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Differential correlation between interleukin patterns in disseminated and chronic human paracoccidioidomycosis

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SUMMARY

In an attempt to understand better the immunoregulatory disorders in paracoccidioidomycosis (PCM), the possible correlation between interleukin pattern, lymphoproliferation, C-reactive protein (CRP) and specific antibody levels was investigated in the polarized clinical forms of this disease. We studied 16 PCM patients, eight with the disseminated disease (four under treatment and four non-treated) and eight with the chronic disease. The patients with disseminated disease exhibited high antibody titres specific to *Paracoccididoides brasiliensis* antigen compared with patients with the chronic form of disease. Tumour necrosis factor (TNF), IL-1, IL-6 and CRP in the serum of non-treated disseminated PCM patients were increased, which correlated positively with the low mitogenic response of peripheral blood mononuclear cells (PBMC) to phytohaemagglutinin (PHA) ($P < 0{\cdot}01$) and with the high antibody titres ($P < 0{\cdot}001$) of these patients. Moreover, we found in the disseminated PCM patients positive correlations between IL-1 and IL-6 ($P = 0{\cdot}0007$); IL-1 and TNF ($P = 0{\cdot}0045$); IL-1 and IL-6 with the high antibody titres ($P = 0{\cdot}0834$ and $P = 0{\cdot}0631$, respectively); IL-1, IL-6 and TNF with CRP levels. By contrast, no correlations were found with those interleukins in the treated disseminated and chronic patients or in controls. It was interesting to find an inverse correlation between IL-4 and antibody production in non-treated disseminated PCM ($r = -0{\cdot}4770$); moreover, a significant correlation ($P = 0{\cdot}0820$) was found in chronic PCM patients with respect to the low level of either IL-4 and antibody titres against fungus antigen. Chronic PCM patients also had IL-2 levels inversely correlated with antibody production ($r = -0{\cdot}6313$; $P = 0{\cdot}0628$). Inverse correlations were also observed between IL-2 and IL-6 levels in non-treated disseminated patients ($P = 0{\cdot}0501$) and between IL-2 and IL-4 in chronic patients ($P = 0{\cdot}0131$). The inflammatory cytokines might have a pivotal role in the genesis and in control of some aspects of the disease, such as granulomatous reaction, hypergammaglobulinaemia and depression of T cell-mediated immunity in PCM.

Keywords paracoccidioidomycosis *Paracoccidioides brasiliensis* interleukins inflammation C-reactive protein

INTRODUCTION

Paracoccidioidomycosis (PCM) is a chronic, progressive fungal infection characterized by granulomatous and suppurative inflammatory reactions and by a depressed cellular but enhanced humoral immune responses [1]. The disease is caused by the imperfect dimorphic fungus *Paracoccidioides brasiliensis*, and has been detected in humid tropical and subtropical regions of continental Latin America. PCM clinical forms range from benign self-limited infection to a severe, progressive and sometimes fatal disease, involving pulmonary and extrapulmonary tissues [2]. Thus, PCM presents two polar clinical forms, namely the hyperegic pole, characterized by localized infection and persistent cellular immune response with positive intradermal tests to paracoccidioidin, compact epithelioid granuloma, and absence or low titres of specific antibodies; and the anergic pole, represented by disseminated infection, decreased cellular immunity with negative test for paracoccidioidin, loose granulomatous inflammation containing high numbers of fungi and hyperactive humoral immunity [3–6].

Several studies have shown that cellular immunity plays an important role in host defence against human PCM [4,7]. One of the key functional parameters determining the outcome of immune responses to infectious agents is the nature of the

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cytokines produced by immune cells, since the patterns of cytokine production have been described for some infectious diseases in man [8–10].

PCM offers an attractive model to investigate the role of cytokines in the face of infection, once it has been observed that disturbances in the immuneregulatory pathways might be involved in the elevation of circulating antibody titre, acute phase proteins such as C-reactive protein (CRP) and depression of cellular immunity, which could be correlated with the intensity of the lesions and with the severity of disease (disseminated PCM). In contrast, a vigorous cellular immune response without elevated specific antibody titres is correlated with restricted disease and a better prognosis (chronic patients) [7]. Here, we examined the pattern of lymphoproliferation of peripheral blood mononuclear cells (PBMC), interleukins, CRP and specific antibody levels present in the serum from 16 patients exhibiting polarized reactivity of PCM, in an attempt at a better understanding of the mechanisms by which interleukins could be correlated with the immunological disturbances presented by PCM patients.

PATIENTS AND METHODS

Paracoccidioides brasiliensis *antigens*

The fungal antigen was obtained from *Paracoccidioides brasiliensis* yeast cells, strain 18, cultivated during 10 days. The yeasts were recovered from FavaNetto's media and resuspended in sterile PBS solution pH 7·2. The suspension was highly disrupted under ultrasonic vibration (16 pulses of 3 min each) and centrifuged at 10 000 ***g*** at 4°C for 30 min. The supernatant was recovered, filtrated at 0·45 and 0·22 μm, respectively, and used as pooled antigen in the assays.

Patients and controls

Twenty-four individuals (16 PCM patients and eight controls) from the peripheral region of Ribeirão Preto (São Paulo, Brazil), were selected and matched according to the criteria of the PCM classification as proposed elsewhere [4,5,11]. From those individuals, Group I was composed of eight males aged 25–40 years, positive for antigen-specific *P. brasiliensis* radial immunodifusion assays with titres higher than 1/16. Group I was further divided into four patients with disseminated disease without any therapeutic treatment, and four patients receiving sulphadiazine (6 g/day); for one of these patients, who exhibited the ganglionic-visceral and disseminated form of PCM, it was necessary to include other drugs such as amphotericin B and ketaconazole to improve the therapeutic response (Table 1). Group II was composed of eight males aged 30–55 years, all of them using anti-fungal therapy, of which 86% presented with irregularity of treatment before evaluation, with a history of reactivation of the infection, but at the time of study 90% of patients had been using sulphadiazine and exhibited chronic PCM, evaluated by immunodiffusion assays with antibody titres <1/16. Group III was composed of 10 healthy males aged 22–26 years, exhibiting no reactivity with *P. brasiliensis*-specific antigen in immunodifusion assays. All patients and controls were examined at the time of blood collection. Individual sera were kept at −70°C until used for assays.

CRP quantification in patients and controls

CRP concentration in patients and controls sera was measured by the nephelometric method using an ICS Analyser II (Immunochemistry System; Beckman, USA). The results are reported as μg CRP/dl serum.

Interleukin determination

Interleukin (IL-1, IL-2, IL-4, IL-6 and tumour necrosis factor (TNF)) concentrations in the sera of the groups studied were determined using a kit for ELISA analysis (R&D System; Quantikine, Minneapolis, MN) according to the manufacturer's directions.

In vitro *lymphoproliferation test*

The lymphoproliferation assay was carried out as described previously [12]. Briefly, blood samples (7 ml) were diluted with an equal volume of sterile RPMI 1640 culture medium containing glutamine, 2·0 g/*l* sodium bicarbonate, 10% heat-inactivated human AB serum, 150 U/ml penicillin, 80 μg/ml streptomycin, and 20 mM HEPES. Samples were then applied to a Ficoll–Hypaque gradient and centrifuged at 400 ***g*** for 30 min at room temperature, and the interface, predominantly composed of PBMC, was harvested. The PBMC were washed twice in RPMI 1640, resuspended in 1·0 ml culture medium, and counted in an automatic counter (CC510; Celm, São Paulo, Brazil). Lymphocyte viability was determined in the presence of 2% trypan blue. The cell suspension was then diluted with RPMI 1640 culture medium to a final concentration of $2{\cdot}5 \times 10^6$ lymphocytes/ml. A 0·1-ml sample of each cell suspension was added to each well ($2{\cdot}5 \times 10^5$ cells/well) of flat-bottomed, 96-well plates (Corning, Miami, FL). The cells from each individual were cultured separately in triplicate in the presence of 20 μg/ml phytohaemagglutinin (PHA; Difco Laboratories, Detroit, MI), 10 μg/ml

Table 1. Paracoccidioidomycosis (PCM) patient data

Characteristics	Group I, disseminated	Group II, chronic	Group III, control
Age (years)	20–40	30–55	22–26
Sex	Male	Male	Male
RIA titres	1/32 to 1/1024	1/2 to 1/8	Negative
Treatment	Four patients with any treatment Four patients, SDZ One patient, AFB/KTZ	SDZ	None

RIA, Radial immunodiffusion assay; SDZ, sulphadiazine during 1 to 2 years of use; AFB, amphotericin B; KTZ, ketoconazole.

concanavalin A (Con A), or in the presence of RPMI medium as control. The plates were incubated at 37°C with 5% CO_2 for 3 days. Sixteen hours before the end of the incubation period, 0·5 μCi ^{3}H-thymidine (specific activity 6·7 Ci/mM; New England Nuclear, UK) was added to each well. Cells were then harvested using an automatic collector (Cambridge Technology Inc., Cambridge, MA) and analysed in a Beckman LS150 liquid scintillation spectrometer. Lymphoproliferation was quantified by determining the stimulation index (SI) which represents the mean quotient between counts of radiation emission per minute in the presence and absence of stimulating agents. SI >2·0 was considered to indicate blastogenesis.

Statistical analysis

Data were statistically analysed by the software INStat (Graph Pad Software Lic. 90485, San Diego, CA) performing one-way analysis of variance (ANOVA). To test the homogeneity of variances Barllet's test was used to compare differences among standard deviations in the groups studied. Bonferroni multiple comparisons test was used to calculate the expected value of P compared with $P < 0·05$. Significant data from analysis of ANOVA and post-tests above were submitted to linear regression, testing the linearity of data with significance level of $P < 0·05$.

RESULTS

Antibody titre

The anti-*P. brasiliensis* antibody titres obtained in radial immunodifusion assays are shown in Fig. 1. The patients with disseminated disease exhibited high antibody titres compared with patients with the chronic form of disease, as well as with controls. The difference between controls, chronic and disseminated PCM patients was significant ($P < 0·001$). The effect of treatment upon antibody production was further checked in those patients without treatment and those disseminated patients treated with sulphadiazine, and the results show that the difference between antibody production in those groups was highly significant ($P < 0·001$), as well as compared with controls ($P < 0·001$).

Lymphoproliferation stimulated by PHA or Con A

The mitogenicity to PHA and Con A presented by lymphocytes obtained from all PCM patients was lower ($P < 0·05$) than that observed for healthy individuals (Fig. 2), mainly in those patients with disseminated PCM without treatment. When the results of the disseminated and the chronic PCM patients are considered separately, it can be noted that responses to either Con A or PHA stimulation were equally non-significant, although higher proliferation was observed in the chronic than in the disseminated PCM patients.

Determination of CRP concentration

The distributions of CRP levels reported as μg/dl of serum were statistically different for both groups of patients studied, as illustrated in Fig. 3. Patients with disseminated PCM had the highest serum CRP concentration (1553·8 ± 861·2); whereas chronic patients had a much lower CRP concentration (661·8 ± 52·4), closer to the levels detected in healthy individuals (627·5 ± 93·4). The difference between those values was significant ($P < 0·001$), suggesting that the reactional

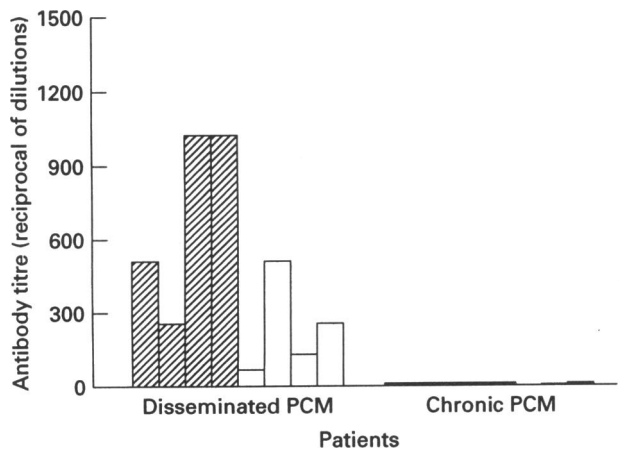

Fig. 1. Anti-*Paracoccidioides brasiliensis* antibody titres in chronic or disseminated paracoccidioidomycosis (PCM) patients receiving (□) or not receiving (▨) anti-fungal therapy. Results are expressed individually as a reciprocal of serum dilutions determined by radial immunodiffusion assay. ANOVA and Barlett's test ($P < 0·05$).

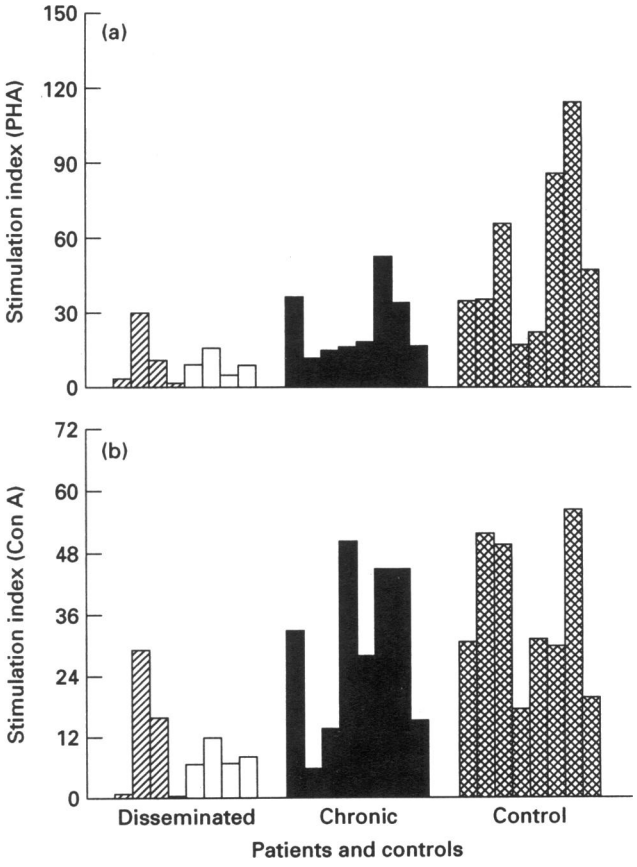

Fig. 2. Proliferative response of peripheral blood mononuclear cells (PBMC) from paracoccidioidomycosis (PCM) patients to phytohaemagglutinin (PHA) (a) and concanavalin A (Con A) (b). The results are expressed individually as stimulation index (SI), i.e. the ratio between the counts (ct/min) of PBMC cultured with mitogens (□) and the counts of PBMC without mitogenic stimulation (▨).

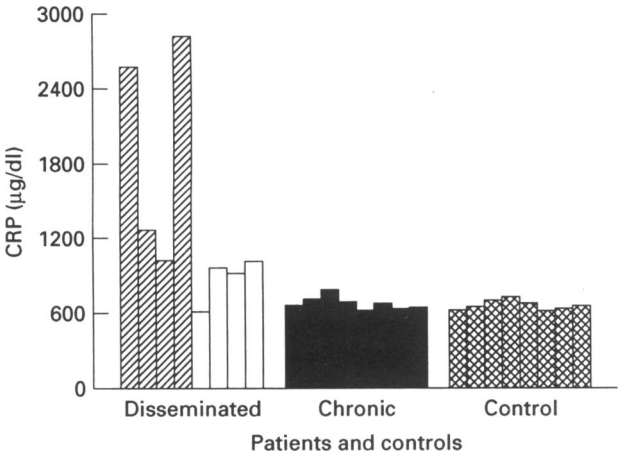

Fig. 3. C-reactive protein (CRP) levels in paracoccidioidomycosis (PCM) patients. Note the prevalence of high levels of CRP in the disseminated PCM patients without treatment (▨).

state of PCM induced an augmentation of CRP production and release compared with chronic disease. In addition, disseminated PCM patients exhibited the highest levels of CRP in the serum, significantly different from those of the disseminated and treated patients ($P < 0.001$) and controls ($P < 0.001$).

Determination of interleukin concentration

Interleukin (IL-1, IL-2, IL-4, IL-6 and TNF) concentrations (pg/ml) in the sera of patients and controls are shown in Fig. 4. It can be seen that serum IL-1, IL-6 and TNF concentrations were significantly higher ($P < 0.001$) in patients with the disseminated form of disease compared with controls. Among disseminated PCM patients, those without treatment presented high IL-1, IL-6 and TNF levels compared with treated patients ($P < 0.01$, $P < 0.001$ and $P < 0.001$, respectively). Moreover, there was no significant difference in interleukin levels between treated disseminated PCM and chronic patients (Fig. 4).

Different patterns of interleukin concentration were observed for IL-2 and IL-4. IL-2 production was higher in chronic patients (54.0 ± 23.3 pg/ml) compared with healthy controls (30.6 ± 8.1 pg/ml) ($P < 0.05$), or compared with disseminated PCM patients receiving treatment (20.6 ± 6.9 pg/ml) and non-treated (17.3 ± 4.5 pg/ml) ($P < 0.01$). However, IL-2 levels were no different between treated and non-treated disseminated PCM patients. Linear regression of data showed an inverse correlation between IL-2 and IL-6 levels in non-treated disseminated patients ($P = 0.0501$) and between IL-2 and IL-4 in chronic patients ($P = 0.0131$). Regarding IL-4 concentrations, no significant alterations were observed in any patients and controls.

Correlation between interleukin and CRP concentrations

The data from linear regression revealed a positive correlation between TNF, IL-1, or IL-6 with CRP levels in non-treated disseminated PCM patients ($r = 0.76$; $r = 0.69$; $r = 0.63$, respectively). The positive correlation between values indicates that when disseminated PCM patients have high TNF ($P = 0.0269$), IL-1 ($P = 0.0620$) or IL-6 ($P = 0.0660$) levels, a proportional increase in CRP concentrations also occurs. A tendency to negative correlations was observed for IL-2 and IL-4 ($r = -0.6462$ with $P = 0.0834$; $r = -0.5785$ without significance) compared with CRP concentrations in the disseminated patients. It is important to stress that when IL-1 and IL-6 levels in the sera of chronic patients were tested for linearity, a significant inverse correlation ($r = -0.6676$) was observed ($P = 0.0474$), and neither interleukin had correlations with CRP levels in the same patients.

Correlation between interleukin and lymphoproliferation stimulated by PHA

The linear regression from data obtained in the lymphoproliferation and interleukin detection assays revealed that IL-1, IL-4, IL-6 and TNF levels in the disseminated PCM patients were not correlated with the mitogenic response of T lympho-

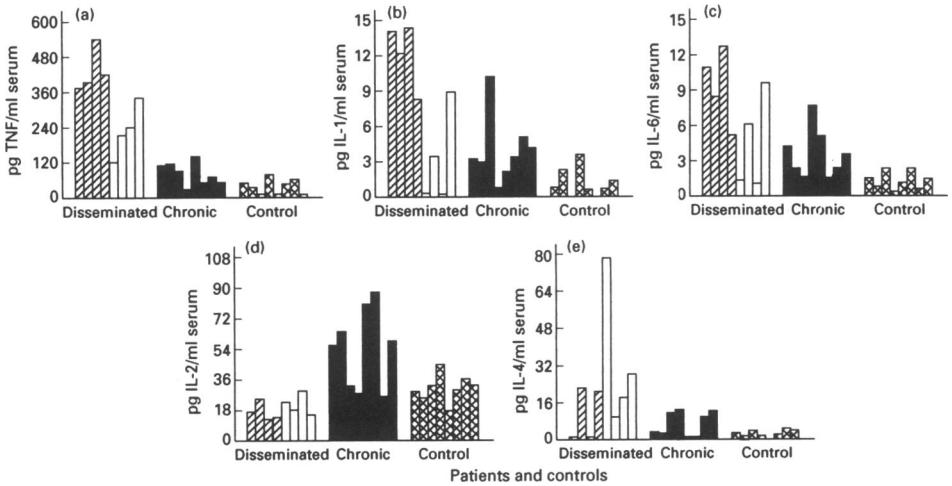

Fig. 4. Interleukin detection in the sera of paracoccidioidomycosis (PCM) patients. (a) IL-1. (b) IL-6. (c) Tumour necrosis factor (TNF). (d) IL-2. (e) IL-4. ELISA anti-human interleukins. All dose × absorbance curves for each interleukin had a r value >0·9. The treatment of disseminated PCM patients reduced the levels of inflammatory interleukins but did not increase IL-2 levels. ▨, Without treatment; □, with treatment.

cytes to PHA ($P = 0{\cdot}7162$; $P = 0{\cdot}7497$; $P = 0{\cdot}9275$ and $P = 0{\cdot}9259$, respectively), showing that these inflammatory interleukins were not related to low mitogenicity of lymphocytes to PHA. However, negative correlations were observed for IL-2 and PHA stimulation index ($r = -0{\cdot}7135$; $P = 0{\cdot}0553$). Thus, non-treated disseminated PCM patients without response to PHA stimulation also showed low levels of IL-2 in the sera. However, chronic PCM patients, who exhibited higher proliferation of lymphocytes, had no positive correlation with increased IL-2 production.

Correlation between interleukin and specific antibody titre

There were straight correlations between IL-1 ($P = 0{\cdot}0834$), IL-6 ($P = 0{\cdot}0168$) and TNF ($P = 0{\cdot}0069$) in non-treated disseminated PCM patients with the highest antibody titres against *P. brasiliensis* antigen. By contrast, no correlations were found with these interleukins in the treated disseminated and in the chronic patients ($P = 0{\cdot}6623$; $P = 0{\cdot}2677$; $P = 0{\cdot}7932$, respectively). An interesting finding was the inverse correlation between IL-4 and antibody production in disseminated patients ($r = -0{\cdot}4770$); moreover, a significant ($P = 0{\cdot}0820$) correlation was found in chronic patients with respect to IL-4 and antibody titres against fungus antigen. Disseminated PCM patients also had IL-2 levels inversely correlated with antibody production ($r = -0{\cdot}6313$; $P = 0{\cdot}0628$).

DISCUSSION

This study showed that PCM patients with the disseminated form of disease presented alterations of some aspects of innate and acquired immunity compared with chronic patients or controls. Some of these alterations were observed in macrophage inflammatory activities reflected by the different levels of interleukins and CRP concentrations, as well as in T cell proliferation and in antibody secretion. The non-treated PCM patients with disseminated disease exhibited higher levels of antibodies, as well as levels of inflammatory interleukins compared with those patients with disseminated PCM but under medication schedule. These results clearly indicate the involvement of these inflammatory interleukins and CRP in the initial phases in the host–parasite relationship in PCM.

CRP is the acute-phase protein occurring in humans which has been studied most extensively [13–16]. In this study, high concentrations of CRP were observed in patients with disseminated disease compared with chronic patients or controls. The elevated CRP levels reflect disseminated diseases such as those observed in tumours, tissue lesions and several infections with different etiological agents [17].

Studies conducted to determine the extracellular signals that increase or decrease the expression of genes coding for acute-phase proteins in hepatocytes have revealed that several interleukins released from activated macrophages, such as TNF, IL-1 and IL-6, are deeply involved in this process [17,18]. Our data showed a positive correlation between TNF, IL-1, IL-6 and CRP levels in the serum of patients with the disseminated form of PCM. Thus, CRP, interleukins and probably the complement system [19–21] may also act synergistically, enhancing the PCM inflammatory process.

TNF has been described as a potent stimulant of leucocyte functions, inducing adherence to endothelial cells and to particles [22], leading to increase of phagocytosis and respiratory burst activity [23], degranulation [24], chemotaxis [25] and granuloma formation [26,27]. The discovery that interleukins may regulate these interactions has potential interest in clarifying the development of disseminated PCM inflammatory reaction. The somatic structure of *P. brasiliensis* has been described as a potent inducer of inflammatory reaction [28,29], enhancing TNF releasing [26] and complement activation [30]. Moreover, in a murine experimental model we also demonstrated that the treatment of mice with antibody anti-TNF prevents the establishment of mature granulomas induced by the fungus or by the cell wall fractions thereof [26].

In view of these statements, it was important to assess what interleukins were presented by PCM patients in a determined period of disease, and to correlate them not only to the innate immune response but also to its consequences upon the humoral and cellular immunity according to the criteria of severity of disease. Thus, of outstanding interest in this study was the positive correlation between IL-1, TNF and IL-6 with the hyperactive antibody response in disseminated PCM patients; this result agrees with the most severe disease [7]. Since overlapping target cell activities of IL-1, TNF and IL-6 include B cell proliferation and antibody secretion [31], we suppose from our data that the strong inflammatory reaction occurring in disseminated PCM patients also affects humoral immunity, as observed for high antibody titres. Giving support to this hypothesis, it is well known that IL-6 from stimulated macrophages and/or Th2 lymphocytes [31], following IL-1 proliferative signals, increases the secretion of IgM by activated B cells [31].

The regulation of interleukin production by T helper cells in experimental systemic fungal infections could be explained partly by the dichotomous relationship between Th1 and Th2 T helper cell subsets [32]. These subsets are distinguishable by their profile of interleukin production [33]. While Th1 cells have been shown to produce IFN and IL-2, and mediate DTH, Th2 cells produce IL-4, IL-5, IL-6 and IL-10. Through the production of IL-4, Th2 cells are potent stimulators of B cell activity and inducers of antibody production, especially IgE and IgG1 subclasses [34]. However, the data obtained here show that these statements may not be entirely applicable to human PCM, at least for the patients studied here. The disseminated patients failed to produce high amounts of IL-4, but exhibited higher antibody titres with low mitogenicity to PHA. Unlike what is proposed for experimental infection, we suppose from our data that there was no apparent preferential induction of Th2 cells in PCM patients, but in fact, a strong inflammatory reaction releasing potent mediators, such as IL-1, IL-6, TNF and CRP, that in turn affected B cell activation and proliferation, which may result in high IL-10 production. This property, together with an inhibitory effect on the expression of MHC class II antigens on monocytes/macrophages results in a strong inhibition of the antigen-presenting capacity of these cells, and a consequent inhibition of IL-2 and IFN releasing and T lymphocyte proliferation [35]. Although IFN was not measured in the PCM patients, IL-2 production was significantly ($P < 0{\cdot}001$) reduced in patients, and their T lymphocytes exhibited low mitogenicity to PHA. In addition, IL-10 was recently shown to induce the proliferation of B lymphocytes and, most notably, their differentiation into plasma cells secreting immunoglobulins at a high rate [36]. IL-10 (although it was not measured in this study) could inhibit cellular

reactions and stimulate humoral responses in disseminated PCM patients. Therefore, the intense inflammatory reaction caused by *P. brasiliensis* could generate the mediators related to the increased B cell activation and immunoglobulin-secreting functions described in PCM [37,38].

The overall analysis of our results leads us to propose the importance of the somatic structure of *P. brasiliensis* in disseminated PCM infection with respect to the strong inflammatory reaction which produces potent mediators, such as IL-1, TNF, IL-6 and CRP, capable of promoting B cell activation (probably in a Th2-independent way), leading to immunoregulatory disturbances such as polyclonal B cell activation and hypergammaglobulinaemia and depressed cell-mediated immunity.

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