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Microbiological and Immunological Features of Oral Candidiasis

Marcos André M. de Oliveira*[,1], Lucas P. Carvalho[1], Marcele de S. Gomes[2], Olívia Bacellar[1], Tania F. Barros[2], and Edgar M. Carvalho[1]

[1]*Serviço de Imunologia do Hospital Universitário Prof. Edgard Santos, Universidade Federal da Bahia, Salvador, BA, Brazil, and* [2]*Laboratório de Micologia e Microbiologia da Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, BA, Brazil*

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***Abstract*: *Candida albicans* (*C. albicans*) is the major infectious agent of oral candidiasis, and both innate immunity and cell-mediated immune response participate in the control of the fungal infections. The aim of this study was to correlate the clinical forms of oral candidiasis with the number of colony forming units (CFU) of *C. albicans* in saliva and to characterize T cell response in patients with oral candidiasis. Participants included 75 subjects: 36 with lesions of candidiasis and 39 without lesions of oral candidiasis. A 2-ml sample of saliva was collected from all subjects for microbiological analysis. Cytokine levels were determined by ELISA in supernatants of peripheral blood mononuclear cells of 25 patients with oral candidiasis, after *in vitro* stimulation with *C. albicans* antigens. In 48% of patients, no association was observed with denture use. *C. albicans* was detected in the saliva of 91.7% of patients with oral candidiasis, and there was an association between the number of CFU and the presence of oral lesions. A type Th1 immune response was observed in supernatants of peripheral blood mononuclear cells stimulated with *C. albicans* antigens. In contrast, IL-5 and IL-10 levels were very low or undetectable. Together, this study shows an association between clinical forms of oral candidiasis and the number of colonies of *C. albicans* in saliva, and that a systemic immune response characterized by the production of TNF-α and IFN-γ is observed in patients with oral candidiasis.**

Key words*: Immunoregulation, Cytokines, Oral candidiasis, *C. albicans

Candida species are ubiquitous human fungi capable of causing a variety of diseases, especially in the oral and vaginal mucosae (3, 28). *C. albicans* is the species most often associated with oral lesions, but other, less pathogenic species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and recently *C. dubliniensis* have also been isolated (10, 11) in the saliva of subjects with or without oral candidiasis. The pathogenesis of oral candidiasis is still not fully understood. The isolation of *Candida* from the oral cavity does not imply disease, since the prevalence of infection in asymptomatic healthy persons ranges from 25% to 50% (2, 24). In dental clinics, denture use has been associated with candidiasis (6, 7). The adherence of *C. albicans* to oral epithelia is the first step in the infection process and enables the yeast to overcome the normal flushing mechanism of body secretions (16, 25). Moreover, the integrity of the immune system is required for efficient control of *C. albicans* infections (3, 10). IFN-γ is the major activating factor for fungal killing by phagocytes. Studies of cytokine gene expression and cytokine synthesis in mononuclear cells derived from healthy individuals that had been stimulated *in vitro* with Candida antigens have shown high levels of IFN-γ (8). Furthermore, an absence of a Th1 immune response as observed in AIDS and in women with recurrent vaginal candidiasis is associated with severe and recurrent disease (3, 6, 10, 13, 20). Humoral immune response has been evaluated in oral candidiasis, and both *C. albicans*-specific IgA and IgG antibodies have been identified (4, 6, 16, 31). Regarding cellular immunity, the cytokine profile has been determined in saliva of patients with oral candidiasis and also after stimulation

**Address correspondence to* Dr. Marcos André M. de Oliveira, Hospital Universitário Prof. Edgard Santos, Serviço de Imunologia, 5° andar, Rua João das Botas, s/n°, Canela, 40110–160, Salvador, BA, Brazil. Fax: +55–71–3245–7110. E-mail: imuno@ufba.br

Abbreviations: CFU, colony forming unit; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer saline; PHA, phytohemagglutinin.

of epithelial cells with *C. albicans* antigens. A mixed Th1 and Th2 pattern of cytokines has been observed in both situations (18). However, very little is known about systemic immunity in patients with oral candidiasis. In the present study, quantitative analyses of *C. albicans* colonies were performed in subjects who sought dental care, and the profile of cytokines produced by mononuclear cells from patients with oral candidiasis was determined after *in vitro* stimulation of cells with *C. albicans* antigens.

Materials and Methods

Patients. This study included 75 adult subjects: 36 with lesions and 39 without lesions of oral candidiasis. They were evaluated in the Dental Clinic of the Brazilian Army Hospital and in the Dental School of the Federal University of Bahia. Exclusion criteria included the following: use of corticosteroids or other immunosuppressive drugs, use of antifungals, diabetes and positive serology for HIV infection. The study was approved by the Ethics Committee of the Hospital Universitário Professor Edgard Santos, and all patients signed an informed consent form.

Oral examination. One dentist performed the clinical examination of the oral mucosa. The diagnosis was based on the clinical appearance of the lesion. A clinical history as well as the frequency of patients who used dentures was recorded. The clinical forms found were erythematous, pseudomembranous and hyperplastic candidiasis. Erythematous candidiasis presents with red and inflamed mucosa. The pseudomembranous form commonly called "thrush" presents as a confluent white plaque material that can be wiped off, revealing a red and inflamed mucosa. The hyperplastic candidiasis, an uncommon form, involves a white macular or plaque lesion that cannot be wiped off.

Microbiological evaluation. A 2-ml sample of saliva was collected from all patients between 9 and 11 a.m. without stimulants or provocation, and culture was performed in Sabouraud agar dextrose (Biobras, São Paulo, SP), supplemented with 0.1 mg/ml of chloramphenicol (17). Briefly, the saliva was diluted 1:3 in sterile 0.9% NaCl, and 1.0 ml was distributed on the plate, with the aid of a Drigalsky loop. The plates were incubated at 37 C for 48 hr and a quantitative analysis of positive cultures was performed. The identification of the yeast species was performed according to standard techniques (24).

C. albicans *antigen preparation*. *C. albicans* isolated from one patient with oral candidiasis were cultured in Sabouraud's medium. The culture was centrifuged at 3,000 $\times$ *g* for 30 min, and the pellet was washed 3 times with phosphate buffer saline (PBS). The pellet was resuspended in 0.5 M sodium hydroxide, and then frozen (-70 C) and thawed (37 C) 20 times (8). After centrifugation at 3,000 $\times$ *g* for 30 min, the supernatant was collected and the pH adjusted to 7.3. The protein content was determined by the method of Lowry et al. (19). The antigens were used for stimulation of PBMC collected from 25 patients. Dose-response curves were performed, and optimal concentration for *C. albicans* antigen was 0.05 μg/ml, and 10 μg/ml for phytohemagglutinin (PHA).

Cell culture and cytokine assay. Peripheral blood mononuclear cells (PBMC) from subjects with oral candidiasis were isolated from heparin-treated venous blood by ficoll-hypaque gradient centrifugation. PBMC were washed three times with 0.9% NaCl and then resuspended in RPMI 1640 culture medium (Gibco BRL, Grand Island, N.Y., U.S.A.), supplemented with 10% human AB serum, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. Cell density was adjusted to 3 $\times$ 10^6 cells/ml, distributed in 24-well plates and stimulated with *C. albicans* antigen (0.05 μg/ml), or PHA at a final dilution of 1:100 (10 μg/ml) (Gibco). After incubation for 72 hr at 37 C in 5% CO_2, supernatants were collected and stored at -70 C. The levels of IL-5, IL-10, TNF-α and IFN-γ were measured by the ELISA sandwich technique (R&D Systems, Minneapolis, Minn., U.S.A.) and the results were expressed in pg/ml.

Statistical analysis. The comparison between the proportions was performed by the chi-square test. Differences in cytokine levels were analyzed by the Mann-Whitney test, and statistical significance was defined as $P < 0.05$.

Results

Of the 75 subjects studied, 48% ($n=36$) had mucosal lesions for *C. albicans* and 39 had no oral candidiasis. The demographic characteristics of patients and the percentage of denture wearers with and without oral lesions are shown in Table 1. The age of subjects ranged from 18 to 59 years and 84% were female. There was no difference in age or gender of the group who had oral candidiasis compared to the group without oral lesions. The majority of the lesions was erythematous and pseudomembranous and were predominantly localized on the palate, cheeks and tongue. The size of the lesions ranged from 2 to 8 cm. There was no significant difference between subjects with and without dentures in relation to the presence of oral lesions due to *C. albicans*. While 50% ($n=24$) of the 48 denture-using subjects had *C. albicans* lesions, 44% ($n=12$) of the 27 without dentures had oral lesions.

C. albicans colonies were identified in the saliva of 91.7% (n=33) of patients who had oral candidiasis and in the saliva of 33.3% (n=13) of subjects without oral lesions. The number of colony forming units (CFU) was quite variable ranging from 1 to 1,200 colonies with a mean ± SD of 145 ± 274 CFU/ml. The number of CFU of *C. albicans*/ml of saliva in subjects with and without oral lesions is shown in Fig 1. A correlation was found between a high number of CFU and the presence of oral lesions. While all subjects who had no oral lesions had less than 100 CFU/ml of saliva, all cases with more than 100 CFU/ml of saliva had oral lesions. The mean CFU level in patients with oral lesions (286 ± 344) was 20 times higher than that in subjects without oral lesions (14 ± 28).

There was also an association between the number of CFU and the clinical form of candidiasis (Table 2).

Of the six patients with the pseudomembranous form, 5 (83.3%) had more than 400 CFU/ml in their saliva. A CFU higher than 400/ml was documented in only 14.2% of the cases of erythematous candidiasis and in none of the patients with the hyperplastic form.

The profile of cytokines secreted by PBMC after stimulation with *C. albicans* in 25 patients who had oral lesions is shown in Fig. 2. TNF-α levels ranged from 0 to 958 pg/ml with a mean of 327 ± 310 pg/ml, and IFN-γ ranged from 7 to 756 pg/ml with a mean of 156 ± 168 pg/ml. In contrast, the majority of patients with oral candidiasis did not show detectable IL-5 and IL-10 *in vitro* after stimulation with *C. albicans* antigen. IL-5 levels ranged from 0 to 53 pg/ml with a mean of 11 ± 19 pg/ml, and IL-10 levels ranged from 0 to 263 with a mean of 41 ± 66 pg/ml. Both TNF-α and IFN-γ levels were significantly higher than IL-5 and IL-10 levels (*P*

Table 1. Demographic, educational and microbiological data of subjects with and without lesions of oral candidiasis

Variables	Subjects with lesions (n=36)	Subjects without lesions (n=39)	*P* value
Age (mean ± SD)	44.8 ± 12.1	40.5 ± 12.0	0.10
Female / male ratio	31 / 5	32 / 7	0.63
Denture use (%)	24 (66.7)	24 (61.5)	0.64
Educational degree			
Elementary	9 (25.0)	7 (17.9)	
High school	24 (66.7)	21 (53.8)	0.08
College	3 (8.3)	11 (28.2)	
% of patients with *C. albicans* in saliva	33 (91.7)	13 (33.3)	< 0.0001

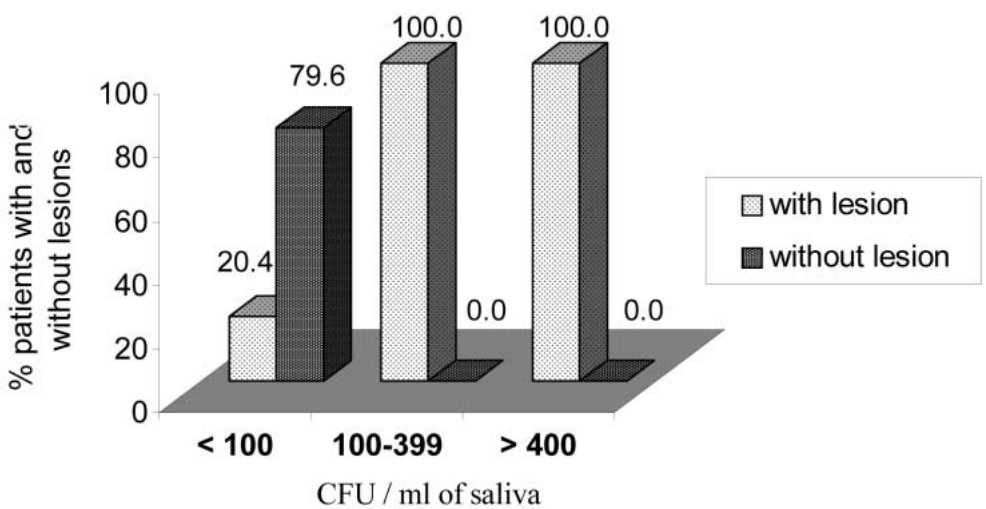

Fig. 1. Association between number of CFU/ml of saliva and presence of oral lesions of candidiasis. The number of CFU of *C. albicans* per ml of saliva was determined in 36 patients with oral lesions indicative of candidiasis and in 39 subjects without lesions. Data represents the percentage of subjects in the 2 groups who had less than 100 CFU, between 100 and 399 CFU and more than 400 CFU per ml.

Table 2. Clinical forms of oral candidiasis categorized according to number of CFU in 36 patients

Clinical forms	Number of patients according to the number of CFU of *C. albicans*		
	< 100	100 – 399	> 400
Erythematous $n = 28$	9	14	5
Pseudomembranous $n = 6$	0	1	5
Hyperplastic $n = 2$	1	1	0

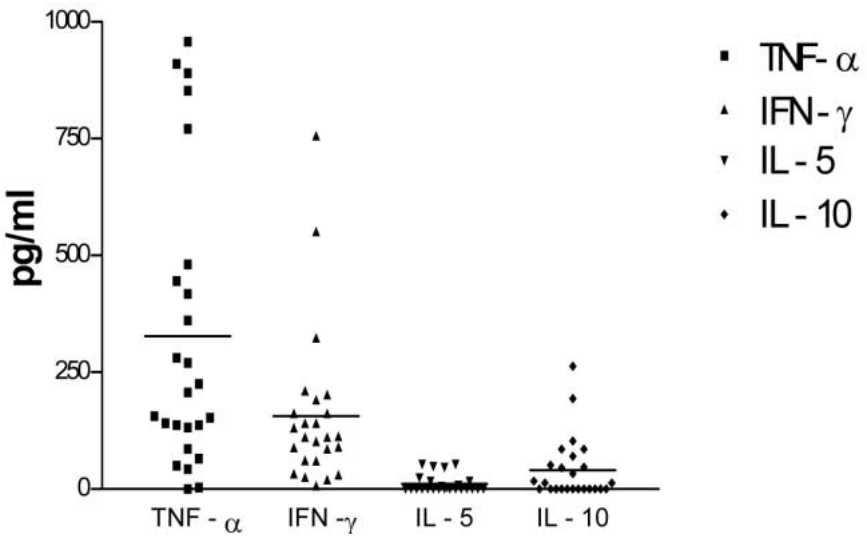

Fig. 2. Cytokine profile in patients with oral candidiasis. TNF-α, IFN-γ, IL-5 and IL-10 were determined by ELISA in supernatants of PBMC stimulated with *C. albicans* antigens.

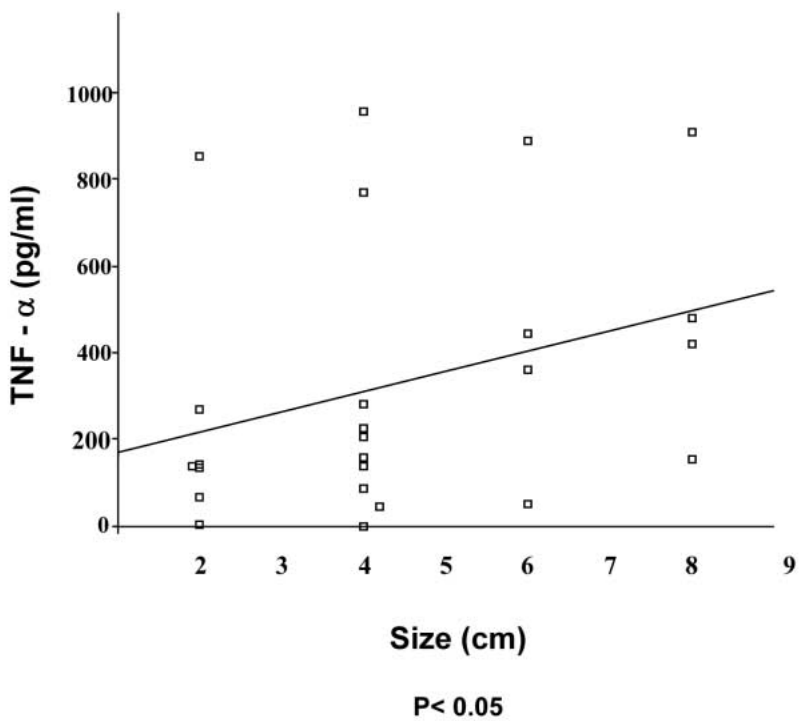

Fig. 3. Correlation between TNF-α levels and size of oral candidiasis. Levels of TNF-α were determined in supernatants of PBMC stimulated with *C. albicans* antigens.

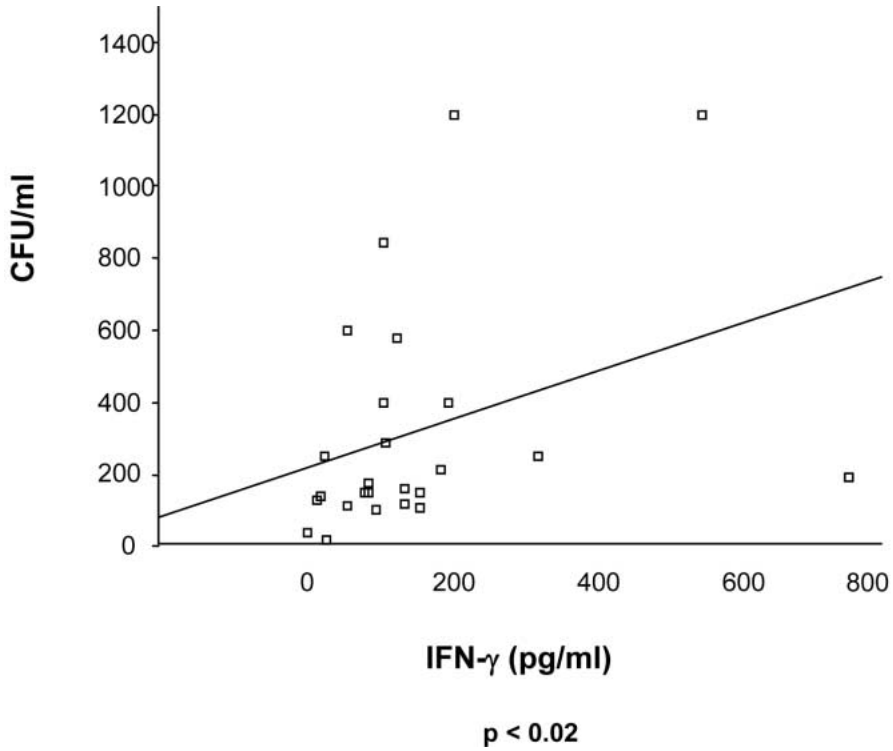

Fig. 4. Correlation between IFN-γ levels and CFU of *C. albicans*. Levels of IFN-γ were determined in supernatants of PBMC stimulated with *C. albicans* antigen, and the number of CFU of *C. albicans* was determined in saliva of 25 patients with oral candidiasis.

< 0.01). While there was a tendency towards a correlation between TNF-α level and lesion size (Fig. 3), there was no association between IFN-γ level and size of oral lesions (data not shown). There was a correlation between the number of CFU of *C. albicans* and IFN-γ level (Fig. 4), but no correlation with TNF-α level (data not shown). Stimulation with PHA was established as a positive control, and as expected, high levels of all cytokines were documented in PHA-stimulated cultures (data not shown).

Discussion

The oral and vaginal mucosae are the major sites of *C. albicans* colonization and disease. While microbiological evaluation is usually performed for vaginal mucosa and immunologic studies have characterized the immune response in patients with vaginal candidiasis, little is known about the immune response in patients with oral lesions due to *C. albicans*. This study emphasizes the high prevalence of oral candidiasis in adult population and shows that an immunological response to *C. albicans* can be established in lymphocytes from the blood of patients with oral candidiasis. This response was predominantly associated with a Th1 immune response characterized by secretion of TNF-α and IFN-γ.

Oral candidiasis is a very common disease in the dental clinic and has been associated with denture use (5, 23). *Candida*-associated denture stomatitis has been reported in 50% to 60% of denture wearers (5, 26). Functional and qualitative factors in denture wear may contribute to the development of lesions in the oral mucosa. In the present study, we found no association between oral candidiasis and denture wear. It is possible that poor oral hygiene, salivary flow and other unknown factors had a strong influence on *C. albicans* colonization and disease which overcame the influence of dentures as previously observed (1, 18). Of note, no association between hyperplastic oral candidiasis, typically seen with trauma, and denture use has been documented (31).

Colonization is not indicative of candidiasis, but it is required for clinical infection. Moreover, there is an association between high density of colonies and development of candidiasis (21). The quantification of the flora is helpful in differentiating between colonization and infection. In this study, all subjects without oral lesions had less than 100 CFU/ml, while 26 of 36 (72.2%) cases with oral candidiasis had more than 100 CFU per ml of saliva. Therefore, a strong association between the number of CFU and disease was demon-

strated. There was also an association between the number of CFU and the clinical form of the candidiasis. Specifically, the pseudomembranous form correlated highly with a CFU level higher than 400/ml.

Keratinocytes, neutrophils, macrophages, eosinophils and basophils are the first line of host defense against mucosal *C. albicans* infections. An important step in the inflammatory response leading to recovery from mycotic infection is a Th1 immune response mediated by CD4+ T cells (27). Activation of effectors cells is provided by cytokines (IFN-γ and TNF-α) secreted by T cells and macrophages (22). It has been shown that an impaired Th1 response may allow the persistence of *C. albicans* and development of disease (15). Cell-mediated immunity conferred by CD4+ Th1 cells is considered the predominant host defense against mucosal candida infections, and Th2 responses are associated with susceptibility (18). A response Th2 was not observed, since low levels of both IL-5 and IL-10 were detected in cultures stimulated with *C. albicans* antigens. Paradoxically, some Th2 cytokine, such as IL-4 and IL-10, are found to be required for the development and maintenance of protective Th1 mediated antifungal immunity. Therefore, in addition to the Th1/Th2 balance, other mechanisms seem to be involved in controlling and sustaining Th1-dependent immune resistance to the fungus (26, 30). We have previously shown that down modulation of cell-mediated immune response to *C. albicans* antigen plays an important role in recurrent vaginal candidiasis (9). In such a case, down regulation of the Th1 immune response was mediated by IL-10, since neutralization of this cytokine enhanced *in vitro* IFN-γ production in lymphocyte cultures stimulated with *C. albicans* antigen (8, 9). The immune response has also been characterized in patients with mucocutaneous candidiasis, a disease associated with T cell deficiency (3). However, very little is known about the immune response of patients who present with localized oral lesions. Previous studies have predominantly studied the cytokines expressed in epithelial cells of the oral mucosa, and it has been documented that epithelial cells of the oral mucosa can produce cytokines such as GM-CSF, IL-8, TNF-α and IFN-γ (12, 30). A mixed Th1 and Th2 pattern of cytokines has also been demonstrated in the saliva of HIV-negative patients with candidiasis (18).

In this study, we demonstrated that oral candidiasis is able to induce a systemic immune response characterized by the production of TNF-α and IFN-γ (14, 30). These cytokines are important for host defense against intracellular pathogens, but these cytokines have also been implicated in the pathogenesis of disease (29). The absence of an association between lesion size and level of these cytokines indicates that the production of these molecules is probably more related to host defense than disease pathogenesis. Therefore, this immunological response may be important in controlling the disease at the oral site, preventing *C. albicans* dissemination.

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