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 (HMI-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 leucophagocytic 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, amoebiasis

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 erythrohagocytosis. 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 contribute 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$_2$O$_2$), which can subsequently be degraded by catalases 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 Tamm, 1994).

The aim of this study was pioneered compare the phagocytic capacity and oxidative stress in interactions between *E. histolytica* and *Entamoeba dispers* 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 (HMI:IMSS) and avirulent (ICB-32) strains of *E. histolytica* and non nonpathogenic *E. dispers* (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 CaCl₂ and 2.0 mM MgCl₂). 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 2×10⁶ cells 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 Miez, 1981). Suspensions of 1 mL containing either MN or PMN leukocytes (2×10⁶ mL⁻¹) and amoebae (1×10⁶ 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 CaCl₂, 2 mM MgCl₂, and Cytochrome C (2 mg mL⁻¹), the cells were incubated for 60 min at 37°C. The O₂⁻ 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 1 mL containing either MN or PMN leukocytes (2×10⁶ mL⁻¹) and amoebae (1×10⁶ mL⁻¹) were mixed and washed in PBS. The pellet was resuspended in 0.5 mL of PBS and the leukocytes and amoeba 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 HMI-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 amoebas 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 mmol). Incubation of PMN with HMI-IMSS strain also caused the level of O₂⁻ from 16.6 mmol, 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 HMI-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 phagocytosis 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 |
|-----------------------------------------------|-----------------------------------------------|
| Infection                                         | E. histolytica | E. dispar |
|                                                  | HMI-IMSS  | ICB-32  | ICB-ADO  |
| Erythrophagocytosis                              |               |
| Number of erythrocytes                          | 16.0±1.5    | 3.00±1.4 | 2.00±1.2  |
| Phagocytic amoebas (%)                          | 92.0±2.9    | 45.0±5.6 | 25.4±2.9  |
| Leukophagocytosis (%)                           |               |
| Adherence to MN                                 | 63.4±5.1    | 60.4±8.0 | 18.4±2.7  |
| Adherence to PMN                                | 62.0±8.6    | 59.0±6.4 | 16.0±2.1  |
| Phagocytosis of MN                              | 50.0±3.6    | 22.0±6.8 | 9.0±1.5   |
| Phagocytosis of PMN                             | 43.0±3.5    | 20.0±4.7 | 9.0±2.3   |
| Death of MN                                     | 40.0±3.5    | 19.0±3.8 | 5.0±1.5   |
| Death of PMN                                    | 31.0±2.7    | 19.0±3.8 | 3.0±1.5   |
| Amoebas killed by MN                            | 21.0±2.1    | 23.0±4.8 | 63.0±8.4  |
| Amoebas killed by PMN                           | 22.0±2.7    | 40.0±4.0 | 76.2±6.8  |

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. dispers* (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. dispers* (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. dispers* 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. dispers* 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 erythropagocytosis, 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 HMI-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 had demonstrated the efficiency of trophozoites of *E. histolytica* (HMI) 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 HMI-IMSS increases O$_2^-$ and H$_2$O$_2$ 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 Kongslevan (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-Martínez 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 HMI-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 HMI-IMSS. This finding explains why superoxide levels in incubations with PMN and HMI-IMSS were lower than in incubations with ICB-32.

The virulent HMI-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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REFERENCES


