Immunocytochemical localization of cytokines and inducible nitric oxide synthase (iNOS) in oral mucosa and lymph nodes of patients with paracoccidioidomycosis

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Abstract

Paracoccidioidomycosis (PCM) is a deep mycosis caused by Paracoccidioides brasiliensis, with high incidence in Brazil. In order to examine the immune response in lesional tissue from patients with PCM, we analyzed cytokines as well as the phenotype of the cell infiltrate. Paraffin-embedded tissue from the oral mucosa of eight patients with the localized adult form (AF) of PCM and from the lymph nodes of 10 patients with the juvenile form (JF) of PCM was analyzed by immunohistochemistry to detect tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGF-β) and interleukin-10 (IL-10). Most of the inflammatory cells in the lymph nodes were CD68+ (macrophages, epithelioid and giant cells), while a mixed infiltrate with macrophages, plasma cells and neutrophils was detected in the oral mucosa. TNF-α as well as iNOS expression was similar in lymph nodes and oral mucosa, whereas TGF-β and IL-10 were observed in a larger number of macrophages, epithelioid and giant cells in the lymph nodes, where numerous yeast cells were visualized. The higher expression of anti-inflammatory cytokines (IL-10 and TGF-β) in lesions of patients with the JF of PCM (lymph nodes) may represent a mechanism by which the fungus evades the host immune response, contributing to a more severe and disseminated form of the disease.

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1. Introduction

Paracoccidioidomycosis (PCM) is a deep mycosis caused by Paracoccidioides brasiliensis, restricted to Latin America, with high incidence in Brazil [1].

According to the current classification, PCM may be considered a disease with two main clinical forms: acute or juvenile form (JF) and chronic or adult form (AF) [1]. The AF mainly affects adult males, who show a high frequency of pulmonary, skin, mucosal and visceral involvement. The JF is characterized by systemic lymph node involvement, hepatosplenomegaly and bone marrow dys-function. From an immunopathological point of view, the JF tends to show necrotizing lesions with abundant fungal cells, impairment of cell-mediated immunity and high titers of circulating antibodies. In the AF, the lesions are seldom disseminated and are associated with tuberculous granulomas and a small number of fungal cells [2].

Experimental and human histopathologic findings have shown that P. brasiliensis in contact with host tissue provoke a non-specific inflammatory response characterized by vascular congestion, edema and accumulation of polymorphonuclear cells. A few hours after inoculation, the number of polymorphonuclear cells decreases and the number of mononuclear cells increases, the first multinuclear giant cells appear thereafter, forming the granuloma [3]. Activation of reactive cells with P. brasiliensis antigens results in the release of cytokines which play a major role in regulating replication and hence control the overall fungal load [4–6].
In previous studies we showed a preferential Th2 type immune response in patients with the JF of PCM, characterized by IL-4, IL-10 and IL-5 production, elevated levels of anti-P. brasiliensis IgG4 and IgE antibodies and a marked eosinophilia [7,8]. These mediators associated with low interferon-γ (IFN-γ) and lymphocyte proliferation levels were correlated with a more severe manifestation of the disease. In contrast, healthy individuals who live in endemic areas and are positive to the paracoccidioidin skin test showed a typical Th1 pattern, with substantial levels of IFN-γ and IL-12 but low or no IL-4, IL-5 and IL-10. As a result, these individuals probably develop an efficient immune response able to prevent the onset of the disease. Intermediate immune responses were observed in AF patients, whose IFN-γ and IL-10 production did not differ from that observed in the JF group, although IL-4 and IL-5 levels were significantly lower [8].

Increased levels of IL-6, TNF-α, and IL-5 levels were significantly different from those observed in the JF group, although IL-4 production was positively to the paracoccidioidin skin test showed a typical Th1 pattern, with substantial levels of IFN-γ and IL-12 but low or no IL-4, IL-5 and IL-10. As a result, these individuals probably develop an efficient immune response able to prevent the onset of the disease. Intermediate immune responses were observed in AF patients, whose IFN-γ and IL-10 production did not differ from that observed in the JF group, although IL-4 and IL-5 levels were significantly lower [8].

Inflammatory cytokines play an important role in the genesis and control of PCM. Increased levels of IL-6, IL-1 and TNF-α as well as the chemokine MIP-1α were detected in sera from adult patients with the disseminated form of the disease [7,9,10].

TNF-α, a monocyte/macrophage-derived factor, is involved in the recruitment and activation of inflammatory cells and in granuloma formation [11]. IFN-γ activates macrophages to secrete TNF-α and to inhibit P. brasiliensis replication [5,12]. Inducible nitric oxide synthase (iNOS), responsible for nitric oxide (NO) synthesis, is induced during macrophage activation by IFN-γ and TNF-α [13]. In vitro studies have demonstrated a direct correlation between NO production and the microbial activity of macrophages in the presence of intracellular microorganisms such as Leishmania major, Mycobacterium bovis and Trypanosoma cruzi [14,15]. In murine models of PCM it was shown that NO is involved in the protective [16,17] as well as in the immunosuppressive response to the fungus [5,18,19]. Transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10) are anti-inflammatory cytokines that antagonize Th1-mediated activation of macrophages. In vitro studies with several cell types have indicated that TGF-β and IL-10 negatively control the expression of iNOS, responsible for the prolonged production of large amounts of NO [20,21].

In this study, we proposed to examine the pattern of cytokine (TNF-α, TGF-β and IL-10) and iNOS expression in lesions of patients with the JF (lymph nodes) and AF (oral mucosa) of PCM in order to clarify the dynamics of the immune response occurring at the site of inflammation.

2. Results

A granulomatous inflammatory reaction was observed in all specimens after routine staining (hematoxylin–eosin) and was confirmed with a panel of antibodies against lymphocytes, macrophages and neutrophils. The oral mucosa (OM) lesions comprised compact granulomas with epithelioid and giant cell aggregates surrounded by a lymphocytic halo. Some granulomas presented a purulent exudate in the central area. The number of yeast cells was lower in OM than in lymph nodes (Table 1). The yeast cells were intensely stained by an anti-TGF-β monoclonal antibody. This antibody reacted non-specifically with the fungal wall probably due to carbohydrate epitopes.

The granulomatous reaction was looser and more extensive and giant cells were more numerous in the lymph nodes than in OM. Some granulomas showed purulent foci. Large areas of necrosis were observed in association with numerous yeast cells. Approximately 80% of the lymph nodes from patients with the JF of PCM showed more than 50 yeast cells per field (Table 1).

No difference was observed among tissues concerning yeast cell viability, which was over 80% in most the biopsies examined (Table 2). Areas with a small number of shell-like empty cells (non-viable) were observed in lymph nodes and OM.

CD4+ and CD8+ T cells were observed in a localized pattern related to granulomas as well as in a diffuse arrangement. The number of CD4+ T cells was higher in OM than in lymph nodes, whereas CD8+ T cells were observed in similar amounts in all specimens examined (JF and AF of PCM) (Fig. 1).

Neutrophils (CD15+) were found around or forming micro-abscesses in the central areas of the granuloma and there was no significant difference among the tissues examined (Fig. 1).

The number of macrophages, epithelioid and giant cells showing strong positive staining by anti-C668 was significantly higher in lymph nodes than in the OM (Fig. 1). In lymph nodes, CD68+ macrophage represented most of the cells (80–100%) of the inflammatory infiltrate, whereas a more polymorphic cell population

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**Table 1**

<table>
<thead>
<tr>
<th>Percentage of Biopsies Presenting 1–25 Yeast Cells/Field</th>
<th>26–50 Yeast Cells/Field</th>
<th>&gt;50 Yeast Cells/Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>1 (10%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Oral mucosa</td>
<td>2 (25%)</td>
<td>4 (50%)</td>
</tr>
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</table>

**Table 2**

| Number of Biopsies Showing 40, 50, 60, or 80% Viable P. brasiliensis Yeast Cells/Field |
|---------------------------------------------|-------------------------|----------------------|
| Lymph node                                  | 1 (10%)                 | 1 (10%)              | 2 (20%)              | 6 (60%)              |
| Oral mucosa                                 | 1 (12.5%)               | 2 (25%)              | 5 (62.5%)             |
was observed in OM, in which macrophages (CD68+) represented 30–50% of the inflammatory cells.

Antibodies to cytokines produced a diffuse staining of the extracellular matrix, although it was possible to discern alterations in the cell distribution of the cytokines analyzed. The intracellular cytokine-staining pattern was predominantly cytoplasmic.

TNF-α reactivity was detected in sparse macrophages, neutrophils and giant cells. A higher number of positive cells was observed in lymph nodes than in OM, although not statistically significant (Figs. 2 and 3).

To confirm the production of NO, we examined iNOS expression in lymph nodes and OM of patients with PCM. A small number of iNOS-positive cells,
identified as macrophages, were detected in the inflammatory sites (Figs. 2 and 4A, B). CD4+ and CD8+ T cells around the granulomas did not express iNOS. In lymph nodes, iNOS was expressed in sparse macrophages and also in giant cells. An interesting difference was consistently observed in relation to the localization of the iNOS-positive cells. In the OM, iNOS expression was restricted to few macrophages around the

Fig. 4. (A) Oral mucosa—There is a small number of iNOS-positive macrophages around the granuloma (streptavidin–biotin-AP method, ×330). (B) Lymph node—Multinucleated giant iNOS-positive cell with fungi in its cytoplasm (streptavidin–biotin-AP method, ×1250).
granuloma (Fig. 4A), while in lymph nodes a positive staining was observed in giant cells, as well as in diffuse macrophages close to the yeast cells (Fig. 4B).

Macrophages, neutrophils, epithelioid and giant cells were positive for TGF-β and the number of TGF-β-positive cells was significantly higher in lymph nodes than in OM (Figs. 2 and 5).

IL-10 expression was observed in epithelioid and giant cells of the granulomas, and in lymphocytes. The number of IL-10-positive cells was markedly higher in lymph nodes as compared with OM (Figs. 2 and 6). Some neutrophils in the OM also stained positive for IL-10.

No specific staining was observed when isotype controls were used in place of primary antibodies.

3. Discussion

The granulomatous inflammatory response in PCM represents a specific immune tissue response of the host to the fungus in an attempt to destroy, block, and circumscribe the parasite and to prevent its multiplication. Numerous yeast cells inside macrophages and giant cells expressing TNF-α characterized the inflammatory response present in lymph nodes of patients with the JF of PCM. In the OM, the number of TNF-α-positive cells was smaller than in lymph nodes, consistent with the low number of *P. brasiliensis* cells in the inflamed area. Entry of monocytes, neutrophils and lymphocytes from the systemic circulation into the tissues is facilitated by TNF-α and other inflammatory cytokines, which induce expression of adhesion molecules, such as ICAM-1, on the surface of endothelial cells [22]. TNF-α also stimulates endothelial cells and macrophages to secrete chemokines that induce leukocyte chemotaxis and recruitment of inflammatory cells [22].

TNF-α is required for granuloma formation [11] and together with IFN-γ activates macrophages to kill *P. brasiliensis* [12]. The microbicidal activity of macrophages is related to the production of reactive oxygen and nitrogen intermediates, with NO being one of the most potent cytotoxic agents [13]. Since NO synthesis is induced by pro-inflammatory cytokines such as TNF-α, we analyzed iNOS expression in lesional tissues from PCM patients. In lymph node infiltrates we observed a small number of macrophages and giant cells expressing iNOS, some of them close to *P. brasiliensis* yeast cells. We expected to find an elevated expression of iNOS in lymph nodes in response to the presence of considerable amounts of TNF-α and numerous yeast cells. iNOS immunoreactivity was also detected to a smaller extent in macrophages around granulomas in OM.

The role of NO in *P. brasiliensis* infection has not been clearly defined. Gonzales et al. [16] showed that

![Fig. 5. Lymph node—TGF-β expressed in numerous macrophages around the granuloma (streptavidin–biotin-AP method, ×330).](image)
cytokine-induced production of NO by macrophages inhibited the transformation from conidium to yeast, a crucial step for the establishment of the infection. The expression of iNOS by macrophages within granulomas is consistent with a role for NO in fungistasis/fungal killing. NO may be protective, acting as an effector molecule of macrophage cytotoxicity and as a regulator of inflammatory responses, but probably it has a secondary effect causing immunosuppression [17,19]. In a recent paper Nascimento et al. [18] demonstrated that NO is essential for resistance since iNOS-deficient mice were extremely susceptible to *P. brasiliensis* infection. Additionally, it was shown that high and persistent NO production is associated with susceptibility to *P. brasiliensis* infection [18].

Inhibitory cytokines are important mediators controlling the inflammatory response. In order to address the role of these cytokines we analyzed TGF-β and IL-10 expression by the infiltrating cells. High levels of TGF-β and IL-10 were detected in lymph nodes from patients with the JF of PCM, probably promoting a local anti-inflammatory response. IL-10 is reported to inhibit cell-mediated Th1 responses mostly by down-regulating the expression of major histocompatibility complex class II and B7-1 (CD80) in antigen-presenting macrophages [21]. The resistance of mice to infection with various microorganisms is also impaired by the administration of recombinant IL-10 [21]. In previous studies from our group we showed that resistance to *P. brasiliensis* observed in individuals living in endemic areas for PCM is associated with a Th1 immune response and little or no IL-10 production [8]. In the present study, the detection of large amounts of IL-10 and TGF-β in the inflammatory lesions of patients with the JF of PCM is in agreement with the severe and disseminated outcome observed in this form of the disease. TGF-β has been shown to exert a profound negative regulatory effect on iNOS expression in murine macrophages [20]. Therefore, it is possible that the low expression of iNOS in PCM lesions may represent a parasite-induced evasive mechanism allowing survival inside the host tissues.

We have demonstrated that TNF-α, TGF-β and IL-10 are expressed simultaneously during the inflammatory response in both lymph nodes (JF) and OM (AF). Coordinate expression of cytokines that up-regulate or down-regulate NO production may provide the means of protecting host tissue from the damaging effects of NO.

In conclusion, our findings suggest that high expression of anti-inflammatory cytokines (IL-10 and TGF-β) in lesions of patients with the JF of PCM (lymph nodes) may represent a mechanism by which the fungus evades
the host immune response, contributing to a more severe and disseminated form of the disease.

4. Materials and methods

4.1. Patients

Biopsy specimens were taken from 18 patients with paracoccidioidomycosis cared for at the Clinical Hospital of the State University of Campinas, São Paulo, Brazil. Due to different tissue localization of the lesions in different clinical forms of the disease, OM biopsies were obtained from patients with the adult localized form ($n = 8$, five males and three females ranging in age from 40 to 70 years) and lymph node biopsies were obtained from patients with the juvenile form ($n = 10$, six males and four females ranging in age from 6 to 30 years) of PCM. The biopsies were obtained before the beginning of treatment for diagnostic purposes.

4.2. Light microscopy

Biopsies were fixed in 10% formalin and embedded in paraffin and histological sections were stained with hematoxylin–eosin (HE). The magnitude of the inflammatory infiltrate, the number of fungi and the percentage of viable fungi were analyzed in all cases. The fungus was considered non-viable when it had the appearance of a shell-like empty cell.

4.3. Immunohistochemistry

Paraffin-embedded sections were submitted to immunohistochemical staining by the streptavidin–biotin technique. Briefly, the sections were deparaffinized, rehydrated in a growing alcohol series, placed in Tris-buffer (TBS) and then submitted to antigen retrieval by boiling at 95°C in citrate buffer, pH 6.0, for 30 min, except for those incubated with the primary anti-CD4 antibody, which were additionally submitted to digestion with proteinase K (1.5 mg/ml, Sigma Chemical Co, St. Louis, MO, USA) for 15 min at 37°C, according to manufacturer’s instructions. The slides were incubated with the avidin–biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA). Non-specific protein binding was blocked by incubating the material with horse serum (Vector) or by serum-free protein block (Dako Corporation, Carpinteria, CA, USA) according to the secondary antibody for 30 min. After the slides were incubated with the primary anti-CD4 (NeoMarkers, Inc., Fremont, CA, USA), anti-CD8 (Dako), anti-CD15 (Dako), and anti-CD68 (Dako), anti-TGF-β1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), anti-iNOS (BD Transduction Laboratories, USA), anti-TNF-α (Serotec, Oxford, UK) and anti-IL-10 (Santa Cruz) monoclonal and polyclonal antibodies for 1 h at 37°C and overnight at 4°C in a humidifying chamber. The next step was an intermediate incubation with biotinylated anti-mouse IgG H+L (Vector) or anti-rabbit IgG H+L (Vector) for 45 min at 37°C in a humidifying chamber, and subsequently with the streptavidin complex (StreptABComplex/AP-Dako), for 30 min at 37°C in a humidifying chamber. Finally, the slides were incubated with a freshly prepared substrate consisting of naphthol AS-MX-Phosphate (Sigma) and fast red dye (Sigma). The slides were counterstained with Mayer’s haemalaun (Merck, Darmstadt, Germany) and covered with glycerin–gelatin (Merck). For control preparations, the primary antibodies were replaced with mouse normal serum or rabbit immunoglobulin fraction (Dako).

4.4. Analysis of positive cells

Serial tissue sections were used and each of the eight different antibodies was applied to a different section from the same block, yielding eight stained sections per case. The analysis of positive cells was semiquantitative. The stained cells were expressed as percentage of all inflammatory cells seen for each stained section. Data were presented as mean ± SEM. Group data were compared by the unpaired two-tailed $t$ test. $p < 0.05$ was considered significant.

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References


