Strategies of the protozoan parasite *Entamoeba histolytica* to evade the innate immune responses of intestinal epithelial cells

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Molecules expressed by the pathogenic ameoba *Entamoeba histolytica* but weakly expressed or absent from the non-pathogenic ameoba *Entamoeba dispar* could be used by intestinal epithelial cells to discriminate between the two species and to initiate an appropriate inflammatory response. Among the possible molecules involved in this identification are the Gal/GalNac lectin and the lipophosphoglycan. Once the inflammatory response is initiated, *E. histolytica* trophozoites have to protect themselves against reactive nitrogen intermediates produced by intestinal epithelial cells, oxygen intermediates, and cytotoxic molecules released by activated neutrophils. By screening the *E. histolytica* genome, we have identified proteins that may play a role in the defence strategy of the parasite. One of these proteins, a serine proteinase inhibitor, inhibits human neutrophil cathepsin G, a key component of the host defence.

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1. Introduction

Entamoeba histolytica is a gastrointestinal protozoan parasite that poses a serious health problem, with 50 million annual infections throughout the world (WHO 1997). Thanks to the development in these last years of accurate *in vivo* and *in vitro* models of disease, and to new methods to perform selected inhibition of gene expression, a consistent picture of *E. histolytica* pathogenesis has been established. The parasite first adheres to the colonic mucus and epithelial cells by a lectin-mediated mechanism. The lectin that appears to contribute the most to this binding activity is the Gal/GalNAc-specific lectin (McCoy *et al* 1994). Following this intimate contact, pore-forming polypeptides called amoebapores are released by the parasite. The amoebapores are capable of killing metabolically active eukaryotic cells and display antibacterial activity (Leippe and Muller-Eberhard 1994). Proteolytic enzymes are secreted by the parasite to disrupt the intestinal mucus and epithelial barrier and to facilitate tissue penetration (Que and Reed 2000). The combination of these molecules and perhaps of some other undiscovered factors, leads to the formation of colonic ulcers and the subsequent migration of the amoeba to the liver where abscesses are formed (Espinosa-Cantellano and Martinez-Paloma 2000; Stanley 2001). Intestinal epithelial cells (IEC), in addition to their role in the absorption of the nutriments, constitute the first line of defence against microbial pathogen (Kagnoff *et al* 1997). These cells are able to sense infection and to initiate a defensive response by producing pro-inflammatory cytokines and soluble factors, including interleukin-1 (IL-1), interleukin-8 (IL-8),

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Abbreviations used: GSNO, S-nitrosoglutathione; HSP, heat shock protein; IEC, intestinal epithelial cells; IL, interleukin; LPG lipophosphoglycan; LPPG, lipophosphopeptidoglycan; NO, nitric oxide; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; TNFα, tumour necrosis factor alpha.

monocyte chemoattractant protein1, granulocyte-macrophage colony-stimulating factor, inducible nitric oxide (NO) synthase and tumour necrosis factor alpha (TNFα) (Jung *et al* 1995). *E. histolytica* is classified as a pathogen by the epithelial cells and it induces a strong inflammatory response whereas *E. dispar* (nonpathogenic) is recognized as a gut commensal. The goal of this article is to review the molecules that could be used by IEC to differentiate *E. histolytica* from *E. dispar* and to describe some strategies developed by *E. histolytica* to evade the defensive response initiates by IEC.

2. Why is *E. histolytica* recognized as a pathogen by IEC?

IEC possess specific receptors termed Toll like receptors that are able to sense the presence of pathogens (Cario and Podolsky 2000). Molecules expressed specifically by *E. histolytica* could be recognized by these systems and used to distinguish between pathogenic and non-pathogenic ameoba. The first step in bowel invasion by *E. histolytica*, is the binding of the trophozoites to IEC. This binding is mediated mainly by the 260 kDa heterodimeric lectin present at the surface of the parasite. The structure of this lectin has been intensively studied and it is composed of a transmembrane heavy (170 kDa) subunit and glycosylphosphatidylinositol anchored light 31 kDa and 35 kDa subunits, which are linked to the heavy subunit by disulfide bonds (McCoy *et al* 1994; Vines *et al* 1998). If the contact mediated by this amoebic lectin is sufficient by itself to initiate the defensive response of the IEC is still hypothetic. In favour is the observation that native Gal/GalNac lectin purified from *E. histolytica* trophozoites stimulates the synthesis of TNFα by naïve bone marrow macrophages (Seguin *et al* 1995). Although Gal/GalNac lectin is found in both *E. histolytica* and *E. dispar*, the cysteine rich region of the Gal/GalNac lectin heavy subunit is hidden at the surface of *E. dispar* (Pillai *et al* 2001) and therefore could represent a putative target used by IEC to discriminate between pathogenic and non-pathogenic amoeba. Lipopolysaccharide of Gram-negative bacteria and peptidoglycan from the cell walls of Gram-positive bacteria are recognized by IEC to induce the defence response (Anderson 2000). *E. histolytica* trophozoites are covered by lipophosphoglycan (LPGs) and lipophosphopeptidoglycans (LPPGs) glycoconjugates. *E. dispar* has less than 10% of the LPGs content in *E. histolytica* (Moody *et al* 1998a) (Bhattacharya *et al* 2000; Moody *et al* 1998b) so LPGs represent also a serious candidate for the induction of the defensive response by IEC.

Cysteine proteinase 5, another membrane-associated protein specifically expressed by *E. histolytica* but not by *E. dispar* (Bruchhaus *et al* 1996; Willhoeft *et al* 1999), could also be used by IEC to identify the pathogenic ameoba.

The damage caused by the parasite to IEC is certainly an important factor for the induction of the inflammatory response. IL-1α released by IEC induces the secretion of the IL-8 by still intact IEC. Neutrophils are then rapidly recruited and activated in response to the proinflammatory cytokine IL-8 (Eckmann *et al* 1995). The killing of IEC by *E. histolytica* is mediated mainly by the action of amoebapores as it has been demonstrated using antisense technology (Bracha *et al* 1999). The lower amount of pore-forming peptides in *E. dispar* compared to *E. histolytica* (Nickel *et al* 1999) contributes certainly to its lower killing activity and therefore to its inability to induce the inflammatory response.

At this stage of the infection, trophozoites also release factors like cysteine proteinases that have IL-1 converting activity (Zhang *et al* 2000) and a chemoattractant activity for neutrophil associated to the membrane of the trophozoites (Salata *et al* 1989): these enhance the inflammatory response. The pathogen parasite may take advantage of the inflammatory stimuli in order to increase its binding to colonic epithelium and its spreading to others organs (Flores-Romo *et al* 1993). Once the inflammatory process is initiated, trophozoites have to deal with reactive nitrogen intermediates (RNI) like NO produced by activated IEC and to reactive oxygen intermediates (ROI), RNI and cytotoxic molecules synthesized by neutrophils. The molecules used by the parasite to evade these various aggressions will be reviewed.

3. Resistance of *E. histolytica* to ROI

Molecules involved in the resistance to ROI in *E. histolytica* have been well characterized. *E. histolytica* produces an iron-containing superoxide dismutase that is induced by superoxide anions to produce H_2O_2 (Bruchhaus and Tannich 1994). Hydroperoxides produced during oxidative stress could be detoxified by a bifunctional NADPH: flavin oxidoreductase containing NADPH-dependent disulfide reductase and H_2O_2-forming NADPH oxidase activities (Bruchhaus and Tannich 1995; Bruchhaus *et al* 1998). *E. histolytica* encodes a 29 kDa cysteine-rich antigen located on the surface of the trophozoites. It has been suggested that this protein which acts enzymically in the detoxification of endogenous reactive oxygen species, might be an important enzyme to protect the amoebae against oxidative attack of activated host phagocytic (Bruchhaus *et al* 1993, 1997; Flores *et al* 1993).

4. Resistance of *E. histolytica* to RNI

Thus, if *E. histolytica* has the enzymatic potential to resist ROI, nothing is actually known about its ability to

resist RNI. Previous *in vitro* and *in vivo* studies have shown that NO plays a critical role in macrophage-mediated killing of *E. histolytica* (Lin *et al* 1992; Seydel *et al* 2000). A pathogen can resist RNI by (i) interfering with the host cell production of RNI; (ii) catabolize them, or (iii) repair their damage. We are actually investigating the existence of these three mechanisms of resistance to RNI in *E. histolytica*.

A simple strategy to evade RNI is to inhibit their synthesis. A previous work has shown that amoebic proteins inhibit the synthesis of NO by mouse bone-marrow-derived macrophages (Wang *et al* 1994). Recently, a small amoebic peptide that inhibits NO production of monocytes and neutrophils has been identified (Rico *et al* 2000). NO is produced enzymatically from L-arginine through the action of NO synthase. The protozoan parasite *Giardia lamblia* and the Gram-negative bacterium *Helicobacter pilori* inhibit the synthesis of NO by consuming arginine present in their environment (Eckmann *et al* 2000; Gobert *et al* 2001). A similar mechanism could take place in *E. histolytica* as it also consumes arginine during its growth in TYI-S-33 media (Zuo and Coombs 1995). Arginase, a key enzyme in the catabolism of arginine, catalizes the conversion of L-arginine to L-ornithine and urea. This activity was detected in a crude lysate of *E. histolytica* trophozoites using the micromethod described by Corraliza *et al* (1994). Thanks to the advance of the *E. histolytica* genome project, a gene homologous to *E. coli* L-arginase has been identified and cloned. Our preliminary results suggest that arginine catabolism probably participates in RNI resistance and represents an important pathway for *E. histolytica* (manuscript in preparation).

We have recently demonstrated that thiol-dependent enzymatic systems in the parasite, like cysteine proteinases and alcohol dehydrogenase 2, are inhibited by S-nitroso-gluthatione (GSNO), a physiological NO donor via a reaction of S-nitrosylation (Siman-Tov and Ankri 2002). Although the inhibition of such critical enzymes could probably impair the virulence and the growth of the parasite, other cysteine rich molecules of less importance for the physiology of the parasite could protect it by scavenging RNI to form S-nitrosothiols. These S-nitrosothiols are themselves toxic, therefore the scavenging of RNI can be protective only if the rate of formation of S-nitrosothiols does not exceed the capacity of the parasite to detoxify them.

Respiratory detoxification of NO by some pathogens, such as *Neisseria meningitidis*, is mediated by NO reductase activity (Richardson *et al* 2000). In *Escherichia coli*, this activity is provided by a periplasmic cytochrome c nitrite reductase (Poock *et al* 2002). Peroxiredoxin activity has been associated to the detoxification of the highly toxic peroxynitrite in various pathogens like *Salmonella typhimurium*, *Mycobacterium tuberculosis* and *Helicobacter pylori* (Bryk *et al* 2000). Peroxiredoxin activity has been characterized in *E. histolytica* (Poole *et al* 1997) and its involvement in the resistance to RNI remains to be established.

Heat shock proteins (HSP) are synthesized by cells in response to heat, as well as to various other stressful stimuli. The cytoprotective capacity of HSP may be attributed in part to their ability to recognize proteins which are not yet in their native conformation or which are denatured. They form a complex with those proteins, which can either lead to the correct folding of the protein or to proteolytic degradation (for reviews see Liang and MacRae 1997; Kregel 2002). The role of NO in the induction of HSP and more precisely HSP70 has been the focus of particular attention in recent years (Bellmann *et al* 1996; Kim *et al* 1997). A member of the HSP family, HSP70, has been previously characterized in *E. histolytica* (Ortner *et al* 1992; Bakatselou *et al* 2000). Interestingly, the expression of HSP70 is induced during encystation of the reptile parasite *Entamoeba invadens* (Field *et al* 2000). We have cloned *HSP*100, an additional gene that belongs to this family. Transcription of *HSP*100 in *E. histolytica* is induced by heat shock and by GSNO but is not significantly induced by H_2O_2. Transfectants in which the expression of HSP100 is inhibited by antisense RNA are more sensitive to heat shock and GSNO than control transfectants, but have the same level of sensitivity to H_2O_2 (manuscript in preparation). We propose that the expression of HSP100 could also contribute to the resistance of the parasite to RNI.

5. Resistance of *E. histolytica* to cytotoxic molecules released by the neutrophils

Human neutrophil azurophil granules contain a family of proteins with structural homology to serine proteinases, the serprocidins. Three members of this family are serine proteinases (cathepsin G, elastase, and proteinase-3) and one is a proteolytically inactive homologue (azurocidin) (Starkey *et al* 1976; Borregaard *et al* 1993). These enzymes are involved in host defence system since they mediate antimicrobial activity (Belaaouaj *et al* 1998), degradation of extracellular matrix, vasoregulation, and processing of IL-8 (Padrines *et al* 1994). Further, cathepsin G acts as a chemokinetic stimulant for T lymphocytes and a chemoattractant for monocytes (Yamazaki and Aoki 1997, 1998; Tani *et al* 2001). The activity of these proteinases is naturally regulated by members of the serine proteinase inhibitor (serpin) superfamily (for review see Ye and Goldsmith 2001). A growing number of examples in literature show that pathogens like *Schistosoma haematobium* (Huang *et al* 1999), *Brugia malayi* (Zang *et al*

1999; Maizels *et al* 2001), and *Toxoplasma gondii* (Pszenny *et al* 2000), may themselves encode serpins to inhibit the host defence functions. By screening the *E. histolytica* genome databank, we have identified a gene that encodes a protein (Ehserp) with strong homologies to members of the serpin superfamily. *Ehserp* is present as a single copy in the amoebic genome and despite an intense screening of the *E. histolytica* genome, no others genes homologous to Ehserp have been detected. Ehserp has a cytoplasmic localization and it is secreted only upon activation of the trophozoites with mammalian cells via a lectin dependent pathway. Ehserp inhibits neutrophil cathepsin G by forming a complex with it that is resistant to denaturants (manuscript in preparation). In view of the important function played by cathepsin G during inflammation (Yamazaki and Aoki 1997, 1998; Tani *et al* 2001), its inhibition by Ehserp may have a central role in the evasion of the immune system of the host. In progress are experiments of up and downregulation of this protein that will certainly help us to better understand its function.

6. Resistance to host defences in the late invasive phase

In the late invasive phase, the inflammatory response leads to the recruitment of additional host defences molecules including the complement system and serum antibodies. *E. histolytica* trophozoites resist to secretory IgA and serum IgG by cleaving them with their cysteine proteinases (Kelsall and Ravdin 1993; Tran *et al* 1998). *E. histolytica* has the ability to activate the serum complement and to resist complement mediated lysis. Cysteine proteinases released by *E. histolytica* trophozoites activate the complement by cleaving C3 and C5 to produce C3b and the proinflammatory factors C3a and C5a, both of which are rapidly degraded (Reed *et al* 1989, 1995). This mechanism is certainly used by the parasite to reduce the inflammatory reaction that activation of complement generates. Gal/GalNac lectins are thought to play an important role in the resistance to the complement since they show similarity and cross reactivity with CD59, a human leukocyte antigen with the capacity to prevent assembly of the C5b9 attack complex (Braga *et al* 1992).

7. Conclusions

E. dispar remain dormant in the human gut and it is not recognized as a pathogen by IEC. In contrast, the strategy followed by *E. histolytica* is to awake the sentinels of the host defence and then to evade these defences by using a remarkable panoply of offensive molecules like the lectins, the amoebapores and the cysteine proteinases and defensive molecules that neutralize the ROI, RNI and toxic factors released during the inflammatory response. We propose that Ehserp, HSP100 and arginase are involved in the parasite evasion mechanism. Our future research in this domain will certainly help us to understand how the expression of these offensive and defensive factors is coordinated during invasion of the host.

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