Role of cysteine proteinase of *Entamoeba histolytica* in target cell death

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SUMMARY

The bacterial flora of the intestine plays an important role in the virulence caused by *Entamoeba histolytica*. Cysteine proteinase (CP), an amoebic virulence factor, plays a major role in host cell destruction. The mechanism of increased virulence following bacterial co-association is not understood. We studied CP of *E. histolytica* HM1:IMSS which was co-associated with *Escherichia coli* K12 strain pre-incubated with GalNAc or CP specific inhibitor E 64. Co-association of *E. histolytica* with bacteria enhanced CP activity 3·6-fold as assessed by azocasein assay and substrate gel electrophoresis showed bands at molecular weights of 28, 35 and 56 kDa. Northern and Western blot analysis showed increase in *ehcp2* and *ehcp5* gene expression. Trophozoites co-associated with *E. coli* showed greater cytotoxicity of BHK cells by a ^{51}Cr release assay than trophozoites that had not been co-associated; this enhancement was abolished by E-64 treatment. The killing of BHK 21 targets by *E. histolytica* was characterized by DNA laddering which was not inhibited with E-64. GalNAc pre-incubation of trophozoites reduced cytotoxicity and DNA laddering, while *E. coli* co-associated *E. histolytica* showed smearing with faint laddering of BHK implicating both necrosis and apoptosis. Hence, bacterial co-association increases CP activity and CP gene expression and contributes to the necrosis of the target cell.

Key words: amoeba, apoptosis, virulence, cytotoxicity, bacteria.

INTRODUCTION

Entamoeba histolytica, an intestinal parasite, is responsible for amoebic dysentery and liver abscesses. It is a major cause of morbidity and mortality worldwide (WHO, 1997). Prolonged axenic cultivation decreases the virulence of *E. histolytica* (Mattern *et al.* 1982); this is restored by manipulations such as *in vitro* incubation of trophozoites with hamster serum, cholesterol, serial passage through hamster liver (Lushbaugh *et al.* 1978; Gupta *et al.* 1998; Das *et al.* 1976) as well as *in vitro* co-association with certain strains of *E. coli* (Sinha *et al.* 1997; Ghosh *et al.* 1998). The mechanism of increased virulence following bacterial co-association is not well understood.

Trophozoites of virulent amoebic strains cause lesions of the bowel by attaching to the epithelium through Gal/GalNAc inhibitable lectins (McCoy *et al.* 1994), degrading the extracellular matrix through the action of cysteine proteinases (Keene *et al.* 1990) and lysing epithelial cells via an amoebapore (Leippe, 1997). Cysteine proteinases (CP) of *E. histolytica* have been shown to be involved in the *in vitro* cytopathic effect of the parasite as well as degradation of extracellular matrix (Keene *et al.* 1990; Jacobs *et al.* 1998); the cytopathic activity depends upon high enzymatic activity of CP (Gadasi & Kobiler, 1983; Jacob *et al.* 1998). CP has also been implicated in parasite virulence since blocking of CP activity by E-64 reduces the liver abscess formation in SCID mice (Stanley *et al.* 1995).

Of the several CP genes and proteins, EhCP5 and EhCP112, have been shown to localize on the surface of *E. histolytica* and hence may play a role in mediating virulence (Jacobs *et al.* 1998; Garcia-Rivera *et al.* 1999).

Approximately 90% of total CP activity present in a lysate of cultured *E. histolytica* trophozoites is present in the 3 cysteine proteinases EhCP1, EhCP2 and EhCP5 (Bruchhaus *et al.* 1996). How important their role is in the cytopathogenic activity of the parasite is not clear. Antisense inhibition of *ehcp5* gene expression did not affect its cytopathic or haemolytic activity while inhibiting phagocytosis by the parasite. This CP-deficient parasite failed to induce liver lesion in a hamster model (Ankari *et al.* 1998, 1999). Inoculation of *ehcp5* deficient *E. histolytica* in a human intestinal xenograft model in SCID mice induced significantly less gut inflammation than that caused by virulent amoeba (Zang *et al.* 2000).

In the host, the parasite mediates its virulence in the presence of the natural gut flora and bacterial associates support *in vitro* growth. The role of these bacteria in parasite virulence is intriguing. Bacterial co-association increases *in vitro* cytopathic activity

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(Sinha *et al*. 1997); however, the mechanism by which it does this is not clear. In this study we have investigated the effect of co-association of *E. coli* K 12 strain with *E. histolytica* on amoebic CP activity and gene expression and the role of co-association in target cell lysis.

MATERIALS AND METHODS

Maintenance of cell culture

Axenic *Entamoeba histolytica* trophozoite strain HM1:IMSS clone 6 was maintained in TYI-S-33 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 15% (v/v) adult bovine serum (Biological Industries, Haemek, Israel), as previously described (Diamond *et al*. 1978).

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 HEPES buffer, 0·2% sodium bicarbonate, 50 μM 2-mercaptoethanol and 10% foetal calf serum (FCS) (Biological Industries, Haemek, Israel) with antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). The cultures were maintained in 6-well tissue culture plates (Nunc, Roskilde, Denmark) at 37 °C in humidified 5% CO_2 atmosphere.

Amoebic trophozoites were harvested by chilling the culture tube for 5 min on ice, followed by centrifugation at 280 ***g*** for 8 min at 4 °C and incubation with log-phase grown *E. coli* at a ratio of 1:1000 (amoeba: bacteria) for 90 min at 37 °C. Then 200 μM E-64 [L-*trans*-epoxysuccinyl-leucyl-amido-(4-guanidino) butane], (Sigma, St Louis, USA) a specific and irreversible inhibitor of CP (Barrett *et al*. 1982) was added to the culture medium for 24 h to block the CP activity. The viability of trophozoites at the end of the incubation was 94% by the trypan blue (Sigma, St Louis, USA) exclusion method.

Adhesion of trophozoites on the BHK-21 monolayer

The BHK-21 monolayer was washed with RPMI-1640 without FCS. *E. histolytica* trophozoites were incubated with 1·0 mM GalNAc (Sigma, St Louis, USA) to block *E. histolytica* lectin or 200 μM of E-64 (Sigma, St Louis, USA) or co-associated with log-phase grown *E. coli*. The trophozoites were added to the BHK-21 monolayer (1:10 *E. histolytica*: BHK-21 ratio) and incubated at 37 °C in humidified 5% CO_2 atmosphere for 60 min. Each well was washed with chilled PBS to dissociate the trophozoites from the BHK-21 monolayer.

Azocasein assay

The proteolytic activity of amoebic proteinase was assayed as described earlier (Scholze & Tannich, 1994; Gupta *et al*. 1998). CP acts on the chromogenic substrate azocasein (Sigma, St Louis, USA) to release low molecular weight soluble peptides into the supernatant fluid, which gives a colour reaction that is measured at 440 nm (A_{440}). Briefly, 250 μl of 0·2% azocasein (w/v) in 100 mM phosphate buffer, pH 6·5, was incubated with 150 μl crude amoebic lysate at 37 °C for 30 min. The reaction was stopped by adding 10% trichloroacetic acid (TCA) for 15 min at room temperature. The mixture was centrifuged at 1400 ***g*** for 5 min at room temperature and 1·2 ml of supernatant was collected in a vial containing 1·4 ml of 1 M NaOH, mixed and the absorbance read at 440 nm.

One unit of enzyme activity was defined as the amount of enzyme required to cause a unit increase in the absorbance across a 1 cm path length at 440 nm. (Sarath *et al*. 1989).

Substrate gel electrophoresis

The molecular weight pattern of CP was assessed by gelatin substrate gel electrophoresis as described earlier (Hellburg, Leippe & Bruchhaus, 2000). Ten μg protein of crude lysates (control and experimental) were electrophoresed at 50 V on 4 °C on a 10% polyacrylamide gel co-polymerized with 0·2% gelatin (Sigma, St Louis, USA). SDS was removed by 2 washings in 2·5% Triton X-100 for 30 min each at room temperature. The gel was incubated in developing buffer (20 mM DTT and 100 mM sodium acetate, pH 4·2 with 1% Triton X-100) at 37 °C with continuous shaking for 3 h and stained with Coomassie blue for 30 min. Zones of protease activity was visualized as clear bands against a blue background.

Northern blotting

Northern blot of *E. histolytica* with and without *E. coli* co-association was probed with ehcp1, ehcp2 ehcp5 and 18S rRNA probes. RNA was isolated using Trizol^{LS} (Gibco BRL, Rockville, USA) according to the manufacturer's instruction. A sample of 20 μg total RNA was separated on 1% agarose–formaldehyde gel and transferred onto nylon membrane. The membrane was hybridized with α ^{32}P labelled ehcp1, ehcp2 and ehcp5 probe and 18S rRNA probe at 42 °C for 18 h and the blot washed with 0·2X SSC with 0·1% SDS at 37 °C. The blot was exposed to Kodak X-ray film at −70 °C for 1 week (Sambrook, Fritsch & Maniatis, 1989).

SDS–PAGE and Western blotting

Crude lysates (20 μg protein) of trophozoites (control and experimental) were added to sample buffer (10% glycerol, 2% SDS, 50 mM Tris–HCl pH 6·8) with 10 mM β-mercaptoethanol and 50 μM E-64, 10 mM

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p-hydroxy methyl benzoate, 3 mM iodoacetamide, heated at 95 °C for 2 min and then chilled in ice. After electrophoresis, the gel was transferred at 0·65 mA/cm^2 of gel at 4 °C onto nitrocellulose membrane and probed with polyclonal antibody specific for EhCP1, EhCP2 and EhCP5 (kindly gifted by Iris Brauchhaus, Germany). The blots were developed by the chemiluminescence system according to the manufacturer's instructions (Amersham Pharmacia Biotech Ltd, Uppsala, Sweden) (Sambrook *et al*. 1989).

Cell cytotoxicity assay

Cell cytotoxicity was tested by ^{51}Cr cytotoxicity assay. BHK 21 was seeded at 50×10^4 cells/well and allowed to grow overnight in 96-well flat-bottom culture plates (Nunc, Roskilde, Denmark) to reach confluence. The medium was removed and 50 μCi/ml ^{51}Cr labelled sodium chromate (BARC, Trombay, India) was added to the culture medium and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 2 h. The plate was washed twice to remove unincorporated ^{51}Cr. *E. histolytica* trophozoites with and without bacterial co-association or with GalNAc treatment or E-64 treatment were added to the BHK monolayer (1 : 10 Eh : BHK-21 ratio) and incubated at 37 °C in humidified 5% CO_2 atmosphere for 60 min. Released ^{51}Cr in the supernatant was counted in a Multigamma Counter (LKB Wallace, New Jersey, USA). All the experiments were done in triplicate and repeated 3 times. Percentage cell cytotoxicity was calculated as follows

$$\% \text{ Cell cytotoxicity} = \frac{\text{Total release} - \text{Spontaneous release}}{\text{Experimental release} - \text{Spontaneous release}} \times 100.$$

DNA fragmentation assay

BHK-21 cells were plated in a 6-well plate (Nunc, Roskilde, Denmark) at a cell density of 2×10^6 cells/well and allowed to grow to 60–70% confluence at 37 °C and 5% CO_2. The monolayer was washed with RPMI without FCS. *E. histolytica* trophozoites with and without bacterial co-association or with GalNAc or E-64 treatment were added to the monolayer (1 : 10 Eh : BHK-21 ratio) and incubated for 1 h at 37 °C and 5% CO_2. Adhered trophozoites were detached by chilling the slide at 4 °C and washing with chilled PBS. A positive control was generated by exposure of BHK 21 cells to UV (256 nm) light for 10 min followed by incubation at 37 °C and 5% CO_2 for 16 h.

Small DNA fragments were isolated as described earlier (Tolskaya *et al*. 1995). Cells were detached by mild trypsinization and collected by centrifugation at 2800 ***g*** for 10 min. The cells were resuspended in buffer containing 20 mM EDTA, 10 mM Tris–HCl (pH 7·4) and 0·5% Triton X-100 and kept at 0 °C for 20 min. The cell lysate was centrifuged at 18 000 ***g*** at 4 °C for 20 min to remove intact chromatin. SDS was added to the supernatant to give a final concentration of 1%, followed by phenol–chloroform deproteinization. Nucleic acids were precipitated with chilled absolute ethanol at −20 °C and the pellet dissolved in 10 μl of water. RNA was removed by treatment with 1 μl of RNase A (10 μg/ml) at 37 °C for 1 h. Glycerol was added to give a final concentration of 8% and samples were subjected to electrophoresis on 2% agarose gel.

Nuclear morphology by propidium iodide staining

BHK-21 cells were plated in 8-chamber slides (Nunc, Roskilde, Denmark) at a cell density of 5×10^4 cells/chamber and allowed to grow up to 60–70% confluence at 37 °C and 5% CO_2. The monolayer was washed with RPMI without FCS. Amoeba trophozoites were added to the monolayer at a ratio of 1 : 10 (trophozoites : BHK-21) and incubated for 60 min at 37 °C and 5% CO_2. Adhered trophozoites were removed by chilling the slide at 4 °C and washed with chilled PBS. A positive control was generated by exposure to UV (256 nm) light for 10 min followed by incubation at 37 °C and 5% CO_2 for 16 h. Cells were fixed with chilled in 70% ethanol for 20 min and washed with PBS and stained with 50 μg/ml propidium iodide with 10 μg/ml RNase A for 30 min in the dark. The slide was mounted with 50% glycerol and examined under a fluorescent microscope.

Statistical analysis

Comparisons between groups were performed by Mann–Whitney sign test and P values $<0{\cdot}05$ were considered as statistically significant.

RESULTS

Protease activity

The protease activity of *E. histolytica* was $16{\cdot}8 \pm 7{\cdot}4$ U/mg of protein; this was almost completely abolished by E-64 ($4{\cdot}0 \pm 1{\cdot}4$ U/mg protein) ($P<0{\cdot}005$). A 3·6-fold increase in protease activity ($60{\cdot}2 \pm 4{\cdot}8$ U/mg protein) was seen when trophozoites were co-associated for 90 min with *E. coli* K-12 strain as compared to *E. histolytica* alone ($P<0{\cdot}005$); and this reduced 2·5-fold when E-64 pre-treated *E. histolytica* were co-associated with *E. coli* ($6{\cdot}7 \pm 1{\cdot}2$ U/mg protein) ($P<0{\cdot}005$) (Fig. 1).

Substrate gel electrophoresis

Crude lysates of *E. histolytica* with and without co-association with bacteria were subjected to gelatin substrate gel electrophoresis. *E. histolytica* co-associated with *E. coli* showed more intense bands at 29, 35 and 56 kDa as compared to *E. histolytica*

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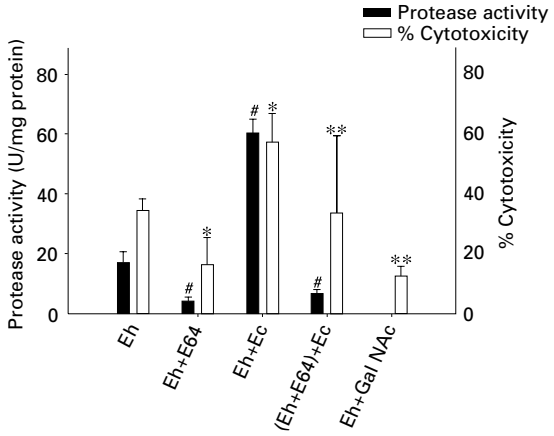

* $P<0{\cdot}0001$ as compared to Eh, ** $P<0{\cdot}005$ as compared to Eh+Ec, # $P<0{\cdot}005$ as compared to Eh

Fig. 1. Protease activity using the azocasein assay and percentage cytotoxicity measured by the ^{51}chromium release assay of *Entamoeba histolytica* (Eh), E-64 and GalNAc treated (Eh + E 64; Eh + GalNAc), *E. histolytica* co-associated with *Escherichia coli* (Eh + Ec), and E-64 treated *E. histolytica* co-associated with *E. coli* ((Eh + E 64) + Ec). The bars represent the mean of 5 experiments, done in triplicate ± S.D.

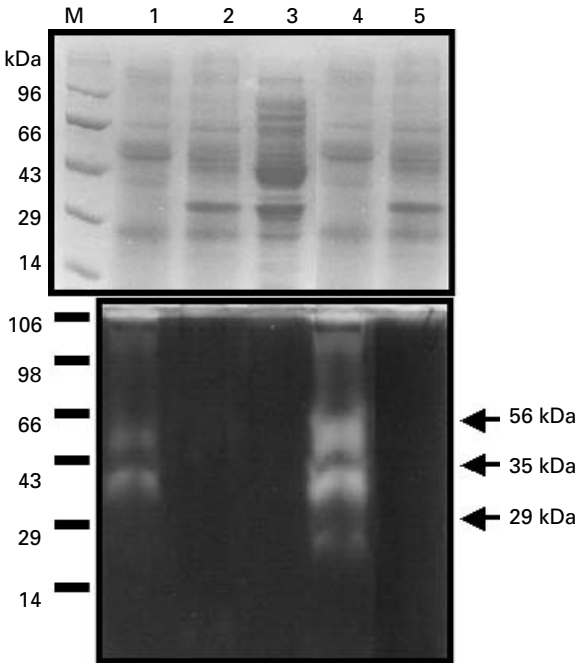

Fig. 2. SDS–PAGE (upper) and substrate gel (lower) of crude lysates of *Entamoeba histolytica* under different experimental conditions. Electrophoresis was performed on 10% SDS–PAGE co-polymerized with 0·1% gelatin. Lane 1: *E. histolytica*; Lane 2: *E. histolytica* pre-incubated with E-64 (200 μM); Lane 3: E-64 treated trophozoites co-associated with *Escherichia coli*; Lane 4: *E. histolytica* co-associated with *E. coli* for 90 min at 37 °C; Lane 5: *E. coli* and Lane M: molecular weight marker.

without bacterial co-association (Fig. 2). The most prominent band was in the region of 56 kDa, which represents neutral CP. No gelatin degradation was seen in *E. coli* alone and E-64 treated *E. histolytica*. The SDS–PAGE confirmed that protein loading in all the lanes was equal.

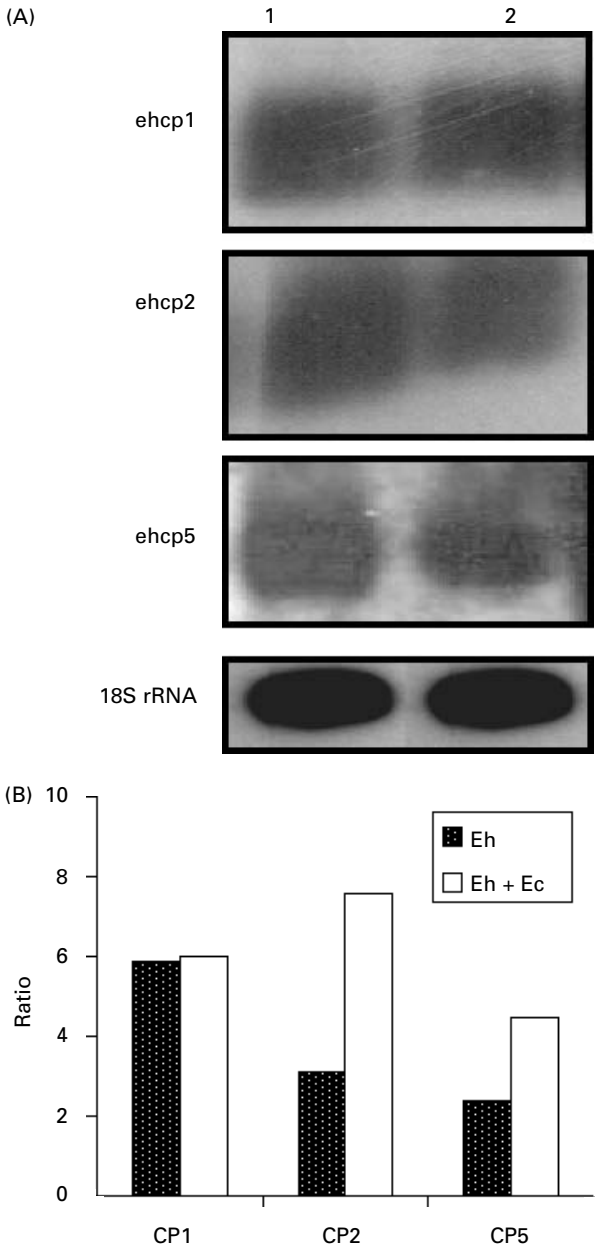

Fig. 3. Northern blot of *Entamoeba histolytica* with (Lane 1) and without (Lane 2) *Escherichia coli* co-association probed with *ehcp1*, *ehcp2*, *ehcp5* and 18S rRNA (A). Bar diagram shows ratio of band intensity of CP to 18S rRNA probe (B).

Northern blot

E. histolytica trophozoites co-associated with *E. coli* K12 strain showed a more intense band than *E. histolytica* alone, when hybridized with *ehcp2* and *ehcp5* probes; there was no difference in band intensity with *ehcp1* probe. The 18S rRNA band intensity was equal in both (Fig. 3). Densitometry analysis showed that bacterial co-association

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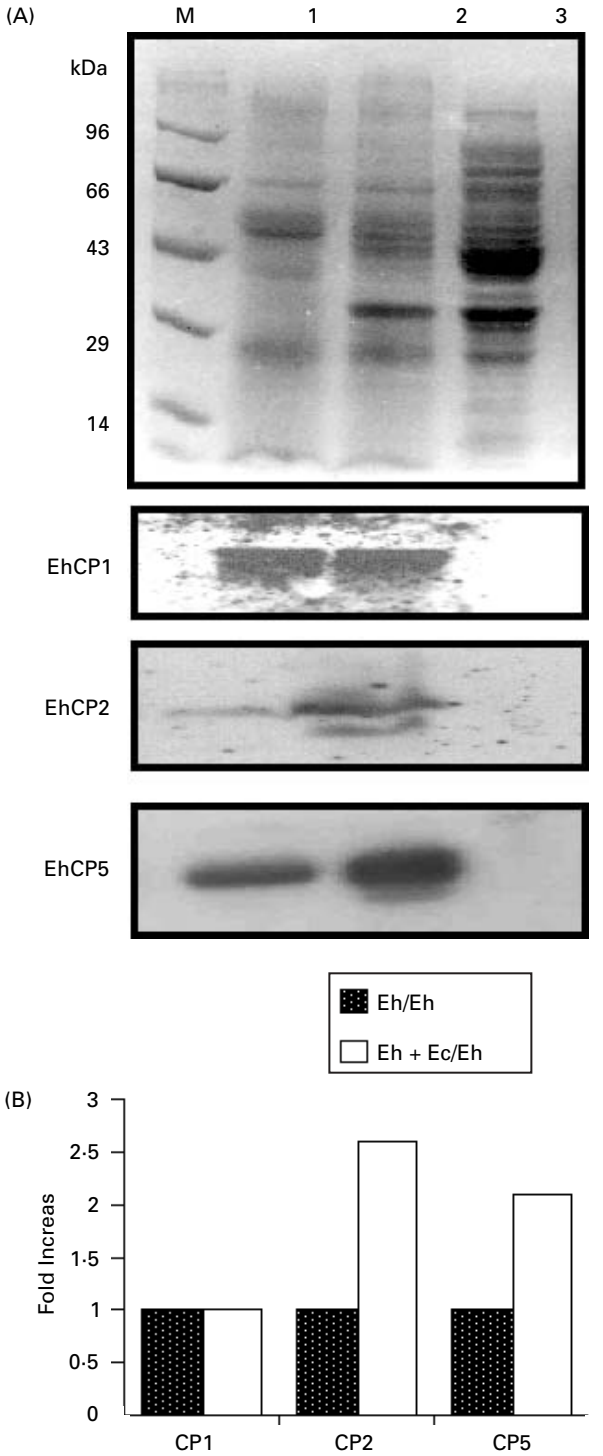

Fig. 4. SDS–PAGE (upper) and Western blot (lower) of crude lysate of *Entamoeba histolytica* without (Lane 1) and with *Escherichia coli* co-association (Lane 2). (A) Lane M is molecular weight marker. Electrophoresis was performed on 12% SDS–PAGE and probed with EhCP1, EhCP2 and EhCP5 specific antibody. Bar diagram shows ratio of band intensity of Western blot of EhCP5 *E. histolytica* co-associated with *E. coli* to *E. histolytica* alone (B).

increased *ehcp2* gene expression by 2·4-fold over that of *E. histolytica* alone; there was also a 1·8-fold increase in *ehcp5* gene expression but there was no change in *ehcp1* gene expression.

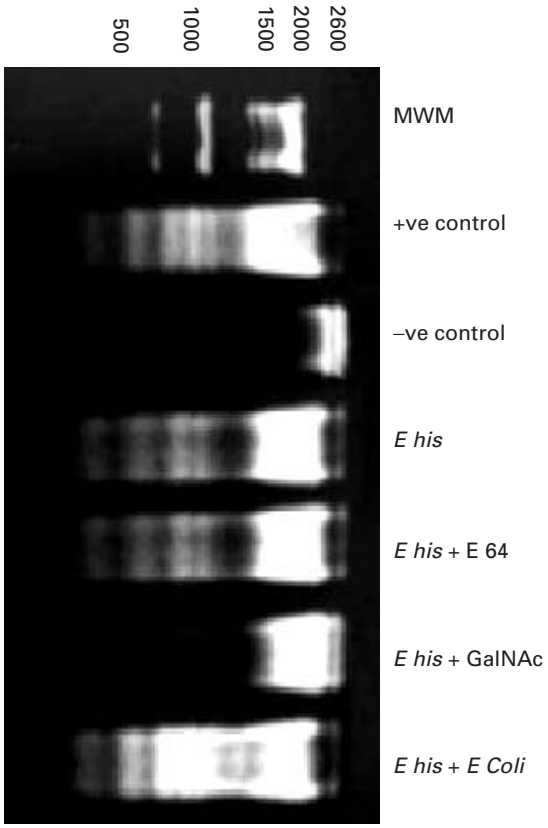

Fig. 5. DNA laddering pattern of BHK 21 cell lines run on a 2% agarose gel. DNA laddering pattern of BHK 21 cells co-cultured with *Entamoeba histolytica* (*E his*), E-64 treated *E. histolytica* (*E his* + E 64), GalNAc treated *E. histolytica* (*E his* + GalNAc) and *Escherichia coli* co-associated *E. histolytica* (*E his* + *E coli*). Positive control generated by UV treatment; negative control without any treatment.

Western blot

The translational modification in CP gene expression was assessed by Western blot, using antibodies specific to EhCP 1, 2 and 5. Expression of *E. histolytica* EhCP2 and EhCP5 was increased by co-association with *E. coli* K12 while expression of EhCP1 showed no difference. Densitometry analysis showed that *E. coli* co-association caused a 2·0-fold increase in EhCP5 and a 2·6-fold increase in EhCP2 gene expression (Fig. 4).

Chromium release assay

The cytotoxicity mediated by *E. histolytica* alone was $34{\cdot}5 \pm 3{\cdot}7\%$ and when trophozoites were pre-incubated with E-64, it was $16{\cdot}1 \pm 9{\cdot}4\%$ ($P < 0{\cdot}0001$) (Fig. 1). Trophozoites pre-treated with 1·0 M Gal/NAc showed reduced cytotoxicity as compared to untreated trophozoites ($34{\cdot}5 \pm 3{\cdot}7\%$ *vs.* $12{\cdot}6 \pm 3{\cdot}1\%$; $P < 0{\cdot}0001$) while co-association of *E. histolytica* with *E. coli* increased cytotoxicity to $57{\cdot}2 \pm 9{\cdot}6\%$ ($P < 0{\cdot}0001$). The increase seen with *E. coli* co-association was reduced to $33{\cdot}5 \pm 25{\cdot}8\%$ ($P < 0{\cdot}005$) when

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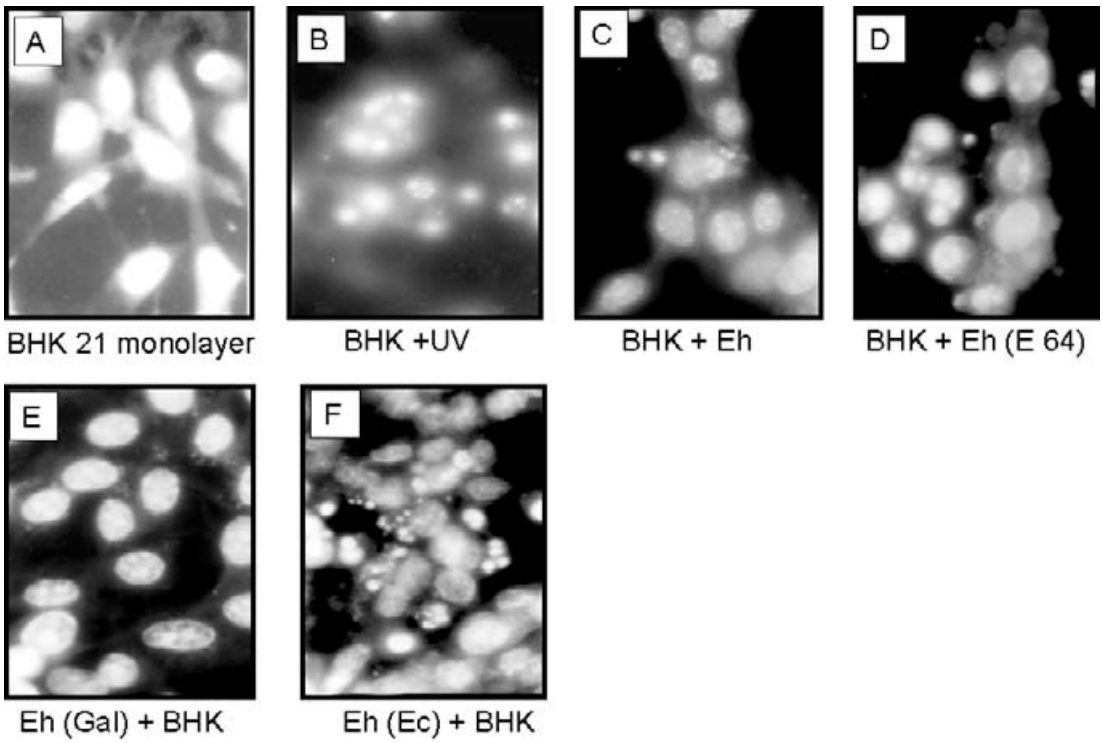

Fig. 6. Propidium iodide staining of BHK-21 monolayer. (A) BHK-21 monolayer (negative control), (B) UV-treated BHK-21 cells (positive control), (C) BHK-21 cells co-cultured with *Entamoeba histolytica* trophozoites, (D) BHK-21 cells co-cultured with *E. histolytica* trophozoites pre-treated with E-64, (E) BHK-21 cells co-cultured with *E. histolytica* trophozoites pre-incubated with GalNAc, (F) BHK-21 cells co-cultured with *E. histolytica* trophozoites co-associated with *Escherichia coli* K12 strain. (Magnifications 40 ×.)

E. histolytica was pre-incubated with E-64 before co-association. This value was similar to that of untreated trophozoites. All the experiments were performed in triplicate and repeated 5 times.

DNA fragmentation assay

BHK 21 monolayer co-cultured with *E. histolytica* trophozoites showed a DNA laddering pattern which was similar to that of positive control generated by UV. When trophozoites pre-treated with E-64 were added to BHK, the pattern of DNA fragmentation was similar to that when untreated *E. histolytica* were added. However, these cells showed reduced laddering when they were exposed to GalNAc treated trophozoites. When *E. histolytica* co-associated with *E. coli* K12 strain were co-cultured with BHK cells, smearing rather than a ladder pattern was observed (Fig. 5).

Nuclear morphology by propidium iodide staining

The BHK-21 cell line was co-cultured with *E. histolytica* trophozoites and stained with propidium iodide for nuclear morphology. The oval nuclei of BHK 21 cells showed a uniform staining (Fig. 6A), while in the positive control the nuclei appeared fragmented (Fig. 6B). Following contact with *E. histolytica* trophozoites nuclei were rounded and fragmented, as was seen in the positive control (Fig. 6C) while the DNA fragmentation was not affected by incubation of trophozoites with E-64 (Fig. 6D). Nuclear fragmentation was not seen when trophozoites were pre-incubated with GalNAc (Fig. 6E). When the BHK 21 monolayer was incubated with *E. coli* co-associated trophozoites, the change in morphology was more prominent with only a few remaining normal cells. (Fig. 6F), and with the majority of cells detached from the surface of the slide.

DISCUSSION

The bacterial flora of the intestine plays an important role in amoebic virulence. A number of studies have shown that association of axenically grown *E. histolytica* trophozoites with certain bacteria enhances its virulence (Anaya *et al.* 1997); however, the biological basis has not been well understood. We have demonstrated for the first time that short-term co-association of axenically grown trophozoites of *E. histolytica* HM1 : IMSS with *E. coli* K-12 strain results in increased CP activity, *ehcp2* and *ehcp5* gene expression and target cell cytotoxicity. This may be the mechanism for the increased virulence.

Co-association of *E. histolytica* with *E. coli* resulted in a 3·6-fold increase in E-64 inhibitable protease activity. The protease activity was of 29, 35 and 56 kDa molecular weights in substrate gels suggesting

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involvement of more than one CP. ACP3 (EhCP1) has been shown to have 2 distinct activities at 27–30 kDa (Luaces & Barrett, 1988), EhCP2 at 35 kDa, EhCP5 at 29 kDa (Hallberg *et al*. 2000) and neutral CP has been reported to have protease activity at 56 kDa (Keene *et al*. 1986). The increased CP activity following co-association therefore involved EhCP1, 2, 5 and neutral CP. We have previously shown that the activity at 56–66 kDa increases after passage of *E. histolytica* through hamster liver, which in turn was related to an increased virulence of the *E. histolytica* (Gupta *et al*. 1998).

The increased CP activity following co-association that we found was accompanied by increased expression of CP at mRNA and protein level. Northern blot analysis showed a 2·4-fold increase in *ehcp2* gene expression and a 1·8-fold increase in *ehcp5* gene expression following bacterial co-association; however, there was no difference in *ehcp1* gene expression. This was supported by the observation of an approximately 2·6-fold increase in EhCP2 and a 2·0-fold increase in EhCP5 expression of the protein. There was no increase in expression of EhCP1. EhCP5 has been shown to be the only CP beside EhCP112 which is expressed on the parasite cell surface and is not found in the non-pathogenic strain *E. dispar* (Willhoeft *et al*. 1999). EhCP2 has been reported to be involved in destruction of CHO cell targets by amoeba (Hellberg *et al*. 2001). Although there was only a 1·8-fold *ehcp5* and a 2·4-fold *ehcp2* gene transcript increase, the 3·5-fold increase in CP activity can be attributed to the increased expression of both EhCP2 and 5 genes. This has also been supported by the substrate gel which has shown bands corresponding to other proteases, i.e. EhCP1, EhCP2, EhCP5 and neutral CP. Increased virulence following co-association of axenically grown *E. histolytica* trophozoites with certain bacteria has been shown previously (Sinha *et al*. 1997; Ghosh *et al*. 1998).

Measurement of *E. histolytica*-mediated cytotoxicity by a chromium release assay, showed an increase following co-association with *E. coli* which was inhibitable by pre-treatment of the parasite with E-64. This observation is similar to the earlier report of increased cytopathic effect on a BHK-21 cell line (Bracha & Mirelmand, 1984; Sinha *et al*. 1997). When trophozoites transfected with EhCP5 antisense were evaluated for cytotoxicity effect, their functional competence was comparable to that of control parasites although 90% of the CP activity was lost. The cytopathic effect was inhibitable by E-64 suggesting that it was mediated by non-EhCP5 proteases. These transfectants did not cause abscess formation in hamsters and failed to induce gut inflammation in the SCID mouse xenograft model (Ankari *et al*. 1998, 1999; Zang *et al*. 2000). Trophozoites with over-expressed *ehcp2* had increased production of CP and increased cytopathic activity; but this increase did not increase the erythrophagocytosis or liver abscess formation (Hellburg *et al*. 2001). Thus, the available evidence indicates that cysteine proteinases are involved in causing lesions and that more than one CP is implicated. Our observations that co-association of *E. histolytica* and *E. coli* results in increased CP activity and target cell killing, both of which are E-64 inhibitable, clearly implicates the increased CP activity following bacterial co-association in the increased target cell cytotoxicity.

Killing of BHK 21 targets by *E. histolytica* was characterized by a DNA laddering pattern in BHK cells, which was not inhibited by E-64 treatment of *E. histolytica* trophozoites. However, cytotoxicity was E-64 inhibitable suggesting that cytotoxicity of target cells was not mediated by apoptosis. Both target cell cytotoxicity by *E. histolytica* trophozoites as well as DNA fragmentation and PI staining was inhibited by pre-treatment of trophozoites with Gal/GalNAc. Hence, lectin mediated cell-to-cell contact is a critical event in target cell killing. The role of amoebic lectin has been well investigated and blocking of these lectins with Gal/GalNAc has been previously shown to reduce both adherence and cytotoxicity (McCoy *et al*. 1994; Ravdin, 1986). Findings similar to ours have been reported by Huston *et al*. (2000), who showed that while Gal/GalNAc inhibited apoptosis of Jurkat cells, E-64 failed to do so.

Contact of *E. histolytica* co-associated with *E. coli* with BHK cells showed smearing rather than a ladder pattern in the DNA fragmentation assay. This may be attributed to the increased CP activity of trophozoites following bacterial co-association. It has been previously reported that target cell killing by contact was apoptotic while purified amoebapore or amoebic cell lysate causes necrotic death. (Berninghausen & Leippe, 1997).

In conclusion, we have shown that axenically grown trophozoites co-associated with bacteria result in increased CP activity of trophozoites. This is accompanied with enhanced *ehcp5* and *ehcp2* gene expression and cytotoxic capability. It may be suggested from these data that normal contact between trophozoites and host cells results in apoptotic death of the latter through lectin-Gal/GalNAc interactions. On the other hand, phagocytosis of the bacteria results in increased expression of CP and necrotic killing of the surrounding cells. The gut milieu is rich in bacterial growth and their role in amoebic disease has not been understood. How relevant our observations are to the *in vivo* situation can only be speculated upon and needs further studies in animal models for amoebiasis.

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REFERENCES

ANAYA-VELAZQUEZ, F., PADILLA-VACA, F., BARRIOS-RODILES, M., VACA-CHAVEZ, A. & GONZALEZ-ROBLES, A. (1997). Selective interaction of *Entamoeba histolytica* with lactobacilli and other intestinal bacteria. *Archives of Medical Research* **28**, 195–196.

ANKRI, S., STOLARSKY, T., BRACHA, R., PADILLA-VACA, F. & MIRELMAN, D. (1999). Antisense inhibition of expression of cysteine proteinases affects *Entamoeba histolytica* induced formation of liver abscess in hamsters. *Infection and Immunity* **67**, 421–422.

ANKRI, S., STOLARSKY, T. & MIRELMAN, D. (1998). Antisense inhibition of expression of cysteine proteinases does not affect *Entamoeba histolytica* cytopathic or haemolytic activity but inhibits phagocytosis. *Molecular Microbiology* **28**, 777–785.

BARRETT, A. J., KEMBHAVI, A. A., BROWN, M. A., KIRSCHKE, H., KNIGHT, C. G., TAMAI, M. & HANADA, K. (1982). *L-trans*-epoxysuccinyl-leucylamido (4-guanidino)butan (E-64) and its analogues as inhibitor of cysteine proteinases including Cathepsin B, H and L. *Biochemistry Journal* **201**, 189–198.

BERNINGHAUSEN, O. & LEIPPE, M. (1997). Necrosis versus apoptosis as the mechanism of target cell death induced by *Entamoeba histolytica*. *Infection and Immunity* **65**, 3615–3621.

BRACHA, R. & MIRELMAN, D. (1984). Virulence of *Entamoeba histolytica* trophozoites. Effects of bacteria, microaerobic conditions, and metronidazole. *Journal of Experimental Medicine* **160**, 353–368.

BRUCHHAUS, I., JACOBS, T., LEIPPE, M. & TANNICH, E. (1996). *Entamoeba histolytica* and *Entamoeba dispar*: differences in numbers and expression of cysteine proteinase genes. *Molecular Microbiology* **22**, 255–263.

DAS, S. R. & GHOSHAL, S. (1976). Restoration of virulence to rat of axenically grown *Entamoeba histolytica* by cholesterol and hamster liver passage. *Annals of Tropical Medicine and Parasitology* **70**, 439–443.

DIAMOND, L. S., HARLOW, D. R. & CUNNICK, C. C. (1978). A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72**, 431–432.

GADASI, H. & KOBILER, D. (1983). *Entamoeba histolytica*: Correlation between virulence and content of proteolytic enzymes. *Experimental Parasitology* **55**, 105–110.

GARCIA-RIVERA, G., RODRIGUEZ, M. A., OCADIZ, R., MARTINEZ-LOPEZ, M. C., ARROYO, R., GONZALEZ-ROBLES, A. & OROZCO, E. (1999). *Entamoeba histolytica*: a novel cysteine protease and an adhesin form the 112 kDa surface protein. *Molecular Microbiology* **33**, 556–568.

GHOSH, P. K., GUPTA, S., NAIK, S., AYYAGARI, A. & NAIK, S. R. (1998). Effect of bacterial association on virulence of *Entamoeba histolytica* to baby hamster kidney cell monolayers. *Indian Journal of Experimental Biology* **36**, 911–915.

GUPTA, S., GHOSH, P. K., NAIK, S. & NAIK, S. R. (1998). Proteinase activity and virulence of *Entamoeba histolytica* on passage though hamster liver. *Indian Journal of Medical Research* **107**, 173–177.

HELLBERG, A., NICKEL, R., LOTTER, H., TANNICH, E. & BRUCHHAUS, I. (2001). Overexpression of cysteine proteinase 2 in *Entamoeba histolytica* or *Entamoeba dispar* increases amoeba-induced monolayer destruction *in vitro* but does not augment amoebic liver abscess formation in gerbils. *Cellular Microbiology* **3**, 13–20.

HELLBERG, A., LEIPPE, M. & BRUCHHAUS, I. (2000). Two major 'higher molecular mass proteinase' of *Entamoeba histolytica* are identified as CP1 and CP2. *Molecular and Biochemical Parasitology* **105**, 305–309.

HUSTON, C. D., HOUPT, E. R., MANN, B. J., HAHN, C. S. & PETRI, W. A. Jr. (2000). Caspase 3-dependent killing of host cells by the parasite *Entamoeba histolytica*. *Cellular Microbiology* **2**, 617–625.

JACOBS, T., BRUCHHAUS, I., DANDEKAR, T., TANNICH, E. & LEIPPE, M. (1998). Isolation and molecular characterization of a surface-bound proteinase of *Entamoeba histolytica*. *Molecular Microbiology* **27**, 269–276.

KEENE, W. E., HIDALGO, M. E., OROZCO, E. & McKERROW, J. H. (1990). *Entamoeba histolytica*: correlation of the cytopathic effect of virulent trophozoites with secretion of a cysteine proteinase. *Experimental Parasitology* **71**, 199–206.

KEENE, W. E., PETITT, M. G., ALLEN, S. & McKERROW, J. H. (1986). The major neutral proteinase of *Entamoeba histolytica*. *Journal of Experimental Medicine* **163**, 536–549.

LEIPPE, M. (1997). Amoebapores. *Parasitology Today* **13**, 179–184.

LUACES, A L. & BARRETT, A. J. (1988). Affinity purification and biochemical characterization of histolysin, the major cysteine proteinase of *Entamoeba histolytica*. *Biochemistry Journal* **250**, 903–909.

LUSHBAUGH, W. B., KAIRALLA, A. B., LOADHOLT, C. B. & PITTMAN, F. E. (1978). Effect of hamster liver passage on the virulence of axenically cultivated *Entamoeba histolytica*. *American Journal of Tropical Medicine and Hygiene* **27**, 248–254.

MATTERN, C. F. T., KEISTER, D. B. & NATOVITZ, P. C. (1982). Virulence of *Entamoeba histolytica* upon continuous axenic cultivation. *Archivos de Investigacion Medica* (*Mexico*) **13**, 185.

McCOY, J. J., MANN, B. J. & PETRI, W. A. Jr. (1994). Adherence and cytotoxicity of *Entamoeba histolytica* or how lectins let parasites stick around. *Infection and Immunity* **62**, 3045–3050.

RAVDIN, J. I. (1986). Pathogenesis of disease caused by *Entamoeba histolytica*: Studies of adherence, secreted toxins, and contact dependent cytolysis. *Review of Infectious Diseases* **8**, 247–260.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

SARATH, G., DE LA MOTTE, R. S. & WANGNER, F. W. (1989). Protease assay methods. In *Proteolytic Enzymes: A Practical Approach* (ed. Beynon, R. J. & Bond, J. S.), pp. 25–I58. IRL Press, Oxford University Press, Oxford.

SCHOLZE, H. & TANNICH, E. (1994). Cysteine endopeptidases of *Entamoeba histolytica*. *Methods in Enzymology* **244**, 512–523.

SINHA, P., NAIK, S., AYYAGARI, A. & NAIK, S. R. (1997). The effect of *in vitro* bacterial association on virulence of *Entamoeba histolytica*. *Indian Journal of Medical Research* **105**, 226–270.

STANLEY, S. L. Jr., ZHANG, T., RUBIN, D. & LI, E. (1995). Role of the *Entamoeba histolytica* cysteine proteinase in amoebic liver abscess formation in severe combined immunodeficient mice. *Infection and Immunity* **63**, 1587–1590.

TOLSKAYA, E. A., ROMANOVA, L. I., KOLESNIKOVA, M. S., IVANNIKOVA, T. A., SMIRNOVA, E. A., RAIKHLIN, N. T. & AGOL, V. I. (1995). Apoptosis-inducing and apoptosis preventing function of Poliovirus. *Journal of Virology* **69**, 1181–1189.

WHO/PAN AMERICA HEALTH ORGANIZATION (1997). Expert Consultation on Amoebiasis: Amoebiasis. *WHO Weekly Epidemiological Record* **72**, 97–100.

WILLHOEFT, U., HAMANN, L. & TANNICH, E. A. (1999). DNA sequence corresponding to the gene encoding cysteine proteinase 5 in *Entamoeba histolytica* is present and positionally conserved but highly degenerated in *Entamoeba dispar*. *Infection and Immunity* **67**, 5925–5929.

ZHANG, Z., WANG, L., SEYDEL, K. B., LI, E., ANKRI, S., MIRELMAN, D. & STANLEY, S. L. Jr. (2000). *Entamoeba histolytica* cysteine proteinases with interleukin-1 beta converting enzyme (ICE) activity cause intestinal inflammation and tissue damage in amoebiasis. *Molecular Microbiology* **37**, 542–548.

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