td>(5)</td>
<td>-1.43 ± .71</td>
<td>-.61 ± .25</td>
<td>-1.57 ± .51</td>
<td>-.87 ± .23</td>
</tr>
<tr>
<td>HM-1</td>
<td>(5)</td>
<td>-1.54 ± .27</td>
<td>-.11 ± .08</td>
<td>-1.97 ± .41</td>
<td>-.25 ± .11</td>
</tr>
<tr>
<td>HM-1 + Indomethacin</td>
<td>(6)</td>
<td>-1.59 ± .28</td>
<td>-.30 ± .11</td>
<td>-2.83 ± .26</td>
<td>-.66 ± .19</td>
</tr>
<tr>
<td>HM-1 (Heated) + Indomethacin</td>
<td>(5)</td>
<td>-1.50 ± .40</td>
<td>-.64 ± .25</td>
<td>+2.31 ± .67</td>
<td>+2.86 ± 1.13</td>
</tr>
<tr>
<td>Laredo</td>
<td>(4)</td>
<td>-.92 ± .23</td>
<td>-.27 ± .13</td>
<td>-1.75 ± .35</td>
<td>-.51 ± .13</td>
</tr>
<tr>
<td>Laredo + Indomethacin</td>
<td>(5)</td>
<td>-.86 ± .34</td>
<td>-.52 ± .23</td>
<td>-2.89 ± .33</td>
<td>-.67 ± .05</td>
</tr>
</tbody>
</table>

Means ± SEM

Negative values indicate absorption and positive values indicate secretion.
Table 2. Net K⁺ fluxes (μEq/10 cm) in rabbit ileal loops exposed to trophozoite supernatant (10⁶/ml) with or without indomethacin (0.1 mg/Kg).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control Loop</th>
<th>Experimental Loop</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N)</td>
<td>0-3h</td>
</tr>
<tr>
<td>PBS</td>
<td>(5)</td>
<td>-8.8 ± 4.2</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>(5)</td>
<td>-9.4 ± 1.9</td>
</tr>
<tr>
<td>HM-1</td>
<td>(5)</td>
<td>-14.6 ± 2.0</td>
</tr>
<tr>
<td>HM-1 + Indomethacin</td>
<td>(6)</td>
<td>-9.7 ± 1.9</td>
</tr>
<tr>
<td>HM-1 (Heated) + Indomethacin</td>
<td>(5)</td>
<td>-5.7 ± 2.0</td>
</tr>
<tr>
<td>Laredo</td>
<td>(4)</td>
<td>-8.0 ± 4.1</td>
</tr>
<tr>
<td>Laredo + Indomethacin</td>
<td>(5)</td>
<td>-9.3 ± 3.1</td>
</tr>
</tbody>
</table>

Means ± SEM

Negative values indicate absorption and positive values indicate secretion.
Table 3. Net Na\(^+\) fluxes (µEq/10cm) in rabbit ileal loops exposed to trophozoite supernatant (10^6/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>0-3h</td>
<td>3-5h</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>-67 ± 44</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>

Means ± SEM

Negative values indicate absorption and positive values indicate secretion.
Four hour net water fluxes of *in vivo* rat colon loops inoculated with PBS or with ameba extract for 1 hour, followed by inoculation with PBS for 3 subsequent hours. Water flux data are plotted semilogarithmically as a function of the trophozoite concentration of the ameba extract used. Closed circles represent results from animals administered 0.1 mg/kg indomethacin subcutaneously 10 minutes prior to inoculation of loops with ameba extracts, while open circles represent loops from animals not administered indomethacin. Means ± SEM. Positive values indicate secretion and negative values indicate absorption.
equivalent to $10^6$ trophozoites/ml or to PBS alone ($P < .05$).

Figure 2 shows that results of an experiment designed to show the relationship between the duration of colonic loop exposure to ameba extract and the luminal water secretion. The results indicate that luminal water secretion could be detected during the first hour when mucosa was exposed to extract equivalent to $10^6$ HM-1 trophozoites/ml for 10 minutes following prior injection of 0.1 mg/kg indomethacin. Inoculation of loops with trophozoite extracts for 30 minutes resulted in water secretion persisting into the second hourly test period. This model shows that secretion could be sustained beyond the period when the extract was actually in the colonic loop. Inoculation of colonic loops with fresh extract hourly for 4 hours did not produce a significant increase in secretion above that resulting from 1 hour ameba extract exposure. The figure illustrates the hourly time course of the water fluxes following a timed exposure of the mucosa to ameba extract.

Figure 3 illustrates the indomethacin dose-response curves for PBS and HM-1 trophozoite extract ($10^6$/ml) exposed loops. In the latter group of experiments the loops were inoculated with the trophozoite extract for 1 hour, then sequentially with PBS for 3 hours. For the 4 hours of the experiment there was net absorption of water in all loops inoculated with PBS alone. The net water absorption was reduced in the PBS treated loops from animals administered 1.0 and 10.0 mg/kg indomethacin. The difference between 4 hour net fluxes in 1 mg/kg indomethacin treated animals and untreated animals was marginally significant ($p = .05$), while the difference between 10 mg/kg indomethacin treated and untreated animals was not significant ($p > .05$). Secretion occurred in loops of animals inoculated with HM-1 trophozoites ($10^6$/ml) and injected with 0.1 mg/kg indomethacin. When 1 mg/kg indomethacin was administered to these animals the secretory response only occurred for
Fig. 2

Hourly net water fluxes in \textit{in vivo} rat colon loops exposed to ameba extract equivalent to $10^6$ trophozoite/ml for 0, 5, 10, 30, 60, and 240 minutes, followed by PBS for the remainder of the 4 hour test period. Animals were administered indomethacin 10 minutes prior to luminal exposure to ameba extract. Means $\pm$ SEM. Positive values indicate secretion and negative values indicate absorption.
HOURLY NET WATER FLUX, ml/10 cm

0 min

30 min

5 min

60 min

10 min

240 min

0-1 h 1-2 h 2-3 h 3-4 h

0-1 h 1-2 h 2-3 h 3-4 h
2 hours and there was net water absorption when the net flux was averaged over the 4 hour period. In animals treated with 10 mg/kg indomethacin absorption took place in all four hour test periods. Animals administered .01 mg/kg indomethacin also never showed colonic secretion during and after mucosal exposure to ameba extracts.

All animals represented in Figure 4 were administered 0.1 mg/kg indomethacin prior to the experiment. As illustrated in earlier figures, in Figure 4 there was a net water secretion in animals inoculated with trophozoite extract (10^6/ml). Heating the HM-1 trophozoite extract at 60°C for 10 minutes destroyed its enterotoxic activity, resulting in a net absorption of water. Injection of the 0.1 mg/kg of indomethacin in the third hour did not allow the manifestation of a secretory response in colonic loops exposed to extracts of 10^6 HM-1 trophozoite/ml during the 0-1 hour time period. There was no secretory response in indomethacin treated animals inoculated with extracts of E. histolytica-like Laredo strain, even when a trophozoite concentration of 10^7 was used.

Figure 5 illustrates that prior exposure of loops of indomethacin treated animals to 100 μg/ml fetuin for 10 minutes inhibited the secretory response to HM-1 extract (10^6 trophozoites/ml). Hourly inoculation with HM-1 extracts for 4 hours failed to overcome this fetuin-induced block of the secretion. When the mucosa was exposed to fetuin following, rather than prior to, the 1 hour exposure to trophozoite extract, this protein failed to inhibit the ameba extract-induced secretion.
Fig. 3  

(a) Four hour net water fluxes in in vivo colon loops of rats exposed to PBS or to extracts of E. histolytica strain HM-1 (10^6/ml) for 1 hour and PBS for 3 subsequent hours, plotted as a function of the dose of indomethacin administered 10 minutes prior to inoculation of loops with extract or PBS. Open circles represent results from loops inoculated with PBS, and closed circles represent results from loops inoculated with extract. Means ± SEM. Positive values indicate secretion and negative values indicate adsorption.  

(b) Hourly net water fluxes in loops exposed to the E. histolytica strain HM-1 trophozoite extract in animals administered 0.01, 0.1, 1 and 10 mg/kg indomethacin subcutaneously 10 minutes prior to inoculation of the loops with extract.
a. $10^6$ /ml HM-I, 1st h

b. INDOMETHACIN

HOURLY NET WATER FLUX, ml/10 cm

INDOMETHACIN, mg/kg
Four hour net water fluxes in in vivo rat colon loops exposed to PBS, heated and unheated *E. histolytica* strain HM-1 trophozoite extract, or *E. histolytica*-like Laredo strain trophozoite extract. HM-1 strain extracts were prepared from suspensions of $10^6$ trophozoites/ml while the Laredo strain extracts were prepared from suspensions of $10^7$ trophozoites/ml. All animals were administered 0.1 mg/kg indomethacin subcutaneously prior to inoculation with extract, except in a group of HM-1 strain extract inoculated animals in which the indomethacin was administered at the beginning of the third hour of the experiment. Mean ± SEM. Positive values indicate secretion and negative values indicate absorption.
NET WATER FLUX, ml/10 cm

4H

10^6/ml HM-1, 1st h

10^6/ml HEATED HM-1, 1st h

10^6/ml HM-1, 1st h, INDOMETHACIN AT 3rd h

10^7/ml LAREDO, 1st h
Four hour net water fluxes in *in vivo* colon loops of rats exposed to *E. histolytica* strain HM-1 extract (10^6 trophozoites/ml) and administered 0.1 mg/kg indomethacin subcutaneously. Some loops were inoculated with a fetuin solution for a 10 minute period immediately prior to or following a 1 hour period of inoculation with the ameba extract. In one fetuin treated group loops were inoculated with fresh ameba extract hourly for 4 hours. Means ± SEM. Positive values indicate secretion and negative values indicate absorption.
DISCUSSION

Enterotoxins produced by a few strains of microorganisms have been demonstrated to induce watery diarrhea in experimental models. One classification of these enterotoxins is based on their heat liability. The heat labile enterotoxins include those of Vibrio cholerae (Banwell et al., 1970) and Escherichia coli (Gyles and Barnum, 1969). The effect of these enterotoxins are mediated through elevated tissue cyclic AMP levels. The only heat stable enterotoxin isolated to date is an E. coli toxin (Sack, 1975; Hughes et al., 1978). The enterotoxic effect of this toxin is mediated through elevated tissue cyclic GMP levels. There are other bacterial enterotoxins which do not have their secretory effect mediated through either cyclic GMP or cyclic AMP secondary messengers, e.g., Shigella dysenteriae I enterotoxin (Keusch et al., 1970).

Pathogenic enteric parasites may also elaborate enterotoxins. Recently Lushbaugh and colleagues demonstrated a cytotoxic/enterotoxic activity associated with a cell free preparation of Entamoeba histolytica. This cytotoxic activity was also studied by Bos (1979). Cell free extracts of E. histolytica have been partially purified, and the enterotoxic activity is believed to be a protein with a molecular weight of about 30,000 daltons (Lushbaugh et al., 1979; Mattern et al., 1980). It is not known what role these trophozoite extracts play in amebic dysentery as not all amebic dysentry involves watery diarrhea (Craig and Faust, 1977; Hashim and Pittman, 1970). Amebic dysentery involves tissue damage which is associated with the ulceration of the mucosa. The postulated mechanisms involved in invasion by this organism may include a surface lectin that causes erythrocyte agglutination (Kobiler and Mirelman, 1980) with possible receptor capping (Takeuchi and
Phillips, 1975), phagocytosis (Trissl et al., 1978) and cell contact killing (Proctor and Gregory, 1972; Eaton et al., 1979; Knight et al., 1975). However, the correlation between any one of these phenomena and strain pathogenicity is not such as to allow one to state that any single event is necessary for invasion.

Lushbaugh and associates (1979) were able to induce watery diarrhea in rabbit ileal loops by inoculating 8 mg protein/ml of cell free ameba trophozoite extract into in vivo rabbit ileal loops. It is very difficult to grow large enough numbers of trophozoites to yield such a high extract protein concentration. Therefore we attempted to develop another model which would use a smaller extract volume. The models used were rabbit ileum and another smaller animal model, rat colon. We then designed our project with the aim of increasing the sensitivity of this animal models to any tested enterotoxic activity. This was achieved by decreasing mucosal cytoprotection. Prostaglandins are involved with gastrointestinal tract cytoprotection, and prostaglandin synthesis inhibition reduces such cytoprotection (Robert, 1976). The prostaglandin synthesis inhibitor, indomethacin, was therefore used on the assumption that there would be an increased cellular sensitivity to ameba extract.

In our experiments we first used the rabbit ileal loop model. With this model only those animals that showed absorption in the control loop were used. There was absorption of water, Na⁺ and K⁺ in loops inoculated with PBS, with or without prior administration of indomethacin. Absorption of water and electrolytes also occurred in loops inoculated with HM-1 trophozoite extract alone in the absence of the administration of indomethacin. Net secretion of water, Na⁺ and K⁺ was seen in only one group of animals, those indomethacin treated animals in which loops were inoculated with HM-1 extract equivalent
to $10^6$ trophozoites/ml. In this model the K$^+$/Na$^+$ net flux ratio measured during secretion in the experimental loop exposed to HM-1 trophozoite extract was higher than that observed in the hypersecretion of experimental cholera (Leitch et al., 1966), raising the possibility of extract associated tissue damage. Heating HM-1 extract equivalent to $10^6$ trophozoites/ml destroyed the enterotoxic activity and net absorption of water and electrolytes occurred. The non-pathogenic *E. histolytica*-like Laredo strain did not produce secretion, even following indomethacin administration. The enterotoxic response was limited to the experimental loop and control loops showed absorption throughout the experiment. In the experimental loops secretion was sustained for 5 hours.

Due to the high trophozoite extract volume required for the rabbit model we then employed the smaller rat model. In this model net K$^+$ fluxes were not measured because the rat colon secretes a substantial amount of K$^+$ under control conditions (Edmonds, 1967). Figure 1 illustrates that there was a net secretion in the colon of animals treated with indomethacin and inoculated with ameba extract equivalent to $10^6$ and $10^7$ trophozoites/ml. The secretory response seen following luminal exposure to ameba extract equivalent to a trophozoite concentration of $10^6$/ml was near the maximal secretory response, while a concentration equivalent to $10^5$/ml was ineffective in producing secretion. In animals not administered indomethacin there was a significant reduction in absorption following luminal exposure to extract equivalent to $10^7$ trophozoites/ml. This may correlate with the experiments of Lushbaugh and colleagues (1979) in which a secretory response was observed when a very high concentration of trophozoite extract was used (8 mg protein). It may be that indomethacin is not essential for ameba cytotoxic/enterotoxic activity, provided that high enough concentrations of the trophozoite extract are employed.
The *E. histolytica* strain HM-1 extracts had to be present in the rat colonic loop for more than 10 minutes before a secretion occurred. When ameba extract exposure lasted for 30 minutes the secretory response was sustained for 2 hours. Exposure of the colonic loop to HM-1 trophozoite extracts for 60 minutes resulted in continuous secretion in the loop for a total of 4 hours (Figures 1 and 2). There was no statistically significant difference between the mean net secretion rates of loops exposed to fresh ameba extracts hourly for 4 hours with those occurring in loops exposed to extract for 1 hour (Figure 2).

High doses of indomethacin have been reported to inhibit intestinal secretion produced in response to a variety of secretagogues (Smith et al, 1981). This was also seen in the present experiments (Figure 3) in which there was no net 4 hour secretory response when animals were treated with 1 mg and 10 mg/kg indomethacin. This suggests that 0.1 mg/kg indomethacin was not anti-secretory *per se* but that it inhibited cytoprotection (Figures 1 and 3).

Heating the HM-1 trophozoite extract at 60°C for 10 minutes resulted in net absorption of water in the rat model, as was the case in the rabbit model (Figure 4). In animals treated with indomethacin in the third hour there was no manifestation of a secretory response in colonic loops with exposure to ameba extract in the first hour (Figure 4). This too is consistent with indomethacin having its effect on mucosal cytoprotection during the period when the mucosa was exposed to ameba extract. There was no enterotoxic response in indomethacin treated animals inoculated with *E. histolytica*-like Laredo strain, even using extract equivalent to a trophozoite concentration of $10^7$/ml (Figure 4). Thus at least with respect to these two strains of ameba there was a correlation between pathogenicity and extract enterotoxic activity.
Fetuin has been shown to inhibit amebic cytotoxic/enterotoxic activity (Mattern et al., 1980). This observation was confirmed in our experiments in rat colon. Prior exposure of loops to fetuin inhibited secretion induced by ameba extract. The inhibition of net water secretion lasted for 4 hours even when the lumen was exposed to fresh ameba extract hourly (Figure 5). This can be explained on the basis that the fetuin and *E. histolytica* enterotoxic activity shared the same receptor on the mucosal surface. It appears that fetuin has a higher affinity for this receptor than does ameba enterotoxic activity. Another possible explanation is that fetuin might bind both the cellular receptor and the ameba enterotoxin, acting like a lectin. The fetuin was not anti-secretory *per se* since secretion by mucosa previously exposed to ameba extract was not inhibited when the mucosa was treated with fetuin (Figure 5).

The above data are consistent with the following hypothesis. Cell free extracts of *E. histolytica* trophozoites elaborate one or more toxic factors that attach to sites on the mucosal surface, which also have a high affinity for fetuin, or that fetuin might have an attachment site for both ameba extract toxic factor and cellular receptor. When the toxic factor(s) is (are) in high enough concentration or if mucosa prostaglandin mediated cytoprotection is impaired, secretion will result.
SUMMARY AND CONCLUSION

1. In the rabbit model 2 loops of 30 cm each were prepared. The upper loop was used as a control and was filled with PBS, and the lower loop was used as the experimental loop. Fluid volume, sodium and potassium concentrations were measured in both loops at the beginning and at the end of 3 hours and 5 hours. Net 0-3 hour and 3-5 hour period water and electrolyte fluxes were then calculated and normalized to loop length (multiples of 10 cm). In the experimental loop there was no net fluid and electrolyte secretion when PBS, heated *E. histolytica* extract (10^6 HM-1 trophozoites/ml), and non-pathogenic *E. histolytica*-like Laredo strain extract (10^6 trophozoites/ml) were inoculated in the loop. Net secretion of water, Na^+ and K^+, was seen only in experimental loops inoculated with HM-1 extract (10^6 trophozoite/ml) in animals previously administered 0.1 mg/kg indomethacin.

2. Due to the large amount of HM-1 trophozoite extract required for this experiment we introduced a smaller animal model, the rat colon loop. Secretion of water was observed in loops inoculated with ameba extracts of 10^6 and 10^7 HM-1 trophozoites/ml following indomethacin administration. The HM-1 extract had to be present in the rat colon loop for a minimum of 10 minutes for secretion to occur. Secretion was observed for 4 hours when the loop was exposed to HM-1 extract for 1 hour. When animals were treated with 0.01, 1 and 10 mg/kg indomethacin there was not net fluid secretion. Secretion of fluid only occurred when animals were administered 0.1 mg/kg indomethacin. No fluid secretion occurred in loops of animals not treated with indomethacin, and inoculated with *E. histolytica* extract or treated with indomethacin and inoculated with *E. histolytica*-like

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Laredo strain extract. There was also no secretion in animals administered indomethacin following rather than prior to HM-1 extract exposure.

3. Secretion was inhibited in indomethacin treated animals when the colonic mucosa was exposed to fetuin prior to its exposure to HM-1 trophozoite extract.

4. In both the rabbit and rat model indomethacin was used to inhibit prostaglandin synthesis and decrease prostaglandin mediated mucosal cytoprotection. This drug was therefore used to increase the cellular sensitivity to ameba extract on the part of the intestinal mucosa.

5. The above data are consistent with the hypothesis that cell free extracts of a pathogenic strain of *E. histolytica* contain one or more enterotoxic factors which appear to bind to sites on the mucosal surface. These sites also appear to have a high affinity for fetuin as fetuin completely inhibited amebic extract toxic factor induced secretion, and prolonged exposure to fresh extract did not reverse the inhibition of secretion. The requirement for indomethacin in the rat and rabbit models suggests that mucosal prostaglandin mediated cytoprotection must be overcome before ameba enterotoxic components can induce secretion.
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