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ORIGINAL ARTICLE

Ectophosphatase activity in *Candida albicans* influences fungal adhesion: study between HIV-positive and HIV-negative isolates

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OBJECTIVE: This study describes the expression of acidic ectophosphatase activity on twenty isolates of *C. albicans* from oral cavities of HIV-infected children (HIV+) and compares them with fifteen isolates from HIV-negative children (HIV−), as well as the fungal adhesion to epithelial cells and medical records.

METHODS: The activities were measured in intact cells grown in BHI medium for 48 h at 37°C. Phosphatase activity was assayed at pH 5.5 using 4-methylumbelliferyl phosphate. Yeast adhesion was measured using the MA 104 epithelial cell line.

RESULTS: Mean values of ectophosphatase activity were 610.27 ± 166.36 and 241.25 ± 78.96 picomoles 4-methylumbelliferone/h/10^7 cells for HIV+ and HIV− group, respectively (P = 0.049). No correlation between *C. albicans* enzyme activity from HIV children with viral load and CD4 percentual was observed. Yeasts with high enzyme activity, isolated from HIV+ children showed greater adherence than yeasts with basal levels of ecto-phosphatases from HIV− (Spearman correlation, r = 0.8). Surface phosphatase activity was apparently involved in the adhesion to host cells, as the enhanced attachment of *C. albicans* to host epithelial cells was reversed by pretreatment of yeast with sodium orthovanadate (1 mM), an acid phosphatase inhibitor.

CONCLUSION: These results show that *C. albicans* from HIV+ has an ectophosphatase activity significantly higher than the other isolates. Yeasts expressing higher levels of surface phosphatase activity showed greater adhesion to epithelial cells. So, the activity of acidic surface phosphatases on these cells may contribute to the early mechanisms required for disease establishment.

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Keywords: *Candida albicans*; ectophosphatase; HIV; virulence

Introduction

Oral manifestations of Human Immunodeficiency Virus (HIV) disease have been recognized since the onset of the pandemic and are an important part of the natural history of HIV disease (Lamster *et al*, 1998; Ramos-Gomez, 2002). The most common oral manifestation of HIV infection in children is candidiasis (Chigurupati *et al*, 1996; Howell *et al*, 1996; Kline, 1996; Scheutz *et al*, 1997; Ramos-Gomez, 2002; Shiboski *et al*, 2009). Oropharyngeal candidiasis is of considerable importance in any condition that results in an immune suppressed state; it is particularly important, however, in individuals with HIV infection. The presence of oropharyngeal candidiasis has been linked to a depressed immune system, more rapid progression to AIDS, and a more advanced stage of disease (Klein *et al*, 1984; Patton *et al*, 1999; Shiboski *et al*, 2009). The species most frequently associated with candidiasis lesions is *Candida albicans* (Patton, 2003). Although the microorganism is a harmless commensal in the oral cavity of healthy children (Costa *et al*, 2003), *C. albicans* can rapidly proliferate, invade tissues, and cause symptomatic mucosal lesions in immunocompromised individuals (Fidel and Sobel, 2002). In a recent study, our group reported that *C. albicans* is the most common *Candida* species present in gingival crevices of HIV-positive children, which could be a novel site in the oral

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cavity that serve as *Candida* reservoir. Moreover, we also observed a strong correlation between the isolation of yeasts and immunosuppression in HIV-infected children (Portela *et al*, 2004).

Various characteristics of the *Candida* species have been implicated to contribute to its virulence, for example the ability to grow both as a yeast as well as in a hyphal form within the host, the phenotypic switching between different colony morphologies, such as phospholipases C and proteinases activities (Cutler, 1991; Hube, 2000). Several studies have reported the presence of surface-located acid phosphatases, called ecto- or extra-cytoplasmic phosphatases in many microorganisms that could influence their pathogenesis (Fernandes *et al*, 1997; Dutra *et al*, 1998; Meyer-Fernandes *et al*, 1999; Braibant and Content, 2001; Kneipp *et al*, 2003, 2004), including the fungi *Saccharomyces cerevisiae* (Mildner *et al*, 1975), *Candida parapsilosis* (Fernanado *et al*, 1999; Kiffer-Moreira *et al*, 2007), *Sporothrix schenckii* (Arnold *et al*, 1986), *Aspergillus fumigatus* (Bernard *et al*, 2002), *Fonsecaea pedrosoi* (Kneipp *et al*, 2004), and *Cryptococcus neoformans* (Collopy-Júnior *et al*, 2006).

Several biological roles for ectophosphatases have been proposed. It is known that protein phosphorylation and dephosphorylation are central events in the cell recognition of external and internal signals, leading to specific responses (Zolnierowicz and Bollen, 1999). These enzymes may provide microbes with a source of inorganic phosphate by hydrolyzing phosphomonoester metabolites (Gottlieb and Dwyer, 1981; Fonseca-de-Souza *et al*, 2008, 2009) and protect them upon entering the macrophage by suppressing the respiratory burst (Remaley *et al*, 1984). These ectoenzymes have also been associated with cell differentiation (Alviano *et al*, 2003; Lee *et al*, 2004) and infection of host cells (Furuya *et al*, 1998; Fernanado *et al*, 1999; Kneipp *et al*, 2004; Kiffer-Moreira *et al*, 2007). In *C. parapsilosis*, a surface phosphatase activity was correlated with fungal adhesion to mammalian cells (Fernanado *et al*, 1999; Kiffer-Moreira *et al*, 2007), indicating that ectophosphatases can also influence the interaction of fungal cells with the host. Kneipp *et al* (2004) demonstrated that ectophosphatase activity in conidial forms of *F. pedrosoi* was modulated by exogenous phosphate. They showed that conidial cells expressing high phosphatase activity were significantly more capable of adhering to epithelial cells and fibroblasts than fungi expressing basal levels of enzyme activity. This then suggests that surface phosphatases may have a role in the interaction of *F. pedrosoi* with host cells, an essential step in the infectious process.

Among the putative virulence factors in *C. albicans* isolated from HIV-infected individuals, the most widely studied in recent years are secreted aspartic proteases (Sap), adherence to buccal epithelial cells (Sweet, 1997; Tsang and Samaranayake, 1999), and influence of the mucosal immune system like as the antimicrobial mucosal protein – salivary secretory leukocyte protease inhibitor (Chattopadhyay *et al*, 2004). The exhaustive use of fluconazole prophylaxis in the treatment of the HIV-positive patients may have contributed to the increasing rates of isolation of more pathogenic species (Martínez *et al*, 2002). In the current investigation, we compared the ectophosphatase activity in *C. albicans* from the oral cavity of HIV-infected children with isolates from HIV-negative children, as well as the fungal adhesion to epithelial cells and medical records.

Materials and methods

Chemicals

All reagents were purchased from E. Merck (D-6100 Darmstadt, Germany) or Sigma Chemical Co. (St Louis, MO, USA). Distilled water was deionized using a Milli-Q system of resins (Millipore Corp, Bedford, MA, USA) and was used in the preparation of all solutions.

Microorganisms and growth conditions

The yeasts of *C. albicans* used in this study were isolated from oral cavity of HIV-infected children (twenty isolates) and healthy children (fifteen isolates) who attended the Instituto de Puericultura e Pediatria Martagão Gesteira (IPPMG) and the Clínica de Odontopediatria, Faculdade de Odontologia, Universidade Federal do Rio de Janeiro, respectively (Costa *et al*, 2003). Stock cultures were maintained in Sabouraud-dextrose agar at 4°C. Transfers were made every 6 months. The yeasts were inoculated into 50 ml Erlenmeyer flask containing 20 ml of brain heart infusion (BHI) medium and grown at 37°C for 48 h in an orbital incubator shaker (200 rpm). Cellular growth was estimated by counting the yeasts in a Neubauer chamber. For experiments, yeasts were washed three times in 0.9% NaCl.

Measurement of phosphatase activity

To compare the acidic ectophosphatase activities between HIV+ (20 strains) and HIV− (15 strains) isolates, the phosphatase activities were determined by measuring the rate of 4-methylumbelliferone (4-MU) production from hydrolysis of 4-methylumbelliferyl phosphate (4-MUP), a fluorogenic substrate (Shakarian and Dwyer, 2000). Briefly, intact cells (10^7 yeasts) of *C. albicans* were incubated at room temperature for 1 h in a reaction mixture (0.1 ml) containing 50 mM sodium acetate buffer, pH 5.5, and 5 mM 4-MUP. To determine the concentration of 4-MU formed through phosphatase activity, the 96-well multi-dishes were centrifuged at 1500 *g* for 10 min (4°C). Reactions were terminated with addition of an alkaline buffer (150 mM NaOH, 150 mM glycine, pH 10.5). Fluorescence was measured in a Turner digital filter fluorometer using a 360-nm excitation filter and a 450-nm emission filter. Concentrations of the products generated in these assays were determined using 4-MU standards. Enzyme activities with 4-MUP are reported as the number of picomoles of this substrate hydrolyzed to 4-MU/h/10^7 cells.

Several experiments were performed in the presence of different phosphatase inhibitors at standard concentrations (Kneipp *et al*, 2004), such as: sodium orthovanadate (1 mM), sodium molybdate (1 mM), sodium fluoride (1 mM), and inorganic phosphate (10 mM). Cellular viability was evaluated by exclusion of Trypan

Blue method and by measurement of lactate dehydrogenase activity in the reaction buffer supernatant. The viability of the cells was not affected by the conditions used in this work.

Mammalian cells
The MA 104 epithelial cell line, from a monkey kidney was purchased from a Rio de Janeiro cell culture collection (BCRJ, registration number CR053). The cells were grown at 37°C in 25 cm^2 culture flasks containing DMEM medium, supplemented with 10% fetal bovine serum (FBS). The pH was maintained at 7.2 by addition of HEPES (3 g l^{-1}) and $NaHCO_3$ (0.2 g l^{-1}) to the medium (Freshney, 1994). The initial inoculum was 5×10^4 cells per ml; cultures were subcultured every 2 days and the cells were maintained in exponential-phase growth as described by Freshney (1994).

Adhesion of Candida albicans *to host cells*
Epithelial cells were plated onto 24-well multi-dishes at a density of 10^5 cells per well. They were then incubated at 37°C for 24 h in the presence of DMEM medium supplemented with 10% FBS. Before interaction with the animal cells, two *C. albicans* isolates with high enzyme activity from HIV-positive children (CAS and PRI) and two strains with basal levels of ectophosphatases from healthy child (ACS-C and RAS-C) (10^6 cells) were incubated for 30 min at room temperature in 0.9% NaCl (control cells) or in the same solution containing 1 mM sodium orthovanadate (Na_3VO_4). The cells were then washed twice with 0.9% NaCl and finally rinsed in DMEM. Fungal cells were suspended in the same medium in a ratio of 10 *C. albicans* per 1 animal cell on monolayers. After the addition of *C. albicans,* the cells were incubated at 37°C for 1 h, washed three times in PBS to remove non-adherence yeast, fixed in Bouin's solution and stained with Giemsa. Adhesion indices were determined with a microscope at a magnification of 100 (Zeiss Axioplan 2, Germany). The adhesion index is the ratio of attached and internalized yeast to the number of host cells per field. For each experiment, 400 animal cells were counted.

Medical data
The systemic conditions of the HIV-infected children, such as $CD4^+$ T-lymphocyte count and viral load, were obtained from the medical records.

Statistical analysis
All experiments were performed in triplicate, with similar results obtained from at least three separate cell suspensions. The data were analyzed statistically using SPSS 11.0 statistical program (SPSS Brazil, São Paulo, Brazil) with the Student's *t*-test, Mann–Whitney test and Spearman correlation test. The *P*-values < 0.05 were considered to be significant.

Results

All isolates of *C. albicans* (HIV+ and HIV−) were able to convert the artificial substrate 4-MUP to the hydrolyzed form 4-MU at pH 5.5. After 60 min incubation in the presence of phosphorylated substrate, the enzyme activity reached 610.27 ± 166.36 and 241.25 ± 78.96 picomoles 4-MU/h/10^7cells for the HIV-positive and HIV-negative group, respectively. A positive correlation between the enzyme activities with the presence of HIV infection was observed. Mean values of ectophosphatase activity of *C. albicans* isolates from HIV+ group were at about 2.5-fold higher than mean values of HIV− group (Mann–Whitney test, $P = 0.049$) (Figure 1). In this condition, the lactate dehydrogenase activity of medium reaction supernatant demonstrated no activity, so the possibility of hydrolysis of 4-MUP by intracellular phosphatase was discarded.

Regarding the correlation between systemic condition of the HIV+ children and ectophosphatase activities values in *C. albicans*, none correlated with $CD4^+$ T-lymphocytes count was observed (Figure 2a). Additionally, the same result did occur with viral load (Figure 2b).

The effects of several well-known inhibitors of phosphatases on the hydrolysis of 4-MUP by intact yeasts isolated from HIV+ are shown in Figure 3. The same results were observed with HIV− strains (data not shown). The classical phosphotyrosine phosphatase inhibitor sodium orthovanadate drastically reduced the enzymatic activity to 5.9%. Sodium molybdate, sodium fluoride, and inorganic phosphate (Pi) reduced the hydrolytic activity to 15.1%, 36%, and 15.9%, respectively. The inhibitor effects by sodium molybdate, sodium fluoride, and inorganic phosphate were reversible, whereas the effect of sodium orthovanadate on phosphatase activity was irreversible (Figure 3). These percentual mean values were obtained from at least three different cell suspensions of yeasts isolated from HIV+ and HIV− groups.

The differential expression of ectophosphatase activity in fungal cells from HIV-positive and HIV-negative

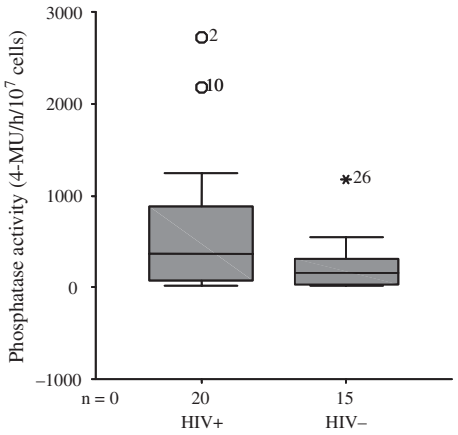

Figure 1 Phosphatase activity of *Candida albicans* isolates from HIV+ ($n = 20$) and HIV− ($n = 15$) subjects. Mean values of 610.27 ± 166.36 and 241.25 ± 78.96 picomoles of 4-MU/h/10^7cells for HIV-positive and HIV-negative group, respectively (Mann–Whitney test, $P = 0.049$). Note: o^2, o^{10}, $*^{26}$: outliner ectophosphatase activities

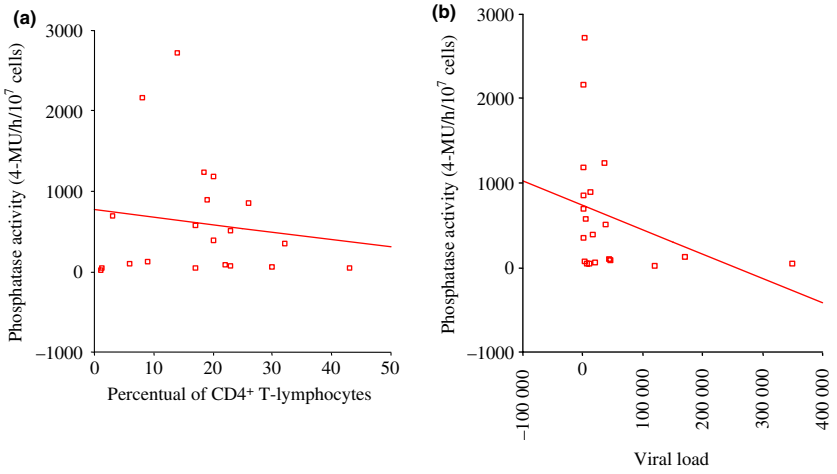

Figure 2 Correlation between the ectophosphatase activities in *Candida albicans* from HIV positive children with the $CD4^+$ T-lymphocyte counts (**a**) ($r = -0.014$; $P = 0.952$) and viral load (**b**) ($r = -0.508$; $P = 0.022$)

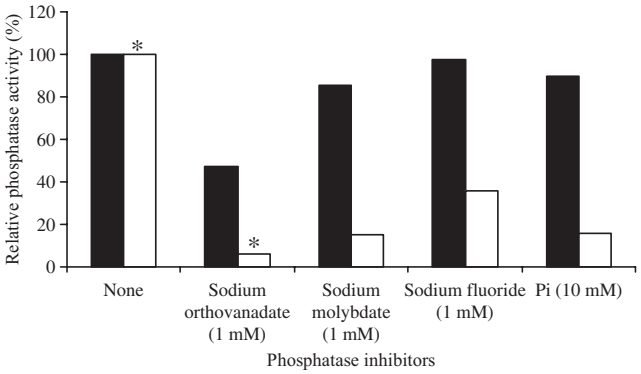

Figure 3 The effect of inhibitors on the phosphatase activity of intact yeasts of *Candida albicans*. Black bars represent the pretreatment of fungi with the inhibitors for 60 min before incubation with 4-MUP substrate. White bars show the relative phosphatase activity when the inhibitors are present in the reaction with 4-MUP substrate. The inhibitor effects by sodium molybdate, sodium fluoride and inorganic phosphate were reversible, whereas the effect by sodium orthovanadate was irreversible (*$P < 0.05$)

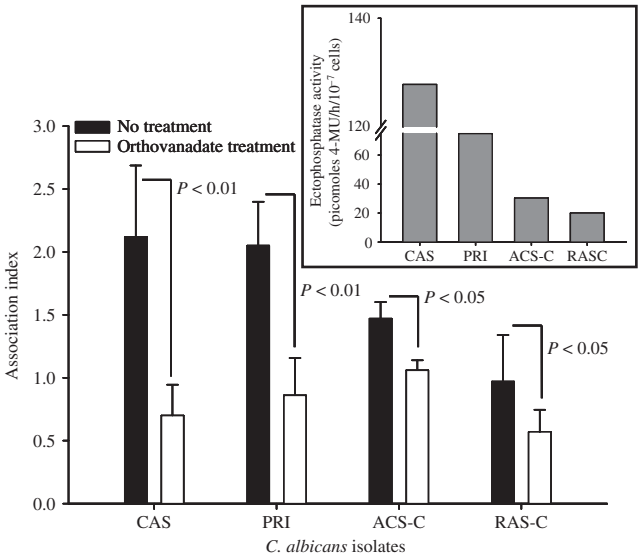

Figure 4 Adhesion of *Candida albicans* strains to epithelial cells is correlated with ectophosphatase activity. Isolates presenting higher levels of enzyme activity (inset) associated more efficiently with host cells ($r = 0.8$; $P = 0.2$). Pretreatment of fungi with the irreversible phosphatase inhibitor orthovanadate resulted in decreased levels of association with epithelial cells ($P < 0.01$ for HIV+ strains; $P < 0.05$ for HIV− strains)

groups provides the basis for new experiments to investigate the possible participation of fungal ectophosphatases in the interaction of *C. albicans* with epithelial cells. Yeasts with greater ectophosphatase activity (HIV+) had higher indices of adhesion to host cells than those of fungi with basal levels of this enzyme (HIV−), which had a high correlation index ($r = 0.8$) (Figure 4). The irreversible profile of enzyme inhibition produced by sodium orthovanadate led us to observe the ability of *C. albicans* to attach to epithelial cells when ectophosphatase activity was fully functional and when surface enzyme activity was inhibited by pretreatment of yeast cells with this inhibitor.

The profile observed in Figure 4 showed that pretreatment of fungi with sodium orthovanadate seemed to inhibit the adhesion of fungi expressing high and basal levels of enzyme activity on epithelial cells. This significant reduction in the adhesion of *C. albicans* isolates to host cells indicates that ectophosphatases can influence the interaction between yeasts and epithelial cells.

Discussion

The HIV epidemic continues to be a world health problem. Reports show that the number of people living with HIV continues to rise, despite the fact that effective prevention strategies exist. Around 33 million people are living with HIV in the world. In 2008, around 2.7 million people were newly infected (UNAIDS/WHO, 2008). Of particular importance to dentistry is the relationship of various forms of oral candidiasis with the presence and progression of HIV infection to full-blown AIDS. Concomitantly with the increase in the number of infected individuals, there has also been an alarming development of resistance against commonly used antifungal agents seen among severely ill patients, such

as patients undergoing treatment for HIV infection (Lopez-Ribot *et al*, 1999).

Although the infections caused by non-albicans *Candida* species have increased, *C. albicans* is still the most common pathogen among fungal infections. To establish an infection, these opportunistic pathogens have to evade the immune system, survive and divide in the host environment, and spread to new tissues (Naglik *et al*, 2003; Yang, 2003). The pathogenic microorganisms have developed alternatives that allow successful colonization or infection of the host (Naglik *et al*, 2003). In a review about virulence factors of *C. albicans*, the author listed some of the major mechanisms like proteinases secretion, hyphal formation, phenotypic switching, and adhesion (Yang, 2003). Enzymes involved in the control of phosphorylation play key roles in these processes of virulence (Hunter, 1995).

Phosphatases are fundamental components in cellular events regulated by the phosphorylation-dephosphorylation processes, which are coordinately controlled through the action of protein kinases and phosphoproteins phosphatases (Hunter, 1995). Ectophosphatases, which are surface molecules whose catalytic site faces the extracellular environment, have also been characterized as bioactive cell wall components in fungi (Kneipp *et al*, 2004; Collopy-Júnior *et al*, 2006; Kiffer-Moreira *et al*, 2007). In this study, all strains of *C. albicans* expressed acidic ectophosphatase activity. The results indicated a strong association between the ectophosphatase activities of *C. albicans* and HIV infection (610.27 ± 166.36 and 241.25 ± 78.96 picomoles 4-MU/h/10^7cells for HIV-positive and HIV-negative groups, respectively). Sweet (1997) showed that more biotypes of *C. albicans* were present in HIV/AIDS groups than in control subjects, and almost all *Candida* species isolated from HIV subjects adhered to buccal epithelial cells in greater numbers than those strains isolated from a healthy group. Concerning the extracellular proteinases, Ollert *et al* (1995) demonstrated a high level and activity of proteinase secretion among *C. albicans* isolated from HIV-positive individuals when compared with healthy individuals. However, no association between ectophosphatase activity of *C. albicans* and HIV/AIDS infection had been carried out.

According to the observation by Hube (2000), the host cellular immuno deficiency is a predisposing factor that could switch the commensal fungi to a pathogenic form. In our research, no association between percentual of CD4 and ectophosphatase activity was observed. This lack of association can be explained by the fact that most subjects were not immune suppressed or had moderate immunosuppression, and most of them had high levels of viral load. However, it must be emphasized that there was a non-significant tendency for higher values of ectophosphatase activity related to a CD4% decrease, indicating that a patient's immune status could have contributed to *Candida* ectoenzyme activity measured *in vitro*.

Among the strains used in this study, we observed distinct levels of phosphatase activity, which allowed the design of the experiment to investigate the putative participation of ectophosphatase activity in the interaction of *C. albicans* with host cells. Before measuring the association between ectophosphatase activity and interaction with epithelial cells, the susceptibility of the *C. albicans* enzyme to several inhibitors was evaluated (Figure 3). The phosphatase activities of all yeasts were inhibited by classical inhibitors of acid phosphatases such as sodium orthovanadate, sodium molybdate, and sodium fluoride. Micromolar concentrations of sodium orthovanadate, a selective phosphotyrosyl phosphatase inhibitor (Hunter, 1995) had the best result in the reduction of the fungal phosphatase activity. Additionally, inhibition of acid phosphatase by fluoride has also been reported by other authors (Kneipp *et al*, 2003). However, in this work, the irreversible profile of enzyme inhibition was produced only by sodium orthovanadate, corroborating a study by Kiffer-Moreira *et al* (2007) that observed the same profile of inhibition caused by sodium orthovanadate in *C. parapsilosis*. This result led us to evaluate if phosphatases were in fact involved in adhesion, and so experiments were performed with yeasts pretreated with an irreversible enzyme inhibitor (sodium orthovanadate). Two isolates of *C. albicans* from each group of HIV-positive and HIV-negative children pretreatment or not with sodium orthovanadate were analyzed to attach to epithelial cells. The profiles observed in Figure 4 show the strains that exhibit the highest levels of enzyme activity and adhesion to epithelial cells. Pretreatment of fungal cells with sodium orthovanadate inhibited the adhesive ability of yeast, confirming the involvement of ectophosphatases in the adhesion of *C. albicans* to mammalian cells. Similar results were found by other authors with *C. parapsilosis* (Fernanado *et al*, 1999; Kiffer-Moreira *et al*, 2007) and with different fungi such as *F. pedrosoi* (Kneipp *et al*, 2004) and *C. neoformans* (Collopy-Júnior *et al*, 2006).

The results obtained in this study suggest that ectophosphatases, besides their possible functions in the biology of fungal cells, may play a role in the interaction of *C. albicans* with host tissues. In *F. pedrosoi*, Kneipp *et al* (2004) attributed an association with adherence of fungal propagules with ectophosphatase activity. The authors suggested that the removal of phosphate groups from surface structures of the host cells could result in conformational changes resulting in the exposure of additional sites for interaction with infectious agents. In *C. albicans*, Lee *et al* (2004) identified the serine/threonine protein phosphatase *SIT4* homologue and demonstrated that this intracellular protein phosphatase is involved in several processes, including the yeast-to-hyphae transition and virulence. It has already become clear that kinases and phosphatases are likely to be important mediators of fungal proliferation and development as well as signal transduction and infection-related morphogenesis. Knowledge of the roles of ectophosphatases in fungal pathogens is still preliminary. Our results demonstrated the involvement of ectophosphatase in *C. albicans* adhesion and consequently in virulence processes.

Knowledge of *C. albicans* biology and pathogenesis involved in the infectious process may reveal new mechanisms of prophylactic and therapeutic anticandidiasis drugs. HIV-infected children, particularly those with

severe immunosuppression, may yield important data on subjects prone to oral candidiasis, an opportunistic infection of great morbidity and an important predictor of HIV infection and AIDS disease evolution. The present investigation showed that ectophosphatases of *C. albicans* influence the interaction between fungi and host cells. Besides, the strains isolated from HIV-infected children had the highest mean of enzyme activity when compared with healthy patients. In particular, our results showed that sodium fluoride affected the phosphatase activity of *C. albicans* isolates. This datum is of great relevance, as all of these strains were isolated from oral cavities, and fluoride is widely used as an anticaries agent in drinking water and a variety of other vehicles (Marquis *et al*, 2003). Fluoride use has resulted in major health benefits with anticaries and probably antifungal effects. Taken together, we believe that the antifungal effects of fluoride could occur *in vivo* principally when our current results indicate that the activity of surface phosphatases influences the interaction of *C. albicans in vitro* is considered. However, the state of biofilms is the common lifestyle for most microorganisms in nature, and so there is a need to consider the interactions of fluoride and weak organic acids with biofilm communities (Marquis *et al*, 2003). So, investigations are in progress to analyze the influence of fluoride on *Candida* biofilms from HIV-positive and HIV-negative isolates (M.B. Portela, T. Souto-Padron, J. Meyer-Fernandes and R.M.A. Soares, unpublished data). Additionally, studies will be needed to reveal new possibilities for drug design and/or production of protective antibodies against these ectophosphatases.

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