The ways of a killer: how does Entamoeba histolytica elicit host cell death?

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Abstract

Entamoeba histolytica is the causative agent of amoebiasis in humans and is responsible for an estimated 100,000 deaths annually, making it the second leading cause of death due to a protozoan parasite after Plasmodium. Pathogenesis appears to result from the potent cytotoxic activity of the parasite, which kills host cells within minutes. The mechanism is unknown, but progress has been made in determining that cytotoxicity requires parasite Gal (galactose)/GalNAc (N-acetylgalactosamine) lectin-mediated adherence, target cell calcium influx, dephosphorylation and activation of caspase 3. Putative cytotoxic effector proteins such as amoebapores, proteases and various parasite membrane proteins have also been identified. Nonetheless the bona fide cytotoxic effector molecules remain unknown and it is unclear how the lethal hit is delivered. To better understand the basic mechanism of pathogenesis and

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to enable the development of new therapeutics, more work will be needed in order to determine how the parasite elicits host cell death.

**Introduction**

*Entamoeba histolytica* is a protozoan parasite and the causative agent of amoebiasis in humans. Parasite cysts are transmitted through contaminated food and water sources, making the incidence of disease especially high in areas of poor sanitation. *E. histolytica* is responsible for an estimated 35–50 million cases of symptomatic disease and approximately 100 000 deaths annually [1]. Infection with *E. histolytica* has a variable outcome, manifesting in asymptomatic colonization, amoebic diarrhoea, amoebic colitis, liver abscess or metastatic disease. These outcomes are not mutually exclusive, and patients may progress from one clinical manifestation to another. Invasive disease develops in 10–25% of cases, with amoebic liver abscesses most commonly seen in adult males [1]. Nitroimidazoles such as metronidazole are available to treat invasive amoebiasis, although shortcomings include toxic side effects and a need for additional drugs to cure infection in 40–60% of patients. Metronidazole resistance has not been reported in the field, but resistant clones have been obtained *in vitro*; hence there is some concern that drug resistance may ultimately emerge [2]. The continued morbidity and mortality caused by amoebiasis indicate that current therapies are insufficient.

**Epidemiology**

There are two related species, *Entamoeba dispar* and *Entamoeba moshkovskii*, that are virtually identical in morphology, with the exception that *E. histolytica* trophozoites are more likely to contain ingested erythrocytes [3]. There is extensive evidence that *E. dispar* is non-pathogenic, but whether *E. moshkovskii* is pathogenic or solely a commensal is not known. *E. dispar* is ~80–95% identical with *E. histolytica* at the nucleotide level, and the available phylogenetic analyses have consistently shown that *E. moshkovskii* is also closely related to *E. histolytica* [4]. However, the basis for the differences in virulence between these species is currently unknown. Combined, it is estimated that they are responsible for infecting approximately 500 million people, or almost 10% of the world’s population [3]. Depending on the geographical location, *E. dispar* and *E. moshkovskii* can be up to ten times more prevalent than *E. histolytica* [3], hence diagnostic tests that can distinguish between these species must be employed clinically.

The majority of morbidity and mortality due to *E. histolytica* infection occurs in Asia, the Indian subcontinent, Central and South America, and Africa. For example, in Dhaka, Bangladesh, a 40% incidence of *E. histolytica* infection per year was documented in a cohort of preschool children [5].
Children may be an especially vulnerable population for disease morbidity since associations with malnutrition, stunting and cognitive defects have been demonstrated [6]. Children with *E. histolytica* infections are about three times more likely to be malnourished and almost five times more likely to be stunted [6].

**E. histolytica and biodefence**

There are several unfortunate properties of *E. histolytica* that could be exploited for misuse in bioterrorism or war. *E. histolytica* has an extremely low infectious dose (<10 cysts), and cysts are resistant to chlorination, making transmission through municipal water supplies feasible. This was recently demonstrated in Tbilisi, Georgia, where faecal contamination of municipal water supplies resulted in *E. histolytica* transmission [7]. Infections can result in invasive lethal disease, and the ability to genetically manipulate the parasite in the laboratory may enable the development of hyper-virulent or drug-resistant strains. Owing to these properties, the NIAID (National Institute of Allergy and Infectious Diseases) Blue Ribbon Panel on Bioterrorism and its Implications for Biomedical Research has determined that the development of a Gal (galactose)/GalNAc (*N*-acetylgalactosamine) lectin prototype vaccine for amoebiasis is an immediate priority.

**Disease pathogenesis**

Following ingestion of parasite cysts, excystation occurs in the small intestine, producing eight trophozoites per cyst. The amoebic trophozoites colonize the large intestine, adhering to the colonic mucus and intestinal epithelium via the parasite surface Gal/GalNAc lectin, which binds to host carbohydrates [8]. Invasive disease outcomes, namely colitis, liver abscess and metastatic disease, are associated with vast tissue damage. For example, amoebic colitis produces characteristic flask-shaped colonic ulcers, with invasion of parasites into the lamina propria and an infiltration of neutrophils and mononuclear lymphocytes [1].

The tissue damage associated with invasive disease is likely to be largely attributable to the potent cytotoxic, or cell-killing, activity of the parasite. Indeed, this activity is literally what the parasite was named for by Schaudinn in 1903 [8a]. *E. histolytica* possesses potent cytotoxic activity towards a variety of mammalian cell types (Figure 1), including neutrophils, T-lymphocytes, macrophages and various tissue culture cell lines. However, the mechanism by which the parasite achieves host cell killing is still an enigma. As outlined below, progress has been made in defining how host cells are killed, and some putative effector proteins have been identified. To better understand the basic mechanism of pathogenesis and to enable the development of new therapeutics, more work is needed to identify the molecular players that specifically mediate host cell destruction.
Mechanism of cytotoxicity

*E. histolytica* kills target cells in a stepwise process by adhering to the target cell, inducing its death and ingesting the killed cell (Figure 2). As outlined below, adherence is mediated by a parasite surface lectin that may also function in cytotoxicity. After adherence, host intracellular calcium becomes dramatically elevated and host proteins become dephosphorylated, both of which contribute to cell death. The subsequent killing of the target cell is primarily via activation of host cell apoptosis, that is to say, the parasite ‘tricks’ the host cell into killing itself.

![Figure 1](image_url)

*Figure 1. Host cell killing and tissue destruction by* E. histolytica


![Figure 2](image_url)

*Figure 2. Schematic diagram of stepwise killing and ingestion of host cells by* E. histolytica

The parasite (blue) first attaches via its Gal/GalNAc lectin (purple) to the target cell (green). Next, *E. histolytica* induces calcium influx, tyrosine dephosphorylation and caspase 3 activation. Finally, the killed host cell is ingested.
Adherence

The parasite surface Gal/GalNAc lectin mediates binding to host carbohydrate determinants containing Gal and/or GalNAc. The Gal/GalNAc lectin has been demonstrated to mediate binding to a variety of substrates, including colonic mucin glycoproteins, neutrophils, erythrocytes, some bacteria and a variety of mammalian cell lines [8]. It is composed of a 260 kDa heterodimer of heavy (170 kDa) and light (35/31 kDa) subunits, which are disulfide-linked, together with a non-covalently linked intermediate subunit (150 kDa) (Figure 3). The intermediate and light subunits are GPI (glycosylphosphatidylinositol)-anchored, whereas the heavy subunit is an integral membrane protein. The extracellular domain of the heavy subunit contains the CRD (carbohydrate recognition domain) that mediates carbohydrate binding [8]. The precise functions of the light and intermediate subunits in adherence and cytotoxicity are unknown. Interestingly, there are multiple genes encoding each subunit that are not identical with one another, with five unlinked genes encoding the heavy subunit (89–95% identical at the amino acid level), two genes encoding the intermediate subunit (84% identical) and six or seven genes encoding the light subunit (79–85% identical) [8]. The 35 and 31 kDa bands of the light subunit are also known to have different post-translational modifications [8].

Figure 3. Subunit composition of the Gal/GalNAc lectin

The *E. histolytica* Gal/GalNAc lectin is present on the plasma membrane and consists of a heavy, intermediate and light subunit. The heavy subunit contains the CRD, and it is an integral membrane protein with a short cytoplasmic tail implicated in intracellular signalling. The heavy subunit is disulfide-bonded to the lipid-anchored light subunit. The intermediate subunit is also lipid-anchored and is non-covalently associated with the heavy–light heterodimer. Reprinted from *Annual Review of Microbiology*, vol. 56, Petri, Jr., W.A., Haque, R. and Mann, B.J., The bitter-sweet interface of parasite and host: lectin–carbohydrate interactions during human invasion by the parasite *Entamoeba histolytica*, pp. 39–64, © 2002, Annual Reviews.
Lectin-mediated adherence to target cells is clearly necessary for cytotoxicity since the addition of an excess of Gal or GalNAc prevents adherence and also prevents target cell killing [9]. Additionally, mutant cell lines that lack surface glycosylation are resistant to amoebic cytotoxicity [10]. Thus adherence to the target cell via the Gal/GalNAc lectin is probably the first step in target cell killing. Since the lectin can mediate adherence to mucin as well as host cells, the adherence to mucin may be an early step in the establishment of infection.

Besides mediating target cell contact, the lectin may also participate in killing of the target cell. Strong evidence for lectin participation in initiating killing comes from the observation that if parasites and host cells are combined in the presence of excess Gal or GalNAc, and are forced together via centrifugation, this results in adherence but not host cell killing [11]. Additionally, anti-Gal/GalNAc lectin monoclonal antibodies have been identified that block cytotoxicity without blocking adherence, suggesting that the lectin has separable roles in adherence and killing [12]. Interestingly, it has been observed that in some cases the lectin is transferred from the parasite to the target cell and this is inhibited by Gal or GalNAc, suggesting that carbohydrate engagement is necessary for lectin transfer [13]. However, although *E. histolytica* is cytotoxic to a variety of targets, lectin transfer has only been observed with epithelial cell lines, hence the significance of lectin transfer in cytotoxicity is presently unclear.

**Calcium influx**

Various inhibitors and chelators of calcium block amoebic cytotoxicity, suggesting that calcium flux contributes to target cell killing. This was first demonstrated by the finding that the slow Na+/Ca2+ channel blockers verapamil and bepridil block amoebic cytotoxicity, whereas the Na+ channel blocker tetrodotoxin does not [14]. Additionally, the extracellular chelators of calcium, EGTA and ethylene glycol, and a putative antagonist of intracellular calcium flux, TMB-8 [8-(diethylamino)octyl-3,4,5-trimethoxybenzoate], also inhibit cytotoxicity [15]. This implies a critical role for influx of extracellular calcium into the target cell. Interestingly, there may be a role for calcium flux in both the parasite and the host, since pre-treatment of either the parasite or the host with calcium channel blockers inhibits killing [14].

Elegant work using the calcium probe Fura-2 demonstrated that there is a dramatic calcium influx in the target cell following parasite contact (Figure 4) [16]. Within 30–300 s of parasite contact, target cell calcium increases from a baseline of ~40 nM to a maximum of ~1240 nM, or roughly 30-fold [16]. This increase in calcium is irreversible and precedes host cell death. Interestingly, in a monolayer of host cells, the cells adjacent to the targeted cell also demonstrate a rapid increase in calcium [16]. This calcium increase is reversed within 240 s and does not lead to cell death, suggesting that calcium influx alone is not sufficient for killing and re-emphasizing that parasite contact is essential for cytotoxicity. A critical role for the Gal/GalNAc lectin in calcium influx is demonstrated by the finding that addition of Gal prevents host cell calcium...
Interestingly, the addition of affinity purified Gal/GalNAc lectin to target cells results in a reversible calcium influx that is also inhibited by the addition of Gal, suggesting a potential role for the lectin in initiating calcium influx distinct from its role in adherence [16].

Thus there is a defined role for calcium influx in the target cell in cytotoxicity, but the role of calcium flux in the parasite itself is less clear. When Fura-2 was used to observe parasite calcium flux, calcium oscillations were detected in the head or tail regions of motile amoebae, but no regional or total changes in amoebic calcium were detected upon target cell contact [16]. Phospholipase A may play a role in cytotoxicity, since pharmacological inhibitors of this enzyme ablate cytotoxic activity. The amoebic phospholipase activity has been separated into two distinct soluble and membrane-associated activities, with the membrane-associated activity exhibiting calcium dependence [17]. Hence there could be a role for calcium-regulated phospholipase A activity associated with the parasite membrane that may contribute to parasite cytotoxicity. Finally, there may be a role for calcium in regulating the transcription of amoebic virulence genes. *E. histolytica* possesses a calcium-regulated transcription factor, URE3-BP (upstream regulatory element 3-binding protein), which modulates the expression of known virulence factors. URE3-BP mutants that constitutively bind DNA result in enhanced parasite invasion *in vivo* [18].

Figure 4. Host cell calcium influx following amoebic contact
(A and B) Time series of Fura-2-loaded Chinese-hamster ovary cells (CHO) before contact (upper panels) and 30 s after contact (lower panels) with an *E. histolytica* trophozoite. (A) Phase images and (B) digitized \( R_{340/380} \) images are shown. Scale bar, 10 μm. (C) Colour bar indicating intensity from background (dark blue) to a maximal \( R_{340/380} \) (red) of Fura-2 fluorescence. From *Infection and Immunity*, 1988, vol. 56, Ravdin, J.L., Moreau, F., Sullivan, J.A., Petri, Jr, W.A. and Mandell, G.L., Relationship of free intracellular calcium to the cytolytic activity of *Entamoeba histolytica*, pp. 1505–1512, reproduced with permission from the American Society for Microbiology.
Thus there are many potential roles of amoebic calcium ion flux in cytotoxicity that clearly warrant further study.

**Dephosphorylation**

Like a variety of other microbes, *E. histolytica* manipulates host cell tyrosine phosphorylation. A global decrease in host cell tyrosine phosphorylation is detected following parasite contact [19]. Pre-treatment of host cells with PAO (phenylarsine oxide), a cell-permeable PTPase (protein tyrosine phosphatase) inhibitor, blocks *E. histolytica*-induced tyrosine dephosphorylation [19]. Pre-treatment of amoebae with PAO does not inhibit host cell dephosphorylation, hence host PTPases appear to be responsible for the observed dephosphorylation. Importantly, pre-treatment of host cells with PAO prevented killing by *E. histolytica*, therefore dephosphorylation contributes to host cell killing [19].

Host cell dephosphorylation may be linked to the aforementioned calcium influx, since a particular calcium-regulated PTPase appears to be involved. Host PTP1B (PTPase 1B) is a PTPase that is activated by the calcium-dependent CP (cysteine protease) calpain in response to elevations in intracellular calcium. The evidence for PTP1B involvement in *E. histolytica*-induced host cell dephosphorylation is that the calpain inhibitor calpeptin inhibits host dephosphorylation [19]. Furthermore, a 42 kDa band corresponding to calpain cleavage of PTP1B is detected following parasite contact, and pre-treatment of host cells with calpeptin inhibits PTP1B cleavage. Note that PTP1B is probably not the only PTPase contributing to host cell dephosphorylation, since calpeptin is only able to inhibit dephosphorylation at extremely low amoeba:host ratios (1:50 and 1:100) [19]. Additional studies of the PTP1B–calpain pathway have shown that amoebae also induce cleavage of the calpain inhibitor calpastatin, which contributes to calpain activation [20]. Finally, there may be potential roles for the host PTPases SHP-1 (Src homology region 2 domain-containing protein tyrosine phosphatase 1) and SHP-2 in host dephosphorylation [21].

**Activation of apoptosis**

Following the observed calcium influx and dephosphorylation, the target cell succumbs to death. The mechanism of host cell killing has been controversial, but the cumulative evidence strongly supports caspase 3-dependent apoptotic death as the major cytotoxic mechanism both *in vitro* and *in vivo*. Initial *in vitro* studies using a murine myeloid cell line reported target cell DNA degradation in an apoptotic pattern, i.e. identical with the ladder of bands detected when target cells were induced to undergo apoptosis via growth factor withdrawal [22]. However, target cells most often appeared to exhibit both necrotic and apoptotic features when examined using electron microscopy, suggesting that the mechanism of killing by *E. histolytica* may not be purely apoptotic. Later studies using human myeloid cell lines (Jurkat and HL60 cells) and freshly isolated human
PMNs (polymorphonuclear cells) contradicted these findings, reporting no evidence for apoptotic killing, either morphologically or via TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) staining [23].

A clearer picture began to emerge when functional studies were used, where the effect of perturbing host apoptotic machinery was examined. These studies demonstrated that caspase 3 is activated in almost all Jurkat cells following contact with *E. histolytica*, and that cytotoxicity is blocked by the caspase 3 inhibitor Ac-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp aldehyde), hence cell killing is caspase 3-dependent (Figure 5) [24]. Inhibition of caspase 8 or 9 via genetic or pharmacological means did not inhibit parasite cytotoxicity, hence killing is caspase 8- and 9-independent [24]. Interestingly, later work demonstrated that the parasite induces apoptosis prior to phagocytosis of host cells, utilizes exposed phosphatidylserine in the recognition of apoptotic cells and preferentially phagocytoses apoptotic cells over necrotic cells [25].

Several lines of evidence demonstrate that apoptosis is critical for pathogenesis *in vivo*. In animal models of amoebiasis, TUNEL staining is observed in both liver abscesses and intestinal colitis [24,26]. Detailed studies of the liver abscess model also demonstrated DNA degradation in the abscessed region [26]. Interestingly, this apoptotic killing was not dependent on signalling

![Figure 5. Host cell caspase-3 activation following amoebic contact](image)

*Figure 5. Host cell caspase-3 activation following amoebic contact*

Time series of *E. histolytica* and Jurkat T-cells. Jurkat cells (arrows) were pre-loaded with the fluorescent caspase 3 substrate rhodamine-G1D2–rhodamine (PhiPhiLux), then incubated with *E. histolytica* trophozoites, and activation of the fluorescent reporter was monitored over time. Images collected at 0, 8, 16 and 20 min are shown. One Jurkat cell is already positive for active caspase 3 (showing green fluorescence) at time zero. The other Jurkat cells show active caspase 3 only following contact with the trophozoites. Reproduced by permission of John Wiley & Sons, Caspase 3-dependent killing of host cells by the parasite *Entamoeba histolytica*, Huston, C.D., Houpt, E.R., Mann, B.J., Hahn, C.S. and Petri, Jr, W.A., *Cellular Microbiology*, vol. 2(6), pp. 617–625, © 2000 John Wiley & Sons.
by the host Fas or TNFα (tumour necrosis factor α) receptors, since liver abscesses and DNA degradation are still detected in mutant mice lacking these receptors [26]. The broad-spectrum caspase inhibitor zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) markedly reduces the formation of liver abscesses [27], and caspase 3-knockout mice are more resistant to intestinal amoebiasis than wild-type littermates [28]. Taken together, these data implicate parasite-induced apoptosis as central to the pathogenesis of amoebiasis. Since apoptosis is potentially an immunologically silent manner of killing, this may explain the relatively weak inflammatory response observed in invasive amoebiasis despite extensive tissue destruction. However, one remaining conundrum is how the parasite is able to accomplish apoptosis within minutes, whereas apoptotic death typically requires hours to complete. This observation, together with the fact that activation of host caspase 3 is independent of Fas and TNFα receptors, caspase 8 and 9 and perhaps Bcl-2 [22,24,26], emphasize that *E. histolytica* may induce a non-classical mechanism of apoptosis.

**What are the cytotoxic effectors?**

How does *E. histolytica* elicit host cell death? The cumulative data demonstrate that target cell killing requires lectin-mediated contact, calcium influx and dephosphorylation of the target cell, and ultimately, caspase 3-dependent killing. However, the specific cytotoxic mechanism remains elusive: i.e. what are the cytotoxic effector molecules and how is the lethal hit delivered? Killing appears to be an active, regulated, process since killed parasites are not cytotoxic (W.A. Petri, Jr, unpublished work) and cytotoxicity requires parasite cytoskeletal remodelling [9]. There does not appear to be an isolated toxin, since parasite detergent extracts are not cytotoxic (W.A. Petri, Jr, unpublished work). Hence, these data support a model where cytotoxic effectors are secreted in a regulated, contact-dependent, manner.

As outlined below, several candidate *E. histolytica* cytotoxic effectors have been implicated in cytotoxicity, but thus far direct evidence for their roles in cytotoxicity is lacking. It will be important to identify the *bona fide* effectors, both to understand the basic mechanism of pathogenesis and to enable the identification of new targets for therapeutic intervention.

**Amoebapores**

The first group of proteins implicated in cytotoxicity are the pore-forming proteins known as the amoebapores. *E. histolytica* possesses three amoebapores, A, B and C, that have sequence similarity to the mammalian membrane-permeabilizing proteins NK-lysin and granulysin [29]. Amoebapore A was originally purified and identified on the basis of its pore-forming activity [30], and indeed all three amoebapores are active on synthetic liposome substrates [29]. Interestingly, amoebapores are only active at low pH (~5.2) [29,30]. On the basis of the solution structure of amoebapore A, it appears
that there is a pH-dependent dimerization event that may act as a molecular switch, with a prediction that this dimerization event allows for the formation of active oligomer pores only at low pH (Figure 6) [31].

It is unclear whether amoebapores are physiologically active on gut bacteria, host cells or both. Amoebapores may play a role in degrading ingested gut bacteria or may play a role in killing host cells prior to their ingestion. Detailed in vitro studies using purified amoebapores demonstrated that all three amoebapores are bactericidal to Gram-positive bacteria at low nanomolar concentrations [29]. There are some differences in the activities of the different amoebapores on different bacterial species, and all three amoebapores are much less active on Gram-negative bacteria than on Gram-positive bacteria [29]. In contrast, purified amoebapores are cytotoxic to eukaryotic cells (Jurkat or U937) at concentrations of more than 10–100 μM [32]. Host cell DNA degradation is not observed [23], suggesting that either the purified amoebapores act by a different mechanism than intact amoebae, or that the purified amoebapores are not sufficient to induce caspase 3-dependent host cell death.

Co-localization studies demonstrate partial co-localization of amoebapore A with ingested bacteria, supporting a potential role for amoebapores in breaking down bacteria [29]. There are currently no data that demonstrate transfer of amoebapores to targeted host cells. In light of these observations, the pH-dependence of the amoebapores is noteworthy. If amoebapores play a
role in the degradation of ingested bacteria, they are likely to encounter a low pH favourable to their activity in the phagolysosome. In contrast, if amoebapores are active on host cells, this would require a low-pH environment, such as a tight synapse between parasite and host that maintains an acidic pH. Whether such synapses form is unknown.

Amoebapore A has been silenced by both antisense and epigenetic approaches [33–36]. However there are major shortcomings to these approaches: both work by unknown mechanisms and have unanticipated ‘off-target’ effects such that the expression of other genes is altered [33]. Thus it is not possible to definitively attribute phenotypic effects in the silenced strains to the specific silencing of the targeted amoebapore gene. Furthermore, in the case of the antisense approach, extremely high levels of drug selection were required [34] that may have created phenotypic artefacts. Nonetheless, in the mouse model, a decrease in the occurrence of liver abscess and/or liver abscess size was noted with both the antisense and epigenetically silenced strains [34,35]. In contrast, the epigenetically silenced strain did not exhibit virulence attenuation in the SCID-HU-INT model of intestinal amoebiasis [37]. Defects in cytotoxicity were noted in vitro, using Trypan Blue exclusion as a readout; however, detailed studies of cytotoxicity towards host cells (e.g. measurements of caspase 3 activation) were not carried out [33–36]. However, since the approaches that were used to silence amoebapore A expression affected other genes, the precise roles of the amoebapores in cytotoxicity and virulence remain unknown.

Other SAPLIPs (saposin-like proteins)
Amoebapores belong to the family of SAPLIPs. SAPLIPs are diverse in primary sequence, but contain a conserved motif of six cysteines that mediate the formation of three disulfide bridges [38]. SAPLIP family members are also diverse in function, ranging from mammalian antimicrobial proteins (e.g. NK-lysin and granulysin) to enzymes (e.g. acyloxyacyl hydrolases and plant aspartic proteases) to enzyme cofactors involved in lipid metabolism (e.g. saposins A–D), but their common feature is association with lipids [38]. Besides the amoebapores A, B and C, 16 additional putative SAPLIPs have been identified in the *E. histolytica* genome [39,40]. Most of these predicted proteins share less than ~30% identity to amoebapore A, although given the typically diverse sequences of SAPLIPs, this is not unexpected. One protein, designated SAPLIP 1, shares 64% identity with amoebapore A [40], which exceeds the percentage identity of amoebapores B and C to amoebapore A (57% and 46% respectively) and thus may represent an additional member of the amoebapore family.

Currently, very little is known about the putative SAPLIP genes in *E. histolytica*. RT (reverse transcription)–PCR analysis demonstrated that all 16 genes are transcribed in vitro [39,40]. Additional characterization has only been carried out for SAPLIP 3 [40]. This protein has been recombinantly expressed and purified from *E. coli*. The purified protein did not exhibit
bactericidal activity in vitro, and tests of potential cytotoxic activity towards eukaryotic cells were not performed [40]. In assays using synthetic liposomes, SAPLIP 3 did not possess pore-forming activity, but did possess membrane-fusogenic activity [40]. Thus it is possible that the other SAPLIP family members may not possess pore-forming activity like the amoebapores. Indeed, it was noted that during the original purification of amoebapore A, no other E. histolytica pore-forming activity was detected [30]; however, it is possible that other pore-forming proteins were not active in the purification conditions and/or their activities may have been below the detection limit. Further studies will be needed to probe the functions of the SAPLIP proteins and to determine whether they function in bacterial and/or host cell killing or if they perform other cellular functions.

**CPs**

E. histolytica possesses ~50 CPs [41]. These proteases act on a variety of host substrates including mucin, villin, laminin, collagen, proteoglycan and an ECM (extracellular matrix) from vascular smooth muscle (e.g. [42,43]). Therefore they may function in tissue invasion by degrading mucus and ECM and may not necessarily function in cytotoxicity per se. Several studies have concluded that CPs contribute to cytotoxicity [44–46], but these data should be interpreted with caution. In these studies, cytotoxic activity of CP overexpressors was measured by pre-loading host monolayers with Methylene Blue, incubating with the parasite, washing and measuring the percentage of dye remaining [44–46]. This assay does not distinguish between host cell monolayer release (reflecting ECM degradation) and host cell death (reflecting cytotoxicity). Therefore additional studies using reliable readouts such as caspase 3 activation are needed. Finally, in vivo studies of EhCP5 (E. histolytica CP5) overexpressors reported that liver abscess formation was modestly increased as compared with wild-type controls, whereas EhCP1 or EhCP2 overexpression had no discernable effect [44,46]. Hence at least one cysteine protease has some role in pathogenesis, but it is not known whether these proteases specifically function in cytotoxicity.

**Other potential effectors**

Besides the amoebapores and cysteine proteases, several additional proteins have been characterized as potential effectors. The proteins themselves are diverse, but a common theme is that they are all membrane proteins, with two of them associating with the Gal/GalNAc lectin, hence it is difficult in some cases to distinguish between a role in host cell adhesion versus a role in host cell killing. The two lectin-associated proteins implicated in cytotoxicity are the 35 kDa light subunit of the Gal/GalNAc lectin itself [47] and the surface-localized thiol-dependent peroxidase [48]. Although the Gal/GalNAc lectin mediates adhesion, antisense inhibition of the 35 kDa light subunit did not inhibit parasite adhesion to BHK (baby hamster kidney) monolayers, but did inhibit
killing of BHK cells, as assessed by Trypan Blue exclusion and also decreased liver abscess size in infected hamsters [47]. Antisense inhibition of the surface-localized thiol-dependent peroxidase also inhibited killing of BHK cells, as assessed by Trypan Blue exclusion and decreased liver abscess size in hamsters [48]. Adhesion was not examined in this case, hence it remains possible that the antisense-inhibited cells have adhesion defects that prevent host cell killing [48].

The membrane proteins implicated in cytotoxicity that have not been reported to be lectin-associated are the family of EhSTIRPs (*E. histolytica* serine-, threonine- and isoleucine-rich proteins) [49], EhTMKB1-9 (*E. histolytica* transmembrane kinase B1-9) [50] and KERP1 (lysine- and glutamic acid-rich protein 1) [51,52]. The family of five EhSTIRP genes was silenced using dsRNA (double-stranded RNA) directed to a common 3′ sequence [49]. Defects in both adhesion and cytotoxicity were noted [49], thus the EhSTIRPs may be required for adhesion to host cells and may not be required for host cell killing, or alternatively they may be required for both processes. Similarly, when EhTMKB1-9 was inhibited by antisense expression, defects in both adhesion and monolayer destruction were noted [50]. Therefore, like the EhSTIRPs, EhTMKB1-9 may function in adhesion. These data should be interpreted with caution, since a monolayer destruction assay was employed that does not specifically assay host cell killing [50].

Finally, KERP1 has been identified as a parasite membrane protein that also binds host cell membranes and therefore may be transferred to target cells [51]. Silencing of KERP1 via antisense inhibition was attempted, but no significant decrease in KERP1 mRNA or protein was detected [51]. A decrease in liver abscess formation in a hamster model was noted, but given the lack of inhibition of KERP1 [51], the significance of this phenotype is unclear. Although a potential role in cytotoxicity is implied by the affinity of KERP1 for host cell membranes, no specific role in host cell killing has yet been experimentally demonstrated.

**Acid vesicle components**

Intriguingly, *E. histolytica* possesses a class of acid intracellular vesicles (pH ~5.4) that appear to play a role in cytotoxicity [53]. Treatment of amoebae with weak bases such as NH₄Cl, or the lysosomotropic drugs primaquine or chloroquine blocks cytotoxicity substantially, such that up to 70% of cells remain viable in a Trypan Blue exclusion assay [53]. Neither base treatment nor drug treatment appeared to alter amoebic viability, protein synthesis or adherence to target cells [53]. Hence there appears to be a critical role for acidic vesicles in cytotoxicity. It is possible that amoebic cytotoxicity is analogous to the mechanism employed by mammalian CTLs (cytotoxic T-lymphocytes), which possess acidic intracellular ‘granules’, pre-loaded with a pore-forming protein (perforin) and proteases (granzymes). However, more work will be needed to determine whether these hypotheses hold true, specifically focusing on identifying vesicle contents and determining if they are involved in target cell death.
Conclusions

_E. histolytica_ is the cause of significant morbidity and mortality in the developing world, with children bearing an enormous burden of developmental consequences resulting from infection. Disease pathogenesis appears to result directly from the potent cytotoxic activity of the parasite. Much progress has been made in demonstrating that host cell killing is primarily via apoptosis and that this apoptotic endpoint requires lectin-mediated host cell attachment, host calcium influx and dephosphorylation. The fact that inhibition of apoptosis _in vivo_ markedly inhibits both invasive colitis and liver abscess formation emphasizes that parasite activation of host apoptosis is fundamental to disease pathogenesis.

Although some putative cytotoxic effectors have been characterized, the _bona fide_ cytotoxic effectors remain unknown and it is unclear how the lethal hit is delivered. Much more work will be needed to identify the effectors and to determine their modes of action. Since _E. histolytica_ appears to induce a non-classical mechanism of apoptosis, any novel effectors will represent candidate drug targets that may offer new hope for therapeutic intervention in amoebiasis. Improved knowledge of the effectors will also clarify the basic mechanism of disease pathogenesis, which may inform the ongoing development of an _E. histolytica_ vaccine. Such work may also shed light on strain-specific differences in amoebic virulence determinants. Since there is an emerging appreciation that infection is extraordinarily common, and is a major cause of morbidity and mortality for children in developing countries, future studies of amoebic cytotoxicity have promise to have a positive impact on global health.

Summary

- _E. histolytica_ possess potent cytotoxic activity that appears to underlie the pathogenesis of invasive disease.
- The parasite Gal/GalNAc lectin mediates host cell attachment and may also play a role in initiating a cytotoxic programme.
- _E. histolytica_ induces massive calcium influx in the targeted host cell that is required for host cell killing. Calcium flux in the parasite itself may also play a role in modulating virulence/cytotoxic activity.
- _E. histolytica_ induces global tyrosine dephosphorylation in the target cell that is required for killing.
- The mechanism for host cell killing is primarily via activation of apoptosis. Inhibition of apoptosis blocks amoebic cytotoxicity in vitro and inhibits disease pathogenesis _in vivo_.
- The amoebapores and other saposin-related proteins are putative cytotoxic effectors that may act on the target cell membrane. However it is unclear whether these proteins are cytotoxic effectors or if they are simply involved in breaking down ingested bacteria.
• Cysteine proteases also represent putative cytotoxic effectors, but they act on a variety of host cell substrates and a specific involvement in cytotoxicity has not been demonstrated.
• Other potential effectors have been characterized, and a role for acid vesicle components has been implied, but no bona fide cytotoxic effectors have yet been identified.

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References

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