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New insights into *Entamoeba histolytica* pathogenesis

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Abstract

Purpose of review—*Entamoeba histolytica* is an important global pathogen and a leading cause of parasitic death worldwide. This article summarizes significant research findings over the last year.

Recent findings—Efforts have focused primarily on identification of novel virulence determinants in *E. histolytica*, transcriptional profiling during tissue invasion and stage conversion, and characterization of basic cell biological processes. Additionally, new techniques for gene silencing have been identified.

Summary—A comprehensive examination of the parasite lifestyle on a whole genome level has been undertaken, allowing identification of new virulence genes and signaling pathways and processes relevant to amebic biology.

Keywords

Entamoeba; gene silencing; microarray; parasite; pathogenesis; protozoa

Introduction

E. histolytica is an enteric protozoan parasite that infects 500 million people per year, causes disease in 50 million, and kills 100 000 individuals annually [1]. Clinical manifestations include asymptomatic infection, diarrhea, dysentery, and liver, lung, or brain abscesses. Why only 10% of infected individuals develop symptoms whereas 90% have asymptomatic colonization is still unknown. Understanding basic biology and genes relating to pathogenesis will give a better understanding of the amebic lifestyle and allow for development of new drug targets and potential vaccine approaches.

The *Entamoeba histolytica* genome

Completion of the *E. histolytica* genome and subsequent analysis has unveiled novel aspects of amebic genome organization [2•]. The genome is predicted to be 24 Mb with 14 chromosomes and is functionally tetraploid. Some interesting features include evidence for lateral gene transfer from bacteria and large numbers of vesicle trafficking genes, transmembrane kinases (TMKs), and proteolytic enzymes. One unprecedented characteristic of the *E. histolytica* genome is the structure and organization of the tRNA genes that are organized into distinct arrays (500 bp to over 1750 bp), of which there are approximately 4500 in the genome [2•,3]. The same type of tRNA array organization is present in other

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Entamoeba species, but individual repeat sequences in these species are quite different. These data suggest that the tRNA organization predates the common ancestor to known *Entamoeba* species. The function of the tRNA arrays remains unclear and warrants further investigation [4].

Transcriptional control

Much progress has been made in transcriptional profiling of *E. histolytica* to study diverse aspects of amebic biology including genes involved in amebic pathogenesis [5], assessing differences between virulent and nonvirulent amebic strains [6], changes during stress response [7], responses to pharmacologic perturbations [8–10], and changes during stage conversion [11••].

Expression profiling of *E. histolytica* trophozoites during log-phase growth was performed and potential promoter motifs associated with specific expression patterns identified [7]. Electrophoretic mobility shift assays demonstrated that amebic nuclear proteins bound specifically to the promoter motifs indicating that in this system, functional transcriptional regulatory networks can be readily identified.

The roles of methylation and histone acetylation on *E. histolytica* gene expression were investigated. DNA methylation hinders transcription factor binding; the effects of 5-azacytidine, an inhibitor of DNA methyltransferase, were examined and minimal changes were identified [8]. Few genes (<2%) tested had significant transcriptional changes suggesting that methylation does not play a significant role in modulating amebic gene expression. Histone acetylation is another epigenetic mechanism used for transcriptional modulation; acetylation of histone proteins removes their positive charge, reducing the affinity between the histone and DNA, thus making the DNA more accessible to RNA polymerase. Two studies revealed conflicting data on the roles of histone acetylation in *E. histolytica* gene expression [9,10]. Ehrenkauf *et al.* examined the transcriptional profile of 200:NIH trophozoites and found that exposure to trichostatin A (TSA), an inhibitor of histone deacetylase, resulted in decreased parasite growth rates and a significant overlap between developmentally regulated genes and those regulated by TSA. These data suggest that histone acetylation is one method by which amebic gene expression is controlled during stage conversion. Isakov *et al.* used the HM-1:IMSS parasite strain and identified significant inhibition of parasite growth but an increase in parasite virulence, hemolytic activity, and resistance to oxidative stress. Overall, very few genes were identified whose transcriptional profile overlapped between the two studies. A number of reasons may account for these conflicting results including the fact that the two studies used different amebic strains and concentrations of TSA. Overall, the data indicate that the effects of histone acetylation in *E. histolytica* are complex and need further investigation.

Gene silencing

One exciting development is the recent identification of epigenetic silencing in *E. histolytica*. Previously, it was demonstrated that the Amoebapore-A (Ap-A) gene was silenced in *E. histolytica* by introduction of a plasmid containing the open reading frame, and the upstream promoter region, which contained a short interspersed nuclear element (SINE) [12]. Silencing of Ap-A occurred at the genomic locus and was permanent, even after plasmid removal; this parasite line is now called G3. Subsequently, additional genes have been silenced in the G3 parasite line using a plasmid containing the Ap-A promoter region with the SINE cloned upstream of the coding region of a gene of interest [13••]. The G3 strain represents a useful new tool for silencing genes in *E. histolytica*. Another technological advance is the development of gene silencing using short hairpin RNAs (shRNA) [14••]. Short hairpin RNAs (~29 bp) were expressed using the amebic U6

promoter and gene knockdown achieved using two out of three shRNAs. Whether the G3 and shRNA approach can be applied to mediate robust and reliable gene silencing in *E. histolytica* remains to be determined.

New insights into stage conversion

The identification of developmentally regulated genes and pathways in *E. histolytica* has been severely limited by the lack of an in-vitro encystation method. One key project involved characterizing the transcriptional profile of *E. histolytica* clinical isolates, which contained cysts [11••]. The clinical isolates were collected from patient fecal samples, grown in tissue culture for less than 8 weeks, and had a vastly different transcriptional profile from trophozoites of various *E. histolytica* strains or parasites from a colitis model. A total of 672 cyst-specific and 767 trophozoite-specific genes were identified. Additionally, this study also identified that a complex diphasic medium induced spontaneous encystation in *Entamoeba invadens* suggesting that this medium may be supportive of stage conversion [11••]. Another study reported that growth of *E. histolytica* in standard TYI-S-33 culture medium supplemented with *Escherichia coli*, *Enterococcus faecalis*, high CO₂ concentration, and histamine resulted in presence of cyst-like structures [15]. However, a number of concerns were noted, and it is not clear that this approach necessarily represents a method to generate mature cysts. One process of interest with regard to encystation is autophagy, a process that allows for degradation of unneeded organelles and proteins in response to various stimuli. Autophagy during both proliferation and encystation of *E. invadens* has been identified [16].

Transcriptional profiling reveals new virulence determinants

Recent approaches to study amebic pathogenesis have focused on comparing virulent and nonvirulent *Entamoeba* strains and species. These comparative studies have uncovered a number of novel virulence determinants [6,17–19,20*]. MacFarlane and Singh compared the transcriptional profiles of virulent and nonvirulent *E. histolytica* strains (HM-1:IMSS and Rahman, respectively) and the nonvirulent species *Entamoeba dispar* and identified a number of genes with lower expression in the nonvirulent strains. They further went on to prove that genes with lower expression in nonvirulent strains may be virulence determinants by functionally studying one such gene, an *E. histolytica* serine, threonine, and isoleucine-rich protein (EhSTIRP) and showing that EhSTIRP downregulation in the virulent strain had a deleterious effect on amebic virulence [19].

Davis *et al.* [6,17] used both a proteomic and microarray-based approach to compare the HM-1:IMSS and Rahman strains of *E. histolytica*. Proteomic approaches identified only six proteins with significantly different abundance between HM-1:IMSS and Rahman including peroxiredoxin, which was in higher levels in the virulent compared with the nonvirulent strain. In order to determine if the differential protein abundance contributed to virulence potential, the authors overexpressed peroxiredoxin in the nonvirulent Rahman trophozoites resulting in an increase in resistance to oxidative stress and increased virulence in the severe combined immunodeficient mouse-human intestinal xenograft (SCID-HU-INT) mouse model of infection [6]. Further studies have shown that this protein is crucial for protection against oxidative stress as gene downregulation causes reactive oxygen species accumulation along with a decrease in parasite viability, cytopathic and cytotoxic ability [21].

Another study examined the transcriptional profile of *E. histolytica* parasites harvested from a liver abscess compared to parasite strains incapable of causing hepatic disease [20•]. A group of four genes encoding lysine and glutamic acid-rich proteins (KERPs) were found to have higher expression in the virulent liver-derived parasites. *EhKERP1* expression was

knocked down using antisense technology, and parasites with decreased KERP1 were unable to form liver abscesses in hamsters, supporting the function of this protein as a potential virulence factor in causing amebic liver disease.

Taken together, these results support the idea that genes or proteins with greater abundance in virulent strains compared with nonvirulent strains may function as virulence determinants in *E. histolytica*.

Proteases and pathogenesis

Of particular interest because of their relevance to amebic pathogenesis are proteolytic enzymes. To date, 86 peptidase genes have been identified in the *E. histolytica* genome including 50 cysteine, four aspartic, 10 serine, and 20 metallo peptidases [2••]. Expression data from different strains and under different conditions identified that only a small subset (*EhCP1*, *EhCP2*, and *EhCP5*) is expressed under tissue culture conditions [5]. A number of cysteine proteases were identified as cyst specific [11••], but the roles of the other cysteine proteinases in amebic biology remain to be determined.

Another class of proteases has recently received particular attention: rhomboid proteases are intramembrane serine proteases that cleave transmembrane proteins in their transmembrane domains and have been implicated in mediating host cell invasion in Apicomplexan parasites [22]. A single functional rhomboid protease *Entamoeba histolytica* rhomboid protease 1 (*EhROM1*) was identified in *E. histolytica* with unique substrate specificity. *EhROM1* targets the heavy subunit of the amebic Gal/GalNAc lectin and relocalizes to the cap when surface receptor capping is induced [23•]. Capping is an immune evasion technique in which receptors on the parasite surface recognized by host antibodies are polarized to the posterior end of the parasite and shed from the surface. Overall, the data suggest a potential new role for rhomboid proteases in immune evasion.

Another avenue of research is identification of protease inhibitors. Recombinant *E. histolytica* cysteine proteinase 1 (*EhCP1*) was used to screen a library of inhibitor compounds. Two inhibitors were tested on amebic trophozoites in the colonic xenograft colitis mouse model and significantly decreased the parasites' invasion potential suggesting that small molecule inhibitors may be one path toward future therapeutics [24].

Transmembrane kinases and phagocytosis

A large gene family of approximately 90 TMKs was identified in *E. histolytica* [2••,25,26]. Recent data suggest that TMK 96, initially identified in phagosome protein preparations, has a role in phagocytosis of human erythrocytes [14••]. TMK 96 is present on the parasite surface and co-localizes with beads being phagocytosed by the parasite. Preincubation of parasites with antibody to TMK 96 resulted in a dramatic reduction of erythrocyte ingestion. Downregulation of TMK 96 or overexpression of a dominant negative version with no kinase domain resulted in a decrease in erythrophagocytosis.

Parasites also ingest apoptotic host cells to prevent inflammatory responses by the host from accumulation of dead cells and signaling of the immune system. An immunogenic surface protein, the serine-rich *E. histolytica* protein, is involved in phagocytosis of apoptotic host cells [27].

Cytoskeleton and vesicle trafficking

The cytoskeleton of *E. histolytica* is crucial to pathogenicity because it controls the ability of the parasite to move, invade host tissues, and phagocytose host components. Recent studies

have characterized filamin, kinesin, and a Lin11, Isl-1 and Mec-3 (LIM)-domain-containing protein. Filamins are cross-linking actin-binding proteins that help to organize filamentous actin networks. *E. histolytica* filamin (EhFLN) associates with signaling molecules *in vitro* suggesting signaling capabilities and overexpression of EhFLN increased motility and chemotactic response, suggesting a role in both motility and signaling [28]. Kinesins are motor proteins involved in spindle formation and genome separation during cell division. EhKlp5 is a divergent member of the kinesin 5 family and silencing of EhKlp5 by dsRNA prevents spindle formation and cytokinesis in *E. histolytica* [29]. LIM domains are protein–protein interaction domains. *E. histolytica* LIM protein A (EhLIMA), the only LIM-domain protein in *E. histolytica*, is in both lipid raft and cytoskeletal fractions, suggesting that it may play a role in connecting the two, which may be important in regulating raft formation and movement [30].

The *E. histolytica* genome encodes 91 Rab guanosine triphosphatases (GTPases) that are involved in diverse aspects of vesicle trafficking. Rab11B is involved in secretion of cysteine proteases as overexpression of Rab11b resulted in a dramatic increase in both intracellular and secreted cysteine protease activity and an increase in cell monolayer destruction [31•]. Other studies have focused on elucidating roles for two of the Rab7 isotypes Rab7a and Rab7b [32]. These studies showed that these isotypes localize to different compartments, and dominant negative versions of these proteins resulted in different phenotypes, suggesting that these two isotypes play different roles in *E. histolytica* biology, further emphasizing the complexity of vesicle trafficking in this protozoan parasite.

Association of *Entamoeba histolytica* and HIV infection

Previously no increased association of amebic disease with HIV infection was noted. However, in the last year, multiple studies have reported a higher prevalence of amebic seroconversion, invasive amebiasis and amebic liver abscesses (ALAs) among HIV-positive individuals compared with comparable groups of HIV-negative individuals [33–36]. Within the HIV-positive population, men who have sex with men (MSM) have higher rates of seropositivity for *E. histolytica* based on a study from Taiwan, which examined HIV patients over an 11-year period [37•]. An overall higher susceptibility to *E. histolytica* specifically amongst HIV-positive individuals in China was also noted, but in this study the incidence of amebic infection was comparable between the sexes [36]. A higher incidence of ALA among MSM than in other HIV-positive populations has been reported [33,37•,38].

Modulation of the host immune response

Understanding which parasite components activate different immune pathways allow for a better understanding of the interplay between the parasite and host immune response during infection and will aid in vaccine development.

Dendritic cells are activated by the amebic Gal/GalNAc lectin driving a T helper cell 1 (Th1)-type immune response [39]. Dendritic cells exposed to amebic lectin had increased expression of activation markers such as CD80, CD86, CD40 and MHC class II and induced Th1-type cytokine production including interleukin-12 and tumor necrosis factor (TNF)- α . Adoptive transfer of Gal/GalNAc lectin treated dendritic cells into naïve mice induced a Th1 response. Activation of dendritic cells is an important step in the induction of cellular immunity required for *E. histolytica* clearance. This work provides further insight into the potential usefulness of the amebic Gal/GalNAc lectin as a vaccine target.

Another trophozoite component that activates the host immune system is lipopeptidophosphoglycan (LPPG), an immunogenic complex macromolecule on the parasite surface [40]. Purified LPPG activated both macrophages and dendritic cells

inducing expression of activation markers and induction of cytokines. LPPG was internalized into these cells in which it may then be processed and loaded into major histocompatibility complex (MHC) molecules for surface presentation and induction of a specific immune response.

Inflammation of the colonic epithelia and infiltration of immune cells characterizes invasive amebiasis. Secreted proteins from ameba induce expression of monocyte chemotactic protein 1, which signals immune cells to come to the site of infection [41]. These data suggest that the parasite manipulates expression of host chemotactic factors to induce an inflammatory response at the site of infection.

E. histolytica induces apoptosis in host cells. Inhibition of neutrophil apoptosis induced by *E. histolytica* can be achieved by incubation of host cells with antibodies to CD18, also known as β 2-integrin [42]. This inhibition increased with incubation of both antibodies to CD18 and galactose, suggesting that a parasite surface protein other than the Gal/GalNAc lectin signals via β 2-integrin to induce apoptosis.

Vaccine development

Efforts for vaccine development for *E. histolytica* have advanced. A combined injection of the Gal/GalNAc lectin, a cell surface immunogenic protein, and CpG oligodeoxynucleotides was protective against *E. histolytica* challenge in both gerbils and mice [43••]. CpG was used as an adjuvant because it is able to activate the ideal branches of the immune system required for a vaccine. Amebic trophozoite DNA activates macrophages in a toll-like receptor (TLR)-9-dependent manner [44]. Immunization of gerbils with *E. histolytica* DNA or Gal/GalNAc lectin showed a protective effect against liver abscess formation. Immunization with a combination of trophozoite DNA and Gal/GalNAc lectin was able to protect completely against *E. histolytica* challenge suggesting that activation of TLR-9 by these amebic components can provide an effective innate response to further *E. histolytica* challenge.

Conclusion

In this review, we have outlined progress made in the field of *E. histolytica* research over the last year including development of new vaccine approaches, new tools and reagents, and identification of a number of novel virulence determinants. With available resources, research on this protozoan parasite will progress steadily in years to come.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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