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Relationship between *Candida* infection and immune cellular response in inflammatory hyperplasia

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Objectives: To analyze and quantify the $CD8^+$ and $CD4^+$ T-lymphocyte populations in inflammatory hyperplasia and to establish the relationship between the frequency and location of these cells and *Candida* infection.
Methods: Samples of inflammatory hyperplasia were stained with PAS for evidence of *Candida* sp. and were classified in two groups, infected and control, according to the presence or absence of infection. After immunoreaction with specific anti-CD4 and anti-CD8 monoclonal antibodies, the distribution and frequency of the positive cells were analyzed in 41 cases (19 controls without *Candida* sp. and 22 infected cases). Lymphocytes were quantified in the three consecutive fields where the inflammatory infiltration was concentrated.
Results: There was no relationship between the frequency and location of $CD4^+$ T cells and *Candida* sp. infection. The number of $CD8^+$ cells close to the fungi hyphae as well as the total number of $CD8^+$ T cells present in inflammatory hyperplasia were higher in the *Candida* sp. group than in the control noninfected group ($P < 0.05$).
Conclusion: Since the $CD8^+$ T cells were distributed according to the location of *Candida* sp. hyphae, and since a higher $CD8^+$/total lymphocytes ratio was observed in the infected group, we suggest a role for $CD8^+$ T cells in the defense against *Candida* in oral infections associated with inflammatory hyperplasia in immunocompetent individuals.

Key words: candidosis, oral; $CD8^+$ T lymphocytes; $CD4^+$ T lymphocytes; hyperplasia, diagnostic

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The term inflammatory hyperplasia denotes lesions that can be caused by long-term mechanical irritation, frequently the result of ill-fitting dentures (6). The mechanical irritation induces an inflammatory response, which manifests clinically as a bulk expansion of the oral cavity mucosa (30). Microscopically, the findings in these lesions are hyperplasia of the epithelium and connective tissue, with a variable inflammatory response (21).

Candidosis is an opportunistic fungal infection that commonly affects immunocompromised patients, and is also associated with the use of complete dentures (2, 8, 12, 23, 24). There is a strong association of *Candida* infection with inflammatory hyperplasia, the infection sometimes being considered in the etiology of that lesion (22). Both innate, represented by macrophages and granulocytes (15, 25), and adaptive immune systems (9–11, 29) are involved in the protection against and response to *Candida* sp. It has been suggested that macrophages and granulocytes are responsible for the prevention of systemic and widespread candidosis, whereas T lymphocytes are concentrated locally in the infected region (10, 15).

Several studies have pointed out an involvement of $CD4^+$ T cells (T-helper lymphocytes) in the response to *Candida* sp., with both an increase in positive cell counts (9) and a relative increase in the number of activated cells in the presence of infection, regardless of alterations in absolute total cell number (7, 20). The role of $CD8^+$ T cells in the protection against candidosis is still controversial; different studies have shown conflicting results regarding the function and frequency of those cells in relation to *Candida* infection (7, 18, 20).

The present study analyzes the presence of *Candida* sp. in immunocompetent patients with inflammatory hyperplasia and quantifies $CD4^+$ and $CD8^+$ T cells, identified by specific monoclonal antibodies, according to their position in the lesions in relation to *Candida* sp. hyphae. Although the study is centered on immunocompetent individuals with inflammatory hyperplasia associated with *Candida* sp. in the oral cavity, the results are also discussed in relation to other models of *Candida* infection as a way of providing a comparative view of the immune responses to this opportunistic fungal infection.

Material and methods

Tissue

Samples were obtained from gingival biopsies with a histopathologic diagnosis of inflammatory hyperplasia stored at the Laboratory of Oral Pathology, Federal University of Rio Grande do Sul (UFRGS). The criteria for inclusion were as follows: a histopathologic diagnosis of inflammatory hyperplasia, lesions obtained from immunocompetent patients, total removal of the lesion by biopsies, and lesions lined with intact nonulcerated epithelium. A further inclusion criterion for the infected group was a clinical diagnosis of erythematous candidosis associated with the lesion, as demonstrated by the presence of Periodic Acid Schiff (PAS)-positive hyphae. The control group met all of the former criteria but had a negative clinical diagnosis of erythematous candidosis, an absence of hyphae and, consequently, an absence of infection.

Four histologic slices (3 μm thin) were made from the selected paraffin blocks and were processed using different staining and labeling techniques.

This study was approved by the UFRGS Committee of Ethics on Research.

Staining and labeling

The first slice was stained by hematoxylin-eosin for the morphologic evaluation of the lesion (Fig. 1A). Another slice was stained by PAS for identification of *Candida* sp. hyphae. On the basis of clinical diagnosis, the samples were classified into two experimental groups: infected and control (Fig. 1). The remaining slices were labeled by the immunohistochemical technique using monoclonal antibodies for CD4 and CD8 cells in a streptoavidin–biotin system (Table 1). For an example, see Fig. 2.

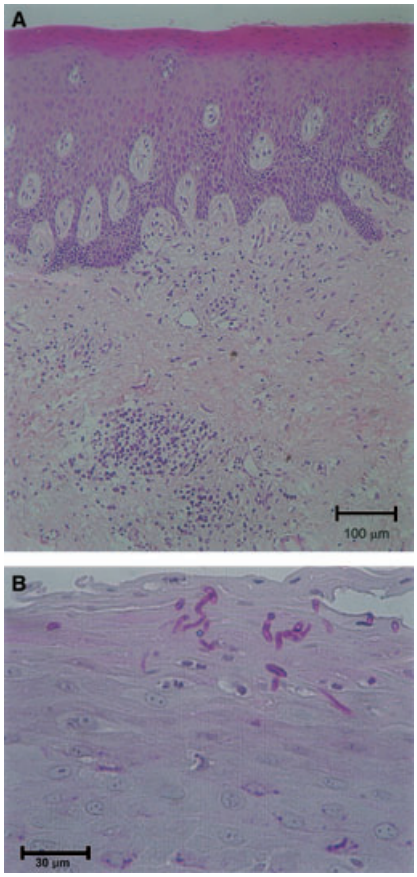

Fig. 1. A) Showing hyperplasia of the epithelium and inflammatory cells in the connective tissue. Stained by hematoxylin-eosin for the morphologic evaluation of the lesion. Magnification, ×100. B) An inflammatory hyperplasia slice with *Candida* sp. hyphae stained by PAS. Magnification, ×400.

Quantitative and qualitative surveys

For the cell count, three microscopic fields were captured (400×) with a mono color JVC® video camera, model TK-C 620 (360 × 480 pixel resolution) (Yokohama, Japan). The first field chosen was in the region of lymphocyte concentration (28), and the other two fields were selected from left to right, resulting in three contiguous, but not superimposed, microscopic fields (19).

The positivity criteria for the monoclonal antibody marking were a plasmatic membrane fully stained brown (16), an evident nucleus (14) and lymphocyte morphology (Fig. 1).

The cell count was carried out with the IMAGETOOL 3.0® program (San Antonio, USA). The count was performed in slides identified by nonsequential numbers so that the analyses were blind. For the assessment of reproductivity, one of the 10 studied fields was re-counted and the researcher was calibrated by Student's *t*-test ($\alpha = 0.05$, $P = 0.6859$).

For the qualitative analysis the marked cells were assessed in an optic microscope (100×) and classified according to their distribution – focal (cells concentrated in one or more areas of the lesion) or diffused (cells evenly distributed throughout the lesion) – and their predominant location – within the epithelium, under the epithelium layer or deep in the lamina propria. The distribution and anatomic location of the positive marked cells in relation to the location of *Candida* sp. hyphae were also noted in the infected patients.

Statistical analysis

The results are shown as the ratio of positive cells to total lymphocytes counted in three individual fields in each slide. Data from the control and *Candida* sp. infected groups were compared using Student's *t*-test ($\alpha = 0.05$).

The data from the present study were normally distributed and, in accordance with the sample size, Student's *t*-test was considered an adequate statistical tool.

Results

Of the 144 cases stored at the Laboratory of Oral Pathology–UFRGS, 86 fulfilled the inclusion criteria for the present study. All selected cases were PAS stained for evidence of *Candida* sp. hyphae. The results of PAS staining confirmed the clinical diagnosis in 67 (everyone with a previous clinical diagnosis of erythematous candidosis) of the 86 cases positive for *Candida* sp. infection, the remaining 19 cases being negative (no previous clinical diagnosis of erythematous candidosis). For the quantitative analysis using

Table 1. Monoclonal antibodies employed and their characteristics

Antibody	Anti CD 4	Anti CD 8
Clone	1F6	C8/144-B
Source	Novocasta Laboratories®UK	Dako®, Denmark
Working dilution	1 : 25	1 : 25
Pre-treatment	Microwave	Microwave
Recovery substance	Target retrieval solution – High pH (Dako®, Denmark)	EDTA pH 8

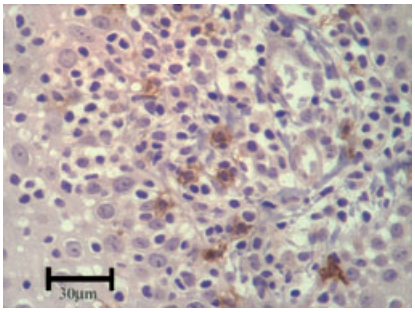

Fig. 2. $CD8^+$ T-cell distribution in the connective tissue of a *Candida* sp. infected region. The tissue sections were labeled with a monoclonal antibody anti-CD8. Magnification, ×400.

the immunohistochemical technique, 22 cases were randomly selected from the infected group and the 19 noninfected samples were used as controls.

Analysis of the data showed no significant statistical differences in $CD4^+$ T-cell count and $CD4^+$/total lymphocytes ratio between the control and infected groups (Table 2). A qualitative analysis showed that the $CD4^+$ T cells showed a preferential focal distribution and location on the subepithelial layer in both control and infected groups. None of the samples showed a pattern suggesting infiltration of $CD4^+$ T cells in the epithelium.

The ratio of $CD8^+$ to total lymphocytes was higher in the *Candida* sp. infected group than in the control group (Table 3). The qualitative analysis showed that $CD8^+$ T cells were preferentially distributed in a focused pattern and that these cells were anatomically positioned preferentially on the subepithelial layer. In seven of the 22 cases in the infected group, $CD8^+$ T cells had penetrated the epithelium, whereas in the control group this cell distribution pattern was absent. The area where $CD8^+$ T cells penetrated the epithelium in the infected group always coincided with the areas of *Candida* sp. infection.

Discussion

Although oral candidosis is an opportunistic fungal infection that commonly affects immunocompromised patients, it is also observed in a variety of other situations. In a previous study we reported an association between candidosis and inflammatory hyperplasia (2). Candidosis is also a common feature in individuals using complete dentures (2, 8, 12, 24) independently of immunosuppression. In the present study, *Candida* sp. was observed in 67 of 86 cases of patients with inflammatory hyperplasia, a prevalence of 79.9%, which is quite similar to our previous results (85%) in a different sample of individuals with hyperplasia associated with denture use (2). Another study also confirmed the strong presence of this microorganism in inflammatory fibrous hyperplasias, although a causative effect of *Candida* infection in those lesions was not demonstrated (27).

The role of T lymphocytes in the defense against candidosis is still controversial. Nevertheless, several studies have shown that these cells accumulate near the areas where *Candida* sp. hyphae are present (7, 26, 28). It was also suggested that the mucosal defense against *Candida* infection involves a cell-mediated reaction in which there is recruitment of macrophages and local production of immunoglobulin with a prominent IgA component (15). Several studies point to the importance of $CD4^+$ T lymphocytes in the response against *Candida* infection. An increased number of activated $CD4^+$ cells and protection against *Candida albicans* were found in a rat model of vaginal infection (7). The fate of cells after an adoptive transfer of vaginal total or purified T-lymphocyte populations was followed in this same model (26). A model was also described for oral candidosis in T-cell-deficient mice via reconstitution with naïve $CD4^+$ cells (9).

Nevertheless, there are also data suggesting that $CD4^+$ T cells are not the only important effector cells in the response against *Candida* infections. For instance, in HIV-1 infected patients suffering from opportunistic *Candida* infection, although a selective defect was postulated to occur, no correlation with viral load, CD4 or CD8 T-cell number or clinical stage was found (20). Also, asymptomatic candidal carriage and relative density were found to be significantly higher in the oral cavity of HIV-positive subjects, but they were not associated with $CD4^+$ counts or the quantity of HIV-1 RNA (4). In the present study, we did not observe any anatomic relationship of the presence, frequency and numbers of $CD4^+$ T cells and areas infected by *Candida* sp. hyphae. Nonetheless, data on the involvement of T lymphocytes in the response to *Candida* is derived from a variety of organisms (e.g. rat, mice, and immunocompromised humans) as well as from a variety of experimental models (systemic, vaginal and oral candidosis). It is worth noting that the genetic background of the animal model was shown to interfere with the type of immune response elicited by *Candida* infection (1, 5). This would suggest that $CD4^+$ T cells are not directly associated with *Candida* hyphae but can lead to humoral responses and activation of macrophages and granulocytes that act as important effector cells in the response to *Candida*, at least in immunocompromised patients (15) and in a rat model of vaginal candidosis (7). We suggest that $CD4^+$ T cells do not play a leading role in the response against *Candida* infection in immunocompetent individuals with inflammatory hyperplasia.

Concerning a possible role of $CD8^+$ T cells in the immune response to *Candida*, a study by Santoni et al. (26) suggests a major role for $CD4^+$ T cells in the response against vaginal candidosis in rats, although $CD8^+$ T cells were also considered to be involved in the clearance of the fungus from the vagina. This group had previously shown, in this same model, a progressively increasing number of $CD8^+$ T cells after the first experimental infection, which decreased during the subsequent infections (7). Along the same lines,

Table 2. Comparison of means and standard deviation between groups for $CD4^+$ cells

Group	$CD4^+$ cells	Total lymphocytes	Ratio $CD4^+$ cells/total lymphocytes
Candida infection	56.95 ± 9.41	136.18 ± 22.46	0.4176 ± 0.0077
Control	60.48 ± 10.13	142.46 ± 24.89	0.4168 ± 0.0065
P-value	0.2503	0.08432	0.3644

Statistical analysis was done using Student's *t*-test ($\alpha = 0.05$).

Table 3. Comparison of means and standard deviation between groups for $CD8^+$ cells

Group	$CD8^+$ cells	Total lymphocytes	Ratio $CD8^+$ cells/total lymphocytes
Infection	29.09 ± 12.66	96.40 ± 43.16	0.3034 ± 0.0085
Control	20.52 ± 9.07	82.73 ± 38.91	0.2514 ± 0.01
P-value	0.0016*	0.2928	< 0.01*

*Statistically significant differences. Statistical analysis was done using Student's *t*-test ($\alpha = 0.05$).

a study with mice with systemic candidosis showed that both $CD4^+$ and $CD8^+$ T lymphocytes contribute to the reduction of tissue damage (1). There is also evidence that $CD8^+$ T lymphocytes inhibit the growth of *C. albicans* hyphae *in vitro* (3). During experimental vaginal candidosis in a murine model, the number of $CD3^+$ T cells (most of them also $CD8^+$) dramatically increased following *C. albicans* administration, suggesting that in this model $CD8^+$ T cells play a protective role (13). Activated $CD8^+$ T cells are involved in the elimination of *C. albicans,* as observed in sections from mice livers with systemic infection after experimental intravenous inoculation with the fungus (17). It was also suggested that $CD8^+$ T cells might be important for the oral host defense against oropharyngeal candidosis, especially when $CD4^+$ T-cell numbers are reduced, as in HIV-positive individuals (18).

In the present study, we observed an increase in $CD8^+$ T-cell count in infected compared with the control cases, and an anatomic relationship between the distribution of these cells and *Candida* sp. hyphae. It is important to point out that, in several cases, these cells were close to yeast (*Candida* sp.) in the epithelium and that the $CD8^+$ T cells penetrating the epithelium were only observed in the infected group, suggesting the development of a local cellular immune response mediated by $CD8^+$ T cells. Considering the high $CD8^+$ T cell count and the higher $CD8^+$/total lymphocytes ratio in *Candida* sp. infected samples, together with the coinciding anatomic distribution of these cells and *Candida* sp. hyphae, we suggest that $CD8^+$ T cells are involved in the response against *Candida* infection in immunocompetent individuals with inflammatory hyperplasia.

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