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# Pathogenesis of infection by *Entamoeba histolytica*

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**Abstract.** ***Entamoeba histolytica,*** **a protozoan parasite, is the etiologic agent of amoebiasis in humans. It exists in two forms—the trophozoite which is the active, dividing form, and the cyst which is dormant and can survive for prolonged periods outside the host. In most infected individuals the trophozoites exist as commensals. In a small percentage of infections, the trophozoites become invasive and penetrate the intestinal mucosa, causing ulcers. The trophozoites may reach other parts of the body—mainly liver, where they cause tissue necrosis, leading to life-threatening abscesses. It is thought that pathogenesis of infection by *Entamoeba histolytica* is governed at several levels, chief among them are (i) adherence of trophozoite to the target cell, (ii) lysis of target cell, and (iii) phagocytosis of target cell. Several molecules which may be involved in these processes have been identified. A lectin inhibitable by galactose and N-acetyl-D-galactosamine is present on the trophozoite surface. This is implicated in adherence of trophozoite to the target cell. Various amoebic pore-forming proteins are known, of which 5kDa protein (amoebapore) has been extensively studied. These can insert into the lipid bilayers of target cells, forming ion-channels. The phagocytic potential of trophozoites is directly linked to virulence as measured in animal models. Factors like association of bacteria with trophozoites also influence virulence. Thus, pathogenesis is determined by multiple factors and a unifying picture taking into account the relative contributions of each factor is sought. Recent technical advances, which includes the development of a transfection system to introduce genes into trophozoites, should help to understand the mechanism of pathogenesis in amoebiasis.**

**Keywords.** ***Entamoeba histolytica;*** **pathogenesis; amoebiasis.**

## 1. Introduction

The protozoan parasite, *Entamoeba histolytica,* is the causative agent of amoebiasis in humans. According to the best estimates (Walsh 1986) approximately 48 million individuals suffer from amoebiasis throughout the world. In 1984, at least 40,000 deaths were attributed to amoebiasis. Amoebiasis is a major problem in developing countries such as India. This is primarily because of inadequate sanitation and contaminated food and drinking water.

Pathogenesis of amoebiasis is believed to be a multistep, multifactorial process. Though a large number of studies have attempted to unravel the factors/molecules responsible for the pathogenesis of amoebiasis, the processes involved in pathogenesis are by no means well understood. The aspects of pathogenesis which have been investigated experimentally can be broadly categorized into mechanisms involving (i) interactions with the intestinal flora, (ii) lysis of target cell by direct adherence, (iii) lysis of target cell by release of toxins and (iv) phagocytosis of target cells. Each of

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these will be discussed after a brief description of the life cycle of *E. histolytica* and pathology of amoebiasis.

## 2. Life cycle of *E. histolytica*

The organism exists in two forms—the trophozoite or the dividing form and the cyst which is the dormant form. Human infection usually begins with the ingestion of the cyst which is present in food and/or water contaminated with human fecal material. Cysts survive the acidic pH of the stomach and pass into the intestine. In the ileo-cecal region, cysts undergo excystment and each cyst gives rise to eight trophozoites. These migrate to and multiply in the colon. In most cases, trophozoites in the intestine live as commensals. Occasionally, however, trophozoites attack and invade the intestinal mucosa causing dysentery and/or progress through the blood vessels to extra-intestinal locations like liver, brain and lungs, where they may form life-threatening abscesses. In the intestine, many of the trophozoites encyst and produce quadrinucleated cysts. Both trophozoites and cysts are excreted along with the feces. Cysts can survive for prolonged periods outside the host while the trophozoites survive only for a few hours. Trophozoites play no role in transmission of the disease but are responsible for producing tissue pathology. The reservoir of human infection is the "carrier" or asymptomatic human host who continuously passes cysts.

## 3. Pathology

Amoebic infection of the human intestine ranges in spectrum from luminal colonization to mucosal invasion (Joyce and Ravdin 1988). Initially trophozoites are found in the intestinal lumen and within mucosa (Brandt and Perez-Tamayo 1970). Following attachment to interglandular epithelium, the trophozoites have been found associated with the microulcerations of the mucosa. Symptoms at this stage include non-specific colitis with edematous mucosa and hemorrhage (Pittman and Henniger 1974). Following attachment of amoeba, there is considerable disintegration of epithelial cell layer followed by invasion of submucosa. The human inflammatory response to amoebic invasion is poor. This may be because *E. histolytica* can lyse inflammatory cells (Guerrant *et al* 1981; Salata *et al* 1985). With time the ulcer extends into *lamina propria* and further into *muscularis mucosa,* where progress usually stops prior to perforation. A plug of necrotic debris accumulates at the center of the ulcer. Trophozoites are found in the leading edge at the base of the ulcer (Brandt and Perez-Tamayo 1970; Prathap and Gilman 1970). Ulcers are typically "flask-shaped" (Brandt and Perez-Tamayo 1970). Inflammatory response may be seen at the edges of the ulcers and involves mononuclear and giant cells with few neutrophils (Brandt and Perez-Tamayo 1970; Pittman *et al* 1973). Ulceration of mucosa is the hallmark of invasive disease. Ulcers develop more frequently in caecum and ascending colon. In about 20% of acute colitis cases, perforations occur which results in peritonitis (Brandt and Perez-Tamayo 1970). Chronic ulceration results in the formation of a proliferative tuft of remaining mucosa that appears as a mass (termed amoeboma) in the lumen (Brandt and Perez-Tamayo 1970; Prathap and Gilman 1970). Occasionally, trophozoites reach the liver by portal venules or intestinal perforation and produce abscesses. Liver abscesses, which may be

up to 10 cm in diameter, occur more frequently in the right lobe. Dead cells are seen in the center of the abscess whereas the trophozoites are found on the periphery. Bacteria are conspicuous by their absence in the abscesses. Ninety five per cent deaths in amoebiasis are due to liver abscess (Brandt and Perez-Tamayo 1970).

## 4. Pathogenesis

The major limitation one faces in studying pathogenesis is the lack of a satisfactory animal model which can duplicate the spectrum of human disease. Nonetheless several species have been used as animal models to study various aspects of pathogenesis (Meerovitch and Chadee 1988). For example, hamsters and gerbils are most commonly used as models for liver disease. Trophozoites produce lesions when injected directly into the liver of these animals. *In vitro* models are also available for studying various steps involved in pathogenesis (Petri and Ravdin 1988). For example, adherence can be scored by using Chinese hamster ovary (CHO) cells, erythrocytes or bacteria. Lysis can be scored as per cent cell culture monolayers disrupted. The number of erythrocytes ingested per trophozoite can be used as a measure of phagocytosis. One or more experimental approaches have been taken to study the killing of target cells by *E. histolytica* trophozoites. The processes/interactions which are thought to influence, or are implicated in, pathogenesis are described below.

### 4.1 *Colonization and interaction with the intestinal flora*

In the gut the trophozoites are constantly interacting with the intestinal flora. Studies have shown that trophozoites undergo changes on interacting with bacteria. Axenic *E. histolytica* which have lost virulence can regain it if associated with bacteria like *Escherichia coli, Salmonella typhosa* or *S. paratyphi.* Bacterial strains which do not attach to, and get ingested by, trophozoites do not affect virulence (Bracha *et al* 1982). Virulence of trophozoites of strain 200:NIH varied depending on culture associates. When cultured with NRS bacteria or rabbit intestinal flora, these trophozoites caused acute disease in animals but very little disease when cultured with *Trypanosoma cruzi.* Reassociation with rabbit flora returned their infectivity. Wittner and Rosenbaum (1970) showed that direct association of *E. histolytica* with viable bacteria was required for virulence. Heat killed or glutaraldehyde-fixed bacteria do not increase virulence. Soluble bacterial factors were not implicated. Bracha and Mirelman (1984) showed that *E. histolytica* exposed to live bacteria (that are known to adhere amoeba) for 30 min, increased in virulence in *in vivo* measurement, however it appears that association with bacteria is not an absolute requirement for invasion by *E. histolytica.* Association of specific bacteria with *E. histolytica* could change the architecture of the cell surface leading to altered properties of the cell (Bhattacharya *et al* 1992a).

### 4.2 *Adherence to establish direct contact between trophozoite and target cell*

Adherence of trophozoites to target cells is a necessary prerequisite for cytotoxicity. Evidence for this is provided by the following observations. Cinemicrography of amoeba interacting with CHO cells on a glass coverslip showed that the CHO cells in

direct contact with amoeba displayed membrane blebbing and release from cover slip, while those not in direct contact, remained viable. When CHO cells and trophozoites were mixed and incubated in the presence of high molecular weight dextran (10%), lysis did not occur as dextran prevented adherence of trophozoites to target cells (Ravdin and Guerrant 1981). In another experiment erythrocytes and trophozoites were mixed so as to allow adherence. Cells were centrifuged through a Ficoll gradient. Trophozoites that banded on top of the gradient had not adhered to erythrocytes. These were found to be much less virulent in a hamster liver model.

Adherence to CHO cells at 37°C is inhibited by cytochalasins B and D, implicating the need for intact amoebic microfilament function in the process (Ravdin and Guerrant 1981). Adherence is also inhibited by the $Ca^{2+}$ channel blocker, Bepridil possibly by preventing intracellular $Ca^{2+}$ flux which is thought to be necessary for microfilament function (Ravdin *et al* 1985b).

Two surface molecules responsible for adherence have been identified—one inhibitable by galactose or N-acetyl-D-galactosamine (GalNAc) (Bracha and Mirelman 1983; Petri *et al* 1987; Ravdin and Guerrant 1981; Ravdin *et al* 1985c) and the other inhibitable by N-acetyl-D-glucosamine (GlcNAc) polymers (Kobiler and Mirelman 1981). Pretreatment of amoeba with galactose or GalNAc inhibits adherence whereas pretreatment with neuraminic acid, maltose, mannose and GlcNAc has no effect. The Gal/GalNAc inhibitable lectin of *E. histolytica* has been characterized in considerable detail (reviewed in McCoy 1994). The following data suggest that this molecule plays an essential role in amoebic adherence to target cells (i) binding of trophozoites to CHO cells was inhibited 90-95% by 50 mM galactose and GalNAc while other sugars had no effect (Chadee 1987, 1988; Ravdin and Guerrant 1981; Ravdin *et al* 1985a; Salata *et al* 1985a; Salata and Ravdin 1986), (ii) a mutant of CHO cell defective in production of N- and O-linked galactose-terminal oligosaccharides was almost completely resistant to adherence, (iii) complex branched polysaccharides containing galactose groups at their termini were 1,000-fold more effective by weight than galactose, in inhibiting adherence to CHO cells (Petri *et al* 1987). The lectin has a molecular weight of 260 kDa and dissociates into heavy (170 kDa) and light (35-31 kDa) subunits in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Petri *et al* 1989). Three genes (*hgl* 1-3) encoding the 170 kDa subunit have been identified and characterized (Mann *et al* 1991; Purdy *et al* 1993; Tannich *et al* 1991). Analysis of deduced amino acid sequences of the three genes indicate that this subunit of the lectin is a transmembrane protein. Northern blot analyses show that all the three genes are expressed in *E. histolytica* and the mRNAs were of the same size (4·0 kb) (Mann *et al* 1991; Purdy *et al* 1993; Tannich *et al* 1991). Two light subunit genes (*lgl* 1-2) have also been identified and characterized (McCoy *et al* 1993a, b; Tannich *et al* 1992). These genes have hydrophobic amino- and carboxy-terminal signal sequences. The 31 kDa isoform of the light subunit has a putative glycosylphosphatidylinositol (GPI) anchor cleavage/addition site while the 35 kDa isoform seems to lack it. Lectin heterodimers have been identified by two dimensional gel electrophoresis. The purified lectin showed atleast two major heterodimers, one containing the 170 kDa subunit.with 35 kDa isoform and another 170 and 31 kDa isoform. Minor heterodimers with 160 and 150 kDa heavy subunit isoforms were also present (McCoy *et al* 1993b). The native lectin probably exists as oligomers of 400 kDa and 660 kDa. Apart from its function in adherence the lectin appears to mediate amoebic resistance to complement lysis.

### 4.3 *Lysis of target cells by release of toxins and introduction of membrane channels*

Prior to mucosal invasion by *E. histolytica* there is depletion of mucous and disruption of epithelial barrier. Cytolysis of the target cell is thought to require amoebic microfilament function, $Ca^{2+}$ flux and phospholipase A, among others. Microfilament function seems to be necessary because lysis is inhibited at 25°C, a temperature at which actin gelation ceases (Pollard 1976); the optimal temperature being 37°C. Studies with the $Ca^{2+}$-binding fluorescent dye FURA-2 showed 20-fold increase in intracellular $Ca^{2+}$ in target cells within seconds of direct contact. Actual cell death occurred 5-15 min after the lethal hit. Possible roles of $Ca^{2+}$ are in contact-dependent release of cytotoxic enzymes and toxins, cytoskeletal changes and activation of $Ca^{2+}$-dependent enzymes, for example, phospholipases.

Bos (1979) proposed that *E. histolytica* has two ways of killing host cells—one is a rapid process occurring at close contact; other is slow, operating through soluble substances. Contact-dependent cytolethal effect of *E. histolytica* is not inhibited by serum but contact-independent effect is inhibited. Lushbaugh *et al* (1978a, b) showed that cell-free extracts from axenically grown trophozoites caused cytopathic effect on cell cultures, in the absence of serum. Lushbaugh *et al* (1979) and Bos (1979) independently purified a "cytotoxic" substance from trophozoite extracts which caused cell rounding and release from monolayer. The activity was associated with a protein (34-40 kDa) activated by thiols (Bos *et al* 1980). It is believed that these thiol-proteases may be one of the molecules involved in pathogenesis (McKerrow 1993). This is based on the fact that there seems to be a correlation between clinical severity with the level of thiol protease in clinical isolates (Reed *et al* 1989). HM- 1:IMSS (more virulent of the two strains) has greater thiol protease activity than HK-9 strain (Gadasi and Kobiler 1983; Lushbaugh *et al* 1989). Patients with invasive disease produce antibodies against this enzyme; those with non-invasive disease do not (Reed *et al* 1989). The enzyme has broad substrate specificity. It can utilize casein, gelatin, insulin, type I collagen, fibronectin and laminin as substrates (Keene *et al* 1986; Luaces and Barrett 1988; Scholze and Schulte 1988; Scholze and Werries 1986; Schulte *et al* 1987). It is a cathepsin B-like enzyme. Similar enzymes are found in extracellular milieu of invasive tumour cells (Lushbaugh 1988). The protease may assist trophozoite to gain access to target cells by degrading the extracellular matrix.

A candidate for the toxin responsible for cytolysis may be a pore-forming peptide. Various amoebic pore-forming proteins (30, 14 and 5 kDa proteins) have been described (Dodson and Petri 1994). A 30 kDa amoebic protein was purified and shown to lyse erythrocytes and insert into and create pores in lipid bilayers. A 14 kDa pore-forming protein was described as an ion-channel forming protein. Of these the 5 kDa protein (amoebapore) has been the best characterized (Leippe *et al* 1991, 1992). The primary structure of the 5 kDa amoebapore from pathogenic *E. histolytica* was determined by sequencing the purified peptide and the corresponding cDNA. It is composed of 77 amino acids, including 6 cysteine residues. Like other membrane-penetrating polypeptides, it too has an all α helical conformation.

The cellular immune response of the host may contribute to destruction of the local host tissue. In hamster liver model recruitment of neutrophils is the initial host response to *E. histolytica* infection (Tsutsumi *et al* 1984). Neutrophils are lysed when they come in contact with *E. histolytica* trophozoites releasing toxic products which lyse distant hepatocytes (Salata and Ravdin 1986).

Leukocytes have the potential to lyse *E. histolytica* trophozoites and *vice versa. E. histolytica* is cytolytic to human leukocytes on contact. Only virulent amoeba can lyse polymorphonuclear leukocytes (PMNs) and lysis is blocked by GalNAc. At a ratio of 1000 PMNs per amoeba, trophozoites of the highly virulent strain HM-1:IMSS were not killed but those of the less virulent strain 303 were killed (Guerrant *et al* 1981). At a ratio of 100 PMNs per amoeba, HM- 1:IMSS trophozoites killed a high percentage of PMNs while killing was less with 303 trophozoites. *E. histolytica* could kill macrophages and T lymphocytes *in vitro*. Conversely, macrophages activated with concanavalin A could kill amoeba. T lymphocytes from immune individuals, following incubation with amoebic antigen, were capable of killing *E. histolytica* trophozoites (Salata and Ravdin 1985b).

### 4.4 *Phagocytosis*

Trophozoites from stools of many invasive patients contain ingested erythrocytes and have much higher rate of erythrophagocytosis than healthy human carrier. Phagocytosis of mammalian tissue culture grown cells was observed by transmission electron microscopy. Cells with intact plasma membrane were phagocytosed, showing that prior cell lysis was not required for endocytosis (McCaul 1977). A phagocytosis-deficient mutant of *E. histolytica* has been isolated by Orozco *et al* (1983). This mutant apart from being poor in phagocytosis, was also found to be low in virulence, when tested in the hamster liver model. Thus there seems to be a correlation between phagocytosis and virulence.

## 5. "Pathogenic" vs "nonpathogenic" amoebae

The vast majority of *E. histolytica* infections are asymptomatic. Are these caused by nonpathogenic strains of the parasite, in contrast with invasive infection resulting from pathogenic strains; or are all *E. histolytica* strains pathogenic and host factors decide the course of infection. If strain differences exist, then the molecular basis of pathogenicity could be elucidated by looking for missing functions in non pathogenic strains. Isoenzyme comparisons (zymodemes) of *E. histolytica* grown from asymptomatic cyst passers and patients suffering from invasive disease showed that clearly different parasite strains were involved (Sargeaunt *et al.* 1978). Infact, the strains found in asymptomatic individuals (nonpathogenic) were so distant from pathogenic strains that they have now been accorded a separate species status, namely *E. dispar* (Diamond and Clark 1993); and the name *E. histolytica* has been retained for the pathogenic strains.

Of the molecules implicated in pathogenesis, the amoebapore and cysteine proteinases from *E. dispar* have been analysed in some detail. Both proteins do exist in *E. dispar,* although differing considerably from the homologous proteins in *E. histolytica.* The specific activity of *E. dispar* amoebapore is less than half that of *E. histolytica* (Leippe *et al* 1993). The two peptides differ in four amino acid residues, of which the substitution of glu in the *E. histolytica* peptide with pro in the *E. dispar* peptide is significant. This change lies in an amphipathic α helix in the $NH_2$-terminal part of amoebapore. Since pro is known to disrupt α-helices, this substitution would shorten the amphipathic helix by 2 residues which could lead to reduction in pore-forming activity.

The natural function of the *E. dispar* amoebapore may be to kill phagocytosed bacteria rather than host cells.

When the 27 kDa cysteine proteinase of *E. dispar* was compared with the homologous enzyme in *E. histolytica,* the two proteins were found to be 83% homologous by deduced amino acid sequence (Tannich *et al* 1991). The residues thought to be important for proteolytic function (by comparison with X-ray crystallographic data on papain) are conserved in the two proteins. Thus, the enzyme from *E. dispar* may not differ functionally from that in *E. histolytica.* However, Northern blot analysis revealed that the *E. dispar* enzyme was expressed at 10-l00-fold lower level than the *E. histolytica* enzyme. The *E. dispar* enzyme may therefore be confined to the vacuolar/lysosomal cellular compartment while the over expressed *E. histolytica* enzyme may be secreted extracellulalry, which may be the crucial difference leading to pathogenicity.

From the limited information available so far, it appears that the property of pathogenesis is determined more by quantitative levels of key molecules than by the total absence of these in nonpathogenic species. Further molecular analysis of absence in *E. dispar* and comparison with *E. histolytica* is required to arrive at meaningful conclusions.

## 6. Future perspectives

A deeper understanding of pathogenesis in amoebiasis would require parallel insights into the cell biology and genetics of *E. histolytica.* This parasite is a fascinating biological system. It seems to lack typical eukaryotic organelles like mitochondria and Golgi bodies. Yet, genes for certain typically mitochondrial proteins, namely pyridine nucleotide transhydrogenase and the chaperonin *cpn60*, can be detected (Clark and Roger 1995). A novel lipophosphoglycan, which is present only in some protozoan parasites, has been discovered in *E. histolytica.* This molecule coats the trophozoite surface and is a variable surface antigen (Bhattacharya *et al* 1992b). Genetic analysis of *E. histolytica* has not been possible so far. However, DNA can now be introduced into this cell by electroporation (Nickel and Tannich 1994; Purdy *et al* 1994; Vines *et al* 1995) paving the way for genetic analysis of specific functions. These developments should ultimately lead to breakthroughs in answering the central question of pathogenesis of infection by *E. histolytica.*

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