Altered Ex Vivo Expression of Caspase 8, Caspase 9, and Bcl-2 Is Associated with T-Cell Hyporeactivity in Patients with Paracoccidioidomycosis[▽]

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To better understand the T-cell hyporesponsiveness of patients with paracoccidioidomycosis, we tested the hypothesis that the T cells were committed to apoptosis. We show here that T cells of patients with paracoccidioidomycosis overexpress caspase 9 and caspase 8 but express low Bcl-2 levels and that interleukin-2 was unable to revert the hyporesponsiveness. These data suggest that the T cells would in vivo be driven to a tolerant state and apoptosis.

Paracoccidioidomycosis (PCM) is the most prevalent systemic mycosis in Latin America, with Brazil contributing the highest number of cases (1). Host resistance to *Paracoccidioides brasiliensis* relies on cell-mediated immune response, predominantly of the Th-l phenotype, and by the ability of the T cells to proliferate in response to fungal antigens and generate effector T cells (1). Several researchers have shown that T cells from patients with disseminated PCM have decreased proliferative responses and production of Th-1 cytokines, such as interleukin-2 (IL-2) and gamma interferon, in response to *P. brasiliensis* antigens, while individuals with cured, inactive disease exhibit high response levels (2). Thus, this defect likely underlies the failure of the host immune responses in controlling the infection (2, 14). The mechanisms responsible for this T-cell hyporesponsiveness are still poorly understood. In previous studies, we detected ex vivo overexpression of several costimulatory molecules in T cells and monocytes from patients with PCM, with most of these molecules downregulating T-cell activation, suggesting that these cells had been activated in vivo (16). In an attempt to revert this in vitro hyporesponsiveness, we carried out experiments in which engagement of these molecules was blocked with monoclonal antibodies specific to each of the molecules. However, this strategy failed to restore the proliferative responses (16).

Thus, to gain improved insight into this T-cell hyporesponsiveness, we tested in the present study the possibility that the T cells were committed to ex vivo apoptosis by measuring the intracellular levels of proteins involved with the apoptosis pathways, such as caspase 9, caspase 8, and Bcl-2, as well as the potential of IL-2 in rescuing this hyporesponsiveness. Caspase

9 and Bcl-2 are associated mainly with the intrinsic pathway, which is typically triggered by deprivation of growth factors, such as IL-2 (12). A lack or removal of growth factors leads to decreased expression of some antiapoptotic molecules (e.g., Bcl-2) and activation of other proapoptotic molecules (e.g., caspase 9), culminating in caspase 3 activation and cell death. The extrinsic pathway depends on the Fas/CD95/APO-1 pathway, which activates caspase 8 and caspase 3, bypassing the mitochondria (12). This pathway is preferentially triggered by repetitive antigen stimulation or by exposure to high antigen loads (11). The ex vivo expression of these proteins was quantified by flow cytometry (EPICS-XL; Beckman-Coulter) with cells isolated by a Ficoll-Hypaque gradient (Sigma-Aldrich, St. Louis, MO) from patients with an untreated, chronic active form (CF) of PCM (age range, 19 to 68 years) as well as from healthy subjects (age range, 24 to 58 years) who had been cured of the mycosis in the past (≥5 years before) (5) and healthy controls (age range, 25 to 56 years) with no past history of PCM or *P. brasiliensis* exposure. Diagnosis of PCM was made by demonstration of characteristic yeast forms of *P. brasiliensis* in biopsy, sputum, or smear specimens of lesions as well as by serological tests, as described previously (5). No patients had comorbidities that could affect their immune statuses. All donors gave written, informed consent. The study was approved by the ethics committees of the Hospital das Clínicas and Fiocruz. The T cells were stained with anti-CD3 monoclonal antibody (Tricolor, Invitrogen, CA) and for caspase 8 (phycoerythrin–IETD-FMK; Calbiochem, CA), caspase 9 (fluorescein isothiocyanate–LEHD-FMK; Calbiochem), or Bcl-2 (phycoerythrin–anti-Bcl-2; Pharmingen, CA). In fact, as shown in Fig. 1a, both caspases were expressed significantly more in ex vivo T cells from patients than in cured controls or healthy controls. There was no difference in the enhanced levels of caspase 8 and 9 in the patients, suggesting that both pathways were equally operative. As shown in Fig. 1b, Bcl-2 expression was significantly reduced in patient T cells

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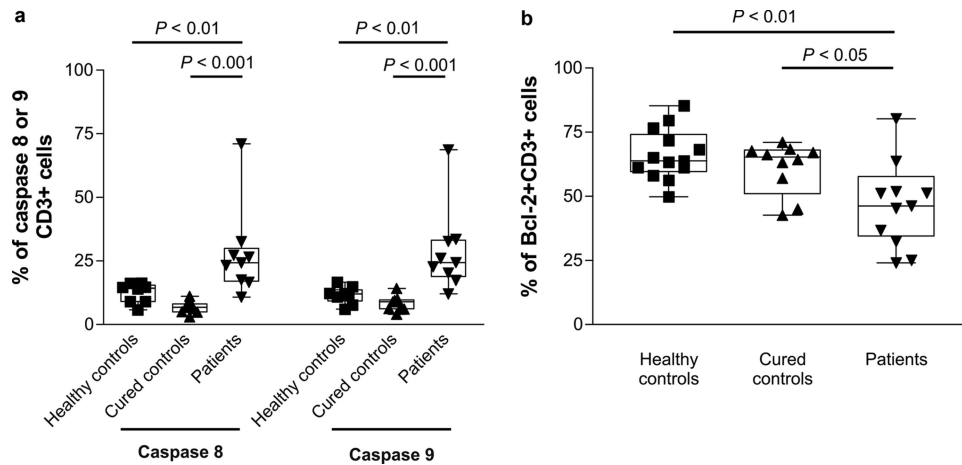
FIG. 1. Ex vivo intracellular expression of active caspase 8 and caspase 9 (a) and Bcl-2 (b) in $CD3^+$ cells from patients with untreated CF PCM ($n = 9$ and 11, respectively), healthy subjects cured of PCM ($n = 8$ and 10, respectively), and healthy subjects ($n = 8$ and 13, respectively), as evaluated by flow cytometry. The Kruskal-Wallis test with Dunn's posttest was used.

compared with the levels for cured subjects and healthy controls. The last two groups exhibited the same levels of Bcl-2 expression. Additional experiments were done with *Candida* metabolic antigen (CMA) and gp43-stimulated peripheral blood mononuclear cell (PBMC) cultures to assess Bcl-2 and caspase 8 and 9 levels. Bcl-2 persisted at lower levels in patients than in cured controls, irrespective of the stimulus, while caspase 8 and 9 expression levels were not further increased in patient cells but were increased in control cells, reaching levels comparable to those in patient cells (data not shown).

Patients with PCM also present profound downmodulation of *P. brasiliensis*-specific Th-1 responses, including IL-2 production (3). Deprivation of growth factors, such as IL-2, accounted for cell death due to activation of the intrinsic pathway in other systems (4) and was implicated in the activation of this pathway in patients with PCM. To address this issue, we performed experiments where IL-2 was exogenously added to PBMC cultures stimulated with the *P. brasiliensis* immunodominant antigen (a 43-kDa glycoprotein) and a control fungal antigen from *Candida albicans* (CMA; Bio-Rad, Marnes-la-Coquette, France). The 6-day proliferative responses and apoptosis rates in cells from untreated patients with CF PCM and cured controls were measured by [³H]thymidine incorporation and annexin V staining, respectively, as previously described (6). Figure 2a and b show that in patients, IL-2 (GE Healthcare, NJ) was ineffective in both consistently redressing the low proliferative response and reducing the apoptosis rate induced with gp43. IL-2, however, increased the proliferative response to CMA in six of seven patients tested. No IL-2 effect was seen in parallel assays with cells from cured controls for either the apoptosis rate (medians of 17.2% [gp43] versus 18.4% [gp43–IL-2] annexin V-positive cells and 25.9% [CMA] versus 21.8% [CMA–IL-2] annexin V-positive cells; $n = 6$) or the proliferative responses (5,068 cpm [gp43] versus 4,933 cpm [gp43–IL-2] and 18,230 cpm [CMA] versus 21,060 cpm [CMA–IL-2]; $n = 4$).

There are very few reports on the participation of caspases 8 and 9 in T-cell apoptosis during chronic infectious diseases. One possible mechanism for apoptosis induction in fungal infections was recently described by Monari et al. (13), who

showed increased Fas-L expression by monocytes loaded with the capsular polysaccharide of *Cryptococcus neoformans*. These cells induced apoptosis in cocultured Jurkat cells by a mechanism involving mainly the intrinsic pathway, with early (24 h)
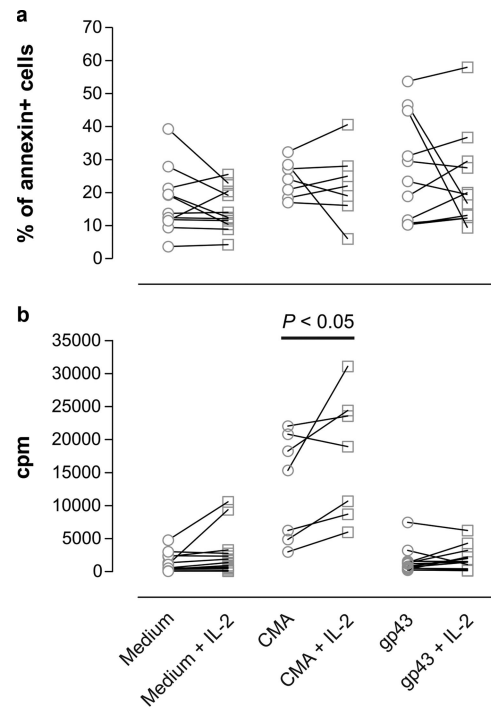

FIG. 2. Effect of IL-2 (5 IU/ml) on the apoptosis rates (a) and proliferative responses (b) of PBMC from patients with untreated CF PCM stimulated with gp43 from *P. brasiliensis* ($n = 10$ and 14, respectively) and CMA ($n = 6$ and 7, respectively) or not stimulated ($n = 10$ and 14, respectively). For apoptosis, the cells were harvested on day 4 of culture, stained with annexin V and propidium iodide, and analyzed on the $CD3^+$ cell gate by flow cytometry. Only cells annexin V positive (annexin+) and propidium iodide negative were considered apoptotic. For the proliferative responses, cells were pulsed with [³H]thymidine on day 5 and harvested on day 6, and the counts per minute (cpm) were measured with a beta counter. The Wilcoxon signed-rank test was used.

enhancement of caspase 8. Caspase 9 also peaked, albeit later (on the fourth day), suggesting a possible cross talk between the two pathways. However, it was recently demonstrated that this cross talk was absent in human CD4$^+$ cells, being restricted to human CD8$^+$ cells (15). In this context, we have previously shown that, in patients with PCM, the T cells that undergo apoptosis are mostly CD4$^+$ (7). We then argue that in PCM, the two pathways are independently activated. In fact, in patients with (bacterial and fungal) sepsis, not only did the lymphocytes present enhanced apoptosis rates, but also both caspase 8 and 9 were overexpressed, while Bcl-2 levels were reduced (10). The persistently high levels of *P. brasiliensis*-circulating antigens that are detected in patients with PCM (1, 17) may well be responsible for the activation of the extrinsic pathway, possibly via Fas-L. Enhanced ex vivo Fas-L expression has previously been reported to occur in patients with PCM (8). These and previous results suggest that the ex vivo T-cell anergy of patients with PCM occurs despite in vivo activation (as demonstrated by overexpression of several costimulatory molecules [5]) and leads preferentially to commitment to apoptosis instead of development of effector functions. Recently, important distinctions were made between the anergy observed in vitro and that observed in vivo (9). The in vivo phenomenon was recognized as an adaptive tolerance, with lack of IL-2 synthesis and proliferative response upon T-cell-receptor stimulation, which, unlike the in vitro phenomenon, cannot be restored by IL-2 replacement and requires persistence of the antigen in the microenvironment for its maintenance. Prior and present data suggest that anergic T cells in chronic disseminated PCM behave as adaptively tolerant. Further supporting this notion is the observation that the proliferative and cytokine responses tend to normalize with treatment, paralleling the gradual clearance of fungal antigens (3).

These data raise two hypotheses that warrant further investigation. The first is that rather than being regarded as unwanted result, the adaptive tolerance may represent the best commitment between the host and the parasite when the host is unable to eliminate the parasite, as we suggest for PCM. Thus, in the tolerant state, the host's inflammatory response, which can otherwise be harmful to the host, is set off. Second, it is tempting to speculate that, for chronic granulomatous infections with persistent antigen exposure, like PCM, vaccine protocols based solely on providing new immunogenic molecules may prove insufficient to revert the tolerant state. Rather, providing appropriate adjuvants or antigen-presenting cells, like dendritic cells loaded with the candidate antigens, which could disrupt the tolerant state, would be necessary to induce potent protective immune responses.

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