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Advances in Amoebiasis Research Emphasizing Immunological and Oxidative Aspects

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ABSTRACT

The purpose of this review is to report the scientific advances of amoebiasis, especially focusing the innate immune response and the oxidative stress related to the infection. Amoebiasis is a significant cause of morbidity and mortality, affects about 50 million persons and leads to approximately 100.000 deaths worldwide each year. As to the virulence, the different strains of *Entamoeba histolytica* and *Entamoeba dispar* can be characterized in several forms. The main ones are: the capacity of inducing liver abscess in hamsters, the erythrophagocytosis and the cytopathic effect on VERO cells. The tissue invasion by *E. histolytica* trophozoites induces a humoral immune response, which may persist for several years, being verified by the levels of antibodies. However, it is the cellular immune response that has been described as the most effective one. It still remains unclear whether, the oxidative stress generated at the inflammatory sites of amoebiasis gives rise to benefits or injuries to the host, or if both possibilities may coexist, depending on other factors such as, for instance, the type of strain and the profile of the host immune response. A better understanding on the relationship between parasite and the host immune response is crucial for the development of vaccines and for the improvement of therapeutic alternatives to amoebiasis.

Key words: *Entamoeba histolytica, Entamoeba dispar*, amoebiasis

INTRODUCTION

Amoebiasis is an important parasitic disease caused by the broadly worldwide disseminated enteric protozoa *Entamoeba histolytica*, yet being more incident in places that did not reach suitable sanitary conditions. In developed countries, this protozoan is seen primarily in travelers to and emigrants from endemic areas. The disease is a significant cause of morbidity and mortality, affects about 50 million persons and leads to approximately 100.000 annual deaths worldwide (WHO/PAHO/UNESCO Report, 1997).

Entamoeba histolytica infects people of both sexes and all ages; however, populations at risk may vary with geographic location, host susceptibility and differences in organism virulence (Pritt and Clark, 2008).

High levels of human infection are found in India, Africa and Central and South America. In Brazil, for instance, the number of infected patients or individuals presenting the symptoms of the disease varies by region, such a variation generally running between 2.5 and 11%. Nevertheless, it is worthy to point out that levels near 20% can be observed at the Amazonian region (Silva and Gomes, 2005).

The purpose of this review was to show the scientific advances in the study of amoebiasis, especially focusing the innate immune response and the oxidative stress related to the infection.

HISTORICAL ASPECTS

The first scientific reports referring to amoebiasis arose on the XIX Century. Through microscopic and clinical studies, Loesch (1875) associated dysentery with the presence of trophozoites in the feces, which received the name of *Amoeba coli*. Koch and Graffki (1887) reported the tissue invasion by amoebae and Councilman and Lafleur (1891) described the pathological process associated with the amoebian invasion of the liver. These authors also described in details the amoebae that are causative agents of lesions, designating them as *Entamoeba dysenteriae*.

At the beginning of the XX Century, the terminology of amoebae was a quite controversial issue. Schaudinn (1903) attributed a new name to *E. dysenteriae*, naming it as *Entamoeba histolytica* and still described another specie of amoeba, the *Entamoeba coli*, which is not pathogenic. Craig (1905) considered that the name *E. dysenteriae* was preferable to *E. histolytica*. However, Walker (1911) re-described the intestinal amoebae, confirming the name *E. histolytica* and distinguishing it from *E. coli* through the number of nuclei inside the cysts of both species.

Brumpt (1925) proposed the existence of two species of the gender *Entamoeba*, which were morphologically similar and infected men, being one of them pathogenic and the other non-pathogenic. Brumpt's studies were grounded on the verification that many of the patients apparently infected by *E. histolytica* were asymptomatic, getting rid of the infection in a spontaneous way. Brumpt called the non-pathogenic specie *Entamoeba dispar*. However, given the identity between the morphological features of both species, the idea proposed by him was not well accepted by the scientific community of his time.

It was only in 1997 that, during the Congress of amoebiasis held in Mexico, the World Health Organization (WHO), in association with the researchers of the area, confirmed the existence of *E. dispar* (WHO/PAHO/UNESCO Report, 1997).

MORPHOLOGICAL AND BIOLOGICAL FEATURES OF THE PARASITE

This protozoan is classified under the Sarcodina subphylum, shows an amoeboid form and possesses pseudopodia for movement. Trophozoites usually measure 20 to 40 μm, being likely to reach 60 μm in the most invasive forms. In general, it has a single nucleus, which is very distinguishable when stained, but rather clear in fresh preparations. Inside these preparations, the trophozoites are pleomorphic and rapidly produce thick and hyaline pseudopodis, which seem to slip over the blade's surface (Silva and Gomes, 2005).

The ingestion of food occurs through pinocytosis (liquid particles) and phagocytosis of debris. However, in the invasive forms of amoebiasis, erythrophagocytosis and leukophagocytosis are also often verified. The process of phagocytosis starts with the ligation of the Gal lectin/GalNAc (Okada *et al*., 2005) and in order to promote degradation, amoebapores and cysteine proteinases are secreted to the phagosomes (Que *et al*., 2002; Andra *et al*., 2003).

The cysts are spherical, with approximately 10 to 16 μm of diameter and present one to four nuclei. When mature, they have four nuclei. There is an energetic reserve (glycogen) in a distinct vacuole inside the immature cyst, becoming diffuse at mature cysts (Diamond and Clark, 1993), whose cell wall is composed by chitin (Ravdin, 1995). In the environment, they can survive for weeks or months, especially under favorable conditions of humidity and temperature. They are subject to degeneration under temperatures lower than 5°C and above 40°C (Tanyuksel and Petri, 2003).

Although, being morphologically indistinguishable, several works showed biochemical and genetic differences between *E. histolytica* and *E. dispar* (Tannich *et al*., 1989; Gomes *et al*., 1997; Srivastava *et al*., 2005). Besides, *E. dispar* has been described as unlikely to cause the disease; in fact, trophozoites colonize the intestine, but the patients remain asymptomatic (Tannich *et al*., 1989; Mak, 2004).

The evidences of genetic differences led to the differentiation between the two species, so that the commensal one was called *E. dispar*, while the pathogenic was designated as *E. histolytica* (Burch *et al*., 1991).

The parasite's life-cycle is monoxenic and relatively simple. Infection starts with the ingestion of cysts from water and contaminated food, which are resistant to the action of gastric juice. During the process of excystation, which takes place at the small intestine, the metacyst escapes from the cyst wall through a tiny pore. The metacyst undergoes successive nuclear and cytoplasmatic divisions and the resulting trophozoites migrate to colonize the large intestine. In this place, they can leave the mucosa, deshydratate and become precysts; subsequently, they secrete a cystic membrane and become cysts, which are initially mononuclear. Then, the single nucleus divides to form the quadrinucleate stage, being eliminated with the feces and so completing the cycle (Ravdin, 1995).

PATHOGENY, VIRULENCE AND IMMUNE RESPONSE

At the early stage of lesions, trophozoites inside the intestinal mucus adhere to epithelial cells through mechanisms mediated by lectins. The lectin which seems to be the major contributive factor to the adhesion is Gal/GalNAc (McCoy *et al*., 1994; Lejeune *et al*., 2009).

Upon intimate contact, polypeptides called amoebapores are released by the parasite. Amoebapores, that are constitutively present at the cytoplasm of trophozoites (Gonzalez *et al*., 2008), are capable of inducing apoptosis and necrosis of eukaryotic cells and also present an antibacterial activity (Leippe *et al*., 1994).

The inhibition of expression of amoebapore in *E. histolytica* trophozoites was responsible for a significant reduction of the lytic capacity on erythrocites and renal cells in hamsters and also for the decrease of capacity of producing zones of hepatic liquefative necrosis (Bracha *et al*., 1999).

There are evidences of the main role of cysteine proteinases as a virulence factor for *E. histolytica*, being involved in the breach of the mucus barrier, which is crucial in the pathogenesis of amoebiasis (Moncada *et al*., 2006; Lejeune *et al*., 2009). The proteolitic enzymes secreted by the parasite breach the mucus and the epithelial barrier, thus facilitating the penetration inside the tissue (Que and Reed, 2000). The combination of these molecules and some other possible unknown factors lead to the formation of ulcers and to the subsequent migration of amoeba to the liver and other sites (Stanley, 2001).

He *et al*. (2010) clonated and characterized a cysteine proteinase (EhCP4), with the specificity of a unique substrate and nuclear localization. It is related to the exposure to colon cells, being

activated and liberated outside the cell. The mapping of the substrate led to the conception of an inhibitor, which mitigated the infection in model of intestinal amoebiasis. It is a possible target of efficient drugs for the treatment of amoebiasis.

As to the virulence, the different strains of *E. histolytica* and *E. dispar* can be characterized in several forms. The main ones are: the capacity of inducing liver abscess in hamsters, the erythrophagocytosis and the cytopathic effect on mammalian cells cultured in monolayers. It is relevant to consider the biologic characteristics or functions of the parasite, since they may be related to pathogenic mechanisms verified during the development of invasive amoebiasis (Gomes *et al*., 1997).

Carranza-Rosales *et al*. (2010) ascertain that animal models animal models may pose ethical issues and are time-consuming and costly. In view of this, they developed a new model of experimental infection and report the infection of precision-cut hamster liver slices with *E. histolytica* trophozoites. The infection time-course, including tissue damage, is shown to be similar to the findings in the animal model. This new model to study Amoebic Liver Abscess (ALA) is simple and reproducible and employs less than 1/3 of the hamsters required for *in vivo* analyses.

As to the interaction with the immune system, the Lipopeptidophosphoglycan (LPPG), a molecule exposed on the surface of *E. histolytica*, was described as being capable to be recognized by Toll-Like Receptor (TLR) 2 and TLR4 of the leukocytes, being likely to trigger an inflammatory response (Maldonado-Bernal *et al*., 2005; Wong-Baeza *et al*., 2010).

The LPPG leads to the release of cytokines from human monocytes, macrophages and dendritic cells, increasing the expression of costimulatory molecules. The LPPG also activates NKT cells, induces antibody production and anti-LPPG antibodies prevent the development of the disease development in animal models of amoebiasis. Because LPPG is recognized by both the innate and the adaptive immune system, it may be a good candidate to develop a vaccine against *E. histolytica* infection (Wong-Baeza *et al*., 2010).

The tissue invasion by *E. histolytica* trophozoites induces a humoral immune response, which may persist for several years with the production of low levels of antibodies after healing. These levels are not necessarily related to the host defense, such data suggesting that humoral immunity does not afford protection against *E. histolytica* infection (Perez and Kretschmer, 1994; Manrique *et al*., 2002).

The morphological and immunohistochemical results disclosed by a recent study performed by Costa *et al*. (2010) suggest that both the complement system and the antibodies may destroy trophozoites in livers of hamsters, which were experimentally infected with *E. histolytica* and *E. dispar*. This first comparative study also showed a higher *in situ* resistance of *E. histolytica* against antibody response and complement activation. Yet being demonstrated that the complement system is not enough to avoid the development and progress of the liver injuries, this system, in association with antibodies, was responsible for a partial control *in vivo*.

Cellular immune response has been demonstrated to be crucial for the infection control, as shown by studies that focused the passive transfer of T cells and experiments of selective immunosuppression. Cellular immunity suppression of laboratory animals was shown to lead to severe clinical symptoms, as well as tissue invasion (Wang *et al*., 1992; Perez and Kretschmer, 1994).

There are evidences showing that amoebas are destroyed *in vitro* by T lymphocytes obtained from ALA healed patients and that CD8+ cells are responsible for the parasite lysis. In order to

perform their amoebicid activity, lymphocytes require not only the contact cell to cell, but also the action of some cytokines such as IL-2 (interleukin- 2) and INF-γ (gama-interferon) (Schain *et al.*, 1992).

E. histolytica induces an intense inflammatory response at the intestinal mucosa. Epithelial cells start a defensive answer by producing pro-inflammatory cytokines such as IL-1, IL-8, chemotactic factors for macrophages and neutrophils and induce production of nitric oxide and tumor necrosis factor alpha (TNF-α) (Ankri, 2002).

During the cellular lysis mediated by the amoeba/cell contact, there is a powerful chemotactic action for neutrophils, which may be destroyed by virulent amoebas at the site of inflammation (Ravdin and Murphy, 1992; Manrique *et al.*, 2002).

By investigating molecular factors involved in amoebiasis, Blazquez *et al.* (2006) showed a relevant role of TNF-α *in vitro*, as it acts as a chemotactic agent for trophozoites, attracting them to the sites where this cytokine can be found.

Another study demonstrated that expression of the inflammatory enzyme cyclooxygenase-2 (COX-2) in trophozoites and macrophages is relevant to the ALA formation, suggesting that, besides macrophages and neutrophils, trophozoites themselves are involved in the inflammatory process related to extra-intestinal amoebiasis (Gutierrez-Alarcon *et al.*, 2006).

The MLIF (monocyte locomotion inhibitor factor) was identified as a peptide produced by *E. histolytica*, being capable of inhibiting the locomotion of human mononuclear phagocytes and the respiratory burst as well, facilitating amoeba invasion (Rico *et al.*, 1992; Kretschmer *et al.*, 2001). Later, a similar study showed that *E. dispar* in axenic cultures does not present the monocyte locomotion inhibitor factor, such a lack being likely to consequently facilitate its elimination by leukocytes (Silva-Garcia *et al.*, 2003).

Stanley (2003) proposed a model for induction of inflammation and tissue damages in amoebic colitis. Some stages were highlighted, as exposed below:

- Adherence of trophozoites to the intestinal epithelial cells (Gal/GalNAc)
- Activation of the virulence program (amoebapores and cysteine proteinases)
- Cell damages and liberation of IL-1β precursor
- Cleaving IL-1β by the cysteine proteinases of amoebas
- IL-1β activates Nuclear Factor Kappa Beta (NF-κB), leading to the liberation of inflammatory mediators such as IL-8 and COX-2
- Neutrophils are attracted by chemotactic substances
- Macrophages liberate TNF-α
- Substances liberated by leukocytes and amoebae cause tissue damages (cysteine-proteinases, other enzymes and free radicals)
- Amoebae invade the tissue

There are evidences showing that innate immunity plays a fundamental role at the healing of amoebic colitis, as the patients who were treated with high doses of corticosteroids, or, in other words, with powerful inhibitors of NF-κB, presented the disease in its most severe form (Stanley, 2003).

The relevance of innate immunity is also evidenced by a recent study *in vivo*, demonstrating that an inflammatory response induced by bacilli Calmette-Guérin and lipopolysaccharide protected the challenged animals, avoiding an invasive amoebiasis (Shibayama *et al.*, 2008).

Nevertheless, it is important to emphasize that the infection can be aggravated by exaggerated immune responses from the host, since elements resulting from activated leukocytes, such as free radicals, may contribute to give rise to lesions (Stanley, 2003; Santi-Rocca *et al*., 2009).

The interaction of amoeba isolates of low pathogenicity with a variety of gram-negative bacteria, mainly *E. coli* strains, which are readily ingested by the amoebae, significantly increased the ability of the trophozoites to ingest and destroy epithelial cells, to secrete cytopathic substances and to cause hepatic abscesses in hamsters (Mirelman *et al*., 1983).

A recent study *in vitro* showed an increased pathogenicity of trophozoites in xenic cultures (Pysova *et al*., 2009), corroborating the data from Furst *et al*. (2002) and Costa *et al*. (2007) who showed that *E. dispar*, yet being considered non-pathogenic, may, in association with the specific microbiota, produce hepatic injuries in hamsters. In xenic cultures, *E. dispar* trophozoites induce severe hepatic lesions and still present lytic activity for VERO cells.

AMOEBAE-LEUKOCYTES INTERACTION AND OXIDATIVE STRESS

Molecular oxygen is indispensable for the life of the most part of organisms. However, considering the chemical characteristics and the metabolic pathways of its use, some possible reactions may produce deletery effects to life itself. Such a destructive aspect is not properly due to molecular oxygen, since this latter shows low reactivity and does not appear as a direct causative agent of oxidative lesions. Nevertheless, the intermediary products from its metabolism, known as reactive oxygen species, are involved in several kinds of oxidative events inside the cells, generating the oxidation of cellular structures (Halliwell and Gutteridge, 1996).

During the process of cellular oxidation, a great part of the oxygen consumed is reduced to water, but about 2 to 5% of this oxygen may suffer a sequential univalent reduction and form superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Alessio, 1993).

A free radical is defined as any chemical specie presenting one or more unpaired electrons or, in other words, an electron that is the unique to occupy an atomic or molecular orbital (Halliwell and Gutteridge, 1996).

The concept of oxygen as being capable of forming free radicals and producing toxic effects is very ancient. By 1960, it was proposed that living organisms may also produce endogen free radicals, since they present an enzymatic complex likely to eliminate superoxide anions, which is known as antioxidant enzyme superoxide dismutase (SOD) (Fridovich, 1995).

The superoxide radical (O_2^-) is formed after the first reduction of O_2. It is produced during the maximum activation of neutrophils, monocytes, macrophages and eosinophils. Despite being considered as not very reactive in water solutions, an excessive production of this radical has been observed to cause secondary biological lesion to systems that generate O_2^- (Halliwell and Gutteridge, 1990).

The hydroperoxil radical ($HO_2\bullet$) represents the protonated form of the superoxide radical or, in other words, possesses the hydrogen proton. There are evidences that hydroperoxil is more reacting than superoxide, given its bigger facility in triggering the destruction of biological membranes (Halliwell and Gutteridge, 1990).

The hydroxyl radical (OH-) is more reactive in biological systems. Its extremely rapid combination with metals or other radicals at the site where it was produced confirms its high degree of reactivity. Therefore, if hydroxyl is produced close to DNA, modifications of purine and pirimidinic bases may occur, leading to DNA inactivation or mutation. Besides, hydroxyl can inactivate several proteins (enzymes and cellular membrane) by oxidizing its sulphidril groups to

disulphide bridges. It can also trigger the oxidation of polyunsaturated fatty acids of cellular membranes (lipoperoxidation) (Halliwell and Gutteridge, 1996).

According to Eaton (1991), the hydrogen peroxide (H_2O_2), yet being not characterized as a free radical due to the lack of unpaired electrons at the last layer, is a deletery oxygen metabolite, since it takes part at the reaction that produces OH^-. The high toxicity is related to the lipoperoxidation of membranes, especially in association with iron, Fenton and Haber-Weiss reaction.

$$Fe^{3+} + O_2^{-\cdot} \leftrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$$

The free radicals can attack all the main classes of biomelocules, among which lipids are the most susceptible ones. The polyunsaturated fatty acids of cellular membranes are rapidly affected by oxidizing radicals. Lipoperoxidation, as a reaction of self-propagation at the membrane, is very harmful (Halliwell and Gutteridge, 1990).

The body system of antioxidant defense plays the main role of inhibiting or reducing damages caused to the cells by the reactive oxygen species. There is a wide range of antioxidant substances, which can be classified in accordance with their source and/or location as antioxidants from nutrition habits and intra and extracellular antioxidants. The action mechanism still allows to classify them as preventive antioxidants (those which prevent formation of free radicals), scavengers (those which prevent attack from free radicals to the cells) and antioxidants for repair (which remedy damages to the DNA molecule and reconstitute the injured cellular membranes) (Jacob, 1985).

The SOD has been reported as a significant antioxidating mechanism in eukaryotic, prokaryotic, strictly aerobic and microaerophilic organisms. In order to play its functional role, this enzyme requires a metal as co-factor, which can be copper (Cu-SOD), manganese (Mn-SOD) or iron (Fe-SOD) (Fridovich, 1995).

This enzyme acts as a catalyzer for the dismutation of superoxide anion. Dismutation is a reaction where two identical molecules are transformed into different composites. In the case of SOD, a superoxide ion oxidizes the other, generating O_2 (normal) and oxygenated water (H_2O_2); this latter may undergo further degradation through catalase or peroxidase (Yu, 1994).

$$2O_2^{-} + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

Entamoeba and other protozoan are considered to be anaerobes because they can be grown *in vitro* only under conditions of reduced oxygen tension, being susceptible to the reactive oxygen species. However, these parasites have been found to be aerotolerant, such data indicating the existence of efficient mechanisms for detoxification (Mehlotra, 1996).

E. histolytica has been described as a microorganism of anaerobic metabolism. Nevertheless, some works report that, even without mitochondria and Krebs cycle, the parasite presents an incomplete respiratory chain with a complex (Fe-S)-protein instead of cytochrome (Weinbach *et al.*, 1980).

Another study identified on *E. histolytica* a mitochondrial homolog organelle known as Crypton/Mitosome and suggested that it may share common ancestry with mitochondria (Chan *et al.*, 2005).

During the invasion of the host, *E. histolytica* trophozoites are exposed to high quantities of reactive oxygen species, such as the superoxide radical. The high toxicity of these molecules causes several injuries to the biologic macromolecules, leading to metabolic damages. In order to survive in this environment, the parasite must be able to inactivate free radicals (Murray *et al.*, 1981; Clark *et al.*, 1986).

Chen *et al.* (1996) showed antioxidants mechanisms in *E. histolytica*, found SOD and catalase enzymes. Lo and Reeves (1980) described the purification of the enzyme NADPH:flavin oxidoreductase in *E. histolytica* lysed cells. The parasite produces Fe-SOD (SOD associated with iron), which is induced by the superoxide anion, as well as H_2O_2, which can also be detoxified by NADPH:flavin oxidoreductase (Bruchhaus *et al.*, 1998).

Studies demonstrated a significant increase in the level of SOD and of the surface enzyme EH29 (thiol-dependent peroxidase) when trophozoites are exposed to high levels of oxygen, thus suggesting the role of said enzymes in the survival of the parasite under oxidative stress (Ankri, 2002; Akbar *et al.*, 2004; Sen *et al.*, 2007).

Wassmann *et al.* (1999) evaluated *E. histolytica* resistance to metronidazole and detected that this data was related to enzymatic changes, as SOD expression appeared to be increased at the resistant amoebae.

Classical studies involving amoebae and leukocytes, such as those performed by Jarumilinta and Kradolfer (1964) and Guerrant *et al.* (1981), demonstrated that virulent amoebae are lethal to leukocytes.

Salata *et al.* (1985) also demonstrated the capacity of *E. histolytica* trophozoites (HM1) as killers of PMN and MN leukocytes. Amoebae were able to kill leukocytes, even those found on previously immunized serum.

Guerrant *et al.* (1981) described the interaction between *E. histolytica* and PMNs. Both *in vitro* and *in vivo* studies showed that trophozoites from less virulent *E. histolytica* strains were surrounded, fragmented and ingested by PMNs. In contrast, contact with trophozoites from more virulent amoebae strains caused loss of motility of these leukocytes, which were phagocited and killed by them.

A study showed that, in the presence of IFN-γ, *in vitro* macrophages presented an increased capacity of killing *E. histolytica* trophozoites. Amoebicidal activity was determined by counting the number of dead trophozoites in cultures containing macrophages and amoebic trophozoites, which were incubated together for 4 h. The treatment with IFN-γ activated murine peritoneal macrophages to kill amoebae, demonstrating that, upon activation, macrophages are significantly more efficient (Ghadirian and Denis, 1992).

The study conducted by Sanchez-Guillen *et al.* (2002) showed that invasive amoebiasis was related to IL-4 production, indicating a Th2 profile and that, among asymptomatic carriers, the disease was correlated with Th1 response, with high levels of IFN-γ.

Another study also evaluated the role of cytokines at the interactions and showed that the immune response to LPPG is mediated by TLR2 and TLR4. The balance between pro-inflammatory and anti-inflammatory cytokines produced by MNs regulates the innate immune responses and an eventual unbalance is harmful to the host. When used to challenge MNs, LPPG stimulated the production of anti-inflammatory cytokines, such as IL-10, indicating that the parasite can modulate the host response on its benefit (Maldonado-Bernal *et al.*, 2005).

A recent study showed that the lesions verified at amoebic colitis presented a high concentration of neutrophils and lymphocytes, such data grounding the authors' theory that the interaction between PMNs and trophozoites contributes to the pathogenicity (Dickson-Gonzalez *et al.*, 2009).

Few data refer to the dose of superoxide in interactions between amoebae and leukocytes and the results are still controversial. Lin *et al.* (1993) investigated the effect of HM1 strain on the oxidative burst of macrophages. The treatment of peritoneal macrophages with amoebic soluble proteins increased a dose-dependent liberation of O_2^-e de H_2O_2.

Ghadirian and Kongshavn (1988) studied the interaction between MN and two *E. histolytica* strains, among which one was virulent and verified that the levels of superoxide produced by macrophages were increased in the presence of both of them, especially of the virulent one.

Gandhi *et al.* (1987) detected that PMN cells from patients showing severe forms of amoebiasis presented high levels of superoxide, in contrast to the data observed in cells from patients affected by non-invasive forms of the disease.

However, Arbo *et al.* (1990) reported that the oxidative response of neutrophils appeared to be reduced in the presence of amoebae. Manrique *et al.* (2002) found no increase in the superoxide production by PMN cells in the presence of antigens from *E. histolytica* pathogenic strains.

During invasion, trophozoites are exposed to elevated quantities of reactive oxygen species, such as the superoxide radical. According to Ramos-Martinez *et al.* (2009), the highly virulent *E. histolytica* phenotype is related to its great skill to reduce superoxide.

Entamoeba histolytica produces SOD with iron and this enzyme is induced by the superoxide anion, leading to the production of H_2O_2. Similarly to SOD, NADPH:flavin oxidoredutase (Eh34) also plays an antioxidant role by converting oxygen into H_2O_2, which, on its turn, may be eliminated by the peroxiredoxin enzyme (Bruchhaus *et al.*, 1998).

Studies showed that both SOD and EH29 surface enzyme this latter acting as a free radical blocker, are significantly increased when trophozoites are exposed to high levels of oxygen, suggesting that both of them are involved in the survival of the parasite under oxidative stress (Akbar *et al.*, 2004; Sen *et al.*, 2007).

Studies confirmed the existence of superoxide in amoebae. Akbar *et al.* (2004) reported the presence of free radicals in trophozoites that encountered high-oxygen environment. Munoz-Sanchez *et al.* (1997) ascertained that amoebae in cultures produce free radicals from oxygen, although these radicals are likely to cause biological damages on them. Crisostomo-Vazquez *et al.* (2002) evaluated the correlation between free radicals produced by *E. histolytica* and proteases (azocasein and azoalbumin), suggesting that free radicals contribute to the proteases action.

Guerrant *et al.* (1981) studied the interaction between *E. histolytica* and polymorphonuclear (PMN) phagocytes. Both *in vitro* and *in vivo* studies showed that trophozoites of less virulent *E. histolytica* strains were surrounded by PMNs which fragmented and ingested them, while virulent amoebae were able to internalize and kill PMNs.

Vinayak *et al.* (1990) evaluated the interaction between peritoneal macrophages and trophozoites of a virulent *E. histolytica* strain (NIH:200) and noted that, in the presence of antibodies, these latter were destroyed by macrophages when opsonized.

Ghadirian and Denis (1992) analyzed the role of IFN-γ at the activation of *in vitro* macrophages to kill *E. histolytica* trophozoites. Amoebicidal activity was determined by counting the number of dead trophozoites in cultures containing macrophages and trophozoites of a *E. histolytica* virulent strain, which were incubated together for four hours. After treatment with IFN-γ, a significant increase in the number of killed amoebae was verified.

França-Botelho *et al.* (2010) showed interactions between leukocytes and amoebae in vitro, as well as the correlated oxidative stress. In the presence of the *E. histolytica* HM1-IMSS and ICB-32

strains, associated with PMN leukocytes, the levels of superoxide were increased. Surprisingly, such augmentation was still more significant in relation to the ICB-32 strain, which may be possibly related to the differences at the stock of detoxification substances. The levels of SOD were higher for this strain, what could justify a lower level of superoxide at the incubations of PMN with HM1-IMSS, when compared to ICB-32. As to the *E. dispar* ICB-ADO strain, O_2^- was found in its lowest levels. Nevertheless, contrarily to what happened to HM1-IMSS, SOD was also disclosed in low levels, such data giving rise to the following possibilities: leukocytes may have used non-oxidative mechanisms to destroy these amoebae, or this particular strain is so susceptible to O_2^- that it was incapable to resist even to the contact with low levels of this element.

Studies showed an important role also played by nitric oxide at the macrophage-mediated death of amoebae, since the survival of this parasite depends on its ability to evade such immune mechanism. This skill is described by works that report the amoeba capacity to inhibit the nitric oxide synthesis and to catabolize this substance (Lin *et al.*, 1992; Seydel *et al.*, 2000; Ankri, 2002).

CLINICAL MANIFESTATIONS

Epidemiological data estimate that about 90% of amoebiasis cases are asymptomatic and that, among the symptomatic forms, the intestinal amoebiasis is the most frequent one (Stanley, 2003).

Patients with non-dysenteric colitis present abdominal colics and intervals of diarrhoea and asymptomatic periods of normal intestinal activity. Some cases involve a dysenteric colitis, characterized by exacerbated dyspeptic symptoms (pain, eructation, burning sensation and nausea), abdominal distension, flatulence, more than ten daily muco-bloody evacuations and constant sensation of evacuation need. Submucosa is then filled with ulcers, that may cause hydroelectrolytic disturbs and energetic-proteic malnutrition (Melo *et al.*, 2004).

In extraintestinal amoebiasis, trophozoites can migrate through the superior mesenteric vessel and reach the liver, where they cause inflammation, cellular degeneration and liquefative necrosis, thus forming the amoebic abscess, generally located at the right lobe. Patients present fever, intense pain at the right hypochondrium, as well as typical irradiations of biliary colic and painful hepatomegaly at palpation, which does not use to be icteric (Thompson *et al.*, 1985).

The most common extraintestinal manifestation, which occurs at the liver, was considered to be invariably fatal in the past. However, since the introduction of more efficient methods of diagnostic and treatment, mortality rates have fallen to 1-3% (Thompson *et al.*, 1985; Barnes *et al.*, 1987).

Amoebiasis may be aggravated by bacterial secondary infections, which are likely to cause an abscess rupture to abdomen, to the lung, to the pleura or the pericardium. The hematogenic dissemination of trophozoites can injure the lung, the skin, the pericardium, the genitourinary system and the brain (Stanley, 2003).

A study conducted in Vietnam reported 21 cases of hepatic amoebiasis in each group of one hundred thousand persons living in the city of Hue (Blessmann *et al.*, 2002). Hepatic lesions seem to be more incident among HIV (Human Immunodeficiency Virus) patients, as described by a study with patients from the National Hospital of Seul, which found that 32% of patients with amoebic hepatic lesions were HIV positive (Muzaffar *et al.*, 2006).

Papavramidis *et al.* (2008) reported a case of acute abdomen due to the rupture of a gigantic amoebic liver abscess, which reflects a severe form of the disease with high mortality rates. The authors emphasized that prompt diagnosis and treatment are fundamental to preserve the patient's life.

Fulminant amoebic colitis is a severe form of the disease, mostly identified among pregnant women and immunocompromised patients and is usually reported in association with diabetes mellitus and chronic alcoholism (Takahashi *et al*., 1997; Stanley, 2003; Suarez-Artacho *et al*., 2006).

DIAGNOSIS AND TREATMENT

Intestinal amoebiasis is traditionally diagnosed through laboratorial tests to detect the parasite on the feces. Cystic forms use to be found on consistent feces, while the trophozoitic ones are present on a diarrheic or pasty fecal material. However, the lack of technical experience, the intermittent elimination of *E. histolytica/E. dispar* cysts (Walsh, 1986) and the non-differentiation from other intestinal amoebae, cells and artefacts may jeopardize the microscopic diagnosis (Bruckner, 1992). At the immunodiagnostic, the reaction of indirect immunofluorescence (RIFI) for the research of parasite-specific antibodies at the serum of patients, as well as the immunoenzymatic assay (ELISA) for detection of coproantigens at the feces have been used as diagnostic alternatives. Both techniques may be recommended for diagnosis of isolated cases or for epidemiological studies (Haque *et al*., 1998), showing higher specificity and sensitiveness if compared to microscopy (Katzwinkel- Wladarsch *et al*., 1994). The Polymerase Chain Reaction (PCR) is reputed as the most specific method for the identification of *E. histolytica* infections, thus offering new perspectives for future use at the laboratorial routine. However, this method still requires optimization to become more practical and less expensive (Silva and Gomes, 2005).

In an effort to improve the diagnosis of intestinal amoebiasis, Gutierrez-Cisneros *et al*. (2010) showed that real-time PCR has been used for the detection and differentiation of *E. histolytica* and *E. dispar* infections. Fecal samples from 130 individuals with positive microscopic examination were analyzed by real-time PCR, which detected *E. histolytica* DNA in materials from only 10 (7.7%), while *E. dispar* DNA was found in samples from 117 individuals (90.0%).

As to the therapeutic perspectives, metronidazole is the most used amoebicidal drug worldwide. Being well tolerated, it is nowadays the drug recommended for both intestinal and hepatic amoebiasis, at the doses of 500-800 mg, three times a day, during five to ten days. If such a treatment does not reach satisfactory results, it is recommendable to associate metronidazole to antibiotics. Apart from drug therapy, fecal contamination of water and food should be avoided by fundamental prophylactic policies such as, for instance, installation of basic sanitary conditions, as well as a sanitary education and a strict control of individuals who manipulate food and may be, sometimes, asymptomatic carriers of amoebiasis (Silva and Gomes, 2005).

CONCLUSION AND FINAL CONSIDERATIONS

Amoebiasis is reputed by the World Health Organization as one of the leading health problems in developing countries, constituting important cause of death. *E. histolytica* is capable of causing devastating dysentery, colitis and liver abscess.

Although, being broadly spread, the disease tends to reach the highest prevalence rates in developing countries, where the investments in basic sanitary conditions are usually insufficient to meet the necessary requirements, thus allowing the expansion of amoebiasis through orofecal transmission. In the lack of significant improvements and progress in the public health area, which would strongly contribute to the reduction of cases of amoebiasis, it is mandatory to advance on the study of the parasite biology and its pathogenicity, in an attempt to develop more efficient methods for the treatment and prevention of the disease.

Many works focused on such aspects have been developed worldwide, but there are still unclear features that require new researches. A better understanding on the relationship between parasite and the host immune response is crucial for the development of vaccines and for the improvement of therapeutic alternatives to amoebiasis.

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