Entamoeba histolytica and *Entamoeba dispar*

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Contents

Abstract Of the various species of amebas that are common inhabitants of the human intestinal tract the only parasitic ameba of medical importance is *Entamoeba histolytica*, the causative agent of invasive amebiasis in humans, a common and potentially fatal infection that may affect the intestinal mucosa and spread to other organs, mainly the liver. *Entamoeba dispar,* an ameba morphologically similar to *E. histolytica*, also colonizes the human gut and is a separate species with no invasive potential. The distinction between the two species of amebas has had profound implications for the epidemiology of amebiasis, since most asymptomatic infections found worldwide are attributed to *E. dispar*.

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1 Host Range and Lifecycle

The motile forms of both species of *Entamoebae*, the trophozoites, live in the lumen of the large intestine, where they multiply and differentiate into cysts, the resistant form responsible for the transmission of the infections. The parasites excyst in the terminal ileum. The most common modes of transmission are by food contaminated with cysts or from person to person. The greatest risk is associated with cyst passers, especially if they are engaged in the preparation and handling of food. The main reservoir is man and human susceptibility to infection appears to be general. *E. histolytica* trophozoites may invade the colonic mucosa and cause dysentery and, through spreading via the bloodstream may give rise to extraintestinal lesions, mainly liver abscess. In contrast, *E. dispar* trophozoites remain in the intestinal lumen as commensals (Fig. 1).

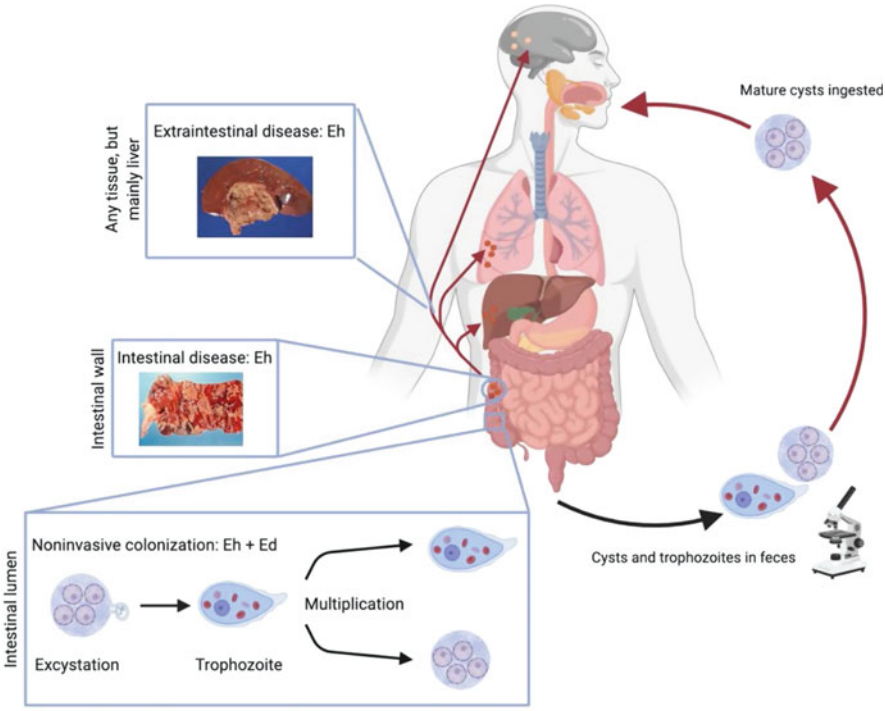

Fig. 1 Life cycle of *E. histolytica* and *E. dispar*

2 *Entamoeba histolytica*. The Trophozoite

The basic structural components of *E. histolytica* as studied with optical and transmission electron microscopy have been defined several decades ago. Below we have summarized previous reviews on the subject containing detailed references (Martínez-Palomo 1982, 1986, 1993).

2.1 The Cytoplasm

The trophozoite of *E. histolytica* is a highly mobile and pleomorphic protozoon, from 10 to 60μm in diameter. Surface and cytoplasmic movements are continuous in amebas, extending large pseudopodia during locomotion, leaving behind a tail or uroid, and even forming occasional slender filopodia. In addition, a prominent endocytic activity is reflected in the frequent observation of varying numbers of phagocytic openings and pinocytic stomas (Fig. 2).

Cultured axenic trophozoites are less active than those recovered from feces. In turn, amebas in mixed cultures display more vigorous movements than those in axenic of monoxenic cultures. Actively moving trophozoites have a well-defined morphological polarity. One or several pseudopodia form and disappear rapidly at the anterior end of the ameba, while a more permanent non-motile uroid occurs at the posterior end of many active amebas. With the light microscope the endoplasm of *E. histolytica* contains abundant vesicles embedded in a cytoplasmic matrix with the appearance of ground glass. Cytoplasmic vesicles in amebas from dysenteric stools contain ingested red blood cells, starch or bacteria. In addition, highly complex vacuolar and tubular systems related to pinocytosis can be revealed by phase-contrast microscopy with the aid of time-lapse microcinematography.

The extreme pleomorphism of *E. histolytica* trophozoites is evident when cultures of the protozoon are examined with the scanning electron microscope, which best reveals their shape and surface morphology (Fig. 3). When preservation is adequate,

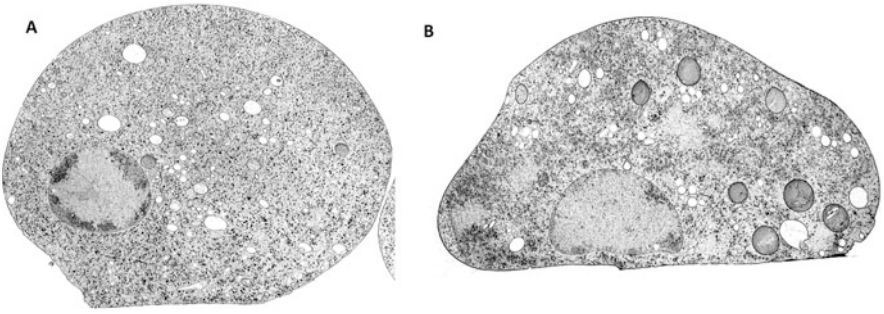

Fig. 2 Transmission electron micrographs of *E. histolytica* (**a**) and *E. dispar* (**b**) trophozoites processed by cryo-fixation

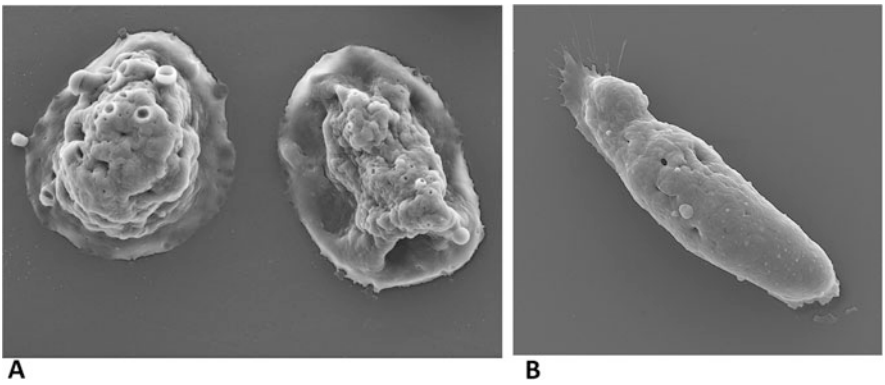

Fig. 3 Scanning electron micrographs of two *E. histolytica* trophozoites (**a**) and one *E. dispar* trophozoite (**b**) adhered to fibronectin

practically every cell has a shape different from that of its neighbors. In general, amebas adhered to a substrate are elongated in form, with protruding lobopodia and a uroid, with varying numbers of small and large membrane openings, ranging from 0.2 to 0.4μm in diameter that correspond to mouths of micropinocytic vesicles. Protruding stomas of macropinocytic channels are much larger, ranging from 2 to 6μm in diameter. When present, the uroid appears as a tail formed by irregular membrane folds and short filiform processes.

Vacuolar System A sizeable proportion of the cytoplasm of *E. histolytica* trophozoites is occupied by a heterogenous population of membrane-limited vesicles and vacuoles, most of which have a circular profile in thin sections, with a variable size raging from 0.5 to 10μm. The contents of these vacuoles vary according to the origin of the amebas. In trophozoites grown under axenic conditions the vacuoles appear mostly empty under the transmission electron microscope, while cellular fragments are readily identifiable in amebas grown with bacteria or with other protozoa, or in amebas from invasive lesions in which debris of epithelial cells, red blood cells and inflammatory cells may be found. The following types of vacuoles can be identified ultrastructurally in the cytoplasm of *E. histolytica*: (a) phagocytic vacuoles, (b) secondary lysosomes, (c) micropinocytic vesicles, (d) primary lysosomes, (e) secondary lysosomes, (f) residual bodies and (g) autophagic vacuoles.

Tubular System Cytoplasmic components similar to the rough endoplasmic reticulum and the Golgi system present in higher eukaryotic cells are lacking in *E. histolytica*. However, in specimens suitably fixed by means of cryofixation, tubular and vacuolar elements resembling a rudimentary Golgi system and endoplasmic reticulum are found.

Ribosomal Helical Arrays Ribosomes appear mostly as helical arrays approximately 300 nm in length and 40 nm in diameter. In cysts and resting cultured trophozoites the helices aggregate in large crystalline inclusions that are up to several

micrometers in length, constituting the classical "chromatoid body" seen with the light microscope.

Cytoskeleton At the ultrastructural level the cytoskeleton is composed of three different types of cytoplasmic fibrillar components involved in cellular shape, movement and support: microfilaments of actin, with an average diameter of 6–8 nm; tubulin-containing microtubules, 20–25 nm in diameter, and intermediate filaments, 10 nm thick. *E. histolytica* trophozoite cytokinesis is dependent upon cytoskeletal elements such as filamentous actin and myosin. A sequence of the participation of actin in the formation of the contractile ring has been shown with rhodamine-phalloidine staining. Ultrastructural analysis reveal the presence of fibrilar aggregates in the cytoplasm of dividing trophozoites. Among them two filaments of different diameter were identified. These aggregates presented repeating assemblies of thin and thick filaments that in cross section reveal a muscle-like appearance. They constitute the contractile ring responsible for the separation of daughter cells.

Cylindrical Bodies One or two rosette-like conglomerates of cylindrical bodies are usually found in thin sections surrounding a finely granular area of the cytoplasm. These conglomerates are about 1μm in diameter and are composed by 10–30 cylindrical bodies. The bodies vary in size but may be up to 250 nm in length and 90 nm in diameter and are limited by a membrane. They tend to be bullet shaped in appearance, flat at one end and round at the other. They are assumed to represent rhabdovirus particles, although their viral nature has not yet been demonstrated.

Electron-Dense Granules Electron dense granules (EDGs) frequently present in the cytoplasm of *E. histolytica* and in other amebas participate in extracellular matrix degradation, an important prerequisite for tissue invasion (Muñoz et al. 1991). The secretion and interaction of EDGs with collagen fibers was demonstrated by ultrastructural studies (Martínez-Palomo et al. 1987). A variety of cationic proteins with proteolytic activity, including proteases that degrade collagen and gelatin, have been identified within these granules. ß-N-acetylhexosaminidase, an enzyme involved in extracellular matrix destruction, was also identified in the granules (Riekenberg et al. 2004). Furthermore, secretion of EDGs has been demonstrated when *E. histolytica* trophozoites are placed in contact with MDCK epithelial monolayers, as well as in liver lesions induced in experimental models of amebiasis (Chávez Munguía et al. 2012).

Plasma Membrane In cultured trophozoites the plasma membrane is covered on the outer surface by a barely detectable surface coat, demonstrable by the use of cytochemical reagents that enhance the electron density of carbohydrate-containing membrane components, such as ruthenium red or Alcian blue.

Surface Specializations Two general types of transient surface structures may be found in *E. histolytica* trophozoites: evaginations (pseudopodia, lobopodia, filopodia, and the uroid) and invaginations (phagocytic and macropinocytic vacuoles and micropinocytic vesicles). All these structures may be present at a given time on

the surface of an ameba. While the evaginations are related to cell motility, the invaginations reflect the various endocytic processes whereby amebas incorporate liquid and particulate material into the cytoplasm. Lobopodia are finger-like protrusion that have a hyaline appearance in living cells. When viewed with the scanning electron microscope the surface of lobopodia appears strikingly smooth in comparison to the irregularity of the rest of the cell surface. Filopodia are long filiform extensions of the cell surface that may extend several micrometers in length, occasionally reaching 100 μm, with a diameter of about 0.1 μm.

Capping The extremely dynamic surface activity of *E. histolytica* is evident not only from microscopic observations of its intense amoeboid movements or its enormous phagocytic capacity, but also from the extremely fast redistribution of surface components in caps. In *E. histolytica*, the surface segregation of surface lectin–receptor complexes forms localized caps (Pinto da Silva et al. 1975). The remarkable ability of these amebas to rapidly regenerate substantial amounts of plasma membrane was demonstrated by the successive capping induced by repeated exposure to antibodies. Capping may be followed either by endocytosis and degradation of the incorporated membrane fragments, or by the gradual formation of a constriction ring and release of membranous cap vesicles (Espinosa-Cantellano and Martínez-Palomo 1991). At the end of the capping process, a spontaneous release of the cap may take place, the released caps containing most of the antibodies that originally bound to the whole cell surface. *E. histolytica* caps have been isolated and subjected to electrophoretic analysis revealing that only half of the surface-immunoprecipitated antigens are enriched in the caps. Co-capping of actin and myosin was documented by immunofluorescent staining of capped amebas using phalloidin, monoclonal anti-actin antibodies, and polyclonal anti-myosin antibodies. Immunoblots confirmed the presence of actin and myosin in isolated caps. These observations demonstrate that amebic cytoskeletal components participate in *E. histolytica* capping of surface receptors.

Specific proteins are mobilized to the uroid following cap formation with concanavalin A (Baxt et al. 2008, 2010). On the one hand, a membrane-embedded rhomboid protease (EhROM1) that cleaves amebic lectins relocalizes to the base of the uroid in *E. histolytica* trophozoites during capping, whereas other membrane proteins remain on the surface of the entire parasite. That protein may play a specific role in late steps of the capping process and/or cap shedding during immune evasion (Baxt et al. 2008), or else, the relocalization may be simply a passive event (Baxt et al. 2010). On the other hand, the endoplasmic reticulum chaperone calreticulin is concentrated to the uroid, following capping of lectin surface receptors in *E. histolytica* (Girard-Misguish et al. 2008). The functional significance of this surface relocation of proteins is not known. It is of interest that also in lymphocytes capping of surface receptors relocates proteins, apparently to facilitate certain cell–cell interactions

In contrast to capping in lymphocytes where the process occurs through the formation of multiple short microvilli (Yahara and Kakimoto-Sameshima 1977), the surface redistribution of plasma membrane components in amebas takes place

through the formation and possibly the planar displacement of small vesicles. The end result is the formation of well-formed caps in the case of *E. histolytica*, and surface patches in *E. dispar*. The difference in mobility of surface receptors between both species of amoebas is striking, at least in culture conditions. As a working hypothesis the lack of capacity of the non-invasive *E. dispar* to displace and eliminate surface antigen–antibody complexes could render this ameba susceptible to the humoral immune response of the host and could be related, at least partially, to the failure of *E. dispar* to produce liver abscesses in experimental models, in contrast to the liver lesions produced under similar conditions by the invasive *E. histolytica* (Pacheco-Yépez et al. 2009). Differences in cellular motility found between the two species of amebas have also been considered as possible factors in the invasive nature of *E. histolytica* (Zaki et al. 2006).

2.2 The Nucleus

In living amebas seen with bright field optics the nucleus is generally inconspicuous but becomes clearly apparent when visualized with phase contrast or Nomarski interference optics. In iron hematoxylin-stained preparations the nucleus of the trophic forms of *E. histolytica* is 4–7μm in diameter. The morphology of the nuclear structures in *E. histolytica* is important because various genus and species of parasitic amebas have been defined largely on the basis of their nuclear structure and their mode of cell division. The nuclear membrane is outlined by a thin uniform layer of granules that gives the nucleus the appearance of a ring in optical sections. Chromatin clumps are usually uniform in size and evenly distributed inside the nuclear membrane. The karyosome or endosome is a small spherical mass, approximately 0.5μm in diameter located in the central part of the nucleus and is apparently a site of DNA condensation. The early suggestion that the peripheral chromatin is the counterpart of the eukaryotic nucleolus has been confirmed with antibodies specific against nucleolar components (Jhingan et al. 2009).

The mechanism of nuclear division of *E. histolytica* remains poorly understood. The only fact that can be considered as settled is that this division proceeds without the dissolution of the nuclear membrane. Very little information has been obtained on the nuclear division of *E. histolytica* using standard techniques of transmission electron microscopy, as no centrioles or spindles can be identified with certainty. Dividing nuclei present microtubules, initially located at random in the karyosome during prophase, that form in subsequent stages a mitotic spindle closely related to the nuclear membrane at the polar regions of the nucleus. In late prophase and in anaphase, chromosomes appear as dense bodies 0.1–0.5μm. At least 15 chromosomes appear in favorable planes of sections, arranged as an incomplete elliptical circle, in close contact with microtubules. No morphological evidence of structures resembling the kinetochores of higher eukaryotes are found. When cut in cross-section, the mitotic spindle is made of 28–35 microtubular rosette assemblies. The latter probably correspond to a similar number of chromosomes, as has been shown

by others with pulse-field electrophoresis and fluorescence microscopy of trophozoite spreads. In turn, each microtubular rosette is constituted by 7–12 parallel microtubules. In later stages of the metaphase, two sets of chromosomes form a pair of elliptical circles (Chávez-Munguía et al. 2006).

2.3 The Cyst

The main reservoir of *E. histolytica* is humans, although morphologically similar amebas may be found in primates, dogs and cats. Surprisingly, the life cycle of *E. histolytica* in humans has not been studied. It is probable that the complete life cycle consists of four consecutive stages; namely, the trophozoite, precyst, cyst and metacyst stages. Trophozoites dwell in the lumen of the colon, where they multiply and encyst producing cysts with four nuclei, present in the formed stools of carriers. They cysts are round or slightly oval hyaline bodies 8–20μm in diameter, with a refractile wall. When ingested, the cyst wall is dissolved in the upper alimentary tract. After a nuclear division, now wall-less cysts give rise to eight potentially invasive trophozoites further down the gut.

With transmission electron microscopy, the walls of mature cysts of *E. histolytica* stained with standard techniques are barely visible. However, in samples treated with ruthenium red added to osmium tetroxide cyst walls appear densely stained, made up by the superposition of five to seven layers of fibrils 7–8 nm in thickness. The dye Alcian blue penetrates the entire thickness of the cyst wall revealing a fibro-granular texture, with fibrils approximately 4 nm thick, some of them disposed perpendicularly to the plane of the plasma membrane. Cationized ferritin particles do not penetrate the cyst wall of *E. histolytica*, but label abundant negatively charged groups at the surface of the cyst wall. In freeze-fracture replicas of cysts the lack of intramembrane particles in the plasma membrane is striking (Chávez-Munguía et al. 2008).

The cytoplasm of mature *E. histolytica* cysts contains abundant vesicles, ranging between 0.3 and 0.6μm in diameter, with a dense fibro-granular content, frequently seen approaching the cytoplasmic aspect of the plasma membrane; others were separated from the plasma membrane by a space 7 nm wide, apparently in the process of depositing the contents to the cell wall (Chávez-Munguía and Martínez-Palomo 2011).

In turn, the surface of *E. histolytica* cysts viewed with the scanning electron microscope appears smooth, with a granular texture when seen at high magnifications (Fig. 4).

It has been generally assumed that the resistance of the cysts of *Entamoeba* in the aquatic environment is due to the impermeability of the cyst wall. However, because ruthenium red penetrates the entire thickness of the cyst wall, the permeability of the mature cystic forms of *Entamoeba* is limited to water and small molecules, not by the cyst wall but by the plasma membrane. Ruthenium red, long used as a stain of cell surface components, also acts as an electron-dense tracer. It reacts mainly with a

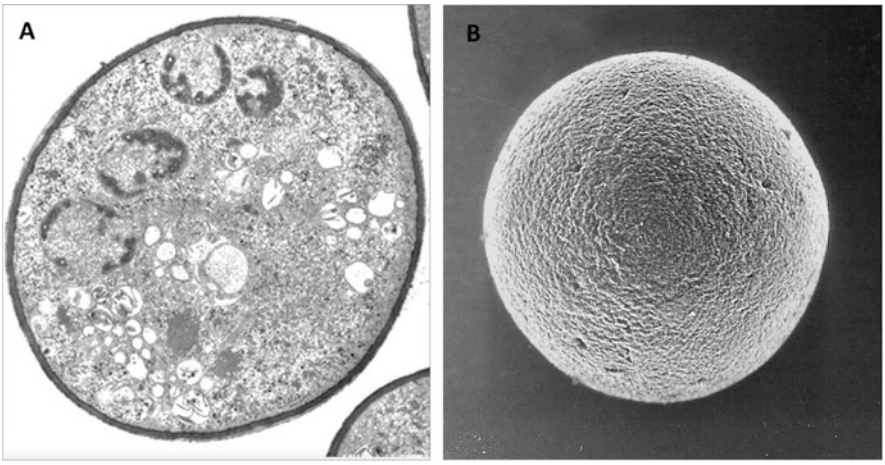

Fig. 4 Cysts of *E. histolytica*. (**a**) Transmission electron micrograph. (**b**) Scanning electron micrograph

large number of polyanions and is therefore non-specific. The stain facilitates the visualization of the structural components of the cystic cell wall and shows that cyst walls of *Entamoeba* are made up by ruthenium red-positive fibrils 7–8 nm thick, giving rise to a tight mesh constituted by five to seven irregular lamellae. When these fibrils are seen in transverse sections, no ordered pattern is apparent. Most probably they correspond to chitin fibrils, as reported previously in transmission electron microscopical observations of purified cyst walls of *E. invadens*.

A second type of fibrils, 4 nm in diameter, apparently running perpendicular to the plane of the wall was revealed in *E. histolytica* cysts stained with Alcian blue, an electron dense stain that reacts with anionic sites in cell surfaces, usually with abundant sulfate, carboxyl and phosphate polyanions. It is tempting to speculate that these fine fibrils arranged in a tight mesh represent some of the cross-linking glycoproteins that constitute the "daub" of the "wattle and daub" model proposed by Chaterjee et al. (2009) for the cyst wall, in which the ruthenium red-positive fibrils constitute the "wattle" of the cyst wall.

In freeze-fracture replicas of *E. histolytica* cysts, intramembrane particles are lacking on both the P and the E faces of the plasma membrane of the encysted cell. This is in contrast to the abundance of these membrane particles reported in the trophozoite form of these parasites. The lack of these membrane proteins in amebic cysts has been interpreted as a reflection of the dormant physiological activity of the parasite.

In contrast to a wealth of information concerning the biology of the motile form of the parasite, the trophozoite, the process of cyst formation of *E. histolytica* remains basically unknown, in spite of the fact that cysts are responsible for the transmission of the infection. One of the obstacles is the lack of an experimental procedure that allows a massive production of *E. histolytica* cysts. In spite of this limitation, some

progress has been achieved. Chitin, an *N*-acetyl-D-Glucosamine polymer, was identified as the principal component of the cyst wall in both human *E. histolytica* and in the reptilian parasite *E. invadens* cysts (Van Dellen et al. 2006). Besides, two abundant encystation-specific proteins have been identified in *E. histolytica*; one of them is the remodeling enzyme chitinase (Ghosh et al. 1999; Van Dellen et al. 2002) and the other is a protein named Jacob, a cyst wall resident glycoprotein composed of five randomly arranged chitin-binding domains. In addition, chitinase gene homologues and two lectins named Jessies were also localized in putative secretory vesicles of *E. histolytica* (Van Dellen et al. 2002). Aguilar-Díaz et al. (2010) have produced in vitro *E. histolytica* cyst-like structures by treating trophozoites with hydrogen peroxide and trace amounts of several cations. Using *E. invadens* as encystation model, ultrastructural studies have shown that the cyst wall delivery to the cell surface takes place through trafficking of specific secretory vesicles (Chávez-Munguía et al. 2003; De Souza 2006). Through the use B4F2 mAb it has been demonstrated that these vesicles and the cyst wall contain enolase. Enolase is also present in the cytoplasmic vesicles produced in *E. histolytica* trophozoites recovered from amebic liver lesions.

3 Pathogenic Factors

The aggressive mechanism of *E. histolytica* is a complex, multifactorial phenomenon that includes adhesion, a contact-dependent "hit-and-run" damage to the plasma membrane of effector cells, phagocytosis, and intracellular degradation of ingested cells (Martínez-Palomo et al. 1985). The adhesion and cytolytic events have been related to three types of molecules: lectins, amebapores, and proteases, previously reviewed (Espinosa-Cantellano and Martínez-Palomo 2000; Martínez-Palomo and Espinosa-Cantellano 2004, 2005; Ralston and Petri 2011).

Following genetic and proteomic searches, many candidate molecules have been postulated to participate in the virulent behavior of *E. histolytica*. However, even though there are some well-defined differences in genomic sequences, up- or down-regulated gene expression, or enzymatic activities between *E. histolytica* and *E. dispar*, these have not yet completely explained the invasive behavior of the former. Estimation of genome size of *E. histolytica* by different methods has failed to give comparable values due to the inherent complexities of the organism, such as the uncertain level of ploidy, presence of multinucleated cells and a poorly demarcated cell division cycle. The genome of *E. histolytica* has a low G + C content (22.4%), and is composed of both linear chromosomes and a number of circular plasmid-like molecules. The rRNA genes are located exclusively on some of the circular DNAs. Karyotype analysis by pulsed field gel electrophoresis suggests the presence of 14 conserved linkage groups and an extensive size variation between homologous chromosomes from different isolates. Several repeat families have been identified, some of which have been shown to be present in all the electrophoretically separated chromosomes. The typical nucleosomal structure has not been demonstrated, though

most of the histone genes have been identified. Most *Entamoebae* genes lack introns, have short 3′ and 5′ untranslated regions, and are tightly packed. Promoter analysis revealed the presence of three conserved motifs and several upstream regulatory elements. Unlike typical eukaryotes, the transcription of protein coding genes is alpha-amanitin resistant. Expressed Sequence Tag (EST) analysis has identified a group of highly abundant polyadenylated RNAs which are unlikely to be translated. The EST approach has also helped to identify several important genes which encode proteins that may be involved in different biochemical pathways, signal transduction mechanisms and organellar functions (Bhattacharya et al. 2000; Wilson et al. 2019).

3.1 Lectins

Adhesion of the parasite occurs mainly through a surface Gal-GalNAc lectin which binds to exposed terminal Gal-GalNAc residues of target cell glyco- proteins (Petri et al. 1989). Other molecules include a 220-kDa lectin, a 112-kDa adhesin and a surface lipophosphoglycan. The Gal-GalNAc adhesin is a multifunctional protein composed of an heterodimer of heavy (170-kDa) and light (35/31- kDa) subunits. Complete inhibition of *E. histolytica* adherence to target cells or colonic mucins is not observed, even when the lectin is blocked with high (100–500 mM) concentrations of galactose or GalNAc monomers (Dodson et al. 1997). In addition to its role in amebic adherence, the adhesin may participate in the cytolytic event, since contact-dependent target cell lysis is reduced in the presence of galactose, and a monoclonal antibody against the heavy subunit is capable of partially inhibiting cytolysis without blocking adherence. Considering that the purified lectin has no cytotoxic effect even at high concentrations, it has been proposed that the adhesin is involved in the signaling of cytolysis, probably through the stimulation of actin polymerization. Furthermore, the adhesin binds to purified C8 and C9 components of complement and blocks the assembly of the complement membrane attack complex on the amebic plasma membrane, suggesting a role in mediating amebic resistance to complement lysis through components C5b through C9

The heavy subunit of the Gal-GalNAc adhesin is encoded by five genes in *E. histolytica.* The derived amino acid sequences suggest that the heavy subunit is an integral membrane protein with a small cytoplasmic tail and a large extracellular N-terminal domain containing a cysteine-rich region. The cytoplasmic tail has sequence identity with ß2-integrin cytoplasmic tails and seems to be involved in the inside-out signaling that controls the extracellular adhesive activity of the amebic lectin. On the other hand, the cysteine-rich region seems to be essential for several activities described for the lectin. With the aid of monoclonal antibodies, several antigenic determinants that affect adherence and complement resistance have been mapped to this region. Moreover, the carbohydrate recognition domain is contained within the cysteine-rich region of the lectin. The light subunit shows two 35- and 31-kDa isoforms, the latter having a glycosylphosphatidyl-inositol anchor. This subunit is also encoded by a gene family that have 79–85% nucleotide sequence

identity. Its function is not yet clear but seems to involve the modulation of parasite cytopathic activity.

Comparison between *E. histolytica* and *E. dispar* reveals very similar Gal-GalNAc adhesins that efficiently bind to target cells and colonic mucins (Dodson et al. 1999). Derived amino acid sequences encoded by homologous genes show that *E. dispar* heavy- and light-subunit clones are 86 and 79% identical in their primary structures, respectively, to their *E. histolytica* counterparts. In vitro, *E. dispar* exhibits adherence and cytotoxicity to target cells that is mediated by the Gal- GalNAc lectin, although to a lesser extent than *E. histolytica.* These observations are in accordance with the ability of *E. dispar* to produce superficial erosions in the colonic mucosa of experimental animals but leaves unanswered the question of why this ameba does not invade further.

Surprisingly, the lectin has attracted attention as a possible candidate for a vaccine against amebiasis. Recent efforts have focused on an internal 578 amino acid fragment, designated LecA, located within the cysteine-rich region of the heavy chain subunit (Barroso et al. 2014).

3.2 Amebapores

Once *E. histolytica* establishes contact with mammalian cells in vitro, a rapid cytolytic event takes place that results in swelling, surface blebbing, and lysis of the inadvertent target cell, including lymphocytes, polymorphonuclear leukocytes, and macrophages, leaving the parasite unharmed. The similarity of this event to the perforin-mediated lysis of target cells by cytotoxic T lymphocytes initially suggested the participation of a channel-forming protein called the amebapore, whose activity was identified in *E. histolytica* lysates.

The amebapore of *E. histolytica* is a channel-forming peptide of 77 amino acid residues; the protein has been sequenced, and the respective genes have been cloned (Leippe 1997). Three isoforms, amebapores A, B, and C, are present at a ratio of 35:10:1, respectively, with the genes showing 35–57% deduced amino acid sequence identity. The molecules share six cysteine residues at identical positions and a histidine residue near the C terminus. Structural modeling suggests a compact tertiary structure composed of four alfa-helical structures stabilized by three disulfide bonds. Thus, amebapores are different from the much larger (65- to 70-kDa) perforins that contain three amphipathic segments, two alfa-helices and one ß-sheet. However, similarities at the structural and functional levels have been found between amebapores and NK-lysin, a polypeptide present in natural killer (NK) cells and cytotoxic T lymphocytes of pigs.

Like other pore-forming peptides, amebapores are readily soluble but are capable of rapidly changing into a membrane-inserted conformation (Leippe 1997). Early observations suggested that the molecule forms multistate channels with similar properties to those found in the barrel-stave aggregates of toxins such as alamethacin (Keller et al. 1989). Amebapores aggregate through the arrangement of their

amphipathic alfa-helices. A model has been proposed by which amebapores bind to negatively charged phospholipids via protonated lysine residues; this is followed by the insertion of the peptide into the lipid bilayer driven by the negative membrane potential of the target membrane (Andrä and Leippe 1994).

In vitro, amebapores exert cytolytic activity against several human cultured cell lines. Amebapore C seems to be the most effective, while amebapore A is not efficient in lysing erythrocytes. In addition, the peptides show potent antibacterial activity against gram-positive bacteria by damaging their surface membranes. Damage to the outer membrane-shielded gram-negative bacteria requires high concentrations of amebapore or removal of the wall with lysozyme. With the use of synthetic peptides, of the four alfa-helices present in the molecule, helix 3 was found to be responsible for the membrane penetration, displaying the highest antibacterial activity. Interestingly, helix 3 is also the most highly conserved domain in the three isoforms (Leippe et al. 1994). The peptide derived from isoform C was the most active of several synthetic peptides studied, reaching a magnitude of activity in the range of the whole molecule. Amebapores are localized in cytoplasmic vesicles, as evidenced by positive immunofluorescence staining and by the presence of typical signal peptides of intracellular transport in translation products. The peptides show maximum activity at acidic pH, which is consistent with previous observations that lysis of target cells by *E. histolytica* required a pH of 5.0 within amebic vesicles (Leippe et al. 1992).

A peptide homologous to *E. histolytica* amebapore A has been identified in *E. dispar.* The molecules have common structural and functional properties, such as insertion into negatively charged liposomes, highest activity at low pH, localization in cytoplasmic vesicles, 95% identity of primary structures, and a high degree of similarity of secondary-structure predictions. In spite of these similarities, the specific activity of the *E. dispar* amebapore is 60% lower than that of the one in *E. histolytica*. This may be related to a shortened amphipathic helix in the former (Leippe et al. 1993).

Surprisingly, in spite of all the advances in the biochemistry and molecular biology of amebapores, their participation in the cytolytic event produced by *E. histolytica* has not yet been demonstrated. Amebapores are not spontaneously secreted from viable trophozoites. Whether the molecule is able to insert into target cell membranes upon adherence in vivo remains to be established.

The presence of pore-forming activity in the noninvasive *E. dispar* suggests that the primary function of amebapores is to destroy phagocytosed bacteria, the main intestinal source of nutrients of amebas; thus, they have a similar function to defensins found in mammalian phagocytes that kill bacteria and fungi to prevent intracellular microbial growth within digestive vacuoles (reviewed in Leippe 1997). The anaerobic environment found in the colon may favor oxygen-independent mechanisms as a means of destroying bacteria, rather than using oxygen metabolites and nitric oxide. Amebapores and other proteins, like the recently characterized ameba lysozyme that colocalizes to the same cytoplasmic granules of amebapores could synergistically enhance the antibacterial activity.

3.3 Proteases

Cysteine proteases are the most abundant proteases in *E. histolytica.* They are encoded by approximately 50 genes and are responsible for epithelial barrier penetration and degradation of host extracellular matrix components. Only four proteases, EhCP1, EhCP2, EhCP5, and EhCP7, are highly expressed by *E. histolytica* in culture and have identifiable cellular localizations. EhCP1 is present in intracellular vesicles, EhCP2 localizes to the internal and external cell membrane, and EhCP5 is on the cell surface. Invasive *E. histolytica* clinical isolates have more CP activity in culture than noninvasive *E. dispar* isolates, suggesting a role for CPs in parasite virulence and invasion (Siqueira-Neto et al. 2018).

Amebic cysteine proteases are active against a variety of substrates and increased activity has been reported in clones of high virulence. Of the extracellular matrix components that *E. histolytica* encounters during colonic invasion, laminin, collagen types I and IV, and FN are good targets for amebapain, histolysin, EhCP5 membrane-bound protease and the neutral 56-kDa protease.

E. dispar seems to possess four homologous cysteine protease genes, although none of the enzymes (EdCP2, EdCP3, EdCP4, and EdCP6) have been purified. In addition, the presence of the neutral 56-kDa protease in *E. dispar* has not been reported yet. If *E. dispar* indeed lacks several of the most potent *E. histolytica* cysteine proteases (EdCP1, EdCP5, and the neutral protease), it is possible that this difference could partially explain its noninvasive nature.

In addition, *E. histolytica* possesses a membrane-bound metallo-collagenase that degrades collagen types I and III and is more active against the former. The degree of collagenolytic activity has been linked to the virulence of different isolates. Activation of the enzyme apparently results in translocation of the enzyme from internal membranes to the plasma membrane.

In summary, *E. histolytica* possesses the necessary machinery to degrade the extracellular matrix components it encounters during invasion. However, the participation of the different proteases during in vivo infections has yet to be demonstrated.

3.4 Adhesion

When the adhesion processes of *E. histolytica* and *E. dispar* to fibronectin (FN) are compared, dramatic differences are observed between the two amebas. Upon incubation on FN-coated surfaces *E. histolytica* tightly adheres to the substrate, maintaining its plasma membrane in close proximity to the FN, while extending focal zones of more intimate contact that seem to fuse with the substrate. This tight adhesion is translated into great stiffness at the site of contact, as revealed by the atomic force microscope in tapping mode. A concentration of filaments is observed in the adjoining cytoplasmic region. These filaments are identified as actin by

confocal microscopy. Furthermore, Western blot analysis reveals that F-actin accounts for 65% of the total actin pool, confirming the fundamental role of the actin cytoskeleton in the adhesion of *E. histolytica* to FN. Extensive degradation of FN is observed not only in the intimate contact sites with the substrate but along the surface that was seen in close proximity to the amebic plasma membrane. In contrast, *E. dispar* do not show relocation or degradation of FN, while F-actin remain distributed throughout the cytoplasmic compartment (Talamás-Lara et al. 2015).

The signaling cues of FN elicit dramatically different responses from the actin cytoskeleton of *E. histolytica* and *E. dispar*. Confocal images of trophozoites stained with rhodamine–phalloidin after incubation with FN suggest that *E. dispar* does not efficiently polymerize G-actin. This may reflect a specific unresponsiveness to the FN stimulus or an inability to form large bundles of polymerized actin. However, several examples seem to indicate that despite having large amounts of cytoplasmic actin, *E. dispar* does not respond with its polymerization when challenged with stimuli that elicit a strong and immediate redistribution of the cytoskeleton in *E. histolytica*.

Binding of *E. histolytica* trophozoites to FN is carried out by an integrin β1-like membrane molecule of 140 kDa which corresponds to the intermediate chain of the Gal–GalNAc lectin (β1*Eh*FNR) (Sengupta et al. 2009). This receptor is present in *E. dispar*, but is not recognized by a monoclonal antibody raised against the β1*Eh*FNR, suggesting differences in conformation and/or posttranslational modifications, which have been reported to occur in β1*Eh*FNR. Binding of the β1*Eh*FNR to FN leads to phosphorylation of several proteins, including FAK, paxillin and vinculin (Flores-Robles et al. 2003). In addition, FN-induced actin rearrangements are activated through a Rho1 and ROCK dependent signaling pathway, while the formation of focalized actin dots requires the participation of Rab21 (Emmanuel et al. 2015).

Any change in the binding with an integrin can turn off the modulation of intracellular signals. It is through integrins that the cells sense the properties of the ECM, such as stiffness, spatial dimension, the nature of the ligand, etc., all of which finally lead to the trigger of signals that result in the restructuring of the actin cytoskeleton (Destaing et al. 2011). FN is a multi- functional protein; studies have demonstrated a mechano-biological interaction with different cell types. Some cells have the ability to mechanically deploy regions that exhibit new binding sites previously hidden within the molecular folds of FN (Bradshaw and Smith 2014). Perhaps *E. histolytica* is capable of finding these binding sites due to its high motility and protease capacity, while *E. dispar,* having a less efficient capacity to secrete lytic components (Talamás-Rohana et al. 1999), would be unable to deploy these sites, therefore showing an inefficient invasive behavior (Oliveira et al. 2015). In sum, adhesion to FN results in dramatic differences in the rearrangement of the actin cytoskeleton in *E. histolytica* in comparison to *E. dispar*.

These observations suggest differences in their ability to organize the actin cytoskeleton and, therefore, to modify its morphology after adhesion to fibronectin. To better understand these observations, the participation of actin cytoskeleton regulatory proteins such as small GTPases (Rho, Rac1 and Cdc42), myosin IB,

paxillin, alpha-actinin, and ARP2/3 on the cytoskeleton rearrangements of *E. histolytica* compared to *Entamoeba dispar* was analyzed during interaction with fibronectin. Results showed higher activation of Rac1 in *E. histolytica*, while Cdc42 and RhoA were equally activated in both amebae. Variations in the amount of myosin IB, paxillin, and ARP2/3 were detected among these species, coinciding with the more frequent formation of lamellipodia in *E. histolytica* and filopodia in *E. dispar*. As a working hypothesis, *E. histolytica* could display its invasive capacity relying on its pleomorphic ability, high motility, migration, activation, and abundance of proteins involved in cytoskeleton arrangement (Talamás-Lara et al. 2020).

3.5 Locomotion

In mammals, cell locomotion involves at least three processes: (1) extension of a leading edge, (2) attachment to the substrate surface through adhesion plaques, and (3) pulling forward of the remainder of the cell. The motile force underlying cytoplasmic streaming is a Ca^{2+}-dependent interaction between actin and myosin, similar to the one described for skeletal muscle contraction. In *E. histolytica*, actin polymerization occurs during pseudopod extension. No reports are available on fluctuations in intracellular Ca^{2+} concentration during locomotion, but the presence of myosin II in leading lamellae suggests that the mechanism for leading-edge extension in amebas is similar to that in mammalian motile cells.

Fibronectin (FN) and other extracellular matrix substrates induce the formation of adhesion plaques in *E. histolytica* trophozoites. Ligand recognition and binding seem to involve a 37-kDa FN-binding protein and a 140-kDa integrin-like receptor. Isolated amebic adhesion plaques are composed mainly of actin filaments and at least four actin binding proteins: vinculin, actinin, tropomyosin, and myosin I. Vinculin may serve as a link between actin and an integral component during anchoring of the cytoskeleton to the plasma membrane in higher eukaryotes. Its identification in amebic adhesion plaques suggests a similar function in this parasite.

The mechanism of the third process involved in cell motility, pulling forward of the remainder of the cell, is still obscure in *E. histolytica*. During locomotion, the cell body of amebas move toward the pseudopod, leaving a trailing uroid. Actin and myosin II have been identified in the uroid, but no regulatory proteins have been reported.

FN binding transduces information into the trophozoites, activating a protein kinase C (PKC) pathway with the production of inositol triphosphate and the phosphorylation of several proteins, probably including PKC itself. Activation of PKC through binding of FN or direct stimulation with phorbol myristate acetate shows a direct relationship to actin polymerization and assembly of various actin binding proteins in the adhesion plaques (reviewed in Meza 2000). Although the targets of PKC have not been identified, its activation sheds some light on one signaling pathway in the parasite.

A second signaling pathway seems to occur via a focal adhesion kinase, $pp125^{FAK}$, identified in immunoblots of isolated amebic adhesion plaques induced by FN. Moreover, adhesion of *E. histolytica* trophozoites to collagen induces the phosphorylation of several proteins, one of which has been identified as $pp125^{FAK}$, further suggesting its participation in signal transduction. It has been proposed that activation of $pp125^{FAK}$ might initiate a signal transduction pathway that activates the mitogen-activated protein kinase cascade, allowing the flow of information from the ECM to the cell interior. This is supported by the identification of $p42^{MAPK}$ in tyrosine- phosphorylated polypeptides induced by collagen-stimulated trophozoites.

Incubation of trophozoites with FN produces a sustained rise in $[Ca^{2+}]_i$ which promotes the stabilization of adhesion plaques and focal contacts by polymerizing soluble actin. External Ca^{2+} influx is responsible for the increased $[Ca^{2+}]_i$ since cytoplasmic Ca^{2+} stores are rapidly depleted (Carbajal et al. 1996). In the absence of Ca^{2+} following the addition of chelating agents, poor adhesion of trophozoites is observed.

Actin organization might have different levels of regulation, namely, changes in actin gene expression and balance between the monomeric G-actin and polymerized F-actin configurations. Treatment of *E. histolytica* trophozoites with drugs known to increase cytoplasmic cyclic AMP levels (forskolin and dibutyryl cyclic AMP) or to activate PKC (phorbol myristate acetate) produces a shift in the actin equilibrium to the F-actin form, which in turn increases actin mRNA levels. This suggests an actin feedback-regulatory mechanism, previously described in other cells, where the gene product and the configuration of the microfilaments could directly regulate actin gene expression (Manning-Cela and Meza 1997). Evidence for another regulatory mechanism in actin organization is provided by the recent isolation and cDNA cloning of the *E. histolytica* profilin basic isoform. Profilin acts as a buffer in actin organization by either inhibiting or promoting actin filament formation.

4 *Entamoeba dispar*

Until recently two of the most puzzling aspects of the biology of *E. histolvtica* were the unexplained variability of its pathogenic potential and the restriction of human invasive amebiasis to certain geographical areas despite the worldwide distribution of the parasite. The main debate centered on the question of whether there are one or two species of *E. histolytica*. Almost one hundred year ago the French parasitologist Emile Brumpt proposed that invasive amebiasis is produced by a species of ameba of restricted distribution, biologically distinct, but morphologically similar to non-pathogenic amebas having a worldwide distribution (Brumpt 1925). Except for some confirmatory studies nothing refuted or confirmed this hypothesis for many decades. However, in the 1970s, differences in surface properties were found between strains of *E. histolytica* isolated from carriers and those obtained from patients with invasive amebiasis (Martínez-Palomo et al. 1973).

Subsequently, the isoenzyme technique was applied to thousands of isolates of amebas isolated in several continents (Sargeaunt et al. 1982) and the application of molecular and immunological techniques confirmed the existence of distinct variations between both species (Strachan et al. 1988; Tachibana et al. 1990). The debate on the existence of two different species of *Entamoebae* was finally solved in 1975 at the XIII Seminar on Amebiasis, held in Mexico City: *E. dispar* formerly known as non-pathogenic *E. histolytica* was classified as a separate species, not capable of causing clinical disease (WHO 1997). The distinction changed our understanding of the epidemiology, control, and treatment of amebiasis.

Trophozoites of *E. dipar* are more elongated in form, their cytoplasmic vacuoles have a patchy distribution and they do not tend to form clumps, as *E. histolytica* does. With Nomarski optics these variations become more evident. In addition, the nuclei of *E. dispar* is lined by a thin layer of regularly distributed dense granules, as reported by Brumpt. In plastic sections of cultured trophozoites, the nuclei of *E. dispa*r appear distinct from those of *E. histolytica*; the two species can be further differentiated by the patchy appearance of the cytoplasmic vacuoles, and also by the presence of large and irregular metachromatic areas apparent when stained with Toluidine blue.

Transmission electron microscopy demonstrates that the metachromatic areas in *E. dispar* represent large cytoplasmic deposits of glycogen. Taken together, these results suggest the possibility of finding, with the use of simple staining techniques, light microscopical differences between the two species of *Entamoebae* in samples of fecal material. However, there was no single ultrastructural feature present in amebas that could be used to distinguish one species from another, except for the plasma membrane of *E. histolytica*, which has a thicker surface coat when compared to that of *E. dispar*.

In cryofixed and cryosubstituted trophozoites of *E. histolytica* a thick uniform surface coat measuring approximately 16 nm can be observed at the outer surface of the plasma membrane, while *E. dispar* displays a loose surface coat, sometimes interrupted in vast areas of the plasma membrane. Scanning electron microscopy of *E. dispar* trophozoites reveal a smoother surface and a polarized shape, with a single prominent pseudopodium at the anterior end. In contrast, *E. histolytica* trophozoites have a rougher cell surface, are more pleomorphic, and show several small pseudopodia.

Under in vitro conditions *E. dispar* displays a much milder lytic effect, compared to *E. histolytic*a (Espinosa-Cantellano et al. 1988, 1998).

In spite of these advances, many questions must be answered before we can fully understand the sequence of events during amebic invasion of human tissues. To name but a few, the signal for the initiation of the invasion process, the mechanism of elimination of the mucus barrier, the role of EDG, and the in vivo participation of the different molecules in this multifactorial process have not been demonstrated. Much work needs to be done to clarify the complex signaling pathways of the parasite and to explain the modulation of the host immune response that results in the establishment and continuation of infection.

In turn, future advances will reveal the biological basis of the differences in pathogenic potential between *E. histolytica* and *E. dispar*. In addition, cultures of these amebas will continue to be valuable models for the cell biologist interested in the study of basic basic phenomena such as the molecular regulation of cell movement and cell shape.

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