Host-Microbe Interactions and Defense Mechanisms in the Development of Amoebic Liver Abscesses

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INTRODUCTION

Entamoeba histolytica (the etiological agent of human amoebiasis) leads to several distinct clinical manifestations: diarrhea, dysentery, and hepatic liver abscesses. Invasion starts when, for hitherto unknown reasons, trophozoites residing in the colon lyse, deplete the mucus, interact with exposed enterocytes, dismantle cell junctions, and lyse host cells. Once the epithelial layer is disrupted, amoeobae cross the basal lamina and the extracellular matrix (ECM); this is a process involving parasite adherence, motility, and cytotoxicity toward host cells. Some trophozoites that reside in the colon penetrate the portal system, resist the stress provoked by this new environment, and follow the bloodstream to the hepatic portal veinule and sinusoids. The latter are the main structures where amoeobae cross the endothelium to reach the parenchyma, with a concomitant initiation of inflammatory foci and abscesses. The typical amoebic liver abscess (ALA) is due to necrotic lysis of the liver tissue, which varies in size from a few centimeters to a large lesion. It is often single, usually in the posterior, superior area of the right lobe. ALA is characterized, in its acute manifestation, by fever (temperature greater than 39°C), pain in the right hypochondrium, and liver tenderness (43). Although they also present pain upon palpation of the liver, patients with chronic ALA exhibit different manifestations: mild or no fever, hepatomegaly, and rales or rhonchi. Upon auscultation, the clinical features of ALA allow one to suspect the amoebic etiology, which, however, needs further exploration, such as ultrasound echography, to be confirmed.

Most patients with ALA are young adult males, although the male/female ratio is equal to 1 in children and infants. Patients with ALA infrequently present concurrent colitis, although they sometimes have a history of dysentery within the last year (1). Once diagnosed, ALA is managed by the administration of metronidazole or tinidazole, followed by treatment with a luminal amoebicide, i.e., paromomycin or diloxanide furoate (35). Responses to amoebic infection depend on the organ’s cell types and architecture; here, we shall describe the interplay between the parasite and the hepatic tissue, which operates during liver abscess formation by E. histolytica.

THE LIVER: VASCULAR AND SINUSOIDAL STRUCTURE

The liver parenchyma includes endothelial cells, hepatocytes, and stellate cells, which together constitute a very complex, three-dimensional tissue that bathes in blood. Important inflammatory cells (such as Kupffer cells [KCs], dendritic cells [DCs], lymphocytes, and leukocytes) are present and support

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immune and homeostasis features. Indeed, besides the liver’s metabolic and amphicrine secretion activities, the organ clears molecules and particles carried by the afferent blood; this notably includes senescent red blood cells, metabolites, and orally ingested antigens that are brought by the hepatic artery from the aorta and by the portal vein from the mesenteric tract. Incoming blood flows centripetally through the hepatic sinusoids and subsequently reaches the efferent centrolobular vein. Figure 1 illustrates the afferent and efferent microvascular connections to the sinusoids within a single hepatic lobule, and Fig. 2 shows the structure of a sinusoid.

Liver Sinusoidal Endothelial Cells and Stellate Cells

The interface between the sinusoid lumen and the perisinusoidal space (also known as the space of Disse [DS]), into which the microvilli of the parenchymal cell (lined up in ribbons) project, is composed mainly of liver sinusoid endothelial cells (LSECs). These are fenestrated endothelial cells that lack tight junctions and a basal membrane, which enables particles less than 12 nm in diameter to cross into the DS (44). LSECs have an enhanced endocytic capacity (with glycoprotein ligands, ECM components, and immunoglobulin G [IgG] Fc receptor immune complexes) and can process antigens for presentation on class I or II major histocompatibility complex (MHC) molecules (27). In addition, they take part in the secretion of cytokines, eicosanoids (derived from arachidonic acid), local inflammation mediators, nitric monoxide (NO), and ECM components. Hepatic stellate cells (SCs) (formerly known as hepatic lipocytes) are found in the DS and fulfill several functions: they can (i) regulate sinusoid blood flow by contraction (performed via smooth muscle α-actin and which can be induced by NO), (ii) store or release retinoids (i.e., vitamin A), and (iii) control the secretion of ECM. Liver injury causes SCs to transform into myofibroblasts that participate in the development of fibrosis. These cells can amplify the inflammatory response by producing chemokines, which, in turn, induce the infiltration of mono- and polymorphonuclear leukocytes (PMNs).

Immune System Cells in the Liver

KCs are monocytes that reside in the liver sinusoids and straddle the endothelium. KCs exert three main functions (antigen presentation, phagocytosis, and cytotoxicity) and thus play a role in clearing the blood of senescent red blood cells and opsonized or complement-coated particles (37). Following phagocytosis of target particles, KCs can secrete potent inflammatory mediators, such as reactive oxygen species (ROS), eicosanoids, NO, tumor necrosis factor (TNF), and other cytokines. TNF activates the presentation of ICAM-1, VCAM-1, and P- and E-selectin by LSECs (56); these proteins are ligands for (among others) natural killer cells (NKC), monocytes, and PMNs. The latter cell types are involved in the inflammatory...
response, and their early recruitment to the nascent inflammatory foci allows the continuation and control of innate immune defenses.

Hepatic lymphocytes consist of different subsets, with a majority of T cells (35%), followed by natural killer T cells (NKTCs) (30%), NKCs or pit cells (20%), and B cells (BCs) (10%) (25). Of these, NKTCs, NKCs, and BCs can be activated directly by pathogen-associated molecular patterns (PAMPs) without prior processing and presentation of antigens by antigen-presenting cells. Thus, NKTCs, NKCs, and BCs have an unquestioned role as fast-responding, potent cytotoxic effectors or modulators of cytokine responses in the early stages of liver infection.

DCs are present in the periportal and sinusoidal spaces (86). Whether DCs are true residents of the sinusoidal compartment is subject to debate, since the presence of N-acetylgalactosamine ligands at the cell surface may be crucial for DC recruitment to liver sinusoids (55). However, increased traffic and the blood-lymph translocation of DCs during infection highlight the cells’ potent role during the initiation and implementation of an adaptive immune response (55). The involvement of DCs in the adaptive immune response via efficient antigen uptake, processing, and presentation is complemented by their role in innate immunity as lymphocyte-activated or -activating cells. Recent publications have described the cross talk between DCs and NKCs, which triggers inflammatory cytokine production by both cell types and the enhancement of the cytotoxic potential of NKCs (85).

Lastly, circulating or lymph-borne leukocytes (such as lymphocytes and PMNs) are cell types that flow through the liver and may be recruited during the various stages of the liver’s reaction to infection. Neutrophil PMNs, lymphocytes, and monocytes can all trigger a so-called oxidative burst. Leukocyte trafficking from the blood vessels to the site of infection occurs in several steps. Following activation by chemokines and cytokines, leukocytes arrive at and adhere to the endothelial cell surface and then transmigrate into the underlying tissue via (i) small gaps at the junctions between endothelial cells and (ii) transcellular migration across the endothelium (28).

**FIG. 2.** *Entamoeba histolytica* trophozoite within a sinusoid. The sinusoidal wall is made of LSECs and, with hepatocytes (HCs), delimitates the DS, in which SCs and components of the ECM are found. Leukocytes (LCs) are present in the sinusoidal lumen (SL), as are KCs, which additionally straddle the endothelium. The diagram presents the major factors involved in the interaction between *E. histolytica* and the liver endothelium. Within the hepatic sinusoids, trophozoites undergo complement attack and oxidative stress provoked by the high-oxygen partial pressure. Amoebae resist these threats by using Gal/GalNAc lectin (blue ellipses), lytic factors such as cysteine proteases (white ellipses), reducing enzymes (purple ellipse), and potentially other molecules of as-yet-unknown function (black ellipses). Trophozoites adhere to the endothelium and cause the apoptosis of LSECs. Amoebae can then migrate to the parenchyma (yellow arrow) through the newly created breach or between LSECs.

**PATHOPHYSIOLOGICAL FEATURES OF CROSS TALK BETWEEN *E. HISTOLYTICA* AND THE LIVER**

Although the host deploys a massive inflammatory response against *E. histolytica*, the parasite manages to survive within this environment. Virulence can be defined as a two-step strategy: invade and survive. The clonal nature of the trophozoite populations used for experimental infection cannot explain the fact that some resist and some succumb in the same microenvironment during host infection. This underlines a potential mechanism of local and individual adaptation, which would hypothetically lead to subpopulation specialization. However, overall virulence traits need to be well defined and can be summed up as the trophozoite’s ability to resist the complement system and oxidative attacks and, concomitantly, invade the host tissues by dismantling the latter’s structure and triggering cell death, processes sustained by parasite cytotoxicity, motility, and phagocytosis.

**Histopathology of Amoebic Liver Abscess Development in Animal Models**

Trophozoite virulence can be assessed in vivo by using animal models for ALA formation; to this aim, three species of rodent animal models are used. The first model features the intrahepatic (17) or intraportal (83) injection of trophozoites.
into Mongolian gerbils. Pathophysiological studies of ALA development in this animal (17) revealed characteristics similar to those of human ALA: the granulomata have a central, necrotic zone (containing inflammatory cells and lysed hepatocytes) surrounded by a ring of trophozoites and inflammatory cells that delimit the abscess from the rest of the hepatic tissue.

In the murine model, amoebae are directly inoculated into the liver of immunodeficient SCID mice (19) or immunocompetent wild-type mice (49). Dead hepatocytes are detected at 12 h postinfection in inflammatory foci surrounded by trophozoites; 24 h after inoculation, both sizes and numbers of inflammatory foci are seen to have increased. Unlike other model rodents, wild-type mice defeat the infection after 2 weeks.

The third model used in ALA development studies is the Syrian hamster. After intraportal inoculation of trophozoites, the histological features of infected livers are found to be similar to those in humans and thus enable the development of hepatic amoebiasis to be studied. Amoebic liver abscesses in humans and hamsters have a common, characteristic structure, as described above and presented in Fig. 3 (69, 82). Three hours after intraportal trophozoite inoculation, the hamster liver harbors a large number of inflammatory foci formed by the recruitment of neutrophils and, later on, macrophages that surround a single trophozoite; at 12 h, a majority of inflammatory foci are trophozoite free. This massive trophozoite killing is attributed to the acute inflammatory response mediated by host immune cells. After this critical point, any surviving amoebae divide, and the inflammatory foci develop into abscesses. New inflammatory foci can result from the metastatic spreading of amoebae throughout the liver tissue, a phenomenon that requires trophozoite motility. After 3 days of infection, abscesses start to coalesce until the formation of necrotic areas resembling those seen in humans (69). *E. histolytica* virulence then relies on the parasite’s ability to resist the host’s multifaceted response to invasion.

**Entamoeba histolytica in Blood and Endovascular Compartments**

Prior to reaching the liver, trophozoites reside in the endovascular compartment, bathing in the blood, where they encounter soluble components, circulating PMNs, and endothelial cells (Fig. 2). A first line of host defense is constitutively maintained by the integrity of the endovascular system. The trophozoites navigate in an environment in which the oxygen partial tension is relatively high (oxygen saturation of 98% ± 1%) (33), along with other factors that may contribute to their death (such as complement system components).

**Amoebic resistance to complement.** Complement is an evolutionarily conserved system composed of circulating zymogens that undergo cascade activation by proteolytic cleavage upon antibody- or lectin-mediated binding to the surface of pathogens. In vitro, *E. histolytica* trophozoites are able to activate the complement system in several ways (66), which leads to the lysis of susceptible parasites by the formation of membrane attack complexes (MACs). Pathogenic strains do still activate the complement pathway but are resistant to killing by MACs (68). Two mechanisms sustaining complement resistance have been identified. On the one hand, galactose/N-acetylgalactosamine (Gal/GalNAc) lectin inhibits the assembly of the
C5b-9 complex and, thus, lysis by MAC (in a way similar to that seen with CD59, a molecule with which the Gal/GalNAc lectin has antigen cross-reactivity and sequence similarity) (12). On the other hand, cysteine proteases (CPs) interfere with the immune response by degrading anaphylatoxin C3a. Moreover, the cleavage of the α-chain of C5b by the extracellular 56-kDa neutral CP leads to the formation of a C3b-like protein, which prevents MAC formation (67). In protozoan parasites, phosphoglycans in the glyocalyx may also act as a physical barrier that protects cells from MAC attack. Interestingly, it has been suggested that the proteophosphoglycans (PPGs) in the amoebic glyocalyx may facilitate in *E. histolytica* pathogenicity, since the closely related, nonpathogenic *Entamoeba dispar* lacks a significant glyocalyx surface layer (8); furthermore, antibodies that bind to PPGs neutralize liver abscess formation (54). PPGs are anchored into the outer leaflet of the parasite cell membrane by a glycosylphosphatidylinositol (GPI) moiety. Synthesis of the GPI anchor requires a cascade of enzymes, including mannosyl transferase 1 (PIG-M1, the enzyme that transfers the first mannose residue onto GPI). The blockage of PIG-M1 reduces GPI synthesis and thus reduces PPG levels in trophozoites (87). The PIG-M1-deficient parasites are highly sensitive to complement and are unable to provoke ALA development in hamsters, highlighting the importance of these GPI-anchored molecules for trophozoite pathogenicity and resistance against complement attack.

**Blood pressure, parasite migration, and adhesion to LSECs.**

In the portal vein, the blood flow is 1.4 liters/min, the blood pressure is 12 to 15 mm Hg, and erythrocyte velocity is 8 to 18 cm/s. In comparison, sinusoidal blood flow is extremely low (3.4 ± 0.16 ml/min), as is red blood cell velocity (0.1 mm/s) (65). The forces exerted on a parasite that adheres to the endothelium are thus much lower in the sinusoids and may partly explain why the parasite crosses the endothelium within these structures. However, the low mean diameter of the sinusoid lumens (6 µm) implies that the latter has to dilate and/or that the parasite must change its morphology in order to cross the endothelium. It is clear that the parasite and host cytoskeletons play a key role at this invasive stage, as does cross talk between *E. histolytica* and sinusoid-resident cells. Nevertheless, little is known about the transmigration of *E. histolytica* through the hepatic endothelium. In particular, we do not know whether the parasite uses specific adhesive molecules to interact with LSECs. The onset of apoptosis in LSECs is observed just 1 h after the intraportal inoculation of parasites in the hamster model of ALA. Twenty-four hours after inoculation, some PMN leukocytes recruited to inflammatory foci then also become apoptotic (9). However, the lack of tight junctions in the sinusoidal endothelium can facilitate crossing by the parasite; the latter may well take advantage of LSEC death (creating a larger breach) when reaching the hepatic parenchyma.

**The Host's Innate and Inflammatory Responses**

During liver invasion by *E. histolytica*, the host will both sequentially and simultaneously deploy a number of mechanisms to kill the parasite. The first line of tissue defense is composed of innate immune system cells that recognize PAMPs and trigger an inflammatory response. It was previously reported that female and male mice differ in their early responses to *E. histolytica* liver invasion (49); females rapidly cleared the parasites, recruited higher numbers of NKTCs to the infection site, and produced higher levels of gamma interferon (IFN-γ). NKTC-deficient females or IFN-γ neutralization led to a greater parasite survival level. IFN-γ is a key regulator of inflammation initiation, since it can activate macrophage production of TNF, which in turn promotes NO synthesis by neutrophil PMNs and the macrophages themselves. In in vitro models, the effects of IFN-γ can be bypassed by the recognition of PPGs of *E. histolytica* by Toll-like receptor 2 (TLR2) and TLR4, which results in direct TNF, interleukin-12p40 (IL-12p40), and IL-8 production (52). This proinflammatory cytokine profile underlines the importance of the early recognition of PAMPs (such as PPGs) and appropriate inflammatory cell recruitment and activation during ALA onset (39).

**Amoebic resistance to reactive oxygen and nitrogen species.**

During liver invasion, *E. histolytica* is exposed to a higher oxygen partial pressure and has to eliminate toxic metabolites, such as ROS produced by activated phagocytes during the respiratory burst (Fig. 4). The role of monocytes in liver infection by *E. histolytica* has not yet been studied in detail. However, it is clear that amoebic factors potentially activate peritoneal macrophages and circulating monocytes, leading to cytokine and ROS production and then parasite and tissue destruction. This self-attack during the oxidative burst is subject to debate, since leukopenic hamsters challenged with wild-type amoebae did not develop liver abscesses (62). Two main (and potentially nonexclusive) hypotheses can be formulated: either PMNs produce an impaired respiratory burst in the ALA context or they are killed by amoebae, thus releasing the content of their cytotoxic granules in a nondirected fashion. In either case, the role of ROS in the development of ALAs in animal models and humans is important and needs further investigation.

In phagocytes, the activation of NADP(H) oxidase generates superoxide (O₂⁻) anions (SAs), which spontaneously combine with water to form efficient oxidants (such as the hydroxyl radical OH⁻ or hydrogen peroxide [H₂O₂]), which are toxic to *E. histolytica* (31). In addition, macrophages produce NO by the action of NO synthase on arginine. The combination of NO with SA produces peroxynitrites (ONOO⁻) that are very toxic for trophozoites (47, 75, 77). Evidence for the role of ROS in human resistance to ALA formation was provided by correlating recurrent onsets of hepatic infection by *E. histolytica* in a patient lacking an oxidative-burst mononuclear phagocyte response (57).

Although intensive work has been undertaken, the chronology of the early events leading to ROS production in amoebiasis remains unclear. However, the production of NO and SA is elicited by TNF stimulation in most types of cells, mainly through an NF-κB activation pathway resulting in enhanced NADP(H) oxidase expression (30). TNF production during *E. histolytica* infection is thus suspected to be an important feature of ALA genesis. *E. histolytica* can resist both reactive oxygen and reactive nitrogen intermediates by using specific enzymes; SA is rapidly dismutated into H₂O₂ and O₂ by iron-containing superoxide dismutase (15). An NADPH:flavin oxidoreductase (p34 thioreredox reductase) converts O₂ to H₂O₂ (14, 18). Hydrogen peroxide could be detoxified by a rubre-
doxin/ruberythrin complex (22), whose activity has not been evidenced yet, although the two genes exist in *E. histolytica*. Furthermore, H$_2$O$_2$ and peroxynitrites are detoxified by a thioredoxin redox system consisting of three enzymes: peroxiredoxin, thioredoxin, and p34 thioredoxin reductase. Peroxiredoxin is a 29-kDa surface molecule that functions as a peroxidase and a thiol-specific antioxidant (64). These activities are performed by two cysteine residues (cysteine 47 and cysteine 170) at the catalytic site; the N-terminal sequence (with numerous cysteines) may constitute a source of free thiol groups. The peroxidase activity leads to a reduction of hydrogen peroxide to H$_2$O. Thioredoxin exhibits antioxidant activity and can reduce peroxiredoxin. This activity is coupled to that of a third enzyme, p34 thioredoxin reductase, which reduces thioredoxin using NADPH as a cofactor.

Amoebae may use different strategies to overcome the production of peroxynitrites. Virulent trophozoites can interfere directly with NO production by host cells: the parasite possesses a gene coding for an arginase that, when expressed by and purified from the bacterium *Escherichia coli*, efficiently hydrolyzes arginine into urea and L-ornithine and thus competes for substrate with the inducible NO synthase enzymes produced by activated phagocytes (26). Furthermore, L-ornithine directly inhibits NO synthesis in macrophages. Using these complementary effects that still need to be demonstrated during infection, amoebae may competently impair NO production in macrophages.

**Immune deregulation by PGE$_2$.** Prostaglandin E$_2$ (PGE$_2$) is an endogenous, anti-inflammatory mediator that inhibits the production of cytokines and class II MHC proteins and thus interferes with antigen-presenting functions (36). In mammalian cells, PGE$_2$ is synthesized by the cyclooxygenase (COX)-catalyzed conversion of arachidonic acid. Two COX isoforms exist: COX-1 is produced constitutively in many cells, and inducible COX-2 is generally produced following exposure to proinflammatory molecules (79). The regulatory actions of PGE$_2$ are mediated by two G-protein-coupled receptors, whose activation in T lymphocytes inhibits cell proliferation (by increasing the intracellular cyclic AMP concentration and protein kinase activity) and reduces class II MHC expression. During the development of ALA in hamsters, *E. histolytica* induces greater PGE$_2$ and COX-2 activity in PMNs and activated macrophages (34). Moreover, a COX-2-like protein has been purified from trophozoites, and a protein that interacts with an anti-mammalian COX-2 antibody has been detected in experimental ALA infection. This COX-2-like protein presents little homology with the mammalian enzymes but is able to produce PGE$_2$ from arachidonic acid; it may thus be responsible for the COX-2-like activity detected during colonic amebiasis (23, 70). As liver infection progresses, T-lymphocyte activity and cytokine (IL-1, -2, and -8 and TNF) and chemokine (monocyte chemoattractant protein 1 and macrophage inflammatory proteins α and β) production continue to fall, suggesting a role for PGE$_2$ in immunsuppression during ALA formation.

**Host Cell Death by Apoptosis**

In liver abscesses in the SCID mouse and hamster models, *E. histolytica* induces apoptosis in LSECs, hepatocytes, and inflammatory cells (9, 61). Apoptosis is divided into extrinsic and intrinsic pathways. The extrinsic pathway is triggered by the interaction of specific ligands with cell surface Fas and TNF receptor family members. The intrinsic pathway leads to the breakdown of mitochondrial outer membrane integrity, followed by the release of cytochrome c and other intermem-
brane space components. Both pathways lead to the activation of caspase-3 and then apoptotic cell death, characterized by membrane blebbing, DNA fragmentation, and exposure of phosphatidylserine on the external membrane (73). The exact mechanism of caspase-3 activation during ALA formation has not been fully resolved. Previous studies reported that liver abscess formation can occur in the absence of either Fas or TNFR1 (78). *E. histolytica* causes apoptosis of cells that lack caspase-8 or have been treated with caspase-9 inhibitors (38), suggesting that classic upstream activation of caspase-8 or -9 is either an accessory for or not involved in the activation of caspase-3. However, upon coincubation with trophozoites, human lymphocytes (Jurkat T cells) exhibited a higher intracellular Ca$^{2+}$ concentration and calpain proteolytic activity, which led (via a mitochondrion-independent mechanism) to caspase-3 cleavage and activation (45). The observed in vitro relevance of amoebiasis in this mechanism needs to be confirmed in vivo during ALA development.

Recent microarray data on SCID mice hepatically inoculated with wild-type (WT) trophozoites evidenced significantly higher levels of expression of genes linked to the two above-mentioned apoptotic pathways (61). After 24 h of infection, the transcription of the genes encoding the Fas receptor, TNFR1/2, and ASK1 was upregulated. The latter is a protein that activates the JNK signaling pathway (upon binding to Jun and the death domain-associated protein DAXX) and ultimately leads to the blockage of Bcl-2 by phosphorylation. The involvement of the mitochondrial apoptotic pathway was also evidenced by the transcription upregulation of the proapoptotic factors Bim, Noxa, Bid, Puma, and tBid.

Transcriptional analysis using infected whole liver tissue also highlighted the upregulation of genes that regulate the cell cycle and its progression (cyclin-dependent kinase inhibitor p21 and cyclin D2). Likewise, the expression of genes linked to hepatic regeneration (such as various transcriptional factors), which are usually activated by IL-6 and hepatic growth factor (HGF) (61), was upregulated. Although liver infection with *E. histolytica* triggers host cell death, the mechanisms of regeneration probably engaged by the host and evidenced at the mRNA level may help to balance tissue destruction and slow down the evolution of the disease, thus preserving the organ functions.

**Adaptive Immune Response**

Trophozoites can infect the human liver for several months or years before abscesses are diagnosed. Expectedly, a host immune response is triggered, leading to the production of circulating immunoglobulins. The major immunodominant antigens located at the parasite surface are the Gal/GalNAc-inhibitable lectin and the serine-rich protein SREHP. Impressively, 80% of patients with ALAs have circulating IgGs that recognize these antigens but do not cure the infection (63); this observation suggests a possible involvement of a defense mechanism against the antibody-dependent control of amoebic invasiveness, such as the concentration of surface-bound antibodies to the posterior edge of the parasite by surface receptor capping, followed by their release via the extrusion of uroids (16).

Another parameter to consider for ALA development is the susceptibility of the host. Few studies to identify host genetic predispositions for amoebic infection have been made. It is noteworthy that the HLA-DR3 haplotype as well as the SC01 complotype have been associated with a greater susceptibility for ALA formation in a Mexican population (6, 7). A higher prevalence of ALA was also linked to HLA-DR5 and HLA-DR6 haplotypes but exclusively in infants. The influence of the genotype on the infection outcome is undoubted; however, as long as the mechanisms sustaining these phenotype traits are not unraveled, it is impossible to state if the different alleles of class II MHC genes are directly responsible for the lower ability to arrest amoebic infection or if they evidence an allele without being involved in the susceptibility to ALA.

**KEY AMOEIC FACTORS IN THE DEVELOPMENT OF LIVER ABSCESSES**

Pioneering studies have demonstrated the impact of *E. histolytica* genotypes on the outcome of infection (2). Furthermore, the purification of trophozoites from different organs of the same patients revealed that their tropism was linked to different genotypes (3). These observations suggest either adaptation by *E. histolytica* to the environment encountered upon invasion or the selection of invasion-prone parasites during the development of pathology. The difference at the genetic level between trophozoites able to cause intestinal or hepatic disease would partly explain the rare occurrence of intestinal symptoms in patients that present ALA (1). Trophozoites from patients can be purified and cultured in vitro, during which time their ability to form ALA in animals decreases (60, 81). However, recurrent passage into animals maintains parasite virulence (59) and involves mechanisms that are still under investigation.

**Virulence-Linked Factors**

The publication of the genome of *E. histolytica* (48) naturally facilitated transcriptome studies of this parasite. Microarrays have been successfully used to compare highly virulent parasites (recently recovered from liver abscesses) with avirulent trophozoites (unable to form liver abscesses) from the same strain (71). This transcriptome comparison highlighted some overexpressed genes that were previously directly linked to virulence and to stress response in invasion-prone amoebae. Among them, the stress-inducible gene ssp1, the asparagine-rich protein-encoding gene *ariel1*, and the 20-kDa factor-encoding gene (all of unknown function) were overexpressed. Interestingly, the expression of the peroxidoxin and rubreythrin genes was also upregulated. Peroxidoxin is involved in protection against ROS in general (18) and H$_2$O$_2$ in particular, and the expression of the gene increases virulence in the nonpathogenic Rahman strain of *E. histolytica* (61). Rubreythrin is involved in H$_2$O$_2$ detoxification and exhibits an NAD(P)H oxidase activity in *Desulfovibrio vulgaris* (22); however, its function remains to be proven in *E. histolytica*. The overexpression of the two latter genes in virulent parasites is of interest for ALA development, since inflammatory cell recruitment and the subsequent setup of inflammation with the production of toxic effectors, like ROS, are early features of liver infection by *E. histolytica*.  

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Amoebic Lytic Factors

A hallmark of tissue invasion by Entamoeba histolytica resides in its ability to directly lyse human cells on contact and to destroy ECM during invasion (51, 80). The molecules potentially involved in cytotoxicity and cytolysis were identified as being CPs (50) and amoebapores (46). Sequencing of the E. histolytica genome has revealed that CPs compose a family of at least 44 genes (20). Most of the latter are not expressed in cultured parasites, in which 90% of the total protease activity is due to CP-A1, CP-A2, and CP-A5 proteins (13). To date, CPs have been identified in intracytoplasmic vesicles (29), bound to the cell surface (40), and in secreted forms (90). Direct evidence for the involvement of CPs in the formation of ALA has been obtained by inhibiting their function with E-64 (90) but also by interfering with cp-a5 gene expression using antisense technology (5) and epigenetic gene silencing (90). Furthermore, ICP proteins, in addition to their activity as CP inhibitors, were shown to downregulate secretion and thus the release of CPs in the extracellular medium; this double effect on the inhibition of CP activity and the impact on the secretion of other factors is thought to repress trophozoite virulence (72).

Amoebapores are intravesicular 77-amino-acid proteins (4) bearing the saposin-like protein (SAPLIP) domain encountered in saposin B, a so-called “physiological detergent” (58). Other proteins exhibiting SAPLIP domains (such as NK-lysin and granulysin) have pore-forming activity. Of the 16 SAPLIPs identified in E. histolytica, only three amoebapores are predicted to have pore-forming activity by sequence analysis (48). Amoebapores are essential for bacterial phagocytosis in E. histolytica and E. dispar. Their cytotoxicity toward eukaryotic (46) and prokaryotic (4) cells adds evidence for a role in pathogenesis, as demonstrated by the absence of ALA formation following hamster or SCID mouse infection with trophozoites whose amoebapore A gene expression has been downregulated by antisense technology (11) or epigenetic silencing (89).

In the transcriptome of virulent parasites, we also identified some so-called virulence factors whose expression was downregulated, such as CP-A6 and amoebapores A and C (71). It is noteworthy that CP-A6 was overexpressed during experimental intestinal infection in CBA/J mice (32). These observations highlight the need for extensive and differential analyses of the role of E. histolytica virulence factors to better understand the pathophysiology of both intestinal invasion and ALA development.

Gal/GalNac-Inhibitable Lectin

The Gal/GalNac lectin is a protein complex comprising a 170-kDa heavy subunit (HgL) and a 30- to 35-kDa light subunit (LgL). On the amoebic surface, this complex binds to a 150-kDa intermediate subunit (IgL). The Gal/GalNac lectin’s importance in amoebic virulence has been demonstrated by the impaired development of experimental ALA infection following the disruption of lectin function in engineered parasites by blocking signaling through either HgL or LgL synthesis (5, 9, 42, 69, 84).

It has been suggested that the Gal/GalNac lectin plays a role in initiating the immune response against E. histolytica. Pioneering work by Seguin et al. demonstrated the potent activation of bone marrow-derived macrophages (BMDMs) by the cysteine-rich region of the 170-kDa Gal/GalNac lectin heavy subunit (74). This activation leads to TNF mRNA upregulation and the production of consistent amounts of TNF. Upon priming with IFN-γ and stimulation by the Gal/GalNac lectin of E. histolytica, BMDMs produce TNF and NO and exhibit amoebcidal activity (75). Priming alone or blockade of the native lectin’s TNF-stimulating region induced neither the upregulation of inducible NO synthase and TNF gene expression nor the production of the corresponding proteins.

The cysteine-rich region of HgL has sequence homology to CD59; blockade by monoclonal antibodies revealed its involvement in complement resistance (12, 53). Other regions of the HgL subunit have been blocked by antibodies; trophozoite adherence and cytotoxicity were inhibited by the 8C12 monoclonal antibody directed against the carbohydrate recognition domain (53), which shares homologies with HGF’s receptor-binding domain (24). Moreover, the carbohydrate recognition domain competed in vitro with HGF for binding to the c-Met receptor and may thus enable trophozoites to adhere to hepatocytes in vivo (24). The upregulation of TLR2 gene expression was observed in vitro in murine macrophages exposed to Gal/GalNac lectin, resulting in proinflammatory cytokine production (41); these effects were confirmed in vivo in mouse ALAs (61).

Gal/GalNac lectin’s contribution to inflammatory cell recruitment and cytokine production has been carefully studied in the hamster model (10). HGL-2 trophozoites (engineered to disrupt the Gal/GalNac heavy chain lectin function) (21) modified the pathophysiology of ALA; HGL-2 parasites produced more inflammatory foci than did the WT strain, but the foci were smaller and contained twice as many trophozoites. This higher survival rate may be a consequence of reduced immune cell activation, a feature correlated with a reduction in the flow of macrophages from the portal veins and sinusoids to inflammatory foci. TNF production was not detected after 24 h of infection with HGL-2 parasites, corresponding to the absence of macrophage/KC activation; this observation correlated with the poor recruitment of PMNs and monocytes to the inflammatory foci, which were reduced in size. Whereas WT trophozoites induced LSEC apoptosis after 1 h of infection and hepatocyte and PMN apoptosis within 24 h, HGL-2 parasites displayed diminished, delayed proapoptotic activity. In the inflammatory foci produced by HGL-2 trophozoites, apoptotic activity was 3.5 times lower than that in the inflammatory foci formed by WT amoebae. This suggests a role for the Gal/GalNac lectin in the signaling pathway leading to apoptosis and the involvement of amoeba-triggered cell death in virulence. HGL-2 parasites also displayed lower motility, which prompted abortive spreading into the tissue (9, 21). Thus, macrophage priming and the subsequent contact with the Gal/GalNac lectin of E. histolytica may be involved in the in vivo killing of trophozoites by BMDMs. However, the first step in the response against E. histolytica infection has not yet been determined. A role for other amoebic molecules and/or a cooperative response involving other host inflammatory cells is plausible. For instance, neutrophil recruitment is observed, along with a dramatic trophozoite death; the amoebcidal ac-
tivity of macrophages may thus depend on their molecular cross talk with neutrophils.

KERP1

As previously cited, invasion-prone parasites overexpress a group of genes that encode lysine-rich factors that we named KRP1s and lysine- and glutamic acid-rich proteins (KRP1s), all of them of unknown function (71). Of these, special attention was paid to KERP1, a KERP thought to be involved in host-parasite interactions (76). In addition to this prominent member, six other krip genes encoding hypothetical proteins of as-yet-unknown function were overexpressed in virulent parasites.

Further studies confirmed that KERP1 was involved in ALA formation in animal models (71); at the mRNA level, kerp1 gene expression was downregulated in the first 3 days of infection and then rose to above initial levels when the abscesses reached confluence; this correlated with a progressive, cortical accumulation of KERP1 protein as the infection progressed. The subtle regulation of kerp1 gene expression during the course of infection evidenced the potential involvement of KERP1 in ALA development. This potential role in virulence was reinforced by the progressive decrease in KERP1 abundance in cultured trophozoites (i.e., those losing the ability to form ALAs in hamsters). Lastly, the hitherto-unknown role of KERP1 in ALA formation was assessed in vivo: the downregulation of kerp1 gene expression in virulent trophozoites (using antisense technology) consistently reduced abscess number and size in hamsters, raising the still-open question of KERP1’s role in early hepatic amoebiasis: is KERP1 involved in (i) parasite resistance to blood components and/or the inflammatory response, (ii) trophozoite interaction with host cells, for instance, adherence, which may lead to host cell death and phagocytosis, and (iii) triggering inflammation during ALA development?

CONCLUSIONS

The development of ALAs is a fatal feature of infection by Entamoeba histolytica and is the most common extraintestinal form of invasive amoebiasis. Indeed, it is estimated that around 100,000 people succumb to ALA each year (88). The host and parasite factors leading to liver infection remain largely undescribed. However, the virulence of E. histolytica isolates is defined by their ability to provoke ALA in animal models, and thus, a number of factors required for virulence and ALA development have been identified, including those necessary for complement resistance (PPGs), ROS resistance (peroxiredoxin), lysis (CPs and amoebapores), and cell adherence (notably, KERP1 and the Gal/GalNAc lectin).

KERP1 stands out since (i) it is specific to E. histolytica and (ii) kerp1 gene expression is increased during ALA development and correlates with the onset of the host inflammatory response. Indeed, analysis of the stress and antioxidative responses of virulent parasites (in which kerp1 gene expression is upregulated) will help decipher the trophozoite’s adaptation to an infectious lifestyle and identify the factors needed for liver invasion. The conclusions of this work will shed new light on the pathophysiology, treatment, and future prophylaxis of hepatic amoebiasis.

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