IL-12 AND NEUTRALIZATION OF ENDOGENOUS IL-10 REVERT THE IN VITRO ANTIGEN-SPECIFIC CELLULAR IMMUNOSUPPRESSION OF PARACOCCIDIOIDOMYCOsis PATIENTS

Carla C. Romano,1 Maria J. S. Mendes-Giannini,2 Alberto J. S. Duarte,1 Gil Benard1,3

Treatment of patients with paracoccidioidomycosis is still a challenge. Patients present defective lymphoproliferation and IFN-γ responses to the main Paracoccidioides brasiliensis antigen (gp43), which correlates with disease severity. Here, we demonstrated that the patients show also a defective synthesis of interleukin (IL)-12. Therefore, we attempted to revert this immune disfunction by adding IL-12 and neutralizing anti-IL-10 antibody to gp-43-stimulated peripheral blood mononuclear cell cultures. Both treatments increased IFN-γ secretion to levels observed with healthy sensitized individuals, but affected proliferation only modestly. When combined, the treatments further increased IFN-γ synthesis and cell proliferation. The addition of suboptimal concentrations of IL-2 also further increased the IL-12-mediated secretion of IFN-γ.

Interestingly, the immune modulation was mostly antigen-specific, since the responses to Candida albicans’ antigen were not affected. These results suggest that appropriate immune intervention with cytokines and/or anti-cytokines may help in the treatment of PCM.

Paracoccidioidomycosis is the most important endemic deep mycosis in Latin America. The disease is caused by the dimorphic fungus Paracoccidioides brasiliensis. The incidence of subclinical infection in populations living in endemic rural areas may be as high as 40% or more.1 However, only a minor portion of the infected individuals will manifest overt disease. The disease evolves as a chronic granulomatous process that can be clinically classified in acute/subacute form (AF) or chronic form (CF). The CF is the most common clinical presentation, affects mainly previously healthy male adult agricultural workers, and is characterized by mucosal involvement, which can vary from an isolated oral ulceration to diffuse pulmonary involvement.1 Usually, these patients present multifocal involvement. In contrast, the more rare, and more severe, acute form affects both genders usually under the age of 30 and is characterized by involvement of the mononuclear-phagocytic system.1 In PCM, as in other chronic granulomatous infectious diseases, the cellular immune response represents the main mechanism of defence. It has been proposed that the balance between Th1/Th2 responses regulates the clinical outcome in many of such infectious diseases.2 Data in support of an association between a dominant Th2 response and severe disease has largely been provided by studies of PCM in mice.3 Only more recently we and others have evaluated the patients’ cytokine scenario underscoring the depression of Th1 responses and the Th2 dominance of the responses to fungal components.4–6 We have utilized a glycoprotein of 43 kDa (gp43) in these studies. This glycoprotein has been shown to elicit strong antibody and cellular immunity responses in humans and experimental models, with little or no cross-reactivity with other fungal antigens, and is considered the main antigenic component of the fungus.7,17 We have previously shown that PBMC from patients with both the AF and CF of PCM proliferate poorly and produce low or undetectable levels of the Th1 cytokines IFNγ and IL-2 from an isolated oral ulceration to diffuse pulmonary involvement.1 Usually, these patients present multifocal involvement. In contrast, the more rare, and more severe, acute form affects both genders usually under the age of 30 and is characterized by involvement of the mononuclear-phagocytic system.1

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in response to gp43 as compared to healthy *P. brasiliensis* sensitized individuals. On the other hand, substantial levels of Th2 cytokines, such as IL-10 and IL-4, were detected.

Treatment of PCM remains a complex and difficult task. Patients need prolonged courses of antifungals that must be continued to variable periods even after clinical manifestations had subsided. Consequently, relapses are frequent. Moreover, patients with more severe disease may need many months or even years to achieve complete remission. They also frequently develop permanent sequels. Recently, for many clinical conditions where currently treatments are unsatisfactory, the proposal of immune-based therapy has become attractive. Several trials of cytokine treatment for patients with cancer, AIDS or some endemic infectious diseases, have been reported with encouraging results. We believe that patients with PCM may also be good candidates for such interventions. However, the lack of more profound knowledge on the immunoregulation of patients with PCM may delay the application of this type of treatment and its potential benefits.

In this study we aimed at modifying the in vitro pattern of Th1 hyporesponsiveness immune response by using IL-12, a cytokine known for its ability to induce IFN-γ production and to promote a Th1 immune response. We also examined the role of IL-2 in enhancing the effects of IL-12. Since IL-10 seems to play a major role in the antigen-specific immunosuppression of several chronic granulomatous infectious diseases, PCM included, we additionally evaluated the effect of IL-10 neutralization. We show that immune modulation with cytokine and/or anti-cytokine treatment can restore the antigen-specific Th1 type immune response PCM patients. The results presented here suggest that appropriate immune intervention may help in the treatment of this severe mycosis.

### RESULTS

In agreement with our previous studies, gp43-induced proliferation and IFN-γ secretion by PBMC from PCM patients were lower than those induced by CMA (*P<0.05* and *P<0.001* respectively) and comparable with those of the non-stimulated cultures (Table 1 and Fig. 1). These results are in agreement with the notion that PCM is characterized predominantly by an antigen-specific hyporesponsiveness. We additionally measured the IL-12 secretion in PBMC cultures from 10 patients and six healthy *P. brasiliensis*-sensitized subjects. While the latter produced on average 69 ± 8 pg/ml of IL-12p70 after gp43 stimulus, patients produced significantly less IL-12p70, 40 ± 5 pg/ml.

#### Table 1. Effect of interleukin (IL)-12 (1 ng/mL) on lymphocyte proliferative responses in cultures of peripheral blood mononuclear cells (PBMC) from 16 patients with paracoccidioidomycosis

<table>
<thead>
<tr>
<th>PBMC culture condition</th>
<th>Proliferative response (n=16)</th>
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<tbody>
<tr>
<td>medium</td>
<td>604 ± 166</td>
</tr>
<tr>
<td>CMA</td>
<td>12380 ± 2995*</td>
</tr>
<tr>
<td>gp43</td>
<td>1533 ± 376</td>
</tr>
<tr>
<td>+IL-12</td>
<td>631 ± 165</td>
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<td></td>
<td>12010 ± 2431</td>
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<td>1720 ± 407</td>
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Five patients presented the acute form and 11 the chronic form. Results (except for medium) are in change in counts per minute of radiolabelled thymidine incorporation. Mean values (± SE) are shown. CMA = *Candida* metabolic antigen. *P<0.01* vs medium and gp43.

![Figure 1.](image-url)
IFN-γ and proliferative response to gp43 in PBMC from patients with PCM. As shown on Table 1, IL-12 was unable to increase the proliferation of non-stimulated lymphocytes or lymphocytes stimulated for 7 days with gp43, and did not change the positive response to CMA. On the other hand, IL-12 strongly enhanced the gp43-induced IFN-γ production, from 129 ± 52 to 631 ± 178 pg/ml (P < 0.01, Fig. 1). A more discrete enhancement of the non-stimulated and CMA-stimulated IFN-γ secretion was also observed but did not reach statistical significance (30 ± 12 vs 159 ± 103 pg/ml with IL-12, P > 0.05, and 525 ± 135 vs 993 ± 214 pg/ml with IL-12, P > 0.05, respectively) (Fig. 1). Non-stimulated PBMC from one of the patients produced high levels of IFN-γ (>500 pg/mL) after IL-12 addition, whereas CMA-stimulated PBMC from six patients generated such levels even in the absence of IL-12. CMA-stimulated PBMC from an additional patient was also found to produce high levels of IFN-γ following IL-12 addition. In contrast, gp43-stimulated PBMC from only one patient secreted high IFN-γ, while six of the 10 patients required IL-12 to yield such levels. Refractoriness to IL-12-mediated IFN-γ synthesis in response to gp43 is apparently correlated to disease severity. Three of the four acute form patients were found to be refractory, while only one of the 6 patients with the chronic form followed this pattern.

We also examined the effect of IL-12 on IL-10 secretion in the same PBMC cultures. There was no down modulation of IL-10 secretion by IL-12 (data not shown). However, low IL-10 levels were detected in any case (medium only: 88 ± 33 pg/mL, CMA: 113 ± 36 pg/mL and gp43: 134 ± 54 pg/mL) compared to those previously reported in 3 days culture supernatants (>200 pg/mL). The present lower levels may be explained by the kinetic of IL-10 secretion by patients’ PBMC, which peaks by 72 h in CMA or gp43-stimulated PBMC cultures and decreases thereafter (Romano CC, and Benard G, unpublished results), whereas in the current experiments supernatants were harvested at day 7. In addition, the lack of a modulatory effect of IL-12 can also be due to the fact that IL-10 produced in response to pg43 is mainly monocyte-derived. This was further confirmed by experiments with PBMC from PCM patients depleted of monocytes by adherence, which showed even lower IL-10 levels after 7 days culture with CMA or gp43 (data not shown).

Because we have previously detected in patients with PCM defective gp43-induced secretion of IL-2, another Th1 driving cytokine, we next asked whether the modulatory activity of IL-12 could be enhanced by addition of this cytokine. IL-2 was used at a sub-optimal concentration (5 UI/mL) to avoid non-specific stimulation of lymphocytes. Interleukin-2 alone neither affected the proliferative response (Table 2) nor enhanced the IFN-γ synthesis in cultures with gp43 (243 ± 129 vs 228 ± 123 pg/mL with IL-2), CMA (514 ± 227 vs 493 ± 203 with IL-2) or medium only (34 ± 15 vs 36 ± 15 pg/mL with IL-2) (Fig. 2). However, the mean IFN-γ levels detected in the non-stimulated and gp43-stimulated cultures to which the combination of IL-2 plus IL-12 (1 ng/mL) was added rose to 344 ± 143 and 761 ± 178 pg/mL, respectively. Such IFN-γ levels were significantly higher (P < 0.05) than those produced in parallel cultures with no cytokine addition or with IL-2 addition, but were not higher than those produced with IL-12 addition (Fig. 2). IL-12/IL-2-mediated increase was observed in four of the five chronic from patients and in 2 of the 3 acute form patients. In contrast, as noted in Figure 2, there was no IL-12/IL-2 mediated increase in IFN-γ synthesis in the CMA-stimulated cultures. On the other hand, IL-12/IL-2 addition promoted a noticeable, but not statistically significant, increase of 129% in the mean IFN-γ production, from 123 pg/ml with IL-2) or medium only (34 ± 15 vs 36 ± 15 pg/mL with IL-2) (Fig. 2). Again, no major effect was seen in the CMA-induced responses.

The data suggest that low dose IL-2 enhanced responsiveness to IL-12. However, the IL-12/IL-2-induced increase in IFN-γ synthesis also in the non-stimulated cultures indicates that this occurred in a less specific fashion than that induced by IL-12 alone. A likely explanation is that both cytokines acted synergistically on T cells that were pre-activated in vivo by

<table>
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<tr>
<th>PBMC culture condition</th>
<th>Proliferative response (n=6)</th>
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<tr>
<td></td>
<td>—</td>
</tr>
<tr>
<td>medium</td>
<td>1013 ± 272</td>
</tr>
<tr>
<td>CMA</td>
<td>19100 ± 5768</td>
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<tr>
<td>gp43</td>
<td>2458 ± 1405</td>
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Two patients presented the acute form and 4 the chronic form. Results (except for medium) are in change in counts per minute of radiolabeled thymidine incorporation. Mean values (± SE) are shown.

**TABLE 2. Effect of a sub-optimal dose of interleukin (IL)-2 (5 UI/mL) on the modulation by IL-12 (1 ng/mL) of lymphocyte proliferative responses in cultures of peripheral blood mononuclear cells (PBMC) from six patients with paracoccidioidomycosis**
circulating *P. brasiliensis* antigens. Finally, in these experiments the low IL-10 levels secreted were not significantly affected by IL-2, IL-12 or the combination of both (data not shown).

To clarify whether or not IL-10 has a role in the down-modulation of anti-*P. brasiliensis* cellular immune response, a set of experiments were performed with PBMC from nine patients with active PCM in the presence or absence of a neutralizing anti-IL-10 antibody. This antibody was added at day 0 to neutralize the high levels of IL-10 since the first days of the culture. Table 3 shows that neutralization of IL-10 was unable to modify the non-stimulated and CMA-stimulated proliferation, but resulted in an increase of 147% in the gp43-stimulated proliferation which, however, did not reach statistical significance. On the other hand, blockade of IL-10, similarly to IL-12 treatment, significantly enhanced the gp43-induced IFN-γ from 112 ± 59 to 764 ± 283 pg/mL (P < 0.05), while IFN-γ enhancement in non-stimulated and CMA-stimulated cultures were slighter and not significant (from 21 ± 7 to 59 ± 30 pg/mL, P > 0.05, and from 381 ± 139 to 907 ± 232 pg/mL, P = 0.07, respectively (Fig. 3). Three of the four patients with the acute form did not show enhancement of IFN-γ secretion whereas only one of the five with the chronic form was refractory. Such results reinforce the previous suggestion that refractoriness correlates to disease severity. Thus, IL-10 neutralization, like IL-12, was able to only partially revert the antigen-specific hyporesponsiveness.

In parallel, we also tested in the experiments above whether the anti-IL-10 modulatory activity could be up-regulated by IL-12 treatment at day 5 of culture. In fact, as can be seen in Table 3 and Figure 3, the combined treatment lead to a more pronounced effect on the gp43-induced IFN-γ from 112 ± 59 to 764 ± 283 pg/mL (P < 0.05), while IFN-γ enhancement in non-stimulated and CMA-stimulated cultures were slighter and not significant (from 21 ± 7 to 59 ± 30 pg/mL, P > 0.05, and from 381 ± 139 to 907 ± 232 pg/mL, P = 0.07, respectively (Fig. 3). Three of the four patients with the acute form did not show enhancement of IFN-γ secretion whereas only one of the five with the chronic form was refractory. Such results reinforce the previous suggestion that refractoriness correlates to disease severity. Thus, IL-10 neutralization, like IL-12, was able to only partially revert the antigen-specific hyporesponsiveness.

![Figure 2](image-url)  
Figure 2. IL-2 at a sub-optimal dose enhances the modulatory effect of IL-12 on the gp43-induced, but not the medium only or CMA-induced IFN-γ secretion by PBMC from PCM patients.

The open dots represent patients with the acute form of the disease and the filled dots represent patients with the chronic form. Horizontal bars represent the mean. IL-12 (1 ng/mL) and IL-2 (5 U/mL) were added to the cultures at day 5 and the supernatants were harvested at day 7. IFN-γ levels were measured by ELISA. *P < 0.05 vs same condition without cytokine addition. **P < 0.05 vs gp43 plus IL-2.

<table>
<thead>
<tr>
<th>PBMC culture condition</th>
<th>Proliferative response (n=11)</th>
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<tbody>
<tr>
<td>medium</td>
<td>2504 ± 700</td>
</tr>
<tr>
<td>CMA</td>
<td>10230 ± 3433</td>
</tr>
<tr>
<td>gp43</td>
<td>2073 ± 918</td>
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</tbody>
</table>

Five patients presented the acute form and six the chronic. Results (except for medium) are in change in counts per minute of radiolabelled thymidine incorporation. Mean values (± SE) are shown. *P < 0.05 vs gp43 alone. **P < 0.05 vs gp43 plus IL-2.
chronic form and the other with the acute form, showed a slight only enhancement when compared to anti-IL-10 alone or no treatment.

DISCUSSION

The main immunologic dysfunctions described in patients with PCM are suppression of lymphocyte proliferation and Th1 cytokine secretion in response to *P. brasiliensis* antigens. Suppression of these responses probably results in the absence of compact granuloma formation capable of restricting *P. brasiliensis* dissemination, and, indirectly, in the large amounts of non-protective antibodies, both of which are phenomena mainly seen in patients with more severe disease. The importance of these immune functions is underscored by the potent lymphoproliferation and secretion of the Th1 cytokines IFN-γ and IL-2 depicted by healthy sensitized subjects or cured patients, contrasting with their low or undetectable antibody response. Indeed, both Th1 cytokine secretion and lymphoproliferative responses were inversely correlated with disease severity and with the antigen-specific antibody production. This study extends the panel of suppressed Th1 cytokines in PCM patients by showing diminished IL-12 secretion in response to gp43, the main *P. brasiliensis* antigenic component. We next demonstrate that in vitro cytokine addition can restore, in the majority of the PCM patients studied here, both the gp43-driven lymphoproliferation and the secretion of IFN-γ. Addition of IL-12 to patients’ PBMC cultures markedly increased IFN-γ secretion. A similar increase was also achieved by neutralizing endogenous IL-10. Both treatments only modestly increased lymphoproliferation. However, when combined, they were able to further increase IFN-γ production and proliferation. In addition, we also demonstrated that suboptimal doses of IL-2, albeit not being able to modulate the responses by itself, further increased the Th1 enhancing effects of IL-12. Noteworthy, the effects of these treatments on the immune system were predominantly on the antigen-specific immune defect, since they did not significantly modulate the already detectable immune responses to a control fungal antigen, from *C. albicans*.

Addition of IL-12, a Th1 driving cytokine, markedly augmented (~5-fold) the mean gp43-elicited IFN-γ secretion by patients’ PBMC, reaching levels comparable to those induced with *C. albicans*. Lymphoproliferation, nevertheless, was not modified. Interleukin-12 exerts its effects on T cells through a heterodimeric high affinity IL-12 receptor that comprises two subunits. The β1 sub-unit is constitutively expressed while the β2 sub-unit is expressed only by activated cells. TCR signalling alone induces low levels of IL-12R expression that would be up-regulated by IL-12 produced by the APC. However, Chang *et al.* reported that IL-2 is crucial for the persistence of the β2 sub-unit expression and for the clonal expansion of lymphocytes induced by IL-12. Others have also

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**Figure 3.** Neutralization of endogenous IL-10 enhances the gp43-induced, but not the medium only or CMA-induced IFN-γ secretion by PBMC from PCM patients. IL-12 further enhances the effect of anti-IL-10 on gp-43 stimulated cultures.

The open dots represent patients with the acute form of the disease and the filled dots represent patients with the chronic form. Horizontal bars represent the mean. The neutralizing anti-IL-10 antibody (10 μg/mL) was added at day 0 of culture and IL-12 (1 ng/mL) at day 5. The supernatants were harvested at day 7. IFN-γ levels were measured by ELISA. *P*<0.05 vs gp43 without cytokine addition.
shown that low levels of IL-2 were able to induce expression of the β2 sub-unit and to increase responsiveness to IL-12. In fact, gp43-exposed lymphocytes from patients express very low levels of the β2 sub-unit compared with cured patients (Romano CC & Benard G, unpublished results). Altogether, these observations may explain the lack of lymphoproliferation seen in our patients even after addition of exogenous IL-12, since we have previously found low or undetectable IL-2 levels in patients' PBMC cultures stimulated with gp43. However, concomitant treatment of patients' PBMC cultures with IL-12 and a suboptimal concentration of IL-2 enhanced further the IFN-γ secretion, but resulted in an only modest increase in proliferation when compared with IL-2 or IL-12 alone. Moreover, the proliferative responses remained lower than those induced by gp43 in lymphocytes from healthy sensitized individuals (~7000–10 000 cpm).

The modest results obtained with the combined IL-2/IL-12 treatment on proliferation could be due to the use of a sub-optimal only concentration of IL-2, or, more likely, by the immunosuppressive effects of IL-10, which is secreted in high amounts by patients’ monocytes a few hours after being challenged with gp43, thus acting since the beginning of the culture. Interleukin-12 acts on T cells by inducing tyrosine phosphorylation of several members of the STAT proteins family, including STAT1, 3, 4 and 5. It has been proposed that STAT 4 is important for the induction of IFN-γ secretion while STAT5 phosphorylation correlates with lymphocyte proliferation, but more recent studies suggested that STAT1, 3 and 5 recruitment and activation may also be necessary for accomplishment of both functions. On the other hand, IL-10-mediated immunosuppression is elicited in lymphocytes by selectively inhibiting the CD28 costimulatory pathway, stopping further signal transduction. This results in suppression of the phosphorylation of STAT1, 3 and 5, and consequently, in inhibition of IFN-γ production and lymphocyte proliferation. However, the observation that, in anergized T cells, IL-12 could only restore the IFN-γ production, while restoration of antigen-specific T-cell proliferation required a direct action on costimulatory molecules such as CD28, may explain why in our anergized patients exogenous IL-12 could override (possibly by inducing STAT4 phosphorylation) the IL-10 inhibitory activity on IFN-γ secretion, but was not sufficient to revert the non-proliferative state of T cells. Therefore, for full recovery of the antigen-specific proliferation, additional costimulatory activity, either by signalling through the CD28 pathway or by blocking the engagement of the negative regulator of T-cell function CTLA-4 on CD80/86 molecules, as well as STAT5 activation, would be necessary. Although we do not know the precise role of these costimulatory pathways in PCM, this issue was in part addressed here by neutralizing the endogenously produced IL-10.

In fact, the immunosuppressive role of IL-10 in PCM was confirmed by the experiments where this cytokine was neutralized by an anti-IL-10 antibody. The marked augmentation of IFN-γ secretion (~7-fold) was comparable to that observed with combined IL-12/IL-2 addition, as well as the modest increase in proliferation. Furthermore, the best results were achieved in the experiments where anti-IL-10 and IL-12 treatments were combined. This combination resulted in the highest mean levels of IFN-γ, with eight of ten patients tested producing >500 pg/mL, and in the highest gp43-induced mean proliferation, which then fell in the range observed for healthy sensitized individuals. Thus, IL-10 neutralization probably sets free the CD28-mediated costimulatory pathway, which, in conjunction with the exogenously added IL-12, resulted in the higher levels of proliferation and IFN-γ. Whatever the mechanism, the balance between IL-10 and IL-12 appears to play a significant role in the antigen specific immune status of patients with PCM.

Of note, our results differ from those reported in coccidioidomycosis, a deep mycosis in many aspects similar to PCM. IL-10 secretion by coccidioidomycosis patients’ PBMC was lower than that observed with our patients, irrespective of their clinical presentation, and neutralization of endogenous IL-10 did not result in enhancement of IFN-γ. Interleukin-12 was also unable to restore proliferation and IFN-γ in patients with active disease and negative delayed type hypersensitivity test in that study. However, in a number of other chronic infectious diseases, the high levels of monocyte-derived IL-10 have been considered a major immunosuppressive factor, and its neutralization, alone or in conjunction with IL-12 addition, resulted in enhancement of the pathogen-specific proliferation and IFN-γ.

Treatment of PCM patients still is challenge. Those with severe disease may need repeated courses of a highly toxic antifungal drug. Amphoterin B, followed by prolonged maintenance treatment that not infrequently is intercalated by relapses. A proportion of them will suffer from permanent sequels. This difficulty may be illustrated in the present study by the few patients, mostly with the acute, more severe form of the disease that responded less well or did not respond at all to the immune modulation. Thus better therapies are warranted, and immune intervention has become a promising modality. An increasing number of trials on the efficacy of subcutaneous or intravenous cytokine administration to patients with different clinical entities have been recently published. In line with our in vitro findings, the most consistent finding in trials of IL-12 in patients with HIV infection, hepatitis C or malignancies was induction of dose-related
increases in serum levels of IFN-γ. Moreover, the problem of attenuation in IFN-γ response by lymphocytes with consecutive IL-12 cycles could be partially overcome by combined stimulation with IL-12 and IL-2. Similarly, trials of low doses IL-2 in HIV patients have resulted in immunological benefits that were comparable to prior trials with higher doses, but with less side effects. On the other hand, anticytokine therapy has also been considered a potentially valuable therapeutic strategy, but its clinical application has been limited to the few humanized antibodies available up to now, e.g. anti-IL-1, anti-IL-6 and anti-TNF-α, with variable results. Nevertheless, with the clinical experience gained in cytokine and anticytokine trials, alongside with our better knowledge of the immune regulation in PCM patients and the ways to redress their Th1 immune responses, it is likely that the current treatments of PCM, based solely in antifungals, would be improved by association with appropriate immune intervention.

MATERIALS AND METHODS

Patients

Thirty-five patients recently diagnosed with active PCM (age range 7–67 years) were studied. Twelve presented the acute form and 23 the chronic form. The acute form patients (age range 7–34 years) had one or more of the following manifestations: superficial and/or deep (mediastinal and abdominal) lymph node enlargement, bone lesions, cutaneous lesions and hepatosplenomegaly. The chronic form patients (age range 24–67 years) presented lesions affecting larynx, oral cavity and/or both lungs. All of them had a positive anti-\textit{P. brasiliensis} counterimmunoelectrophoresis antibody test (titers range: acute form: 1/32–1/1204, chronic form 1/32–1/256). Since the patients were consecutively admitted during development of the study and the volume of blood drawn from each patient was limited, not all tests were performed for all patients. The exact number of patients tested is indicated in the results. For IL-12 determination experiments we additionally studied six healthy \textit{P. brasiliensis} sensitized subjects as controls, as previously described. All subjects gave written informed consent and the study protocol was approved by the institutional ethics committee of the Hospital da Clínicas da Faculdade de Medicina da USP.

Antigens

\textit{gp43} was obtained from a crude exoantigen preparation of \textit{P. brasiliensis} (113-FMUSP) and fractionated by affinity chromatography in columns of protein A-purified rabbit anti-gp43 IgG coupled to CNBr Sepharose, as previously described. The fraction eluted from this column was concentrated and further purification was achieved by gel filtration in a Sephacryl column to eliminate high-molecular mass contaminants. gp43 was used at 20 µg/mL, as described. A \textit{Candida albicans} metabolic antigen (CMA), purchased from Institut Pasteur (Paris, France), was used (5 µg/mL) as a control fungal antigen.

PBMC isolation and cultures

PBMC were isolated from heparinized venous blood by Ficoll-Hypaque gradient (Pharmacia, Sweden) and were resuspended in RPMI supplemented with gentamicin (40 µg/mL) and 10% of AB serum (Sigma, USA). Cells (2.5 × 10^6/well) were cultivated in 96 well flat bottom plates (Costar, Cambridge, MA, USA) with medium only, CMA (5 µg/mL) and gp43 (20 µg/mL) at 37°C and 5% CO2. For proliferative response analysis, the cells were cultivated in triplicate for 6 days and pulsed with 1 mCi/well of 3H-Thymidine (New England Nuclear, USA) per well for an additional 18 h before harvest. Cell-bound radioactivity was measured by liquid scintillation counting (1205 Betaplate, Wallac, USA). Data are represented as mean counts per minute of triplicates (cpm). For cytokine analyses, supernatants were harvested at day 7 and stored at −80°C until use.

Modulation by IL-12, IL-2 and anti-IL-10

To determine the role of cytokines in the proliferative response and cytokine production, PBMC were incubated as above in the presence of recombinant human IL-12, purified human IL-2 and/or a neutralizing anti-IL-10 monoclonal antibody (MoAb). Recombinant human IL-12 (a generous gift from Dr M. Gately, Hoffman LaRoche, NY) was added at day 5 of the cultures as previous reports have shown that optimal T cell IL-12 receptor expression and responsiveness occur after 5 days in antigen stimulated cultures. Preliminary experiments adding IL-12 at day 0 confirmed the lack of major modulatory activity in our system. Interleukin-12 was added at 1 ng/mL, determined to be optimal in previous dose-response experiments (0.1 to 10 ng/mL) with PBMC from six patients (data not shown). Purified human IL-2 (Genzyme, Boston, MA, USA) was used at the sub-optimal dose of 5 UI/mL, as determined in preliminary experiments with PBMC from 4 patients, to avoid inducing high, non-specific, proliferation in unstimulated PBMC. A rat anti-human IL-10 MoAb (IgG1, 10 ng/mL) (Endogen, Boston, MA, USA) was added at day 0 of the cultures. This dose completely neutralized the cytokine, as determined by ELISA. An irrelevant rat anti-IgG1 MoAb (Pharmingen) was used in some experiments as isotype matched control and showed no effect on the proliferative or IFN-γ responses (data not shown).

Detection of IFN-γ, IL-10 and IL-12p70

IFN-γ, IL-10 and IL-12p70 were detected by standard ELISA. Antibody matched pairs and respective standards were purchased from Endogen (Cambridge, MA, USA) and used following the manufacturers’ recommendation for IFN-γ and IL-10 detection, as previously described. Antibody matched pairs and respective standards from Pharmingen were used for IL-12p70. The detection limit was 10 pg/mL for all cytokines.

Statistical analysis

One-way analysis of variance with the Newman-Keuls test as post-test were used to compare non-paired continuous
data among three or more groups; Student’s t-test was used to compare data between two groups. GraphPad Prism 3.0 software was used (GraphPad Software Inc., San Diego, CA, USA).

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**REFERENCES**


