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PARASITOLOGY (A VAIDYA, SECTION EDITOR)

Molecular Basis of Pathogenesis in Amoebiasis

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Abstract Amoebiasis is one of the major public health problems in developing countries. In spite of the availability of an effective drug and absence of overt drug resistance, the disease is still prevalent among large population and spread over a number of countries. It is caused by the protist parasite *Entamoeba histolytica* that essentially infects humans, though other species that infect a few animals have been reported. A number of molecular techniques have recently been developed. These have helped in understanding biological processes in *E. histolytica* and in the identification of key molecules that are involved in amoebic virulence and invasion. Moreover, developments in the area of disease and invasion models have allowed understanding of these processes at molecular level and circumvented lack of a good animal model of amoebiasis. All these knowledge will help us to design better therapeutics and allow us to control this important disease.

Keywords *Entamoeba histolytica* · Amoebiasis · Cysteine proteases · Phagocytosis

Entamoeba histolytica was first described by Schaudinn in 1903 as the *Entamoeba* species associated with human dysentery [1]. The species found in healthy individuals was originally named as *Entamoeba coli*. The concept of the two species of *Entamoeba*, one involved in the human disease and the other, a nonpathogenic form though capable of infecting human, was felt even in early days [2]. After the advent of molecular tools, it became clear that there are two morphologically indistinguishable closely related species, *E. histolytica* (pathogenic) and *Entamoeba dispar* (nonpathogenic) [3•, 4]. Not all *E. histolytica* infected individuals display invasive disease as a large fraction of infected individuals remains healthy, although capable of spreading infection [5, 6]. It is still not clear under what conditions *E. histolytica* cells turn invasive. Both parasite and host factors may have important roles to play in tissue invasion. However, no invasive disease has been clearly documented in individuals who are infected with *E. dispar*, though there are occasional reports of *E. dispar* causing amoebiasis [7–10]. Since it is difficult to rule out complete absence of *E. histolytica* in these studies, further validation is required. Currently, the disease caused by *E. histolytica* is referred to as "amoebiasis" and includes both intestinal and extraintestinal forms. In this article, we will not describe pathophysiology and epidemiology of the disease as these have recently been covered [11–13, 14•, 15–17].

E. histolytica exists in two developmental stages, four-nucleated cyst form and the trophozoites. Infection is initiated by ingestion of the cysts through food or water. Cysts can survive acidic environment of the stomach and excyst to form trophozoites in the terminal ileum or colon. On the other hand, trophozoites are highly motile, multiply by binary fission, and encyst in the colon. Cysts excreted out in the feces can infect new individuals through contamination of food and water. Unlike many other parasites, *E. histolytica* multiplies and differentiates only in humans. The life cycle described here is generally seen in most individuals who do not show any symptoms of invasive disease. However, in a fraction of infected humans, amoebic trophozoites do become invasive, spreading to the epithelial tissue or blood vessel after making

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lesions through colonic layer. Therefore, the steps that play a critical role in pathogenesis are attachment to the colonic wall and destruction of basement membrane and tissues. Molecular analysis of pathogenic mechanisms has been possible due to unprecedented development, not only in our ability to identify, purify, and characterize individual molecules but also in our ability to manipulate parasite cells to express desired proteins and/or downregulate the expression of a specific gene. Though there has not yet been an animal model that mimics closely human disease, many in vitro cell-based assays, ex vivo systems, and tissue mimics have been developed. These can be used, in addition to animal models, to answer some of the questions related to pathogenesis. These studies have helped to identify some of the pathogenesis-related molecules and establish the role of some of the identified molecules in amoebiasis.

Host Genotype and Susceptibility to *E. histolytica* Infection

It has been recognized for some time that not all individuals are equally susceptible to infection and invasive disease as a result of an exposure to *E. histolytica* [17]. Only those humans who carry susceptible allele are more likely to get invasive disease. The host can contribute in number of ways to determine the outcome of *E. histolytica* infection. The gut environment may be highly favorable for *E. histolytica* to either excyst or multiply. Among other host factors, human intestinal microflora is thought to be involved in amoebic invasion as it plays an important role in metabolism and host immunity. Early studies have shown a clear relationship between bacterial association and pathogenesis of *E. histolytica* [18]. The trophozoites multiply in the lumen of the gut and obtain food through phagocytosis of the resident flora. It is therefore not surprising that gut microbiome has tremendous influence on amoebic physiology. Co-culturing of the trophozoites of *E. histolytica* with *E. coli* 055 or *Shigella dysenteriae* showed that these bacteria could change the cytopathic effect of *E. histolytica* and increase the expression of Gal/GalNAc lectin and cysteine proteinase activity [19]. Qualitative and quantitative alterations in gut flora have been reported in many intestinal diseases [20–22], but there are very few reports currently available on the status of gut flora in *E. histolytica*-infected individuals. Studies with selected bacterial species have shown that there is a significant decrease in absolute number of *Bacteroides*, *Clostridium coccoides*, *Clostridium leptum*, *Lactobacillus*, and *Campylobacter* and an increase in *Bifidobacterium*, while there is no change in *Ruminococcus* compared to healthy patients [23•, 24], indicating that some of the pathology observed during amoebiasis may be driven by an altered microbiome.

The host genotype may determine the nature of intestinal epithelial lining, thereby influencing the ability of *E. histolytica* to invade epithelial tissues. Since HLA-allelic polymorphism is associated with diversity in immune response, attempts have been made to associate susceptibility to amoebic infection with the presence of a specific HLA allele. Protection against amoebic infection was observed with HLA class II allele DQB1*0601/DRB1*1501 haplotype. Moreover, among heterozygous and homozygous haplotypes, the former were ten times less likely to be infected than the latter [25]. Genome-wide association studies have been used to identify susceptibility alleles for *E. histolytica* infection. In one of these studies, a SNP in leptin receptor, associated with invasive disease, was identified [26•]. Adipocytokine leptin was shown to have a protective role in mucosal resistance to *E. histolytica* infection. The Q223R allele showed fourfold higher susceptibility in children [26•, 27] which is thought to be due to attenuated STAT3 signaling. Moreover, leptin or leptin receptor deficient mice were found to be also more susceptible to infection [28]. Mice with non-functional leptin receptor were highly susceptible to *Entamoeba*-mediated mucosal destruction. Both STAT 3 and SHP2/ERK signaling are involved in leptin-mediated resistance. Host complement system also plays a protective role against *Entamoeba* infection; however, the pathogen has shown to develop a reversible resistance to complement lysis. A cell surface calreticulin-like protein that binds human C1q has been identified and is likely to play a role in complement resistance [29]. Matrix metalloproteinases (MMPs) play an important role in the invasive process. Genes encoding MMP 1 and MMP 3 were found to be overexpressed in colonic biopsies of patients with acute colitis [30]. Metallosurface protease EhMSP1, a novel M8 surface family metalloproteinase, was also shown to be involved in regulating amoebic adherence as silencing of the gene increases adherence to Chinese hamster ovary cell monolayer while having less impact on cell motility, phagocytosis, and monolayer destruction [31].

Molecules Involved in Pathogenesis

E. histolytica is a highly motile cell and motility is a pivotal factor for invasion. Trophozoites interact with the extracellular matrix and adhere to human cells as a result of binding to surface receptors. Amoebic motility appears to be one of the key features needed during tissue invasion. *E. histolytica* has an actin-rich cytoskeleton which plays a critical role by providing the necessary motive force during invasion and cytolysis. Some of the molecules that have been identified to modulate cytoskeleton are myosins (myosin II and myosin IB) [32, 33], gelation factor ABP 120 [34], small GTPAse Rac G [35], and PAK [36]. In general, any interference with cytoskeleton-associated molecules in *E. histolytica* blocks virulence [37].

Cell Surface Molecules of *E. histolytica*

Lipophosphopeptidoglycan and Other GPI-Anchored Cell Surface Molecules Trophozoites abundantly express lipophosphopeptidoglycans (LPPG), a major glycosylphosphatidylinositol (GPI) containing complex carbohydrate on their surface [38, 39]. These molecules are thought to make up the glycocalyx layer of *E. histolytica* and constitute the major surface component that interacts with the target cells, including human tissues, through terminal sugar molecules. Fresh trophozoites isolated from patients displayed higher thickness of LPPG-based glycocalyx layer [40]. Conversely, LPPG was not detected in nonpathogenic *E. dispar* [41], indicating an important role of LPPG in amoebic pathogenesis. LPPG is also thought to initiate inflammatory and immune response in animal and in patients [42•, 43]. EhLPPG can recognize TLR-2 and TLR-4 on the surface of macrophage and dendritic cells stimulating the expression of cytokines and co-stimulatory molecules [43, 44]. It mediates pro-inflammatory response after it enters the intestinal barrier [45]. Antibodies against LPPG prevent disease progression in animal models of amoebiasis. It has also been suggested as a possible ligand of natural killer T cells (NKT). The purified PI moiety of this molecule was found to induce IFN gamma, but not IL4 production by NKT cells [42•]. Exposure to LPPG also reduced the severity of liver infection in mouse models. All these results suggest that LPPG could be a good vaccine and therapeutic candidate for amoebiasis.

The *E. histolytica* cell surface displays a number of other GPI-anchored molecules including subunits of Gal/GalNAc lectin described below. Many of these molecules may have a role in attachment and virulence of *E. histolytica*. Though most of the molecules have not been characterized in relation to pathogenesis, their role in virulence has been demonstrated using indirect approaches. Reduction of cell surface GPI-anchored glycoconjugates was achieved by downregulating the expression of either mannosyltransferase or GlcNAc deacetylase [46, 47]. In both cases, the cells lost their ability to invade, as determined by different assays of pathogenesis, suggesting that GPI-anchored molecules play a crucial role in amoebic pathogenesis.

Gal/GalNAc Lectin Galactose/N-acetyl D-galactosamine-inhibitable (Gal/GalNAc) lectin is one of the major cell surface molecules that is involved in adherence of *E. histolytica* to basement membrane and epithelial tissues [48]. It has been proposed as a major vaccine candidate and a key molecule in amoebic pathogenesis [49–56]. The heavy subunit (Hgl) of the Gal/GalNAc lectin is a type 1 transmembrane protein while the light (Lgl) and intermediate (Igl) subunits have GPI anchors [57]. Genome analyses revealed redundancy in the genes encoding different subunits of the lectin. The orthologs of Hgl and Lgl subunits have been identified in the nonpathogenic *E. dispar* and in other *Entamoeba* species (*E. invadens*, *E. moshkovskii*, and *E. terrapinae*) [58]. Therefore, it appears that in addition to pathogenesis, these molecules may have a basic role in *Entamoeba* biology. The role of these molecules in attachment to target cells and resistance to complement has been amply demonstrated using monoclonal antibodies that were able to block attachment and subsequent invasion [59]. Moreover, dominant-negative phenotype of the Hgl subunit of the Gal/GalNAc lectin showed reduced cell adhesion activity and tissue invasion capacity, indicating a signaling pathway involving the cell-surface lectin molecules [60]. Although details of the pathway are not clear, it is thought that Gal/GalNAc lectin initiates signaling through the participation of phosphoinositides, lipid rafts, and cytosolic Ca^{2+} [61].

Other Cell Surface Molecules A number of other cell surface molecules with roles in amoebic pathogenesis have been identified and partially characterized. Among these, the EhCP-ADH complex has been studied most extensively [62–67]. EhCP-ADH complex encodes two proteins, a cysteine protease, EhCP112, and an adhesion protein, EhADH112 [68]. These proteins are involved in invasion, phagocytosis, and cytolysis, and the complex has been suggested to be a potential vaccine candidate [69]. In a screen to identify amoebic cell surface molecules that participate in phagocytosis, a serine-rich protein (SREHP) was identified [70]. Though the precise function of this abundant cell surface protein is not clear, results suggest that SREHP is likely to be part of the PI3K pathway [71]. Genomic polymorphisms in SREHP gene have been noted [72]. A lysine and glutamic acid-rich cell surface adhesion molecule KERP1 is reported to be involved in amoebic virulence [73]. The nature of the ligands and the mechanisms by which these molecules function is not clear. Genome analysis has revealed a large array of putative, cell surface transmembrane kinases (TMKs) in *E. histolytica*. These have been categorized into nine families and a few have been functionally characterized. Members of EhTMKB1 family, such as EhTMKB1-9 and EhTMKB1-2, have been shown to be involved in cellular proliferation and serum response [74, 75]. PATMK (EhTMKB3-96) co-localized with human erythrocytes at the site of contact and is involved in erythrophagocytosis and was identified during screening for proteins involved in the recognition and ingestion of apoptotic cells [76•]. TMK39 also participates in amoebic phagocytosis though in non-overlapping manner [77]. On the other hand, TMK54 interfered with the growth of *E. histolytica* and may have indirect effect on virulence as it regulated the expression of heavy subunit of Gal/GalNAc lectin [77].

It is not clear whether adhesion to target cells and phagocytosis require the participation of only a few key molecules or the combined action of a large number of cell surface components. It appears likely that participation of a large number

of molecules is needed, as blocking any one molecule gives only partial inhibition. The rules by which different molecules collaborate with each other and the various pathways that are involved in transducing signals after attachment to target cells need to be understood.

Secreted Molecules Involved in Pathogenesis

Damage of extracellular matrix, tissues, and cells are the hallmark of amoebic pathophysiology. As pointed out in the previous section, contact with *E. histolytica* cells is one of the primary events in the initiation of invasion. Besides direct contact, amoeba also invades by degrading or damaging the relevant tissues/cells by secreting a number of enzymes that are capable of digesting even hard tissues or extracellular matrices. Some of the well-known enzymes are described here.

Cysteine Proteases Cysteine proteases constitute the major class of secreted hydrolases of *Entamoeba*. Genome mining helped to identify about 50 genes coding for cysteine peptidases belonging to C1 papain superfamily, C2 (calpain-like cysteine proteases), C19 (ubiquitinyl hydrolase), C48 (Ulp1 peptidase), C54 (autophagin), and C65 (otubain), respectively (reviewed in Clark et al. [78]). Out of these, only 20 cysteine peptidase genes are expressed in *E. histolytica*, of which EhCP1, EhCP2, and EhCP5 constitute 90 % of the transcripts arising out of all cysteine proteases (CPs) [79•]. A number of experiments suggest that CPs play a major role in amoebic pathogenicity [80–86, 87•, 88, 89]. CPs can degrade extracellular matrix (ECM) proteins as well as mucin 2, a major component of colon mucus [90]. They also invade the immune system by degrading host antibodies and complement [91]. Among different CPs, EhCP5 has emerged as one of the major virulence-related CP. EhCP5 is present on the surface of the amoeba and is thought to participate in the disruption of the mucin barrier of the colon [92]. It also interacts with the colonic epithelial cell integrins, thus activating NFkappaβ-mediated inflammatory response in host cells [93]. Overexpression of cysteine protease genes *ehcp-b8*, *ehcp-b9*, and *ehcp-c13* results in transformation of a nonpathogenic *E. histolytica* to more pathogenic type [94]. CPs may have a direct role during tissue invasion and cell killing. Overexpression of cysteine proteinase 2, EhCP2, increases amoeba-induced monolayer destruction in vitro but has no effect on amoebic liver abscess (ALA) formation [86]. CP-specific inhibitor, *trans*-epoxysuccinyl-L-leucyl-amido-4-guanidino-butane (E-64), interfered with EhCP-A5 gene expression, thus showing involvement of CPs in ALA formation [85]. EhCP5 has interleukin-1b convertase activity suggesting that these enzymes use a mechanism that is novel in microbial pathogenicity [95]. A recent report by Thibeaux et al. showed that EhCP-A5 activates matrix metalloproteinases pro-MMP3 by cleaving. This in turn activates pro-MMP1 resulting in mucosal invasion. Ex vivo incubation with recombinant EhCP-A5 was able to rescue the deficient trophozoites, thus showing that cysteine proteases regulate the activities of MMPs for colonic invasion [96].

All these studies clearly show that CPs are major virulence-associated molecules and are a good target for developing novel therapies for amoebiasis. Cysteine protease EhCP4 has a key role in invasive amoebiasis and therefore a potential therapeutic target [97]. Recombinant EhCP1 has been used to identify a potent inhibitor of amoebic invasion in human colonic model [98]. High throughput screens for the identification of CP inhibitors have been developed, and small inhibitor molecules that blocked CP activity were identified. Auronafin is several times more potent than metronidazole and a few other inhibitors, such as paromomycin and tinidazole against *E. histolytica*. Auronafin works by altering the nucleotide metabolism, signal transduction, and mitosis. In the mouse models of amoebic colitis and hamster model of amoebic liver abscess, oral auronafin markedly decreased hepatic damage and inflammatory response [99••, 100], thus providing a potential drug to treat amoebiasis.

Amoebapores Amoebapores are a family of pore-forming peptides that is thought to be involved in cytolysis of target cells. There are three different amoebapores (a, b, c) encoded by separate genes, and these are structurally and functionally related to granulolysins and natural killer (NK) lysins produced by mammalian T cells [101•, 102, 103]. Trophozoites of *E. histolytica* lacking the major isoform amoebapore A, whether through antisense inhibition [104••] or epigenetic silencing of the gene [105], became avirulent demonstrating that this protein plays a key role in pathogenesis. Recent studies indicate that these peptides are more involved in killing phagocytosed bacteria or other organisms rather than target cells/tissues. These are discharged by *E. histolytica* into bacteria-containing phagosomes in order to kill and lyse engulfed microorganisms [106].

Other Molecules Some of the molecules reported to be involved in pathogenesis other than the ones described in the previous section are lysine and glutamic acid-rich protein KERP 1 [107], peroxiredoxin [108, 109], and arginase [110]. Since oxidative stress is harmful for amoeba, it is not surprising to find some of the enzymes that participate in removing different O_2/H_2O_2 ions/radicals.

Molecules Involved in Phagocytosis and Cell Surface-Associated Signaling

Phagocytosis plays a critical role in amoebic pathogenesis. Therefore, the molecules associated with phagocytic

pathways would be required for successful amoebic invasion. Though a number of cell surface molecules, such as Gal/GalNAc lectin, SREHP, KERP1, and EhCPDH, have been implicated in the attachment of target cells, it is not clear what role these play and the nature of signaling event initiated by these after ligand attachment [17]. It also appears that there is some sort of specificity in the choice of ligand by a cell surface receptor. This has particularly been seen in the Gal/GalNAc lectin where uptake of certain ligand was not inhibited by anti-lectin antibody [111, 112]. There is a need for detailed studies on the recognition of different cellular ligands by *E. histolytica* and the early signaling events initiated after attachment.

The mechanism of phagocytosis of RBCs by *E. histolytica* has been investigated in order to understand the process of phagocytosis in this organism. Ca^{2+} signaling was found to be important, as chelation of intracellular Ca^{2+} blocked erythrophagocytosis [113]. A number of calcium-binding proteins, such as EhCaBP1 and EhCaBP3, were identified as key molecules during erythrophagocytosis. These two molecules were found to be recruited early to the phagocytic cups. Both molecules bind actin and, in addition, EhCaBP3 also interacted with myosin 1B [114], thereby regulating cytoskeleton dynamics.

Recent work by Somlata et al. suggests that recruitment of a C2 domain-containing protein kinase (EhC2PK) at the ligand attachment site is one of the early signaling events in phagocytosis. This leads to recruitment of EhCaBP1 and subsequently actin [115•]. EhCaBP3 joins the phagocytic site independently. It is suspected that increase in local Ca^{2+} level on the attachment of the ligand helps EhC2PK binding to the PS-containing inner leaflet of the plasma membrane through C2 domain. The kinase activity of EhC2PK is essential for the progression of phagocytic cup to phagosome, as overexpression of a kinase-dead mutant resulted in reduced erythrophagocytosis [115•]. Since the substrate for EhC2PK has not been identified, the nature of downstream signaling pathway propagated through EhC2PK remains unknown. Since both EhCaBP1 and EhC2PK leave the phagocytic cup before it closes to form the phagosome, these two molecules appear to be required primarily in the initial stages of cup formation and stabilization. On the other hand, EhCaBP3 is present in nascent phagosomes and is also present along with myosin 1B at the site of cup closure [114]. It has been speculated that EhCaBP3 is involved both in cup progression as well as phagosome closure and scission. Therefore, the two calcium-binding proteins EhCaBP1 and EhCaBP3 have distinctly different functions. Apart from these two CaBPs, an alpha kinase EhAK1 is also involved in the amoebic phagocytic pathway [116]. EhAK1 phosphorylates actin and thereby regulates cytoskeletal dynamics. It is recruited at the phagocytic cups and is present till phagosomes are fused, but is absent in nascent phagosomes. A number of approaches, such as antisense inhibition, overexpression of kinase-dead mutant, and phosphorylation-defective actin molecules, were used to demonstrate that EhAK1 participates in phagocytosis. These results point to a novel pathway of phagocytosis in *E. histolytica*, and the key molecules described here offer good targets for developing novel therapeutics.

A number of other signaling molecules are believed to participate through interaction of cell surface-signaling molecules with RBCs/bacteria/epithelial cells/ECM. These involve cytoskeletal dynamics and are important in the pathogenesis of amoebiasis [117, 118]. Transduction of these signals may be mediated through heterotrimeric G proteins [119] and subsequently, GTPases, multifunctional kinases, and phosphatases may be involved [120]. Some of the known cytoskeleton-modulating proteins, such as PAK1 [36]; EhFP4 [121]; EhGEF1 [122], EhGEF2 [123], and EhGEF3 [124]; myosin 1b [125] and myosin II [126], have also been identified in *E. histolytica*, and preliminary functional characterization suggests that these also participate in cytoskeleton dynamics and consequently phagocytosis in amoeba. However, detailed mechanisms are still lacking.

Traditional concept about target cell killing and invasion has been seriously challenged in a recent study. Instead, it has been suggested that cell killing and invasion follows a process called trogocytosis, nibbling bits of cells and tissues similar to that seen in the immune system [127]. Live cells undergo trogocytosis whereas phagocytosis operates for dead cells. Since EhC2PK was also found to be involved in trogocytosis, it appears that it follows the same pathway as phagocytosis. The conditions that determine whether trogocytosis or phagocytosis will take place is not yet clear.

Mucus Layer

Goblet cells in the colonic epithelium secrete mucins into the lumen, which form a gelatinous barrier and present the first line of defense against invading pathogens. Studies with MUC2-deficient mice suggested that mucin layer provides resistance against infectious colitis by maintaining a balance between pathogenic and commensal organisms associated with the mucosal surface [128]. They also help to block adherence of trophozoites to epithelial cells by providing ligands for the binding of Gal/GalNAc lectin, resulting in competitive inhibition [48]. CPs are thought to bind less glycosylated regions of the MUC2 polypeptide, facilitating solvation of the colonic mucus gel and invasion of the colonic epithelial cells [90]. When these mice were challenged with *E. histolytica* trophozoites, it displayed pro-inflammatory response as shown by increased expression of pro-inflammatory markers such as TNFα and IFNγ [128]. In the absence of MUC2, mice became vulnerable to EhCP-A5-mediated secretory and pro-inflammatory responses [129]. PGE2 was secreted [130], by the activation of prostaglandin receptor EP4 [131]. The

resulting disruption of tight junctions allowed invasion of trophozoites into the basolateral surface of intestinal epithelial layer [132]. Apart from CPs, other molecules may also be involved in the disruption of the colonic mucus layer, such as an "occludin- like" protein that is thought to play a role in pathogenesis [133].

Models of Pathogenesis

In Vitro Models The extracellular matrix consists of basement membrane and interstitial connective tissue, dominated by the three-dimensional (3D) network of collagen I fibers [134]. Fibrillar collagen is the most abundant protein of the extracellular matrix, others being elastin, fibronectin, and laminins. The visualization and interpretation of these 3D collagen matrices using advanced techniques of 3D time lapse microscopy [135] has provided a great boost in understanding pathogenesis. The 3D collagen architecture with or without intestinal epithelial cells (mostly using epithelial tumor cells) define mechanical properties of tissues and provide models which partially mimics the way amoeba invade through basement membrane in the intestine. Thibeaux et al. showed that collagenolytic activity important for amoebic invasion is mainly due to the CPs, particularly EhCP5 [136], and that the parasite follows amoeboid migration through the 3D collagen matrix and has unique features as compared to mesenchymal and small cells, such as T lymphocytes and dendritic cells. In a dense 3D collagen matrix, amoebic cells adopt a protease- and amoeboid-dependent mode of migration [136, 137].

During liver invasion, *E. histolytica* trophozoites are in contact with liver sinusoidal endothelial cells (LSEC). A LSEC cell line has been generated from immortalized cells of human liver endothelial cell primary cultures [138]. Cells do not transform and express phenotypic markers of primary cultures [139] and respond to inflammatory molecules such as TNF [140] and to hypoxia/reoxygenation stress by inducing necrosis and apoptosis [141]. Incubation of virulent trophozoites of *E. histolytica* with LSEC interfered with host cell adhesion signaling and leads to diminished adhesion and target cell death. This is a direct result of contact with parasites that induces the disruption of actin stress fibers and focal adhesion complexes of target residues [142]. A 3D in vitro liver model generated using a monolayer of Huh-7 hepatocytic cells embedded in a 3D COL-I matrix and an LSEC monolayer plated on top of the matrix was incubated with trophozoites and monitored by two-photon microscopy. This model revealed the role of human galectins in aiding invasion. Human galectins have been reported earlier to play a role in innate immune response to microbial infections. Human galectins 1 and 3 play a dual role: promoting hepatic adhesion and then triggering a pro-inflammatory response in hepatic infection by *E. histolytica* [143•]. Some of the amoebic factors that appear to participate are Gal/GalNAc lectin, cysteine proteases, and KERP1.

Animal Models Animal models, such as mice models of amoebic colitis and gerbil model of liver abscess, have been extensively used to study amoebic pathogenesis. Mice carrying this humanized *Lepr* 223R allele using homologous recombination (equivalent to 222R in the mouse; for simplicity, it will be referred to as 223R) were generated by injecting ES clone into C57BL/6J blastocysts [26•, 144]. The amoebae were injected intracecally and the transfected cell lines were mixed in 1:1 ratio. This induction was maintained for 4 days and then sacrificed post 4 days of infection, and amoebae were isolated from luminal gut and mucosal surface of mice. These studies helped to confirm a relation between amoebic invasion and leptin receptor. Cells overexpressing the kinase dead mutant of EhTMKB1-9 showed its inability to survive and did not show invasive phenotype in murine model of amoebic colitis [145, 146].

In the gerbil model liver, abscesses are induced by injecting trophozoites in different lobes of 50–60-day-old Mongolian male gerbil. These are sacrificed 7 days post infection and the abscess weights measured [146]. Though EhTMKB1-9 did show its involvement in tissue invasion in the mice model, there was no effect seen in the gerbil model [146]. Similarly, amoebae overexpressing EhCP-2 induced monolayer destruction in vitro but did not show any marked effect on liver abscess formation in gerbils [86]. Guinea pig model of intestinal amoebiasis was generated by intracecal injection of *E. histolytica* in 4–6-week-old cholesterol-fed animals, and it consistently produced amebomas [147]. This model has been used to see the effect of heparin sulfate-binding proteins of *E. histolytica*. These were found to provide partial protection against challenged infection [148]. Unavailability of good animal models precludes detailed study on the progression of this disease and is difficult to monitor.

Ex Vivo Models These provide intermediate complexity between in vitro and animal models. The results can explain some of the features that mimic a potential tissue. Cysteine protease-deficient *E. histolytica* showed less inflammation and degradation of tissues in the severe combined immunodeficient mouse/human intestinal xenograft (SCID-HU-INT) model of amoebic colitis [95, 149]. CPs mimicked the interleukin 1-beta-converting enzyme activity of human and breaks down interleukins [95]. Bansal et al. used human colon explants for the first time to show that dynamics of tissue invasion, destruction, and induction of early inflammatory response can be reproduced [150, 151••]. Porcine colonic explants [152•] behave in the same ways as the human colonic explants. Studies on human colon explants also suggest carbohydrate sources may affect the ability to invade the intestine

[137]. Precision cut liver slices has been proposed as a useful model for studying amoebic liver abscess [153, 154]. It is possible to observe cytokine production and apoptosis after incubation with *E. histolytica* and study mechanisms using this model.

This brings out an important issue of using multiple models, as a single model may not give accurate picture as none of the models do really reflect actual infection cycle in humans (by cysts through oral route).

Conclusion

The pathogenesis of amoebiasis involves interplay of various molecules secreted by *E. histolytica* such as LPPG, lectins, cysteine proteases, and amoebapores. Lectins help in the attachment of the parasite to the mucosal layer of the host during invasion. The amoebapores destroy the ingested bacteria present in the colonic environment. Cysteine proteases lyse the host tissues. Other molecules such as PATMK, myosins, G proteins, C2PK, CaBP3, and EhAK1 play an important role in the process of phagocytosis. Unraveling of new molecules in understanding the process of phagocytosis will help a great deal in developing therapeutics for this disease, but there are still many questions that remain unanswered. We have also seen that host genotype also play an important role in susceptibility of the infection. All the animal models available till now have not been able to replicate the actual infection cycle in the humans. Extensive work has been done in understanding the complex mechanisms of infection, but there still a long way to go before we could completely understand the basis of the pathogenesis.

Compliance with Ethics Guidelines

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article contains no studies with human or animal subjects performed by the author.

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- •• Of major importance

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