Contact of *Entamoeba histolytica* with baby hamster kidney-21 (BHK-21) cell line on cysteine proteinase activity

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**Background & objectives**: *Entamoeba histolytica*, the causative agent of amoebiasis and amoebic liver abscess, lyses host cells by direct contact using surface lectins and releases cysteine proteinase (CP). Virulence of *E. histolytica* is directly related to activity of its CP. The relationship of CP activity and cytotoxicity has not been established. The present study was carried out to explore the events following contact of *E. histolytica* with target cells.

**Methods**: Protease activity of *E. histolytica* was measured by azocaseine and haemoglobin assays, and cysteine proteinase activity was assessed by substrate gel electrophoresis. Target cell lysis was measured by chromium release assay.

**Results**: Protease activity of *E. histolytica* was increased 2.5-fold following contact with BHK-21 cell line. CP activity of trophozoites alone was visualized at position 56, 35 and 29 kDa in substrate gel electrophoresis. Contact of trophozoites with target cells augmented the cytotoxic activity of amoebic CP. The increase in CP activity seen by substrate gel electrophoresis and cytotoxicity assay was blocked by pretreatment with E 64, a specific CP inhibitor and GalNAc, a contact inhibitor.

**Interpretation & conclusion**: The present data showed the involvement of amoebic CP in cytotoxicity and that the CP activity was enhanced on lectin-mediated contact of *E. histolytica* to the target cells. Further studies need to be done to understand the mechanism at the molecular level.

**Key words** BHK-21 - *Entamoeba histolytica* - GalNAc - lectin - virulence

*Entamoeba histolytica*, an intestinal parasite responsible for amoebic dysentery and liver abscess, is a major cause of morbidity and mortality worldwide¹. *E. histolytica* damages the colonic mucosa and other tissues of the human host through direct contact, which is mediated by Gal/GalNAc lectins²; inhibition of lectin activity with N-acetylgalactosamine (GalNAc) blocks contact-mediated cytotoxicity³.

Several virulence factors of *E. histolytica* have been described such as Gal/GalNAc inhibitable lectins⁴, amoebapore⁵, and cysteine proteinase (CP)⁶-⁸. CP of *E. histolytica* is involved in several steps of invasion and tissue damage⁹. Though, amoebic CP has been implicated in the cytopathic effect of *E. histolytica* on cultured cell monolayer⁶, partial inhibition of CP gene by antisense had no effect on cytopathic or haemolytic activities⁸. The sequence of events, which follow contact between *E. histolytica* and target cells⁹ is not clearly understood. The aim of the present study was therefore to explore the events following contact of *E. histolytica* with target cells. The effect of *E. histolytica* strain HM1: IMSS contact with baby hamster kidney cells (BHK-21) on CP and the consequences of this cell-to-cell interaction, were studied.
Material & Methods

Maintenance of cell culture: Axenic E. histolytica trophozoites strain HM1:IMSS clone 6 was maintained in TYI-S-33 medium supplemented with penicillin (100U/ml), streptomycin (100 µg/ml) and 15 per cent adult bovine serum (Biological Industries, Haemek, Israel), as previously described10.

BHK-21 cell line (National Center for Cell Sciences, Pune, India) was cultured in RPMI-1640 (Gibco BRL, Rockville, USA) supplemented with 2 mM L-glutamine, 25 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer, 0.2 per cent sodium bicarbonate, 50 µM 2-mercaptoethanol (Sigma, USA) and 10 per cent foetal calf serum (FCS, Biological Industries, Israel) with antibiotics penicillin 100U/ml and streptomycin 100 µg/ml (Gibco BRL, Rockville, USA). The culture was seeded and grown in six-well and 96-well tissue culture plates (Nunc, Roskilde, Denmark) as required for different experiments, at 37ºC in humidified 5 per cent CO₂ atmosphere.

Adhesion of trophozoites on BHK-21 monolayer: BHK-21 monolayer was washed with RPMI-1640 without FCS. E. histolytica trophozoites were incubated with 0.5 M or 1.0 M GalNAc (Sigma, USA) to block E. histolytica lectin and 200 µM of L-trans-epoxysuccinyl-leucyl-amido-(4-guanidino) butane (E 64) (Sigma, USA) a CP inhibitor11 to block CP activity. The trophozoites were added to BHK-21 monolayer (1:10 E. histolytica:BHK-21) and incubated at 37ºC in humidified 5 per cent CO₂ atmosphere for 60 min. Each well was washed with chilled phosphate buffered saline (PBS), pH 7.2, to separate the trophozoites from BHK-21 monolayer. Trophozoites were sonicated and crude lysate was used for measuring CP activity. All experiments were done in triplicate and repeated three times.

Chromium release assay12: Culture medium was aspirated from the wells of 96-well plate seeded with BHK 21 cells followed by addition of 20 µl each of foetal bovine serum and 51Cr labeled sodium chromate (50 µCi/ml) (BARC, India). Plate was incubated at 37ºC in humidified 5 per cent CO₂ atmosphere for 1 h. Plate was centrifuged and released 51Cr in 100 µl of supernatant was counted in a gamma counter (Multigamma, LKB, Sweden). All experiments were done in triplicate and repeated five times. Per cent cell cytotoxicity was calculated by the following formula:

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\text{% cell cytotoxicity} = \frac{\text{total release} - \text{spontaneous release}}{\text{experimental release} - \text{spontaneous release}} \times 100
\]

Azocasein assay: The proteolytic activity of CP was assayed as described earlier13,14. CP acts on the homogenic substrate azocasein (Sigma, USA) to release low molecular weight soluble peptides into the supernatant, which give a colour reaction that is measured at 440 nm. Azocasein (250 µl, 0.2% w/v) in 100mM phosphate buffer, pH 6.5 was incubated with 150 µl crude lysate of E. histolytica at 37ºC for 30 min and phosphate buffer without crude lysate was used as control. The reaction was stopped by adding 10 per cent trichloroacetic acid (TCA) for 15 min at room temperature. Mixture was centrifuged at 15,000 g for 5 min at room temperature; 1.2 ml of supernatant was collected in a vial containing 1.4 ml 1 M NaOH, mixed and absorbance read at 440 nm.

Haemoglobin assay: Haemoglobinase activity of CP releases soluble low molecular weight peptides into the supernatant, which is measured at 280 nm (A₂₈₀)15. In brief, 500 µl of haemoglobin (2% haemoglobin powder with 5 M urea) was incubated with 150 µl crude amoebic lysate at 37ºC for 15 min; blank was prepared by incubation of amoebic crude lysate with 10 per cent TCA15. The reaction was stopped by adding 5 ml of 10 per cent TCA. Mixture was centrifuged at 15,000 g for 5 min at room temperature; supernatant was collected and absorbance read at 280 nm.

One unit of enzyme activity was defined as the amount of enzyme required to cause a unit increase in absorbance across a 1 cm path length, at 440 nm for azocaseine and 280 nm for haemoglobin assay15.

Substrate gel electrophoresis: Crude lysate of control and experimental groups (10 µg protein) was electrophoresed at 50V at 4ºC on a 10 per cent polyacrylamide gel co-polymerized with 0.2 per cent gelatin (Sigma, USA). Rainbow molecular weight
markers (Invitrogen, Carlsbad, USA) were used for detection of correct size of cysteine protease of *E. histolytica*. Sodium dodecyl sulphate (SDS) was removed by two washing in 2.5 per cent triton X100 for 30 min each at room temperature. The gel was incubated in developing buffer [20 mM dithiothreitol (DTT) and 100 mM sodium acetate *pH* 4.2 with 1% triton X100] at 37°C with continuous shaking for 3 h; and stained with coomassie blue for 30 min. Zone of protease activity was visualized as clear band against a blue background.\(^1^6\)

**Statistical analysis:** Comparisons between groups were performed by Mann-Whitney sign test and *P* value <0.05 was considered as statistically significant.

**Results**

**Chromium release assay:** Cytotoxicity of BHK-21 targets mediated by *E. histolytica* alone was 34.5±3.7 per cent, while that of trophozoites pre-incubated with E 64, was 16.1±9.4 per cent (*P*<0.0001). The trophozoites pre-treated with 0.5 *M* and 1.0 *M* GalNAc showed reduced cytotoxicity of 25±7.0 and 12.6±3.1 per cent (*P*<0.0001) respectively.

**Cysteine proteinase activity:** The protease activity of *E. histolytica* was 20.9±1.7 and 21.8±0.5 U/mg protein by azocasein and haemoglobin assays respectively. This activity was significantly inhibited by E 64 pre-treatment of trophozoites (2.7±1.7 and 2.7±0.9 U/mg protein) (*P*<0.0001). Trophozoites incubated on BHK-21 cell line to allow cell contact showed significantly increased activity of 53.1±0.6 and 56.4±0.3 U/mg protein by azocasein and haemoglobin assays respectively (*P*<0.0001). This increase was inhibited to 11.6±3.4 and 11.1±2.8 U/mg protein with 0.5 *M* GalNAc pre-treated trophozoites; and to 4.0±1.0 and 4.9±0.3 U/mg protein with 1.0 *M* GalNAc pre-treated trophozoites by azocasein and haemoglobin assays respectively (*P*<0.0001; Fig. 1).

**Substrate gel electrophoresis:** Crude lysates of *E. histolytica* with and without contact with BHK-21 monolayer were subjected to gelatin substrate gel electrophoresis. The bands depicting protease activity were more intense in lane containing crude lysate of *E. histolytica*, which had contact with BHK-21 monolayer; bands were visible at 29, 35 and 56 kDa. Highest activity was seen in the region of 56 kDa, which represents neutral CP. No gelatin degradation was seen in BKH-21 alone or E 64 treated *E. histolytica* (Fig. 2).

![Fig. 1. Results of protease activity of *Entamoeba histolytica* by azocasein (■) and haemoglobin (●) assays. (Result expressed as Units/mg protein; bars represent mean±SD of three separate experiments). *P*<0.001 compared to Eh - *E. histolytica*, BHK-baby hamster kidney, E-64 - a cysteine proteinase inhibitor, Gal-N-acetyl galactosamine.](image)

![Fig. 2. Substrate gel showing cysteine proteinase (CP) activity of crude lysate of *Entamoeba histolytica* trophozoites (lane 1), crude lysate of BHK-21 (lane 2), *E. histolytica* trophozoites treated with E64 following contact with BHK-21 monolayer (lane 3), *E. histolytica* trophozoites following contact with BHK-21 monolayer (lane 4), 0.5 *M* GalNAc treated *E. histolytica* trophozoites following contact with BHK-21 monolayer (lane 5) and 1.0 *M* GalNAc pretreated *E. histolytica* trophozoites following contact with BHK-21 monolayer (lane 6). 10 per cent polyacrylamide gel was co-polymerized with 0.2 per cent gelatin and 10 µg of protein was added per well. Abbreviations are the same as given in Fig.1.](image)
Discussion

The role of CP of *E. histolytica* in the degradation of extracellular matrix, in vitro destruction of monolayer and tissue damage has been well established. *E. histolytica* is believed to degrade target cells through Gal/GalNAc mediated contact but factors responsible for activating amoebic CP are not clear. Proteases which are present inside cells in inactive form are converted to active form by unknown signals. Similarly amoebic cysteine proteases are synthesized as precursor proteins with predomains, prodomains and catalytic domains. Prepro enzymes are subsequently processed to mature enzyme. The signal which activates conversion of inactive enzyme to mature active enzyme is not known. The findings of the present study showed that CP activity of *E. histolytica* was dependent on Gal/GalNAc mediated contact with target cell.

The target cell cytotoxicity was inhibited by pre-treatment of *E. histolytica* trophozoites with the CP specific inhibitor E64 or GalNAc. It has been previously shown that blocking of amoebic lectins with Gal/GalNAc reduces both adherence and cytotoxicity, however, the cytotoxicity was not completely abolished, as is the case in our study also. Our results suggested that inhibitable contact between *E. histolytica* and BHK-21 was important for the cytotoxicity, which was mediated by CP, since the effects were inhibited by E64. However, other factors are also involved in the cytotoxicity.

CP activity of *E. histolytica*, which had been in contact with BHK-21 monolayer, was greater than that of *E. histolytica* alone; pre-treatment of *E. histolytica* by GalNAc blocked the increase in CP activity suggesting that GalNAc mediated signals were required for conversion of prepro form of cysteine proteinase into active enzyme. CP activity was almost completely blocked by E64 treated *E. histolytica* trophozoites.

Cysteine proteinase activity seen in substrate gel electrophoresis at 28, 35 and 56 kDa region suggests involvement of more than one cysteine proteinase. ACP3 (E. histolytica CP1) has been shown to have two distinct activities at 27-30 kDa, CP2 at 35 kDa and CP5 at 29 kDa and neutral CP has been reported to have proteinase activity at 56 kDa. The increased CP activity following contact with monolayer therefore involved CP 1, 2, 5 and neutral CP. We have earlier shown that the activity at 56-66 kDa increased after passage of *E. histolytica* through hamster liver, which was related to increase in virulence of *E. histolytica*.

Drugs in current use for amoebiasis have problems of toxicity and drug resistance. Cysteine proteinase is an attractive potential target for new anti-amoebic drug development. However, better understanding of its mechanism of action is required to exploit its full potential. In conclusion, the present findings suggest that direct contact of *E. histolytica* with target cells through GalNAc results in increased CP activity. Further studies are required to elucidate the molecular basis for this observation.

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References


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