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Relationship Between Oxidative Stress Production and Virulence Capacity of *Entamoeba* Strains

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Abstract: This study aimed to evaluate the phagocytic ability of *Entamoeba histolytica* and *Entamoeba dispar* strains and the oxidative stress response. Erythrocytes and mononuclear (MN) and polymorphonuclear (PMN) leukocytes, were separated from peripheral blood. The samples were fractionated over a Ficoll-Paque density gradient. Then they were incubated with trophozoites of the virulent *E. histolytica* (HM1-IMSS) and avirulent (ICB-32) strain and *E. dispar* (ICB-ADO) nonpathogenic. Superoxide anion was determined by its reaction with cytochrome chromogen and SOD release by the nitroblue tetrazolium reduction test. The results show that the virulence of *E. histolytica* was correlated to their phagocytic activity. The virulent strain indeed presented greater erythrophagocytic and leuchophagocytic activity. The interaction between MN and *E. histolytica* provoked larger SOD release and smaller superoxide concentration compared to the values obtained in the PMN and *E. histolytica* interaction, where the largest concentration of superoxide was observed in the PMN and ICB-32 interaction, which caused significant amoeba death. The results indicate that these *E. histolytica* virulent strains are more efficient in inhibiting the oxidative burst of MN and that avirulent strains stimulate the production of superoxide for PMN, making the latter more susceptible to death.

Key words: Phagocytosis, superoxide, erythrocytes, leukocytes, amoebiasi

INTRODUCTION

Amoebiasis, an important parasitic disease caused by the protozoan *Entamoeba histolytica*, provokes significant morbidity and death in infected people. The disease affects about 50 million people and accounts for around 100,000 deaths a year, constitutes a serious public health problem (Stanley, 2003).

Virulence characterization of the different strains of *E. histolytica* can be performed using different methods, but they often provide conflicting results. The main parameters

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evaluated are the ability to induce liver abscess, the cytopathic effect and erythrophagocytosis. However, besides virulence, the biological characteristics and parasite functions should be considered to determine the pathogenic mechanisms involved in the development of invasive amoebiasis (Gomes *et al.*, 1997).

A few studies have compared the ability of amoebae to hold leukophagocytosis. *In vitro* and *in vivo* assays showed that trophozoites of less virulent strains of *E. histolytica* are surrounded by PMNs, fragmented and ingested by leukocytes. In contrast, trophozoites of more virulent amoeba strains block PMN motility, engulf and kill them (Guerrant *et al.*, 1981).

The cytopathic effects of amoebae are not attributed to a single factor, but to a combination of many chemical factors such as toxins and enzymes. There are indications that *E. histolytica* can produce free radicals that contributes to damage organic tissues (Crisostomo-Vazquez *et al.*, 2002).

The superoxide radical is produced after the first O_2 reduction. It occurs in nearly every cell and is produced in large amounts during the maximum neutrophil, monocyte and macrophage activation (Halliwell and Gutteridge, 1990; Ferrari *et al.*, 2009). The enzyme superoxide dismutase catalyzes the dismutation of O_2^- into oxygen and hydrogen peroxide (H_2O_2), which can subsequently be degraded by catalyses and peroxidases (Yu, 1994).

Entamoeba and other protozoa have been classified as aerotolerant anaerobic, which indicates the existence of effective mechanisms for oxygen detoxification. The extraintestinal amoebiasis is the most serious form of this disease, the parasites are exposed to large amounts of oxygen; and in these cases detoxification becomes crucial to parasite survival in the presence of oxygen intermediates (Bruchhaus and Tannich, 1994).

The aim of this study was pioneered compare the phagocytic capacity and oxidative stress in interactions between *E. histolytica* and *Entamoeba dispar* strains and leukocytes.

MATERIALS AND METHODS

The study was conducted from August 2007 to August 2009 in Laboratory of amoebiasis of the Universidade Federal de Minas Gerais (Belo Horizonte, Brazil) and the Clinical Analysis Laboratory of the Centro Universitário do Planalto de Araxá (Araxá, Brazil).

Parasites

Trophozoites of the virulent (HM1:IMSS) and avirulent (ICB-32) strains of *E. histolytica* and non nonpathogenic *E. dispar* (ICB-ADO) were grown axenically in a TYI-S-33 medium (Diamond *et al.*, 1978). The parasites were kept with three sub-cultures weekly, ensuring their use in the exponential growth phase.

Subjects

After volunteer consent, 45 blood samples were obtained from healthy male donors aged 18-35 years. Blood samples (10 mL) were collected from each volunteer and used in the different experiments. All procedures were submitted to ethical evaluation and obtained institutional approve.

Blood Cell Separation

Blood samples were collected from volunteer donors 18 to 35 years of age whose serology for HIV, Syphilis and Hepatitis B were negative. Immediately after the donation, the donors gave informed consent to donate 10 mL of blood for the study, collected into heparinized (1 U mL^{-1}) tubes. The samples were fractionated over a Ficoll-Paque (Pharmacia, Upsala, Sweden) density gradient (density 1.077 g L^{-1}). The mononuclear (MN) cells were separated and resuspended independently in serum-free medium 199. Polymorphonuclear (PMN) cells were obtained by the dextran (4 g L^{-1}) sedimentation hypotonic lysis method (Boyum, 1968). The cells (MN and PMN) were washed separately twice in phosphate buffered saline (PBS with 2.6 mM $CaCl_2$ and 2.0 mM $MgCl_2$). This procedure resulted in 95% pure mononuclear (MN) and 93% pure polymorphonuclear (PMN) preparations as analyzed by light microscopy (Honorio-França *et al*., 2001; França *et al*., 2009a). The resulting MN and PMN phagocyte suspensions were adjusted to 2×10^6cells mL^{-1}.

Erythrophagocytosis Assay

The trophozoites were left to interact in PBS with human erythrocytes in a ratio of 1:100 for 20 min (Trissl *et al*., 1978). Results were expressed as percentage of phagocytic amoebae, as well as, the mean number of phagocytized erythrocytes per amoeba (out of 100 amoebae counted on each slide). Five replicates were carried out.

Leukophagocytosis Assay

Leukocytes and amoebae were interacted in a ratio of 2:1 in PBS at 37°C for 60 min, to allow the blood cells to interact with the trophozoites. Assays were carried out with MN and PMN cells according to a protocol adapted (Bellinati-Pires *et al.,* 1989). The reaction was interrupted by incubating the tubes in ice for 10 min. Leukocytes and trophozoites were then stained with 200 μL of acridine orange (14.4 mg L^{-1}) for two minutes and washed twice in iced PBS (França-Botelho *et al*., 2006). Adherence, ingestion and death indices of the trophozoites and leukocytes were determined by fluorescence microscopy observation (TIM-4000, Germany). One hundred amoebae were counted on each slide. The death numbers of trophozoites or phagocytes were calculated as presence of orange stained (dead) and green stained (alive). All the experiments were performed in duplicate or triplicate.

Anion Superoxide

Superoxide release was measured by reducing cytochrome C (Sigma), as previously described (Pick and Mizel, 1981). Suspensions of 1 mL containing either MN or PMN leukocytes (2×10^6 mL^{-1}) and amoebae (1×10^6 mL^{-1}) in PBS were mixed and incubated for 60 min at 37°C. After incubation, pellets were resuspended in PBS containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$ and Cytochrome C (2mg mL^{-1}), the cells were incubated for 60 min at 37°C. The O_2^- rate was measured by absorbance at 630 nm.

Superoxide Dismutase

The concentration of the enzyme SOD was determined using the adapted Crouch *et al.* (1981) protocol, which is based on the ability of this enzyme to inhibit NBT (Nitro Blue Tetrazolium) reduction (Novelli *et al*., 1993). Suspensions of 1mL containing either MN or PMN leukocytes (2×10^6 mL^{-1}) and amoebae (1×10^6 mL^{-1}) were mixed and washed in PBS. The pellet was resuspended in 0.5 mL of PBS and the leukocytes and amebae were incubated for 1 h at 37°C. To extract SOD produced during incubation, a 0.5 mL sample of each incubated suspension was placed in test tubes containing 0.5 mL of chloroform-ethanol and 0.5 mL of reactive mixture, consisting of 0.98 mg mL^{-1} of NBT and 58.4 mg L^{-1} of EDTA (ethylenediaminetetraacetic acid). A total of 2 mL of carbonate buffer (pH 10.2) with hydroxylamine (26 g L^{-1}) was added to the solution. The samples were allowed to rest for 15 min before absorbance measurement at 560 nm. A unit of SOD is defined as the amount of enzyme that inhibits the initial speed of NBT reduction by 50% under trial conditions. Results were expressed in International Unit of SOD (IU).

Statistical Analysis

Through the program GraphPad InStat (3.0 for Windows) Analyses of Variance (ANOVA) were used to compare the groups. Significant differences were compared using the Tukey test. Statistics were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Table 1 shows HM1-IMSS was the most effective for internalizing erythrocytes, reaching 16±1.5 erythrocytes phagocytosed and 92±2.0 of phagocytic amoebas. In leukophagocytosis, these amoebae also had the highest adherence and phagocytosis rates compared to the other strains. They were more effective in killing the internalized white blood cells, especially MN and had the lowest death rate during phagocytosis. The highest amoeba death rate during leukocyte internalization was observed in ICB-ADO, followed by ICB-32 when interacted with PMN.

Figure 1 shows the concentrations of superoxide anion in the interactions between trophozoites and blood cells, overall rates were higher for PMN, the highest level was found with incubation of PMN with the ICB-32 (33.8 nmol). Incubation of PMN with HM1-IMSS strain also caused the level of O_2^- from 16.6 nmol, which was significantly higher than the interactions of three strains with MN and ICB-ADO with PMN.

The SOD concentrations during the interactions between leukocytes and amoebae are Fig. 2, the highest concentration of SOD was observed when the HM1-IMSS was incubated with MN cells (76.38 IU). Incubations of PMN cells with *E. histolytica* strains resulted in higher levels of SOD than those obtained with *E. dispar* incubations.

Amoebiasis is the second cause of death among parasitic diseases in the world. In amoebiasis, the interaction between host immune response and parasite establishment has important implications for controlling the transmission of this parasitic disease (Carrero *et al.*, 2007).

The course of this infection begins with an inflammatory process that recruits eosinophils, lymphocytes, macrophages and neutrophils (Olivos-Garcia *et al.*, 2004) and destroys the host tissue by means of the secretion by parasite of proteinases, kills the target-cells by contact and phagocytisis of erythrocytes (Santos and Soares, 2008).

The erythrophagocytosis is characteristic of invasive amoebiasis, whereas the inability to perform this is an avirulence factor (Boettner *et al.*, 2005). Differences in phagocytic activity have already been described between *E. histolytica* and *E. dispar* in terms of

Table 1: Erythrophagocytosis and leukophagocytosis performed by *E. histolytica* and *E. dispar*

	E. histolytica		*E. dispar*
Infection	HM1-IMSS	ICB-32	ICB-ADO
Erythrophagocytosis			
Number of erythrocytes	16.0±1.5[ab]	3.00±1.4[a]	2.00±1.2[b]
Phagocytic amoebas (%)	92.0±2.9[ab]	45.0±3.6[ac]	25.4±2.0[bc]
Leukophagocytosis (%)			
Adherence to MN	63.4±5.1[a]	60.4±8.0[b]	18.4±2.7[ab]
Adherence to PMN	62.8±8.6[a]	59.6±6.4[b]	16.6±2.1[ab]
Phagocytosis of MN	50.6±3.6[ab]	22.8±3.6[ac]	9.00±1.5[bc]
Phagocytosis of PMN	43.0±4.3[ab]	20.6±4.7[ac]	9.60±2.3[bc]
Death of MN	40.4±3.3[ab]	19.2±4.8[ac]	5.40±1.5[bc]
Death of PMN	31.2±2.7[ab]	19.0±3.3[ac]	3.00±1.5[bc]
Amoebas killed by MN	21.8±2.1[a]	23.6±3.6[b]	63.0±8.4[ab]
Amoebas killed by PMN	22.8±2.7[ab]	40.2±4.0[ac]	76.2±6.6[bc]

Same letters in groups indicate significant differences ($p<0.05$)

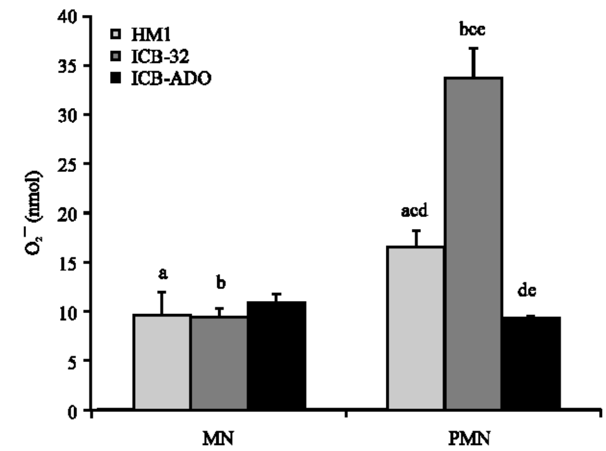

Fig. 1: Superoxide concentrations during interactions between mononuclear (MN) and polymorphonuclear (PMN) leukocytes with *E. histolytica* (HM1-IMSS and ICB-32) and *E. dispar* (ICB-ADO) trophozoites. Same letters in groups indicate significant differences ($p<0.05$)

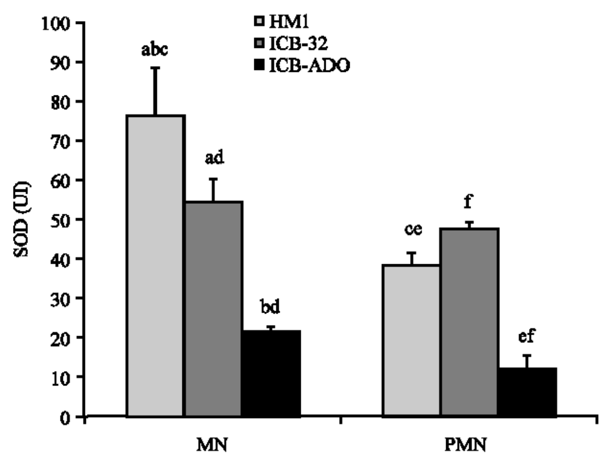

Fig. 2: Superoxide dismutase released during interactions between mononuclear (MN) and polymorphonuclear (PMN) leukocytes with *E. histolytica* (HM1-IMSS and ICB-32) and *E. dispar* (ICB-ADO) trophozoites. Same letters in groups indicate significant differences ($p<0.05$)

changes in phagosome morphology, acidification and degradation (Mitra *et al*., 2005). Study comparing these species sought to determine which ligands were recognized on erythrocytes for ingestion by *E. histolytica* and whether *E. dispar* also recognized these ligands (Boettner *et al*., 2005). The authors found that, before engulfing them, *E. histolytica* changes erythrocytes by phosphatidylserine exposure, which is recognized by amoebae, whereas *E. dispar* is inefficient in erythrophagocytosis.

Initially the erythrophagocytosis used to assess the virulence of the strains. It was confirmed that the HM1-IMSS *E. histolytica* strain has a great ability for internalizing erythrocytes that ICB-32 and *E. dispar* both presenting low virulence. Phagocytosis plays an important role in *E. histolytica* pathogenicity. Phagocytic activity and superoxide alterations have been considered major factors of infectious diseases (Honorio-França *et al.*, 2009) and the generation of free radicals has been reported as an important mechanism for protecting the body during the infectious processes, mainly in intestinal infections (Honorio-França *et al.*, 1997; França *et al.*, 2009b).

Therefore, a broader assessment of the abilities of phagocytic amoeba should consider leukophagocytosis as well as erythrophagocytosis, given that amoeba *in vivo* also phagocyte leukocytes, which must in turn destroy the amoeba to survive.

This pioneering study compared strains of *E. histolytica* and *E. dispar* in terms of the adherence, phagocytosis and death of internalized leukocytes and amoeba death during leukocyte internalization.

There was no significant difference in the adhesion of two strains of *E. histolytica* to PMN and MN; however, significant differences were observed in leukophagocytosis. The HM1-IMSS strain had higher phagocytosis rates than did the other strains. This amoeba strain was also more adept at killing the internalized leukocytes, especially MN and had the lowest death rate. These results corroborate with classic studies involving amoebae and leukocytes, such as those by Jarumilinta and Kradolfer (1964) and Guerrant *et al.* (1981), who showed that virulent amoebae are lethal to leukocytes. Another study also demonstrated the efficiency of trophozoites of *E. histolytica* (HM1) in killing leukocytes, irrespective of the addition of immune serum to the interactions (Salata *et al.*, 1985). The low virulence of ICB-32 and ICB-ADO can also be reinforced by their low rates of adhesion and leukophagocytosis.

Considering the importance of phagocytosis in the virulence of *E. histolytica*, we investigated the ability of this species to induce oxidative burst in PMN and MN. Information on superoxide determination in amoeba-leukocyte interaction is scarce and the available data are controversial. With respect to macrophage oxidative burst, it is known that treatment of peritoneal macrophages with soluble proteins from the amoeba strain HM1-IMSS increases O_2^- and H_2O_2 release on a dose dependent basis (Lin *et al.*, 1993). PMN cells can produce high levels of superoxide in patients infected with serious forms of invasive amoebiasis, but it may not happen in patients infected with noninvasive ones (Gandhi *et al.*, 1987). In contrast, the oxidative response of neutrophils may be reduced in the presence of amoebae (Arbo *et al.*, 1990) and superoxide production by PMN cells may not increase in the presence of antigens from pathogenic strains of *E. histolytica* (Manrique *et al.*, 2002).

Ghadirian and Kongshavn (1988) studying the interactions of MN with two strains of *E. histolytica*, a virulent and the other not, found that levels of superoxide produced by macrophages were higher in the presence of both, especially with the virulent strain.

During the mucosal invasion, the trophozoites are exposed to high amounts of superoxide. According to Ramos-Martinez *et al.* (2009) the phenotype of *E. histolytica* highly virulent is related to high ability to reduce superoxide. Oxidative stress diminishes the activity of endogenous antioxidant enzyme defense system (SOD and Catalase levels), which play a significant protective role (Soni *et al.*, 2009). Others studies have shown that SOD produced by amoebae has an antioxidant role by converting oxygen in hydrogen peroxide and levels are increased significantly when the trophozoites are exposed to oxygen (Bruchhaus *et al.*, 1998; Akbar *et al.*, 2004; Sen *et al.*, 2007).

In the present study incubations of PMN cells with *E. histolytica* strains resulted in higher levels of SOD than those obtained with *E. dispar* incubations. Interestingly, this effect was more pronounced for the ICB-32 strain interacting with PMN. The HM1-IMSS, the most virulent form, may have other mechanisms to avoid the adverse effects produced by the oxidative metabolism of leukocytes. The virulent strain may inhibit the oxidative burst of PMN or develop a different arsenal of detoxifying substances. Indeed, the highest SOD concentrations were obtained in the assays involving HM1-IMSS. This finding explains why superoxide levels in incubations with PMN and HM1-IMSS were lower than in incubations with ICB-32.

The virulent HM1-IMSS strain can resist oxidative stress by superoxide dismutase production, suggesting an adaptation of this parasite to oxygen-rich environments. In contrast to these results, SOD levels were low in ICB-ADO. Perhaps the low level of adhesion to leukocytes is indicative of non-oxidative mechanisms involved in the destruction of this species. Another possibility is that ICB-ADO is so vulnerable to O_2^- that low levels of this element are enough to destroy them.

The present study was to widen knowledge on the interactions between blood cells and amoebae, by explaining how this protozoan, specialized in phagocytosis, behaves towards human cells, which are in turn highly adapted to phagocytosis. This study of new methods to assess the virulence of *E. histolytica* and *E. dispar* may contribute to a better understanding of their biology and pathogenesis.

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