Luminal bacteria and proteases together decrease adherence of Entamoeba histolytica trophozoites to Chinese hamster ovary epithelial cells: a novel host defence against an enteric pathogen

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Abstract

Background—Factors that prevent colonic mucosal invasion by pathogenic Entamoeba histolytica are not understood. A key initial step in pathogenesis of injury induced by amoeba is adherence to target cells mediated by a surface glycoprotein lectin on E histolytica. Mucin degrading bacteria, alone or in combination with proteases present in colon lumen, can decrease E histolytica adherence to target epithelial cells by degrading E histolytica adherence lectin.

Methods—The effects of exposure of E histolytica trophozoites strains HM1:IMSS and 200:NIH to faecal culture supernatant fluids, culture supernatant preparations of mucin degrading bacteria, and luminal proteases on their adherence to Chinese hamster ovary (CHO) cells were determined.

Results—Incubation of E histolytica with culture supernatant preparations or proteases alone did not modify their CHO cell adherence. However, 24 hour incubation of trophozoites with culture supernatant preparations together with pancreatic proteases decreased CHO cell adherence of HM1:IMSS strain by 71-1% (p<0.001) and of 200:NIH strain by 95% (p<0.05). Incubation of trophozoites for 24 hours with faecal extracts which contain bacterial and host hydrolases decreased the adherence of the HM1:IMSS strain by 69-2% (p<0.01) and of the 200:NIH strain by 83-0%. Reduction of trophozoite adherence to CHO cells by hydrolases was promoted by 7-5 mM cycloheximide, and was irreversible on incubation in an enzyme free medium. Decrease in CHO cell adherence of trophozoites was associated with decreased lectin on trophozoites as determined by immunofluorescence using a monoclonal antibody to the lectin.

Purified lectin was degraded by the mixture of faecal culture supernatant preparations and proteases, but not by either alone.

Conclusion—Mucin degrading bacterial glycosidases and colonic luminal proteases together, but not alone, degrade the key adherence lectin on E histolytica trophozoites resulting in decreased epithelial cell adherence. These in vitro findings suggest a potential novel host defence mechanism in the human colon wherein the invasiveness of a pathogen could be curtailed by the combined actions of bacterial and host hydrolases. This mechanism may be responsible for preventing mucosal invasion by pathogenic E histolytica.

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Keywords: Entamoeba histolytica, intestinal amoebiasis, enteric bacteria, bacterial glycosidases, host defence mechanisms.

The enteric protozoan Entamoeba histolytica infects 10% of the world’s population, resulting annually in 50 million cases of invasive amoebic diseases (colitis and liver abscess) and 100,000 deaths. Based on biochemical, molecular, and immunological analysis of isolates and clinical manifestations in infected people most researchers now separate E histolytica into two morphologically indistinguishable varieties (species), the non-pathogenic E dispar and the potentially pathogenic, true E histolytica. Other researchers still caution against separation into two species. It has recently become clear that most non-invasive infections are caused by E dispar. However, even in most patients infected with true E histolytica the organism does not invade colonic mucosa and clinical invasive diseases develop infrequently, although the potential for developing invasive diseases exists in everyone. Factors which prevent tissue invasion in vivo by pathogenic E histolytica are not understood.

Our knowledge of the key steps in the pathogenesis of invasive amoebiasis has advanced considerably in recent years. E histolytica adheres to colonic mucus and epithelial cells mainly by a galactose/N-acetylgalactosamine inhibitable lectin, which is a major surface glycoprotein with subunits of 170 kDa and 31–35 kDa. Lectin mediated...
adherence is a required step for target cell cytolysis.\textsuperscript{13-14} Factors capable of decreasing trophozoite lectin activity or adherence might prevent mucosal invasion by \textit{Entamoeba histolytica} in vivo.

A subset of normal colonic anaerobic flora ('mucin degrading bacteria') produces extracellular glycosidases that degrade carbohydrate moieties of soluble and cell surface glycoconjugates.\textsuperscript{15-19} Degradation of the carbohydrate moieties enable proteases to subsequently cleave the polypeptide core of the glycosylated regio cell adherence glycoprotein.\textsuperscript{20} Thus, hydrolases normally present in the colon ecosystem—bacterial glycosidases and host and bacterial proteases—might degrade the amoebic lectin and thereby decrease adherence of \textit{Entamoeba histolytica} to epithelial cells and prevent invasive amoebic diseases. To investigate this possibility, the effects of glycosidase containing faecal bacterial culture supernatant preparations and pancreatic proteases on the adherence of \textit{Entamoeba histolytica} to Chinese hamster ovary (CHO) epithelial cells were studied.

**Methods**

Pathogenic \textit{Entamoeba histolytica} strains HM1:IMSS and 200:NIH (American Type Culture Collection, Rockville, MD, USA) were maintained in Diamond's TYI-S-33 medium.\textsuperscript{21} The CHO cells were maintained in minimum essential α medium (GIBCO Biologicals, Grand Island, New York, USA). Partially purified bacterial glycosidases were prepared from cell free supernates of early stationary phase anaerobic cultures of fresh faeces from healthy human subjects (\(10^5\) g/ml final concentration of inoculum) and cultures of mucin degrading bacteria isolated from human stool (\textit{Ruminococcus} torques, \textit{R gnavus}, \textit{Bifidobacterium bifidum}, and \textit{B infantis}) by ice cold 60% v/v ethanol or 3-5 M ammonium sulphate precipitation and Sephadex G-200 gel filtration chromatography as described previously.\textsuperscript{15,16} Faecal extracts (20% w/v) containing bacterial glycosidases and endogenous (host and bacterial) proteases were prepared as described previously\textsuperscript{16} by homogenisation of fresh stool, precipitation by ice cold acetone, and Sephadex G-200 gel filtration chromatography. The enzyme preparations were assayed for protein (modified Lowry method) and their major glycosidase activities (blood group A antigen degrading activity and paranitrophenyl α-N-acetyl galactosaminidase activity).\textsuperscript{16,17} Faecal culture supernatant preparations contain various glycosidases active against simple glycoside and complex oligosaccharide substrates,\textsuperscript{16,18} but lack protease activity against azoalbumin substrate.\textsuperscript{16} Faecal extracts contain glycosidases and protease activity.\textsuperscript{16} Trypsin, α-chymotrypsin, and cycloheximide were purchased from Sigma Chemical Co, St Louis, MO, USA. All other chemicals were reagent grade or better. \textit{Entamoeba histolytica}-CHO cell adherence assay was performed as described by Ravdin \textit{et al.}.\textsuperscript{12-14} \textit{Entamoeba histolytica} trophozoites harvested from 48 hour cultures were washed in ice cold M-199 medium (GIBCO Biologicals, Grand Island, New York, USA) supplemented (M-199s) with 5-7 mM cysteine, 25 mM sodium - (2-hydroxyethyl) 1- piperazine ethane sulfonic acid (HEPES), 0-5% bovine serum albumin, and 1% (v/v) antibiotic mixture (final concentration penicillin (100 units/ml), streptomycin (100 μg/ml)) and suspended in M-199s. CHO cells, harvested by trypsinisation, were washed and suspended in ice cold M-199s. Quadruplicates of \(2 \times 10^5\) CHO cells in 12 mm\(\times\)75 mm plastic tubes were mixed with \(1 \times 10^7\) \textit{E histolytica}, centrifuged at 150 g for three minutes, and incubated in an ice water bath. At various incubation times all but 0.1 ml of supernatant fluid was discarded, the pellet was gently vortexed, and the suspension loaded on a haemacytometer and examined under a microscope. \textit{Entamoeba histolytica} trophozoites with ≥3 adherent CHO cells were considered adherent and the percentage of adherent trophozoites determined by counting all trophozoites in the four corner chambers (n=50). Specificity of adherence was determined by adding 20 mM galactose, which inhibited adherence in all studies by >96% and in most by 100%.

**EFFECTS OF FAECAL CULTURE SUPERNATANT PREPARATIONS, PANCREATIC PROTEASES, AND FAECAL EXTRACTS ON \textit{E HISTOLYTICA}-CHO CELL ADHERENCE**

\textit{Entamoeba histolytica} trophozoites harvested from 48 hour cultures (>90% viability as determined by trypan blue exclusion) were incubated in fresh TYI-S-33 medium containing 0.2 μm filter sterilised faecal culture supernatant (50 μg-100 μg/ml protein), 25 μg/ml trypsin, and 50 μg/ml α-chymotrypsin. At these concentrations activities of the glycosidases and proteases approximated to their activities in a 2%-10% (v/v) faecal extract. Additionally, trophozoites were incubated in (a) TYI-S-33 medium alone, (b) medium with similar concentrations of pancreatic proteases and heat inactivated (100°C for three minutes) culture supernatant preparations ('control'), and (c) medium with culture supernatant preparations. Cultures were incubated at 35-5°C and gently inverted periodically. Samples were removed at various intervals, the trophozoites were harvested and counted, and their viability and adherence to CHO cells determined. Changes in adherence were calculated as percentage of mean adherence of control cells.

**EFFECTS OF FAECAL CULTURE SUPERNATANT PREPARATIONS AND PANCREATIC PROTEASES ON \textit{E HISTOLYTICA} ADHERENCE LECTIN**

\textit{Entamoeba histolytica} adherence lectin on control and enzyme treated trophozoites was detected by indirect immunofluorescence. Trophozoites washed ×3 in M-199s were incubated for one hour at 4°C with monoclonal antibody to the lectin (500 μg/ml), washed ×3 in M-199s, and incubated for one hour at 4°C with fluorescein labelled goat antimmuno-
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globulin (50 μg/ml), washed ×3 in M-199s, and finally washed with phosphate buffered saline. Trophozoites, untreated, and after one hour incubation at 4°C with either monoclonal antibody or fluorescein labelled goat antimouse immunoglobulin alone, were used as controls. Fluorescence was detected qualitatively (grades of 0 to 4+) under an immunofluorescence microscope. Paraformaldehyde fixed cells (>30,000 in each) were analysed by flow cytometry in a fluorescence activated cell sorter (FACScan, Becton Dickinson).

Purified E histolytica lectin (1·4 μg/μl) was mixed with an equal volume of faecal culture supernatant preparation (50 μg/ml) and pancreatic proteases (50 μg/ml α-chymotrypsin, 25 μg/ml trypsin) and incubated for six hours at 37°C. Controls included incubations of lectin with faecal culture supernatant preparation alone and with heat inactivated faecal culture supernatant preparation plus pancreatic proteases. Proteins from samples were separated by electrophoresis by the method of Laemmli22 on a 5%-15% gradient polyacrylamide gel (PAGE). Untreated lectin and a mixture of faecal culture supernatant preparation and pancreatic proteases were run in additional lanes.

STATISTICAL ANALYSIS

The E histolytica–CHO cell adherence values under control and experimental conditions were compared by Student's t test for unpaired data.23 Values are given as means (SEM).

Results

E histolytica trophozoite counts and viability in culture were not influenced by 24 hour incubation in medium containing partially purified faecal culture supernatant preparation or pancreatic proteases. Thus trophozoite counts in medium containing faecal culture supernatant preparation were 105·2±2·5% of those in control medium (four experiments, p>0·1) and their viabilities (trypan blue exclusion), which ranged from 92% to 98%, were similar to those of trophozoites grown in control medium. Trophozoite counts in medium containing faecal culture supernatant preparation and pancreatic proteases in two experiments were 87·5% and 91·2% of those in control medium and their percentage viabilities were 94% and 96%.

Components of the TYI-S-33 medium did not affect glycosidase activity of faecal culture supernatant preparations as determined by their ability to degrade hog gastric mucin (HGM) carbohydrates; per cent degradation of HGM carbohydrates at 24 hours and 48 hours of incubation (measured by anthrone assay, of HGM precipitate in 60% v/v ethanol,24) in TYI-S-33 medium was similar to that in standard assay buffer (86% v 94% at 24 hours, 90% v 95% at 48 hours). E histolytica trophozoites lacked blood group antigen degrading activity and glycosidase activities against various synthetic glycoside substrates, except for β-N-acetylglucosaminidase. Incubation with E histolytica for 48 hours and 72 hours did not change the A blood group antigen degrading activity of faecal culture supernatant preparations.

EFFECT OF FAECAL CULTURE SUPERNATES, PANCREATIC PROTEASES, AND FAECAL EXTRACTS ON EH-CHO CELL ADHERENCE

The percentage of E histolytica trophozoites with CHO cell adherence increased with time of incubation. At two hours the adherence was 68·1±0·4 for HM1:IMSS trophozoites and 42·4±0·5 for 200:NIH trophozoites. The HM1:IMSS strain, which exhibits greater virulence in experimental animals, showed greater adherence. These results are similar to those previously published.14 Adherence was >96% inhibitable by galactose.

Neither faecal culture supernatant preparations nor pancreatic proteases alone appreciably modified E histolytica–CHO cell adherence when trophozoites were exposed to them for 24 hours. Incubation of E histolytica trophozoites with faecal culture supernatant preparation, trypsin, and α-chymotrypsin for periods up to seven hours did not change their adherence to CHO cells. Thereafter, percentage adherence rapidly decreased (Fig 1), and at 24 hours CHO cell adherence of the HM1:IMSS strain decreased by 71% (p<0·001) and of the 200:NIH strain decreased by 95% (p<0·05; Fig 1 and Table I) from control values (100% adherence). Incubation with faecal extracts, which contain

Figure 1: Entamoeba histolytica (HM1:IMSS strain, except where indicated) were harvested after exposure at 35-5°C to hydrolase preparations as indicated and their adherence to CHO cells compared with that of trophozoites exposed to pancreatic proteases and heat inactivated faecal culture supernatant preparation (control). Concentration of faecal culture supernatant protein 60 μg/ml, trypsin 25 μg/ml, α-chymotrypsin 50 μg/ml. Adherence is expressed as mean (SEM) of the percentage of trophozoites with rosettes. That is, 2±3 adherent CHO cells at two hour incubation in an ice water bath. • = faecal culture supernatant preparation and pancreatic proteases; ○ = faecal culture supernatant preparation, pancreatic proteases, and cycloheximide 7·5 mM; △ = faecal culture supernatant preparation only for 24 hours, then pancreatic proteases added. ■ = pancreatic proteases only for 24 hours, than faecal culture supernatant preparation added; □ = faecal culture supernatant preparation and pancreatic proteases for 24 hours, followed by wash ×2 in enzyme free medium and incubated in enzyme free medium at 35-5°C (200:NIH strain).
**TABLE I**  Mean (SEM) decrease in Entamoeba histolytica (EH) adherence to Chinese hamster ovary cells by glycosidases and proteases

<table>
<thead>
<tr>
<th>EH strain</th>
<th>Before exposure of EH to enzymes</th>
<th>After 24 h exposure of EH to α-Chymotrypsin, trypsin and heat-inactivated faecal cell sup (control)</th>
<th>Faecal cult sup prep</th>
<th>Faecal extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM1:IMSS</td>
<td>(67-8 (1-2))</td>
<td>67-8 (5-3) (28-9%)</td>
<td>19-6 (1-6)***</td>
<td>20-9 (5-9)**</td>
</tr>
<tr>
<td>200:NIIH</td>
<td>(40-6 (4-1))</td>
<td>2-0 (0-05)*</td>
<td>6-8 (2-46)</td>
<td>(17-0%)</td>
</tr>
</tbody>
</table>

† EH (%) with ≥3 adherent CHO cells at two hours.  ‡ Compared with control adherence (%). § Number of experiments performed, 5±2 replicates in each experiment. *Faecal cult sup prep=faecal culture supernatant preparation.  *p<0.05; **p<0.01; ***p<0.001 v control.

**TABLE II** Decrease in Entamoeba histolytica adherence to Chinese hamster ovary cells by culture supernatant preparations from mucin degrading bacteria and pancreatic proteases*

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Decrease in adherence† (% mean (SEM))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruminococcus torques</td>
<td>35-0 (7-1)</td>
</tr>
<tr>
<td>Ruminococcus gravis</td>
<td>82-2 (3-5)</td>
</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>73-4 (1-6)</td>
</tr>
<tr>
<td>Bifidobacterium infantis</td>
<td>89-8 (1-6)</td>
</tr>
</tbody>
</table>

* HM1:IMSS strain trophozoites incubated for 24 hours with mucin degrading bacterial culture supernatant preparation and pancreatic proteases.  † Per cent of Entamoeba histolytica with ≥3 CHO cells at two hours. EFFECT OF FAECAL CULTURE SUPERNATANT PREPARATIONS AND PANCREATIC PROTEASES ON E HISTOLYTICA ADHESION LECTIN

Before incubation of trophozoites with the enzymes the adherence lectin was detectable on the surface of most HM1:IMSS strain trophozoites with a grade 3 + and some with lower grades. After incubation with faecal culture supernatant preparation and pancreatic protease together, the trophozoites exhibited no adherence. However, when 5 mM cycloheximide was added to the medium, the HM1:IMSS trophozoites failed to return to control adherence values and had only 45-0 (1-1)% of the adherence of control trophozoites at two hours.
Figure 3: A–G Flow cytometry analysis of HM1:IMSS trophozoites before (control) and after incubation with faecal culture supernatant preparation and/or pancreatic proteases and reacted with mouse anti-lectin monoclonal antibody and fluorescein labelled goat antimouse immunoglobulin. Cell counts (ordinate) and arbitrary fluorescence units (abscissa) in log scale. A–D Control trophozoites; A = reacted with monoclonal antibody only; B = reacted with fluorescent antibody only; C = reacted with both antibodies; D = reacted with both antibodies. E–G Trophozoites after incubation with enzymes and reacted with both antibodies; E = incubated with faecal culture supernatant preparation only; F = incubated with pancreatic proteases only; G = incubated with faecal culture supernatant preparation and pancreatic proteases.

Table III: Flow cytometry analysis of Entamoeba histolytica adherence lectin.

<table>
<thead>
<tr>
<th>Trophozoites</th>
<th>Incubated with</th>
<th>Cells (%) with arbitrary fluorescence units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M-Ab†</td>
<td>F-Ab‡</td>
</tr>
<tr>
<td>Control cells:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Untreated</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Untreated</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolases treated:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faecal cult, sup and proteases</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Faecal cult sup</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteases</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*HM1:IMSS trophozoites used.
† Concentration of hydrolases same as Table I.
‡ M-Ab = mouse monoclonal antibody to lectin.
§ F-Ab = fluorescein labelled goat antimouse antibody.

Figure 4: Polyacrylamide gel electrophoresis of E histolytica adherence lectin (5 μg) before and after treatment with faecal culture supernatant preparation, pancreatic proteases, or both, stained with Coomassie blue. Lanes: (1) untreated lectin; (2) mixture of faecal culture supernatant preparation and pancreatic proteases with no lectin; (3) lectin treated with pancreatic proteases and heat inactivated faecal culture supernatant preparation; (4) lectin treated with faecal culture supernatant preparation and pancreatic proteases; (5) lectin treated with faecal culture supernatant preparation only; (6) molecular weight standards. Numbers in margin represent molecular mass in kDa. Arrow indicates the heavy subunit of the lectin.

Discussion

Our studies show that adherence of E histolytica trophozoites to CHO epithelial cells is decreased by the combined effects of faecal bacterial culture supernatant preparation and endogenous (host and bacterial) luminal proteases. The levels of enzyme activities used in these studies were similar to their activities in the right colon contents in that they were 2% to 10% of their activities in a faecal extract. Adherence of E histolytica to colonic epithelial cells occurs by a mechanism similar to their adherence to CHO cells. Therefore, it is likely that these enzyme activities would similarly decrease adherence of E histolytica to colonic epithelial cells. Adherence is a key step in mucosal invasion and it is a required step for target cell cytology. Thus the normal
Luminal hydrolases that promote to the layer, by its luminal indicating humans effect of mucin prevent mucous defined to be enzyme turnover promote to mucin. EH-CHO mucin.

phospholipase-C activity (data on preincubation levels ensued when prevented turnover by preincubation). 25 Components.25 Cycloheximide, enzyme components were expected and membrane indicate adherence lectin. The rapid restoration of trophozoites,34 mechanisms that colonic mucin could have been developed by exposure to preincubation levelsensued. Enteric bacteria in faecal culture supernatant fluids are sizeable (Table 3). It has been shown that colonic mucin plays a protective role in preventing invasive amoebic disease. It may be argued that the protective effect of mucin degrading bacteria might be offset by their ability to degrade colonic mucin. But, in the normal colon a well defined mucous layer is demonstrable when enzyme activities are present in the lumen, indicating that mucin synthesis keeps up with its luminal degradation. Loss of the mucus layer, by mechanisms that may include activity of \( E. histolytica \) trophozoites,5 could also be a factor that promotes amoebic invasion. Luminal hydrolases may also have effects on lectin receptors on colon epithelial cells, similar to the effects of O-glycosidases on such cells, such effects could modify amoeba-epithelial cell interaction.

The role of enteric bacteria in the natural history of human \( E. histolytica \) infection is not clear. Previous studies in vitro and in animal models have generally led to the conclusion that enteric bacteria promote colonization and increase amoebic virulence.36-38 Such studies have generally determined the roles of facultative bacteria and have been limited in studying the role of strict anaerobes such as the mucin degrading bacteria. The studies reported here support a novel, protective role for enteric bacteria in the development of invasive and amebic colon flora. Recent studies have shown that association of virulent axenic \( E. histolytica \) trophozoites with mixed bacteria leads to decreased proteolytic activity39 and to loss of carbohydrate dependent \( E. histolytica \) surface antigen.40

Faecal populations of mucin degrading bacteria in healthy humans are generally stable.41 Occasionally, transient losses of their activities have been noted after diarrhoeal illness or antibiotic use.43 The current studies showing reduction of \( E. histolytica \) adherence to CHO epithelial cells by faecal bacterial culture supernatant fractions indicate that indigenous proteases suggest that alterations of normal enteric flora which include a loss or decrease in activity of mucin degrading bacteria could lead to invasive amebiasis. Indirect support for such a hypothesis is provided by two outbreaks of invasive amoebiasis, one in an airborne camp42 and another from colonial irrigation.43 Several airforce camp personnel developed amoebic dysentery after contamination of their water supply by sewage.42 Faecal prevalence of \( E. histolytica \) was similar among camp personnel before and after development of dysentery, indicating that the sewage contamination did not result in new cases of \( E. histolytica \) infection. Invasive amebiasis occurred more often in those who developed a diarrhoeal illness before manifesting dysentery. Presumably, the diarrhoeal illness led to alterations in normal enteric flora and amoebic invasion. The second outbreak was traced to ‘therapeutic’ colonic irrigation.43 Dysentery occurred in 72% of infected patients, a rate considerably higher than the usual occurrence of dysentery in <10% of those infected. Alterations of bowel flora or trauma from irrigation presumably resulted in high desentery rate in this outbreak. Whereas these outbreaks of invasive disease in the rat studies reported in this study support a protective role for normal enteric bacteria in preventing invasive amebiasis, longterm studies of the functional activities of normal enteric flora in persons infected with pathogenic \( E. histolytica \) are necessary to define their protective role.

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Host defence against Entamoeba histolytica

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