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The Role of Apoptosis in the Antigen-Specific T Cell Hyporesponsiveness of Paracoccidioidomycosis Patients

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Paracoccidioidomycosis is a deep endemic mycosis associated with an antigen-specific immunodeficiency. To examine the role of apoptosis in this immunodeficiency, peripheral blood mononuclear cells (PBMC) of patients with paracoccidioidomycosis and controls were stimulated with the main antigen of *Paracoccidioides brasiliensis* (gp43) and an unrelated fungal antigen (from *Candida albicans,* CMA) and analyzed for annexin V and propidium iodide staining by flow cytometry. Control PBMC proliferated well with both antigens. Patients' PBMC proliferated only with CMA, but presented higher levels of apoptosis with gp43 and CMA than in their own unstimulated cultures. Moreover, gp43-triggered apoptosis in control PBMC was lower than in those of the patients. Thus, patient but not control gp43-stimulated T cells apparently remained anergized and subsequently underwent apoptosis. While CMA-induced apoptosis is likely triggered by activation-induced cell death, this is apparently not the case in gp43-induced apoptosis because of the lack of cell cycling and IL-2 in the gp43-stimulated cultures. However, higher IL-10 levels were found in gp43-stimulated patient PBMC cultures. Addition of a neutralizing anti-IL-10 antibody to the cultures resulted in increased apoptosis levels only in gp43-stimulated patient PBMC cultures. Our results suggest that apoptosis plays a role in the patients' antigen-specific hyporesponsiveness and that IL-10 may have an antiapoptotic role. © 2002 Elsevier Science (USA)

***Key Words: Paracoccidioides brasiliensis*; apoptosis; immunosuppression; paracoccidioidomycosis.**

INTRODUCTION

Paracoccidioidomycosis (PCM), the leading endemic deep mycosis in Latin America (1), is caused by the dimorphic fungus *Paracoccidioides brasiliensis*. It is generally accepted that the infection is acquired through inhalation of conidia produced by the mycelial phase of the fungus. Most people living in endemic areas acquire infection only, while a few will develop disease characterized by a chronic granulomatous inflammatory process. Such patients may present a spectrum of clinical manifestations, ranging from mucocutaneous lesions to visceral involvement, especially of the mononuclear–phagocytic system (1). PCM has an important social and economic impact as it affects mainly healthy men during their productive years, requires prolonged treatment, and, not infrequently, results in important sequelae (2).

In PCM, active disease is associated with profound imbalance of the anti-*P. brasiliensis* immune responses, whereas responses to an unrelated fungal antigen, from *Candida albicans,* are not infrequently preserved. The *P. brasiliensis* antigen-specific Th-1-type responses are down modulated, as illustrated by highly reduced IL-2, IL-12, and IFN-γ secretion and lack of lymphoproliferation in response to the main antigenic component of *P. brasiliensis* (3–6). Concurrently, Th-2-type responses are up regulated, especially in patients with severe, disseminated disease, and characterized by the presence of IL-10 and IL-4 secretion and exacerbated anti-*P. brasiliensis* antibody synthesis, particularly of the IgG4 and IgE anti-gp43 subclasses (4, 6–8). The mechanisms by which Th-1 responses are suppressed are not fully known, but we and others have provided evidence that, as in other chronic granulomatous diseases such as tuberculosis and leprosy (9, 10), IL-10 may play an important role (11, 12, and T. Costa and V. L. G. Calich, unpublished results). IL-10 has the ability to induce a prolonged state of anergy in T cells (13). On the other hand, it has also been recently shown that apoptosis of antigen-specific reactive T cells is also an important mechanism in anergy induction (14). The role of apoptosis in infectious diseases has been less well studied. Interesting data come from studies of malaria and schistosomiasis patients, in which *in vitro* challenge with parasite antigens led to apoptosis of mononuclear cells (15–17). In tuberculosis, it has been demonstrated that the mycobacteria has apparently advanced mechanisms that induce the infected monocytes to evade apoptosis, a mechanism pos-

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sibly implicated in the perpetuation of the infection (18). However, the possible connection between apoptosis and the inability of the immune system of patients with endemic mycoses to control fungal dissemination has not yet been addressed.

Therefore, in this paper, we examined the hypothesis that apoptosis might contribute to the Th-1 hyporesponsiveness of PCM patients. Our data support such an assumption, since peripheral blood mononuclear cells (PBMC) of patients with active untreated disease, but not of healthy PCM-cured individuals, display an impaired proliferative to the main antigenic component of the fungus response that is associated with higher percentages of apoptotic cells. The same did not occur when the control fungal antigen, from *C. albicans,* was used. We additionally provide evidence showing that IL-10 has the potential role of inducing protection against apoptosis.

MATERIAL AND METHODS

Patients and controls. Patients ($n = 23$) with newly diagnosed active PCM from the Instituto de Infectologia Emilio Ribas and the Hospital das Clínicas de São Paulo were studied. The diagnosis was established by identification of yeast forms of *P. brasiliensis* in clinical specimens and serology (Wilcoxon matched pairs test). The control group comprised 8 healthy individuals who had had PCM in the past and had been considered cured. These individuals were selected from the records of the Mycosis Out-Patient Unit of the Hospital das Clínicas de São Paulo and recalled. Only those who persisted as paracoccidioidin skin-test positive (>10 mm of induration) and had negative or low-titer serological results, indicating that they were free of active PCM, were recruited, as described earlier (19). None of these individuals had presented with a relapse since the end of treatment (>6 years before inclusion in this study) nor did they have signs or symptoms of any other debilitating disease at the moment of the study. The study was approved by the ethics committee of both institutions and informed consent was obtained from each subject before blood collection.

PBMC isolation and culture. PBMC were isolated from heparinized venous blood by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) gradient and resuspended in RPMI supplemented with gentamicin (40 μg/ml) and 10% pooled AB normal human serum, as previously described (3). PBMC (2.5×10^5/well) were cultivated in microculture flat-bottom plates (Costar, Cambridge, MA) at 37°C and 5% CO_2, in the presence of medium only or optimal concentrations of gp43 (20 μg/ml) and, as an unrelated fungal antigen from (*C. albicans*), CMA (10 μg/ml; Institut Pasteur, France), to which most individuals are sensitized (19). Purified gp43 was prepared from a *P. brasiliensis* culture filtrate as described (20). For lymphoproliferation assays, cells were incubated for 6 days and pulsed for an additional 18 h with 1.0 μCi/well [^{3}H]thymidine (Radiochemical Center, Amersham, UK) before harvest. Cell-bound radioactivity was measured using a β-plate scintillation counter (Wallac Oi, Turku, Finland). Results were expressed as mean counts per minute of triplicates. In some cultures a monoclonal anti-IL-10 antibody (10 μg/ml, mouse anti-human IgG1; Endogen, Cambridge, MA) was added on day 0.

IL-10 determination. IL-10 was quantified by ELISA as previously described (6). The antibody-matched pair and the respective standard were purchased from Endogen and used following the manufacturer's recommendations. The detection limit was 10 pg/ml. As prior kinetic studies indicated that IL-10 secretion occurs predominantly during the first days of culture, results from supernatant harvested on days 3–4 are presented.

Evaluation of apoptosis. Apoptosis was assessed by the binding of the protein annexin V to phosphatidylserine residues, a phospholipid of the inner side of the membrane that moves to the outer side at the initial stage of the apoptotic process, using a previously described protocol (21) modified from Vermes *et al.* (22). Briefly, PBMC from controls and patients were cultured as above, gently harvested from the wells at the indicated times, washed twice with cold PBS, and then resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at 10^6 cells/ml. An aliquot of the cell suspension was transferred to a 5-ml cytometer tube, and 5 μl of annexin V–FITC (BD Pharmingen, Los Angeles, CA) and 10 μl of propidium iodide (PI) (Sigma) at 50 μg/ml were added to each tube. Cells were gently vortexed and incubated in the dark for 15 min at room temperature, followed by the addition of 400 μl of binding buffer for analysis in the flow cytometer using the software System II (Coulter Epics XL-MCL). The results were expressed as the percentage of apoptotic cells per 10,000 counted cells. The apoptosis analysis from a representative patient is illustrated in Fig. 1. Viable cells were visualized in the lower left quadrant as cells that did not stain with either annexin V or PI, as previously reported (22). Cells that stained with annexin V only were considered to be at an early apoptotic stage and were seen in the upper left quadrant, whereas cells that stained with both markers were considered to be at a late apoptotic stage and were seen in the upper right quadrant. Necrotic cells, stained with PI only, were found in the lower right quadrant. Apoptotic cells could also be visualized in the PBMC forward-scatter and side-scatter dot-plot graphs by their reduced size and enhanced granularity compared to live cells (insets

of Fig. 1). Apoptosis was analyzed in the whole PBMC population. However, although detailed cell subtype determination was not performed, cells harvested and gated for apoptosis were mostly lymphocytes, with $<3\%$ $CD14^+$ cells (monocytes) and $<5\%$ $CD19^+$ cells (B cells), because most monocytes and B cells remained adhered to the bottom of the plates (unpublished observations).

Statistical analysis. Since percentages were not normally distributed, Wilcoxon signed-rank paired test was used to compare the apoptosis scores (%) in the different culture conditions among patients or controls. The Mann–Whitney test and the Kruskal–Wallis test with Dunn's posttest were additionally used to compare nonpaired continuous data between two and three sets of data, respectively. Data are presented as medians, 25th and 75th percentiles, and maximum and minimum values. Differences were considered significant when $P < 0.05$.

RESULTS

Apoptosis of gp43-stimulated PBMC. Previous results of kinetics studies with eight patients showed that the percentages of cells at early apoptosis stage ($annexin^+/PI^-$) increased from day 4 to 6 in CMA- and gp43-stimulated cultures (medians of 17.8% vs 23.3% and 14.6% vs 19.3%, respectively). In contrast, the percentages of early apoptotic cells did not increase in the unstimulated cultures (data not shown). These results are similar to those of Toure-Balde *et al.,* which showed that the number of apoptotic PBMC from malaria patients increased from day 3 to day 6 in cultures with parasite antigen (15). On the other hand, late apoptotic cells, i.e., those cells that stained $annexin^+/PI^+$, were always found in smaller numbers than early apoptotic cells (Fig. 1). These numbers increased from day 4 to day 6, but in similar fashion in stimulated and unstimulated cultures (data not shown). Thus, this cell death probably reflected a physiological process that occurs during cell culture independent of antigenic stimuli. As our aim was to analyze specifically the cell death associated with antigenic stimuli, we focused on and present results of the analysis of cells that were at the early apoptosis stage at day 6 (cells within upper left quadrant in Fig. 1). However, the overall differences observed between the different culture conditions or patient groups did not differ if the data below were presented as the sum of early and late apoptotic cells.

In patients with active, untreated PCM, both study antigens, gp43 and CMA, induced significantly higher levels of apoptosis (medians of 26.4%, $P = 0.0015$, and 26.1%, $P = 0.003$, respectively, Wilcoxon matched pairs test) than in unstimulated cultures (21.0%) (Fig. 2). In control individuals, while gp43-stimulated cultures showed percentages of apoptosis (14.8%) similar to those of unstimulated cultures (14.8%), higher levels were found in the presence of CMA (26.0%, $P = 0.031$) (Fig. 2). Interestingly, when patients and controls were compared, there was no significant difference in the levels of spontaneous and CMA-induced apoptosis ($P = 0.29$ and $P = 0.68$, respectively, Mann–Whitney test). In contrast, gp43-induced apoptosis was significantly higher in patients than in controls ($P = 0.045$).

Because apoptosis and cellular activation followed by proliferation may be related phenomena, we sought to measure the rate of proliferative responses in parallel cultures. In agreement with the notion of an antigen-specific immune deregulation in PCM (3, 11), PCM patients displayed significant lymphoproliferative responses to CMA but not to gp43 (Fig. 3). Conversely, the control group presented strong lymphoproliferative responses to both antigens that were significantly higher than those of the patients (Fig. 3). Thus, the high apoptosis scores with gp43 in patients' PBMC occurred mostly in the absence (or presence of low levels) of cell proliferation, whereas those observed in CMA-stimulated cultures were found in association with significant cellular proliferation.

Modulation of apoptosis by IL-10 neutralization. Several groups have recently demonstrated that IL-2-deprived lymphocytes can be rescued from apoptotic death by IL-10 (23–25). Since IL-10 is secreted in high amounts by PBMC from PCM patients, we decided to investigate whether it plays a role in the *in vitro* gp43-induced apoptosis.

First, we determined the levels of IL-10 secretion in the supernatants of PBMC cultures upon CMA and gp43 stimuli in this series of patients. In agreement with previous observations (6), we found a gp43-induced overproduction of this cytokine in patients compared with that induced by CMA (212.0 vs 50.0 pg/ml, $P < 0.05$, Kruskal–Wallis with Dunn's posttest) (Fig. 4). In contrast, gp43- and CMA-induced IL-10 secretion by PBMC from control individuals did not significantly differ (79.5 and 119.5 pg/ml, respectively, $P > 0.05$) (Fig. 4).

Next, we set up PBMC cultures as above with the addition at day 0 of a neutralizing anti-IL-10 antibody. Previous experiments with PBMC from three patients showed that addition of an isotype-matched control antibody had no effect on the apoptosis scores (CMA, 27.0% vs 27.3%, and gp43, 32.4% vs 31.3%). As shown in Fig. 5, IL-10 neutralization significantly increased the score of day 6 gp43-induced apoptotic cells in patients ($P = 0.0085$, Wilcoxon matched-pairs test). This increase was seen for some patients with CMA-stimulated and unstimulated cultures, but it was in general less marked and not statistically significant ($P = 0.080$ and 0.067, respectively) (Fig. 5). In the control

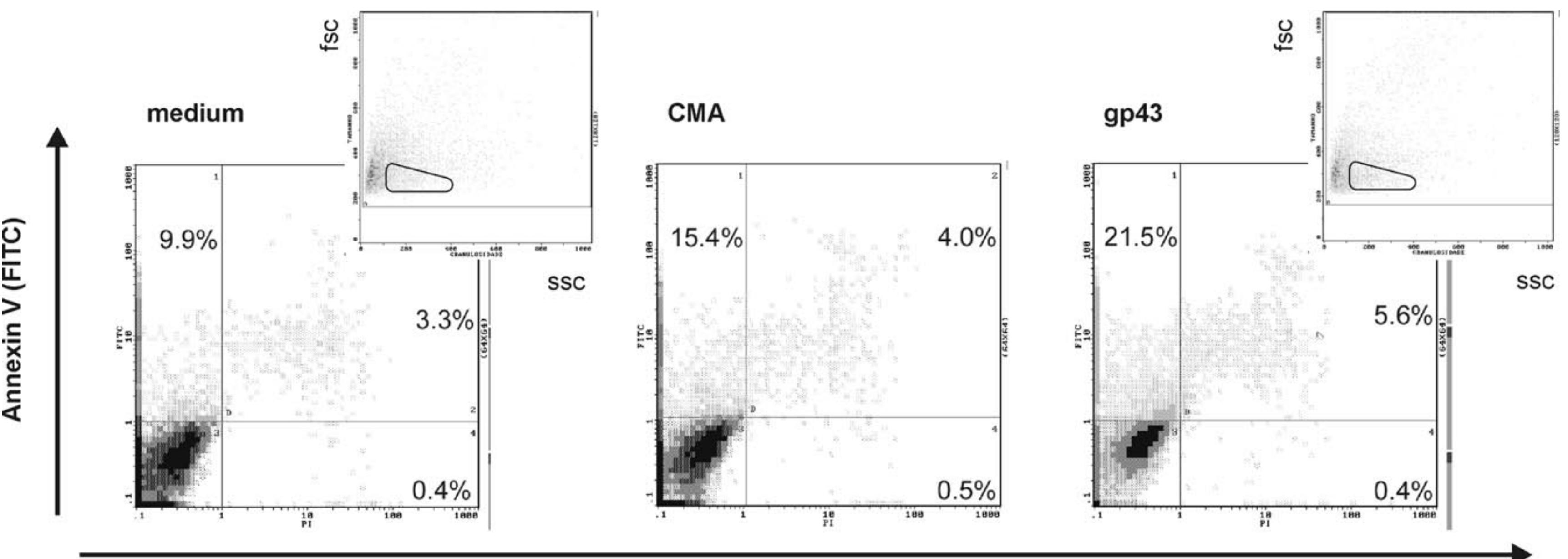

FIG. 1. Two-color annexin V–PI analysis of apoptosis in PBMC cultures from a representative patient with paracoccidioidomycosis stimulated for 6 days with gp43 or CMA or not stimulated (medium). The PBMC were harvested at day 6 as described under Material and Methods and the whole PBMC population was analyzed. The percentage of cells in early apoptosis (annexin V^+/PI^-, upper right quadrant) was higher in the gp43- and CMA-stimulated cultures than in the unstimulated culture. The percentages of cells at late apoptosis stage (annexin$^+/PI^+$, upper left quadrant) were lower but increased slightly in the stimulated cultures over the unstimulated culture. Note that the number of necrotic cells (annexin V^-/PI^+, lower right quadrant) was negligible in all three analyses. The two insets show the FSC × SSC dot plots of the PBMC population harvested from unstimulated and gp43-stimulated cultures. Within the gate are the cells that have migrated toward the right and down as they entered apoptotis, enhanced their granularity, and decreased their size.

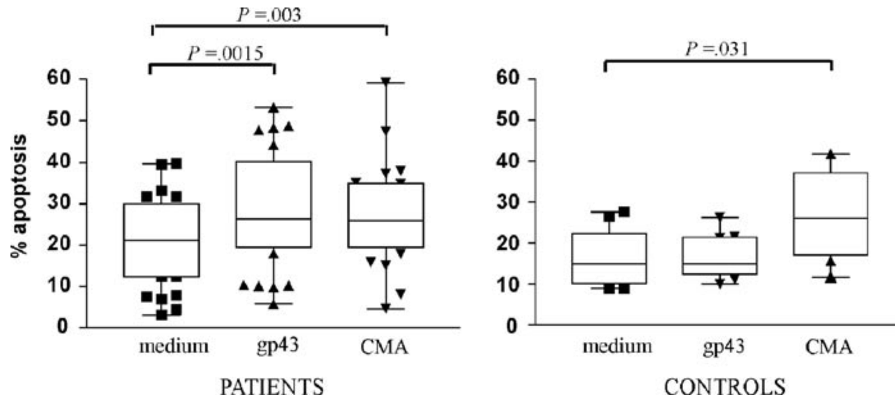

FIG. 2. Increased apoptosis rates in gp43- and CMA-stimulated cultures of PBMC from patients with PCM. gp43 (n = 23) and CMA (n = 20) stimulation significantly increased the percentage of apoptotic cells in patients' PBMC compared to that seen in cultures without stimulus (medium, n = 23) (Wilcoxon matched pairs test). In controls, CMA (n = 7), but not gp43 (n = 8), stimulation significantly increased the percentage of apoptotic cells compared to that seen in cultures without stimulus (n = 8).

group, on the other hand, there was no enhancement of the apoptotic scores in gp43- (P = 0.11), CMA- (P = 0.93), and unstimulated cultures (P = 0.85) (Fig. 5).

DISCUSSION

In this study, we verified that PBMC of patients with untreated, active PCM do not proliferate in response to the main antigenic component of *P. brasiliensis,* but, instead, a significant proportion of them (~26%) underwent apoptosis. This higher score of apoptotic cells was comparable to that observed in CMA-stimulated cultures, which, however, exhibited a high degree of lymphoproliferation. Unstimulated cells, in turn, did not proliferate, like the gp43-stimulated cells, but exhibited significantly lower apoptotic scores. We believe that understanding the role played by apoptosis under these different culture conditions may help to elucidate the mechanisms underlying the antigen-specific anergy of PCM patients.

Apparently, most gp43-stimulated cells of PCM patients remain anergized, i.e., do not undergo clonal expansion, but, instead, undergo apoptosis after 6 days of culture. Since gp43 elicits highly specific, non-cross-reactive, *in vitro* T cell responses (3), it is likely that the difference in cell death numbers between gp43-stimulated and unstimulated cultures represents

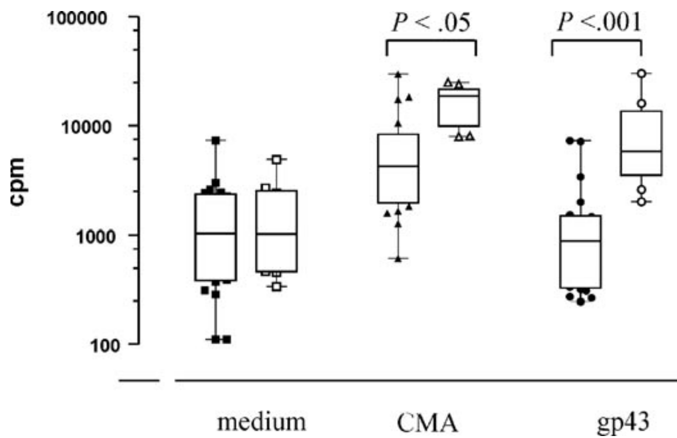

FIG. 3. Decreased lymphoproliferative response to gp43 of PCM patients as measured by [^{3}H]thymidine incorporation (counts per minute, cpm). Proliferative responses to gp43 (n = 22) and CMA (n = 16) of patients' PBMC (solid symbols) were significantly lower than those of controls (open symbols, n = 8) (Mann–Whitney test). With CMA, both patients' and controls' lymphoproliferative responses were above those of the respective unstimulated cultures (medium) ($P < 0.001$, Kruskal–Wallis with Dunn's posttest); with gp43, only the controls presented lymphoproliferative responses above those of the unstimulated cultures ($P < 0.05$).

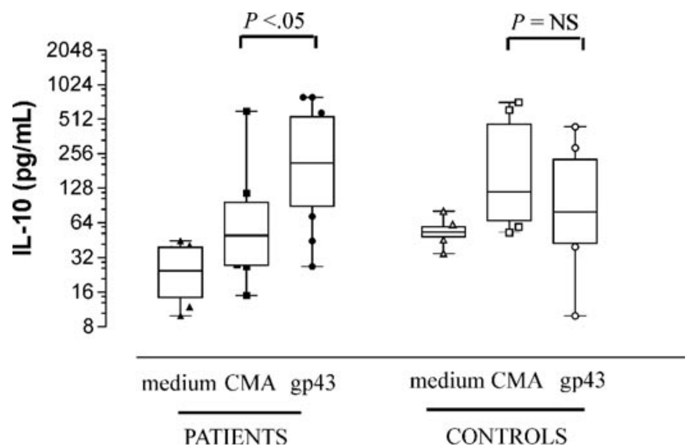

FIG. 4. IL-10 levels secreted by PBMC of patients with paracoccidioidomycosis and controls upon gp43 and CMA stimulation. IL-10 secretion induced by gp43 in patients (n = 12) was significantly higher than that induced by CMA (n = 10), whereas in controls (n = 8) both secretions were statistically comparable (Mann–Whitney test).

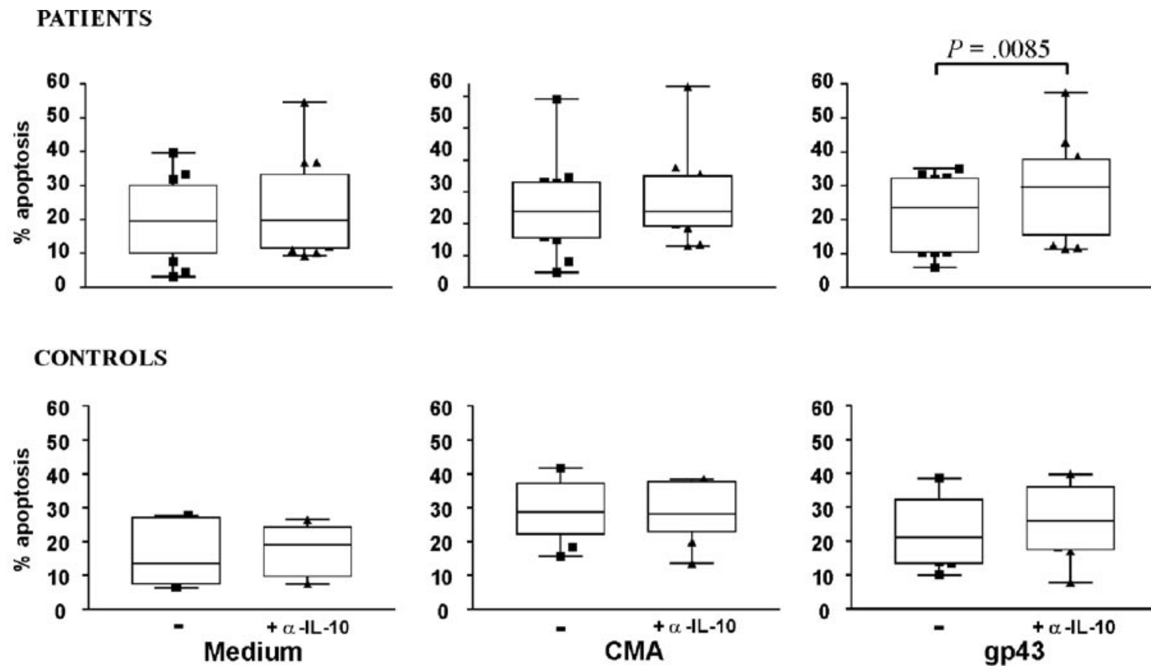

FIG. 5. IL-10 neutralization modulates the apoptosis induced by gp43 in PBMC of patients with PCM. IL-10 blockade by addition of an anti-IL-10 (α-IL-10) monoclonal antibody at day 0 increased the apoptosis percentages at day 6 of patients' PBMC cultured with gp43 (n = 14), but not with CMA (n = 13) or not stimulated (n = 14) (Wilcoxon matched pairs test). It also did not significantly affect the apoptosis percentages of control PBMC cultures (n = 7).

mainly the death of activated antigen-specific T cells and could account for the antigen-specific hyporesponsiveness of such patients. The mechanism by which these cells are driven to apoptosis remains elusive, but is apparently different from that of CMA-stimulated cells. According to Lenardo *et al.*, two main mechanisms trigger apoptosis of T cells during an ongoing immune response, activation induced cell death (AICD) and cytokine-deprivation-associated cell death (26). Recently, Usherwood *et al.* have shown that proliferation per se generates apoptotic cells at every cell division, thus suggesting an additional mechanism for T cell apoptosis (27). Cytokine-deprivation cell death seems an unlikely mechanism because the optimal time of proliferation in *C. albicans* antigen-stimulated cultures is in the range of 6 to 9 days (28). Rather, it is likely that apoptotic cell death seen in the context of CMA stimulation may be due in part to the intrinsic generation of apoptosis associated with cell division and in part to AICD. This is illustrated by the observation that, in the control group, the higher proliferation rates observed in CMA- (median, 18,870 cpm) vs gp43-stimulated cultures (median, 5317 cpm) occurred concomitant with the higher CMA-generated apoptosis levels.

In contrast, the mechanisms by which T cells die in gp43-stimulated cultures cannot be readily ascribed to classical AICD, because of the lack of IL-2 secretion and cell cycling in the PBMC cultures with gp43 (3, 6, and this paper), which are required for triggering AICD (26). It has recently been shown that the lack of IL-12 is an important factor leading to anergy in the context of high antigen load, through the induction of apoptosis of the antigen-specific T cells, and that this anergy is associated with highly reduced IL-2 and IFN-γ secretion and increased IL-4 secretion (14, 29). PCM was also shown to be associated with lack of IL-12, IFN-γ, and IL-2 secretion and, in severe cases, with enhanced IL-4 and IL-10 secretion (4–6, 11). Thus, it is tempting to suggest that the lack of IL-12 in our patients could also be leading to enhanced apoptosis of antigen-specific T cells. This hypothesis is consistent with our results showing that *in vitro* IL-12 addition to patients' PBMC cultures rescued the antigen-specific IFN-γ and lymphoproliferative responses to gp43 (11). Also in support to our hypothesis are the findings that Th-1 T cells are more prone to Fas-mediated apotosis than Th-2 T cells (30, 31) and that IL-12 has a regulatory role in preventing apoptosis of Th-1 T cells (32).

On the other hand, IL-10, a cytokine better known for its anti-inflammatory and Th-1 down-modulating properties, has paradoxically been shown to protect T cells from IL-2-starvation-induced cell death, an effect that was shown to be mediated by up regulation of Bcl-2 (25). In infectious mononucleosis, for example,

IL-10 was shown to rescue T cells from apoptotic death and to be crucial for the establishment of memory T cells and long-term immunity (33). IL-10 is actively secreted by gp43-stimulated PBMC, as shown here, but its role in T cell apoptosis in PCM has not so far been investigated. To address this question, we neutralized IL-10 activity by adding an anti-IL-10 antibody to the cultures. We were able to demonstrate an increase in the apoptotic scores over those of the same cultures without IL-10 neutralization (Fig. 4). Importantly, this augmentation was (a) specific for gp43-stimulated cultures, in that it was not found, at least at the same levels, in CMA-stimulated and unstimulated cultures, and (b) associated with active disease in that it was not seen in cured controls' cell cultures with gp43. Similar findings were reported in malaria patients, in which PBMC apoptosis induced by *in vitro* coculture with parasite antigen was enhanced by addition of neutralizing anti-IL-10 antibody (16). Thus our results suggest that IL-10 also acted by preventing even higher scores of cell death. This activity may be important because it may lead to part of the gp43-reactive lymphocytes of the patients remaining in an anergic state but not undergoing apoptosis, thus allowing them to be rescued to an active state by the addition of Th-1-driving cytokines such as IL-12 and IL-2, as previously demonstrated (11).

In conclusion, in this study we provided for the first time evidence that T cell apoptosis may be one of the mechanisms leading to the antigen-specific T cell anergy of patients with an endemic deep mycosis. Although the pathways triggering apoptosis were not determined in this study, the anergy of PCM patients resembles in many aspects that of the high-dose antigen-induced anergy in which extinction of the IL-12 signaling pathway is an important mechanism for enhancement of peripheral T cell apoptosis. In addition we report that, in addition to its immunosuppressive effects, IL-10 acts to prevent even higher *in vitro* apoptotic T cell death. This protective activity may allow the survival of a fraction of the antigen-specific T cells which can be restored to an active immunological state later on during the immune response in an appropriate cytokine environment. Further studies on the mechanisms of antigen-specific T cell apoptosis and anergy are warranted, as they might help to pave the way to alternative, immunobased, therapies of this still difficult-to-treat mycosis.

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