Patients With Active Infection With
Paracoccidioides Brasiliensis Present a Th2
Immune Response Characterized by High
Interleukin-4 and Interleukin-5 Production

Luane Marques Mello, Mario Leon Silva-Vergara, and
Virmondes Rodrigues Junior

ABSTRACT: Paracoccidioidomycosis (PCM) is caused by
the dimorphic fungus Paracoccidioides brasiliensis. In hu-
mans, the disease presents a broad spectrum of clinical
manifestations, ranging from localized mucocutaneous le-
sions to a widespread manifestation involving the mononuclear phagocyte system. In attempt to better un-
derstand the regulation of immune response during the
infection, this study analyzed the production of regulatory
and inflammatory cytokines in 25 infected patients and
19 health controls. Regulatory and inflammatory cyto-
kines were analyzed in supernatants of peripheral blood
mononuclear cells (PBMC) stimulated with mitogens or
soluble P. brasiliensis antigens. A pattern of Th2 immune
response was observed in patients, mainly attributed to a
higher production of IL-4 and IL-5 than to a lower pro-
duction of IFN-γ. Patients with disseminated infection
presented undetectable levels of IFN-γ after antigen stim-
ulation and high levels of IL-1, which were probably
associated with the inflammatory reaction observed in
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KEYWORDS: paracoccidioidomycosis; cytokines; T
helper cells; mycosis

INTRODUCTION
Paracoccidioidomycosis (PCM) is the most prevalent sys-
temic mycosis in Latin America, being endemic in Bra-
zil, Argentina, Venezuela, and Colombia [1]. It is caused
by the dimorphic fungus Paracoccidioides brasiliensis [2].
In humans, the disease presents a broad spectrum of clinical
manifestation, ranging from localized mucocuta-
neous lesions to a widespread manifestation involving the
mononuclear phagocyte system [3]. The disease predom-
inantly affects agricultural workers [1, 4] and is found
more frequently in males due to the protective effect of
estrogens in females after puberty [5]. The outcome of
infection, as demonstrated in animal models, depends on
the virulence of the fungus, the route of inoculation, and
the resistance of the host [6]. Host resistance to P.
brasiliensis is related to the ability to develop a cell
immune response characterized by a positive skin test to
paracoccidioidin and formation of a compact epithelioid
granuloma. On the other hand, patients with dissemi-
nated infection present a negative skin test to paracoc-
cidioidin, a poorly defined granulomatous reactions with
large numbers of fungi and high titers of antifungal
antibodies [7]. These properties are closely related to the
outcome of infection, are utilized for clinical follow-up of
patients under treatment, and as criteria of cured disease.
Cytokines are pleotropic peptides that regulate many
aspects of the immune response and inflammatory reac-
tions [8, 9]. T-helper cells are the major source of reg-
ulatory cytokines and can be divided into at least two
distinct polar subpopulations according to their cytokine
pattern [10]. In humans, Th1 cells produce IFN-γ,
TNF-β and TNF-α, whereas Th2 cells produce IL-4,
IL-5, and IL-13. Due to their strong influence on the
immune effector mechanism, preferential activation of
one population or another is closely related to resistance/
susceptibility to infectious disease. Th1 activation is
related to resistance to intracellular pathogens and fungi, such as Mycobacteria, Leishmania, Trypanosome, and Histoplasma, whereas Th2 responses are associated with resistance to worm infections such as schistosomiasis [11, 12]. The dichotomy of T-helper cell subsets offers an attractive model to explain the pattern of immune response observed during PCM infection and may allow us to address the failure of the immune response to control fungal growth and spread in internal organs. Thus, the present investigation studied the cellular immune response of patients with active chronic infection. Regulatory and inflammatory cytokines were analyzed in the supernatants of peripheral blood mononuclear cells (PBMC) stimulated with mitogens or soluble P. brasiliensis antigens. A pattern of Th2 immune response was observed in the patients, mainly attributed to a high production of IL-4 and IL-5 than to a lower production of IFN-γ. Patients also presented high IL-1β levels, which was probably associated with the inflammatory reaction observed during disseminated infection.

MATERIAL AND METHODS

Subjects

Patients from the teaching hospital of “Faculdade de Medicina do Triângulo Mineiro,” with a diagnosis of paracoccidioidomycosis, were invited to participate in this study. Twenty-five patients and 19 control subjects, matched for age and gender, were analyzed. In patients with chronic disease, severity was classified as mild (unifocal infection or without extensive pulmonary involvement) or severe (with pulmonary and lymph node, or mucocutaneous commitment) [13]. Blood was collected by venipuncture into a 20-ml heparinized syringe (5 ml was collected without an additive for serum separation). Serum samples were stored at −70°C until analysis and heparinized blood was used for PBMC purification. This study was approved by the Ethical Committee of the school.

Cell Culture

PBMC were isolated from heparinized blood by centrifugation on Ficoll-Paque (400 × g, 20 minutes at RT), washed three times in RPMI medium, and then resuspended in RPMI (Gibco, Grand Island, NY, USA) supplemented with 50-mM 2-ME, 2-mM L-glutamine, 40-μg/ml gentamicin, and 10% fetal calf serum (complete medium). For supernatant production, 2 × 10^6 cells per well per milliliter were cultured in a 24-well microplate in presence of medium alone, 5-μg/ml PHA, 5-μg/ml lipopolysaccharide (LPS), or 5μg/ml cell-free antigens. Plates were incubated at 37°C in a 5% CO₂ atmosphere. At 48 and 120 hours supernatants were collected, cen-

trifuged, and then stored at −70°C until analysis for cytokine production.

Cytokine Titration

For cytokine titration, microplates (Nunc, Roskilde, Denmark) were sensitized overnight with anti-IL-1β mAb (Genzyme, Cambridge, MA, USA), anti TNF-α (Genzyme), anti-IL-4 (Mabtech, Nacka, Sweden), anti-IL-5 mAb (Pharmingen, San Diego, CA, USA), anti IL-10 mAb (Pharmingen), or anti-IFN-γ mAb (Mabtech) overnight. Nonspecific binding was prevented by incubating the plates with 3% BSA (Sigma, St. Louis, MO, USA) in PBS. Plates were incubated overnight with 100 μl of 1:2 dilution of serum or culture supernatants in PBS, 2% BSA and standard cytokines (Pharmingen and R&D [Minneapolis, MN, USA]). Plates were then washed four times with PBS and 0.05% Tween and incubated with rabbit anti-IL-1β Ab (Genzyme), rabbit anti-TNF-α Ab (Genzyme), biotinylated anti-IL-4 mAb (Mabtech), biotinylated anti-IL-5 mAb (Pharmingen), biotinylated anti-IL-10 mAb (Pharmingen), or biotinylated anti-IFN-γ mAb (Mabtec) for 4 h. Plates were then washed and incubated for 2 h with alkaline phosphatase conjugated goat anti-rabbit IgG (Immunootech, Marseille, France) or phosphatase alkaline conjugated to streptavidin. Finally, plates were washed four times and enzymatic activity was developed by incubating the plates with p-nitrophenyl phosphate (Sigma). Absorbance was read at 405 nm in a microplate reader apparatus (Biostar, Hercules, CA, USA). The sensitivity of the tests was 10 pg/ml to 30 pg/ml.

RESULTS

Cytokine Levels in Serum From Patients and Healthy Controls

Serum cytokine levels were assessed by ELISA, using antibody pairs for each cytokine. The IL-1β levels were significantly higher in infected patients (799 pg/ml) than in healthy controls (178 pg/ml). IL-10 and IFN-γ levels were of 371 pg/ml and 296 pg/ml in patients, and 36 pg/ml and 59 pg/ml in healthy controls. IL4, IL-5, and TNF-α levels did not differ significantly between groups (Figure 1).

PBMC from Infected Patients Present a Th2 Response

PBMC derived from patients and from healthy controls were stimulated with PHA or LPS for 48 and 120 hours, and the supernatants were tested for the presence of regulatory and inflammatory cytokines. After PHA stimulation, IL-4 was not detected in the supernatants from healthy controls and was present in maximum amounts of 402 pg/ml (median: 67 pg/ml) in the supernatants
from infected subjects. IL-5 levels were markedly elevated in infected subject, with a median value of 1025 pg/ml as opposed to 107 pg/ml for control individuals. Th2 bias was due to an overproduction of Th2 cytokines, because IFN-γ levels did not differ significantly between groups (Figure 2 [top]). The results obtained after LPS stimulation confirmed the observation made on serum. IL-1 reached higher levels in patients (median: 4100 pg/ml) compared with controls (median: 360 pg/ml). TNF-α levels did not differ significantly between groups (Figure 2 [bottom]).

**DISCUSSION**

The present results provide clearly evidence for a Th2 imbalance in patients infected with *P. brasiliensis*. A Th2 bias was observed when PBMC supernatants from infected patients and healthy controls were stimulated with the mitogen PHA, and in this situation, the Th2 response was due to an overproduction of IL-4 and IL-5 rather than to a lower production of IFN-γ. Comparison of antigen-stimulated PBMC supernatants revealed that when patients were grouped according to the extension of lesions, those who were able to better control their lesions, presented higher IFN-γ levels than those with more severe infection. Various reports have described that Th1 or Th2 lymphocytes play a central role in immunity to infectious pathogens, and some reports correlate Th1 response with protection against intracellular pathogens [14, 15] and fungi [16–18], although other reports point to a protective role of the Th2 response mainly against worms [19, 20]. A Th1/Th2 balance seems to be crucial in protecting against infectious agents, and it can be obtained by modulation of cytokines from both subsets. In the present study, the Th2 balance was attributed to an overproduction of IL-4 and IL-5 without commitment of IFN-γ production. This kind of favorable Th2 balance was already observed at T-cell clone level in Schistosomiasis patients [20]. Another interesting observation presented here was cytokine production after antigen stimulation. Among infected

![Cytokine Levels in Serum](image)

**FIGURE 1**  Serum Levels of cytokines. Levels of cytokines were determined in serum samples obtained from patients (hatched bars) and controls (gray bars). Results are expressed in pg/ml and bars represent the average of cytokines levels and vertical line the standard error. *Statistical significance between groups is indicated *p* < 0.05 (Mann-Whitney).
patients, IFN-γ levels were higher in those with unifocal lesion, which represent patients able to better control *P. brasiliensis* infection. This suggests that although these patients present a strong response in terms of IL-4 and IL-5 production, they concomitantly developed a Th1 response that was able to limit fungal dissemination, or that the balance of T-helper subset tended to Th0 until dissemination. Conversely, patients with disseminated infection produced more IL-4 and IL-5 after stimulation with *P. brasiliensis* antigens than those with unifocal infection. This result confirms our knowledge of *P. brasiliensis* infection and other fungal pathologies, that result in elevated specific antibody levels during active infection and reduced antibody titers after successful treatment. Furthermore, the high IL-1 levels observed in serum and in PBMC supernatants after LPS and antigen stimulation can explain the clinical features observed, such as loss of weight, fatigue, fever and malnutrition. The marked production of IL-1 may also be associated with the extension of lesions, because patients with disseminated infection presented high levels of this cytokine in the supernatants. Taken together, the present results support the idea that *P. brasiliensis* infection is associated with a Th2 response and that dissemination of infection is related to a lower ability of specific T lymphocytes to...
produce IFN-γ. The production of IL-1 is also associated with the extension of the lesions, and it may be speculated that high levels of IL-1 are a consequence of strong stimulation of mononuclear phagocytes due to a large number of fungi present in patients who were unable to control dissemination.

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