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Increased virulence of *Candida albicans* after antineoplastic chemotherapy

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ABSTRACT

Although yeasts of the genus *Candida* are part of the normal human oral microbiota, in cancer patients they may be associated with invasive fungal infections. Antineoplastic therapy, with its adverse side effects, increases the likelihood of such infection. One of the important virulence factors is the capacity of yeast cells to adhere to the surface of medical devices inserted into patients. In this study, we tested the *in vitro* adhesive and film-growing capacity of four *Candida albicans* samples, isolated before and during chemotherapy from the oral cavity of a patient with adenocarcinoma. Adherence to the orotracheal tube (OTT) and biofilm production were assessed spectrophotometrically and the amount of film growth was estimated by measuring fungal metabolism. Also, the frequency and size of germ tubes were evaluated. The results showed a significant increase in the germ tube size of *C. albicans* and also an increase in yeast adherence to the OTT after the start of chemotherapy with mitoxantrone.

Keywords: Virulence. Adherence. *Candida* spp. Antineoplastic therapy.

INTRODUCTION

Antineoplastic chemotherapy is one of the principal forms of treatment for cancer and is given to more than 10 million people each year worldwide. This type of treatment has many side effects, among them neutropenia and mucositis. Such conditions predispose the patients to infection by bacteria and fungi of the normal microbiota (Elting et al., 2003; Blijlevens, 2005; Shokohi et al., 2010). The oral cavity is colonized by yeasts of the genus *Candida* and the risk of developing an infection is directly related to the intensity of colonization (Fanelo et al., 2006; Al-Attas & Amro, 2010).

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The adhesion of a microorganism is the first stage in its relationship with the host and it is a prerequisite for the pathogenesis of infectious diseases; thus, adherence capacity is considered an important virulence factor in yeasts (Hasan et al., 2009). Yeasts of the genus *Candida* are capable of adhering to and producing biofilms on the surface of a large number of medical devices inserted into patients. Microorganisms in biofilms are less vulnerable to antimicrobial therapy (Niimi et al., 2010) and frequently associated with the origin of invasive infections. Several groups have studied the adhesion of yeasts and the formation of biofilms *in vitro* on the surface of medical devices (Camacho et al., 2000; Tamura et al., 2003; Camacho et al., 2007; Seneviratne et al., 2009), but adhesion to the orotracheal tube (OTT) has not yet been investigated. Recently, we showed a significant and continuous enhancement of *C. albicans* virulence in two radiotherapy patients as treatment progressed and we believe the therapy may have facilitated the development of more virulent *C. albicans* strains (Dambroso et al., 2009).

The objective in the present study is to follow the colonization of the oral cavity by yeasts in a patient undergoing antineoplastic chemotherapy and to assess some virulence factors of these yeasts.

MATERIALS AND METHODS

Clinical information and yeast identification

G.G.R., a 77-year-old male, signed the consent term, in accordance with the requirements of the Human Research Ethics Committee of the State University of Maringá, PR, Brazil (Protocol 254/2006). This patient received treatment in March 2007, at the Cancer Hospital of Maringá, for bone and lung metastases due to prostatic adenocarcinoma. From March 2001, he was treated with Tecnoflut® (flutamide). In addition, palliative chemotherapy with 20 mg Novantrone® (mitoxantrone) was given. Oral rinses were collected prior to chemotherapy (a) and also at monthly intervals, prior to each new treatment cycle (b, c and d). These samples were collected, processed and cultured as described by White et al., (2004). The yeasts were identified by conventional

methods (Larone, 1995). The four samples (a, b, c, d) showed the presence of *Candida albicans* in pure culture, with a large number of colony-forming units (>50 CFU/mL) (Table 1), indicating that this patient was intensely colonized. In order to confirm the identity of this yeast, genomic DNA was isolated from these samples and DNA sequencing was carried out.

Table 1: Intensity of colonization by *Candida albicans* in oral rinses from a patient undergoing antineoplastic chemotherapy and respective capacity to produce biofilm.

Samples	Number of colonies*	Biofilm**
a	392	++
b	750	+
c	698	+
d	800	+

* Numbers of colony-forming units/mL in oral rinses: a prior to chemotherapy; b, c, d, monthly samples taken during chemotherapy
**Biofilm assessed by method proposed by Shin et al., (2002).

Antifungal susceptibility testing

Minimal inhibitory concentration (MIC) was determined exactly as described in CLSI (2008) document M27-A3, for azoles and amphotericin; for nystatin, we used the same protocol as for amphotericin B. Briefly, powders of fluconazole (Galena Química Farmacêutica), nystatin (Sigma Aldrich, Steinheim, Germany), amphotericin B (Fungizon®, Bristol-Myers Squibb Brazil), itraconazole and ketoconazole (Janssen Pharmaceutical, Titusville, NJ, USA) were used. Panels of wells, containing serial twofold dilutions of each antifungal agent in RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS), frozen in 96-well plates at −70°C for no more than 3 months, were thawed and inoculated with a suspension of yeast cells adjusted to give a final density of $1.5 \times 10^3 \pm 1.0 \times 10^3$ cells/mL. The panels were incubated in air at 35°C and observed for the presence and absence of growth at 48 h. The MIC was taken as the lowest concentration that produced an evident decrease in turbidity (ca. 50% reduction in growth) relative to that of the drug-free control.

DNA extraction, amplification and sequencing

DNA was extracted from the four isolates as described by Amberg et al. (2006), with modifications. Isolates of *C. albicans* were intra-specifically differentiated by amplification with the following primers: forward 5´- TCAACTTGTCACACC AGATT-3´ and reverse 5´-TTTTTGGTTAGACCTAAGCC -3. Sequencing reactions were performed with the DYEnamic ET Dye Terminator kit (GE). The sequences obtained were identified in the NCBI *Candida albicans* database, by using BLASTn software. Alignment was carried out with ClustalW2 (EMBLL-EBI) software.

Determination of biofilm production

Adherence was tested as previously described by Shin et al., (2002). The experiments were carried out in quadruplicate. Isolates a, b, c, and d were cultured in Sabouraud dextrose broth (SDB – Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 8% glucose. A yeast suspension of each isolate was prepared, counted in a Neubauer chamber and adjusted to 3×10^7 CFU/mL, to induce biofilm formation on a 96-well microtitration plate (TPP®, Switzerland). Subsequently, the microplate was incubated for 24h at 35°C. *C. albicans* ATCC 90028 was used as a positive control in this experiment. Adherence and biofilm formation were estimated spectrophotometrically (ASYS Expert Plus microtitration reader, Biochrom, Cambridge, UK). The results were recorded as absorbance at 405nm and then converted to transmittance.

Germ tube growth of *C. albicans* – frequency and size

A yeast suspension of each isolate (a, b, c, d) was obtained from individual colonies grown on Sabouraud dextrose agar (SDA) for 18-24 h. The inoculum was prepared in 0.85% sterile saline solution (SSS) and diluted to match the No. 4 McFarland turbidity standard. The suspension was centrifuged at 1170×g for 10 min. The sediment was resuspended in 500µL RPMI 1640 medium (Gibco, Grand Island, NY, USA), containing 50% fresh heat-inactivated human serum, and incubated for 3 h at 37°C. Next, the cultures were vigorously homogenized for 30s. Germ tubes were counted and measured with a light microscope (Nykon, TMD; Nippon Kogaku Inc), equipped with a calibrated eyepiece graticule. Their frequency and size were determined blind in 200 cells of each of the samples a, b, c and d. The results were expressed as average ± S.D. of three independent experiments, carried out in quintuplicate.

Colony-forming unit (CFU) adherence assay

For this assay, OTT units, made of transparent PVC, with an outer diameter of 13 mm and an inner diameter of 9.5 mm (Portex® - Hythe, Kent, UK - ref. 100/199/095), were cut aseptically into 20-mm sections, named “test OTT”. The yeast cells were reactivated by culturing in SDB, followed by two sequential subcultures on SDA, for 24 h at 37ºC. Suspensions of these recently subcultured cells were prepared in SSS, adjusted to contain 5 to 6 x 10^5 cells/mL. The test OTTs were transferred aseptically to individual sterile 22 x 180 mm test tubes, containing 10 mL of each yeast suspension; these were incubated for one hour at 37ºC, with continuous shaking at 60 oscillations per minute (Shaker NT-712). After incubation, the yeast suspensions were aspirated and discarded and the test OTTs were gently washed three times with 20 mL of SSS, to remove the non-adherent or weakly adhering yeasts. Next, the test OTTs were transferred to clean test tubes of the same dimensions, containing 10 mL of SSS and 4 g of 2 mm-diameter glass beads (Laborglass, Brazil), which were vortexed for one minute to remove the strongly adhering yeasts. The resulting suspensions were spread on SDA plates and incubated for 48 h at 37ºC. The colonies were counted and the results expressed as CFU/mL. Three independent assays were carried out, each one executed in triplicate. The colony counts obtained, which represent the

adhesion capacity of the yeasts, were analyzed statistically by Student's t test, with the program Graph Pad Prism® version 4 (Graph Pad Software Inc.).

Estimation of growth by fungal metabolism measurement

The fungal metabolic rate of samples a, b, c and d was determined. Four replicates were used for each sample. Firstly, an inoculum of each sample was prepared and adjusted to a density of 3×10^7 CFU/mL. After that, 20µL of each inoculum was separately disposed in the wells of 96-well microtiter plates (TPP®, Switzerland), containing 180µL of SDB supplemented with 8% glucose. After 1, 2, 4, 8, 24 and 48h of incubation, 100µL of SSS, containing 100µg/mL of 2H-tetrazolium-5-carboxanilide (XTT) and 10 µg/mL phenazine methosulfate (PMS) (Sigma Chemical Co., St. Louis, MO, USA), was added to each well. The microtiter plates were further incubated for 3 h at 35°C in order to allow conversion of XTT into its formazan derivative. Absorbance was read at 492nm with a microplate spectrophotometric reader (ASYS Expert Plus, Biochrom, Cambridge, UK).

RESULTS

Yeasts isolated before and during chemotherapy were identified as *C. albicans* by conventional methods and confirmed by molecular techniques (Figure 1). The sequences obtained were 99% identical to the *C. albicans* 5.8S rRNA gene (strain accession number AM 998790.1) (Scanlan & Marchesi, 2008). The E-value observed was 3.10^{-101}.

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b           ------------------------------------------------------------
d           ------------------------------------------------------------GT 2
a           ----------------------------------------------------GATCTCTTGGT 11
DNArCa      TCAACTTGTCACACCAGATTATTACTAATAGTCAAAACTTTCAACAACGGATCTCTTGGT 60
c           ------------------------------------TTTCAACAACCGGATCTCTTGGT 23

b           ----------------------------AATGCGATACGTAATATGAATTGCAGATATTCG 33
d           TCTCG-CATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCG 61
a           TCTCG-CATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCG 70
DNArCa      TCTCG-CATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCG 119
c           TCTTGACATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCG 83
                                     **********************************

b           TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGGTATTCCGGAGGGCATGCCTG 93
d           TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGGTATTCCGGAGGGCATGCCTG 121
a           TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGGTATTCCGGAGGGCATGCCTG 130
DNArCa      TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGGTATTCCGGAGGGCATGCCTG 178
c           TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGGTATTCCGGAGGGCATGCCTG 143
            ************************************************************

b           GTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTTGGTGTTGAGCAATACGACTTGGGT 153
d           GTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTTGGTGTTGAGCAATACGACTTGGGT 181
a           GTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTTGGTGTTGAGCAATACGACTTGGGT 190
DNArCa      GTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTTGGTGTTGAGCAATACGACTTGGGT 237
c           GTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTT---------------------------- 179
            ************************************

b           TTGCTT-------------------------------------------------------- 159
d           TTGCTTGAAAGACGGTAGTGGTAAGGC---------------------------------- 208
a           TTGCTTGGAAAGACG----------------------------------------------- 205
DNArCa      TTGCTTGAAAGACGGTAGTGGTAAGGCGGGATCGCTTTGACAATGGCTTAGGTCTAACCA 297
c           ------------------------------------------------------------


b           ----
d           ----
a           ----
DNArCa      AAAA 301
c           ----
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Fig. 1. Alignment of the sequences obtained from samples collected (a) prior to chemotherapy and (b, c, d) at monthly intervals during chemotherapy with the ribosomal DNA region of *Candida albicans* (DNArCa), using the ClustalW2 program (EMBL-EBI). * nucleotides that are identical in all aligned sequences.

A great increase in yeast cell density was noted in the oral rinse after the first session of chemotherapy (Table 1), but the colony count did not change significantly after subsequent sessions.

In the antifungal drug susceptibility tests, no difference was detected among the *C. albicans* isolates (**a**, **b**, **c**, **d**) collected before and during the treatment. The observed MICs were identical for all 4 isolates: 0.125 µg/mL for nystatin and fluconazole and 0.03 µg/mL for amphotericin B, itraconazole and ketoconazole.

The percentage of cells that produced germ tubes did not change significantly during the chemotherapy; thus, 57%, 55%, 52% and 61% of the yeast cells had germinated after 3 hours in a, b, c and d, respectively. On the other hand, the 3-hour tube length increased as the chemotherapy proceeded (Fig. 2A). No significant change was seen after the first session (a × b; p = 0.1025), but after two applications there were significant differences between a × c (p=0.0031), and a x d (p= 0.0009).

The ability of *C. albicans* to adhere to surfaces is positively correlated with its pathogenicity. According to Figure 2B, there was a significant increase in the capacity of yeast cells to adhere to the OTT, after the beginning of chemotherapy ($p<0.05$). However, the same was not observed for amount of biofilm produced on polystyrene (Table 1). It is important to point out that this methodology, proposed by Shin et al. (2002), tests the capacity to form a biofilm within 24 h and only gives the number of adhering microorganisms, without distinction between dead and living cells. However, the CFU test (Fig. 2B) shows the capacity of yeast to adhere to a medically interesting surface (the OTT), during a 1h exposure, and selects only live yeasts, making it a more specific test. *C. albicans* a, isolated before chemotherapy, showed a mean adhesion of 2,876 CFU/mL, whereas the isolates from samples b, c and d, collected during chemotherapy, adhered much more (6,930; 7,570 and 7,278 CFU/mL, respectively). The adhered CFUs for isolates b, c, and d did not differ significantly among themselves ($p>0.05$).

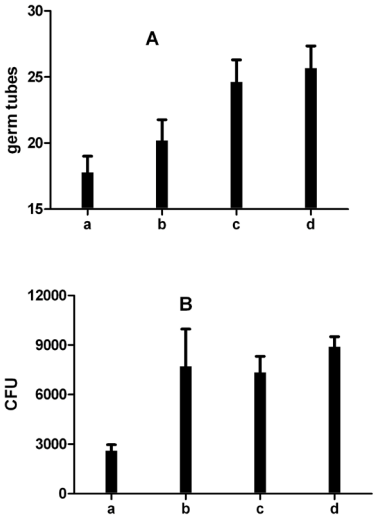

Fig. 2. (A) Germ tube length (µm) and (B) adhesion capacity of *Candida albicans* to the orotracheal tube, expressed in CFU/mL. Mean of three experiments executed in quintuplicate. Sample a collected prior to chemotherapy; b, c, d collected one, two and three months after start of chemotherapy, respectively.

DISCUSSION

The results reported here suggest that chemotherapy induced changes in yeast colonization, since there was a significant increase in the CFU count in oral rinses after treatment had started and also in some virulence factors: size of the germ tubes and capacity to adhere to the test OTT. On the other hand, the drug susceptibility profile and biofilm-forming capacity did not change. It should be recalled that yeasts isolated from the oral cavity before and after chemotherapy were genetically similar, indicating that the yeast population isolated before chemotherapy probably survived in the cavity during the treatment.

In relation to virulence factors, there are some interesting points to be discussed. The germ tube is considered an important virulence factor for *C. albicans*, since it is associated with both a higher capacity to invade tissues and a higher capacity to adhere to surfaces (Grubb et al., 2008). Recently, we reported a significant increase in the size of yeast germ tubes during radiotherapy treatment (Dambroso et al., 2009). In the present study this also occurred: the tube size increased steadily after each session, especially after the first two (Figure 2A). However, the percentage of cells producing germ tubes did not change significantly during the chemotherapy.

The development of *Candida* spp. biofilms on medical devices, such as catheters and prostheses, has been recognized as an increasing clinical problem. Figure 3 shows the adherence profile of the yeast against time of exposure. It was observed that the biofilm grew exponentially, suggesting a great potential of these yeasts to adhere to and remain viable on a surface, thus offering a risk of infection. According to Sellam et al., (2009), the biofilm is visible at 40min and a structure, in which hyphae at the edges extend into the surrounding medium, can be seen at 60min. Between 60 and 90 min, there is a sharp transition in which most cells lose their surface adhesiveness. Biofilm formation is a strain-specific property that plays an important role in the persistence of infection by yeast (Hasan et al., 2009). In the present case, it was not affected by chemotherapy.

The biofilm growth of samples a, b, c and d, estimated by the XTT reduction assay, showed good correlation among XTT absorbance measurements in replicate wells (Figure 3). This method is a good tool to compare the biofilm production of various species because it can be shown that distinct species or strains of this genus may metabolize XTT differently (Silva et al., 2008).

This study was conducted in light of the fact that antineoplastic drugs are capable of modifying several characteristics of a yeast, including its virulence potential. Linares et al., (2006) demonstrated that exposure to methotrexate increased the catalase activity of *C. albicans*. In addition, according to Buschini et al., (2003), cytotoxic drugs were capable of provoking biological changes in Saccharomyces cerevisiae, which depended on cell-specific physiological or biochemical conditions. Ueta et al., (2001) showed that several antineoplastic drugs acted on *C. albicans*, leading to greater adhesion to HeLa cells, resistance to antifungal drugs, enolase and aspartic proteinase activity and yeast resistance against neutrophils.

We have shown not only a significant increase in *C. albicans* germ tube length after the second chemotherapy session with mitoxantrone, but also an increased capacity for adherence of the yeast to the model OTT, starting from the first session. Medical devices such as stents, prostheses, and various types of catheter, endotracheal tubes and pacemakers have demonstrated a capacity to promote the colonization process and biofilm formation (Ramage et al., 2005). Biofilms formed on these devices act as a reservoir or yeast source for future infections, owing to their structure and embedding of these microorganisms in an extracellular material matrix, and this could have a negative impact on the patient (Uppuluri et al., 2009). Thus, the findings presented here may have important implications for yeast adhesion and biofilm formation on the OTT (Inglis et al., 1995), especially in cancer patients who are undergoing chemotherapy and require mechanical ventilation, increasing the risk of pulmonary infections.

Our results suggest that even though yeasts of the genus *Candida* belong to the normal human oral microbiota, their presence should be taken seriously in cancer patients, since they may be associated with invasive fungal infections. Antineoplastic chemotherapy is a common cancer treatment, but it usually has diverse side effects, which might predispose patients to severe fungal infections.

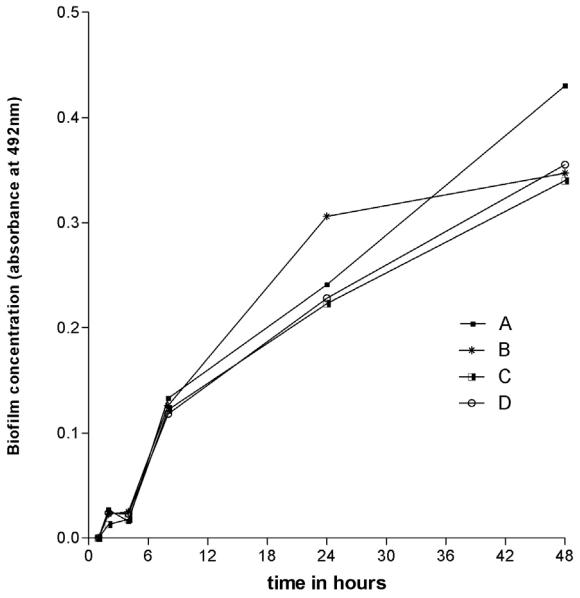

Fig. 3. Estimation of growth by measurement of fungal metabolic rate by the XTT method, after 1, 2, 4, 8, 24 and 48h (product absorbance read at 492nm). Mean of three experiments executed in quadruplicate. Sample A collected before chemotherapy; B, C, D collected one, two and three months after start of chemotherapy, respectively.

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providing the clinical data on the patient; and also Arthur Svidzinski, for the statistical analysis.

RESUMO

Aumento da virulência de Candida albicans após tratamento de quimioterapia

Embora as leveduras do gênero *Candida* pertençam a microbiota humana oral normal, em pacientes com câncer a sua presença pode estar associada a infecções fúngicas invasivas. O tratamento quimioterápico, com seus efeitos colaterais, aumenta as chances desta possibilidade. Um dos principais fatores de virulência consiste na capacidade das leveduras de se aderirem a superfície de instrumentos médicos inseridos nos pacientes. O presente estudo avaliou a capacidade *in vitro* de quatro isolados de *Candida albicans*, obtidos antes e após o início da quimioterapia de um paciente com adenocarcinoma. Estudos de adesão a tubo endotraqueal; produção de biofilme e quantificação por meio do metabolismo fúngico pelo XTT; frequência e tamanho de tubos germinativos foram realizados. Os resultados demonstraram aumento significante no tamanho do tubo germinativo, assim como o aumento da aderência dessas leveduras ao tubo endotraqueal após o início da quimioterapia com mitoxantrone.
Palavras-chave: Virulência. Aderência. *Candida* spp. Tratamento quimioterápico.

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