Immunosuppression in Paracoccidioidomycosis: T Cell Hyporesponsiveness to Two *Paracoccidioides brasiliensis* Glycoproteins that Elicit Strong Humoral Immune Response

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To assess human cellular immune response to paracoccidioidomycosis (PCM), lymphocyte proliferative responses to purified antigens from *Paracoccidioides brasiliensis* were determined in healthy persons previously infected by the fungus (positive donors), in healthy noninfected persons (controls), and in PCM patients. Affinity-purified gp70 and gp43, the two major antigens in humoral immune responses, were used. Both induced lymphocyte proliferation (gp43 species-specific) in positive donors but not in controls; healthy persons previously infected by *Histoplasma capsulatum* reacted to gp70 and not to gp43. A similar cross-reactivity in antibody response to gp70 was previously reported; however, antibody response to gp43 has been considered specific. Lymphocytes from PCM patients, who, unlike positive donors, have high levels of anti-gp43 and anti-gp70 antibodies, proliferated poorly with gp70 and gp43 but better with other stimuli. This dichotomy between humoral and cellular antigen-specific responses suggests a Th2 immune response in PCM, which may be related to failure to control the infection.

Paracoccidioidomycosis (PCM), which is caused by the dimorphic fungus *Paracoccidioides brasiliensis,* is the most frequent deep mycosis in Latin America involving previously healthy subjects [1]. Patients may present with a spectrum of clinical manifestations, ranging from localized mucocutaneous lesions to widespread visceral involvement, especially of the mononuclear-phagocytic system. PCM has an important social and economic impact. Most patients are adult male agricultural workers with low economic status, and although less frequent, the disease in children is generally severe and disseminated.

The clinical heterogeneity of PCM has been correlated with the intensity of immune impairment. The cellular immunity, considered the main defense mechanism in PCM [1], has been extensively studied in murine models. However, the interaction between *P. brasiliensis* and the cellular immune response in humans remains poorly studied. We recently described a cellular immune hyporesponsiveness to a *P. brasiliensis* cell wall extract in patients [2, 3]. However, this crude insoluble cell wall preparation showed significant cross-reactions, particularly with *Histoplasma capsulatum* [4], and could not be further purified. We therefore investigated lymphocyte proliferative responses to two glycoproteins from *P. brasiliensis* (43 and 70 kDa) previously shown to be major antigens in human humoral immune responses [5, 6]. We first evaluated the immunogenicity and specificity of the responses in healthy sensitized and nonsensitized donors and then compared the responses to those in PCM patients with different clinical presentations.

Materials and Methods

Healthy donors and patients. We studied 3 groups of healthy donors and 1 group of patients. Group 1 was 12 healthy *P. brasiliensis*–sensitized persons (Pb-positive; age range, 30–59 years) selected from persons who had PCM in the past who had been considered cured. All had completed antifungal treatment 4–18 years before this study and were free of signs of PCM and other debilitating diseases. All had strongly positive paracoccidioidin skin tests (>10 mm induration) and negative histoplasmin tests. Group 2 (n = 10) were *H. capsulatum*–positive (Hc-positive; age range, 20–50 years) with positive (>10 mm induration) histoplasmin skin tests and negative paracoccidioidin tests. These subjects were urbanites whose hobby was speleology. Group 3 (n = 12) were laboratory personnel with negative paracoccidioidin and histoplasmin skin tests (controls; age range, 24–48 years). The PCM patients (n = 20; age range, 11–58 years) were studied before or within 15 days of treatment. Five presented with acute PCM, 5 had chronic localized PCM, and 10 had chronic disseminated dis-

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ease. PCM is considered more severe in the acute or chronic disseminated forms [1]. The 4 groups did not differ in age (Kruskal-Wallis test, $P > .05$).

Preparation of antigens. *P. brasiliensis* antigen (PbAg) is a cell wall crude insoluble extract prepared and standardized for lymphocyte proliferation assays as previously described [4]. Purified gp70 and gp43 solutions were obtained from, respectively, a crude exoantigen preparation of *P. brasiliensis* (113 FMUSP) and a cell-free *P. brasiliensis* suspension as previously described [6, 7]. gp43 and gp70 were fractionated by affinity chromatography in columns of protein A–purified rabbit anti-gp43 IgG and anti-gp70 IgG coupled to CNBr-Sepharose. The fractions eluted from these columns were concentrated; further purification was achieved by gel filtration in a Sephacryl column to eliminate high-molecular mass contaminants. The molecular masses of the two fractions was estimated by SDS-PAGE. The presence and purity of gp43 and gp70 molecules were further monitored by SDS-PAGE, silver staining, and immunoblotting. In addition, purified gp43 was treated with periodate as follows: Sodium metaperiodate (Sigma, St. Louis) was diluted in acetate buffer, 50 m*M,* pH 4.5, and added to the purified gp43, resulting in a final concentration of 5 m*M.* The solution was left at 28°C for 10 min. An equimolar amount of glycerol was added for another 10 min to consume the remaining periodate. The solution was then dialyzed against PBS at 4°C and concentrated to the initial volume with polyethylene glycol. Paracoccidioidin and histoplasmin were prepared and used in skin tests as described [4].

Lymphocyte proliferation assays. Mononuclear cells were isolated from heparinized venous blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) and resuspended in RPMI supplemented with gentamicin (40 μg/mL) and 10% pooled AB normal human serum as previously described [3]. Cells (2×10^5/well) were cultivated in triplicate in microculture flat-bottom plates (Costar, Cambridge, MA) at 37°C with 5% CO_2 in the presence of medium only or optimal concentrations of pokeweed mitogen (PWM; 5 μg/mL; Sigma), *Candida albicans* metabolic antigen (CMA; 1 μg/mL; Institut Pasteur, Paris), PbAg (75 μg/mL), and differing concentrations of gp70 (0.01–50 μg/mL) and gp43 (0.01–100 μg/mL). Cells were incubated for 6 days and pulsed for an additional 18 h with 0.5 μCi/well [^{3}H]thymidine (2 mCi/m*M;* Radiochemical Centre, Amersham, UK) before harvest. Cell-bound radioactivity was measured by scintillation beta counter (model LS3150T; Beckman, Palo Alto, CA). We calculated the mean counts per minute of triplicate results and expressed the results as the difference between the counts per minute of stimulated and nonstimulated cultures.

Statistical analysis. The Kruskal-Wallis test with Dunn's post test was used to compare ≥ 3 groups. Student's *t* test was used to compare patient and healthy *P. brasiliensis*–sensitized groups.

Results

Proliferative responses of lymphocytes from healthy donors. The gp43 and gp70 used in this study were affinity-purified from *P. brasiliensis* preparations. Each specific fraction emerged from the chromatographic column as one peak, and SDS-PAGE showed distinct bands of 43 and 70 kDa, indicating the homogeneity of the fractions (figure 1A). A single distinct band was also obtained when the fractions were studied by immunoblotting with a pool of PCM patient sera (figure 1B, lane d) and with rabbit hyperimmune sera (figure 1B, lanes a–c).

Sera from the 3 groups of healthy donors were tested with a range of concentrations of gp70 and with control antigens (CMA, PbAg) and PWM. Responses to PWM and CMA, as well as background proliferation, were comparable among the 3 donor groups (data not shown). Pb- and Hc-positive donors, as expected, presented strong and comparable responses to PbAg (mean ± SE, 19,958 ± 3112 and 14,626 ± 2371, respectively), whereas controls did not respond. Both Pb- and Hc-positive donors reacted strongly and equally to the higher concentrations of gp70 tested (respectively, 7748 ± 1197 and 6651 ± 2197 [50 μg/mL]; 7057 ± 1425 and 6233 ± 2243 [10 μg/mL]) and more discretely or not to the lower concentrations (not shown). In contrast, controls did not react to any concentration.

In preliminary experiments, gp43 failed to induce a proliferative response in its untreated form. We then used a periodate-treated gp43. Periodate treatment was used to oxidize its carbohydrate chains and abrogate its enzymatic-associated activity [8]. gp43, in contrast to gp70, induced proliferation only with lymphocytes from Pb-positive donors. Responses of compara-

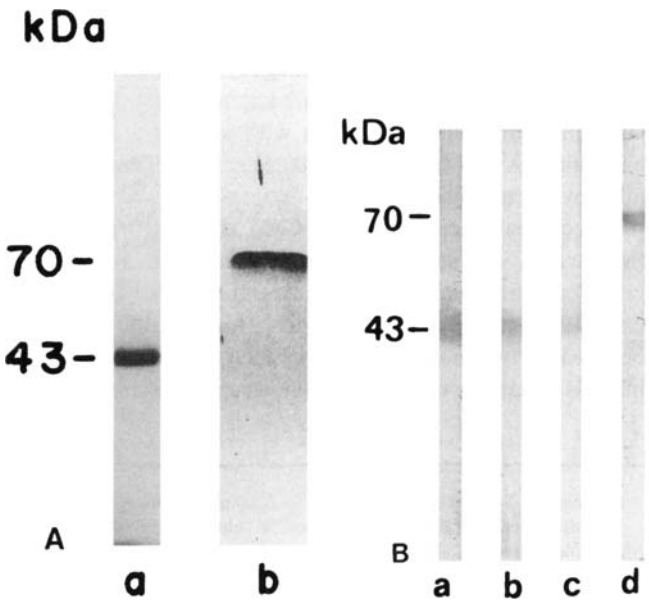

Figure 1. **A**, SDS-PAGE and silver staining of gp43 (lane a) and gp70 (lane b) solutions used in proliferation assays. **B**, Immunoblot analysis of gp43 and gp70 solutions probed with hyperimmune and patient sera; lane a, untreated gp43 probed with rabbit anti–*P. brasiliensis* culture filtrate serum; lane b, untreated gp43 probed with rabbit anti-gp43 serum; lane c, periodate-treated gp43 probed with rabbit anti-gp43 serum; and lane d, gp70 probed with pool of sera from patients with paracoccidioidomycosis.

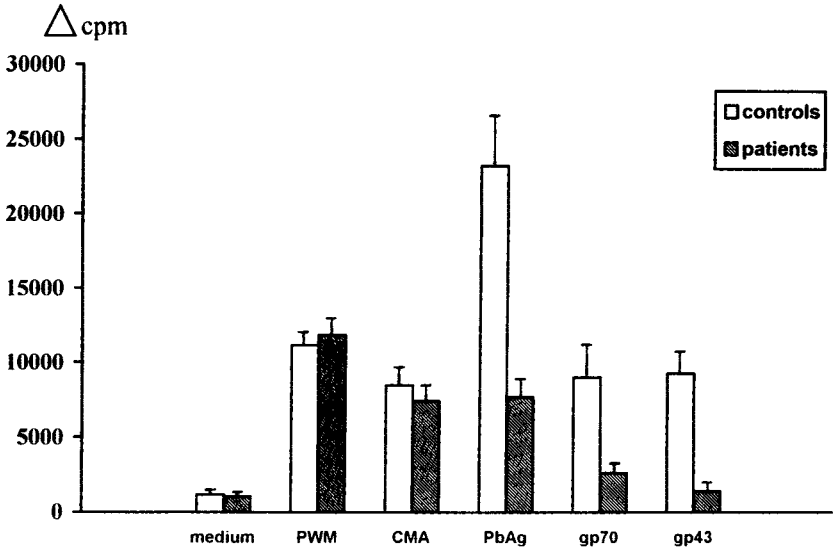

Figure 2. Proliferative responses of lymphocytes from healthy *P. brasiliensis*–positive donors (controls; $n = 9$) and from patients with paracoccidioidomycosis (patients; $n = 17–20$) with medium only, pokeweed mitogen (PWM), *Candida* metabolic antigen (CMA), and *P. brasiliensis* cell wall extract (PbAg), gp43 and gp70. Results (except for medium) are in change in cpm.

ble magnitude to gp70 were obtained with 100 (11,639 ± 1211) and 10 μg/mL (8784 ± 1784).

Reproducibility and stability of the responses were checked by retesting 4 Pb-positive donors at intervals of 4–16 months with the same range of concentrations of gp70 and gp43. Results were comparable to those of the first assay (data not shown).

Proliferative responses of lymphocytes from PCM patients. Results of assays with lymphocytes from PCM patients and Pb-positive donors (controls) are illustrated in figure 2. Spontaneous and PWM- and CMA-induced responses of patients' lymphocytes was similar to that of Pb-positive donors ($P > .05$, Student's t test). In contrast, patient responses to PbAg, gp70, and gp43 were significantly lower (gp70, $P = .002$; PbAg and gp43, $P < .001$).

The different clinical forms of PCM may reflect different underlying immune mechanisms [1]. In figure 3, which shows responses of patients with the different clinical forms, lymphocytes from most patients (~60%), regardless of the clinical form, proliferated in response to PWM and CMA with levels comparable to those of controls (usually >10,000 cpm for PWM and ≥5000 for CMA). In fact, there was no statistical difference in the response to these stimuli among the clinical forms. In general, patients with low responses to the *P. brasiliensis*–nonrelated stimuli were in poor clinical condition but did not necessarily have more severe fungal disease (as previously reported [3]). In contrast, reactivity to PbAg differed by clinical form ($P < .05$); patients with the chronic, localized, less severe form had higher responses (>10,000 cpm). Of note were the very low responses (<2500 cpm) to gp43 in 16 of 19 patients tested and the low responses (<4000 cpm) to gp70 in 13 of 17 patients tested, regardless of clinical form. However, patients' lymphocyte proliferation induced by gp70 was significantly higher ($P < .05$) than that induced by gp43, whereas lymphocytes of healthy Pb-positive sensitized donors proliferated equally with both (figure 2).

Discussion

We demonstrated that both gp43 and gp70 elicited lymphocyte proliferative responses in healthy *P. brasiliensis*–sensitized donors but not in nonsensitized persons. However, only gp43 generated species-specific responses, since gp70 was also recognized by *H. capsulatum*–sensitized donors. Anti-gp70 antibodies have also been detected in sera of persons with histoplasmosis [5]. Polysaccharide components, the predominant components in gp70, may contain epitopes responsible for that cross-reactivity. However, the strong cross-reactivity in the cellular immune responses suggests the presence of cross-reactive epitopes also in polypeptide components of the molecule. Some cross-reactivity with gp43 was also detected in sera from patients with other mycoses, particularly histoplasmosis [6, 7, 9]. This cross-reactivity was abrogated by oxidation or enzymatic digestion of the polysaccharide components of the molecule, suggesting that mainly peptide epitopes are responsible for the species-specific reactivity of the antibody response [9]. According to our results, species-specific T cell epitopes are also present in this molecule. Whether the T and B cell epitopes are overlapping remains to be determined. In addition, our unpublished observations using flow cytometry showed that gp43 and gp70 proliferating cells were mostly CD4.

Analysis of T cell responses to gp43 in PCM patients is useful because persons may be exposed to both *H. capsulatum* and *P. brasiliensis*. This increases the need for antigens that discriminate between the 2 pathogens. In addition, in several patients, we observed that hyporesponsiveness to PbAg recovered relatively early, much before the initial high-dose treatment could be replaced by maintenance therapy ([10], unpub-

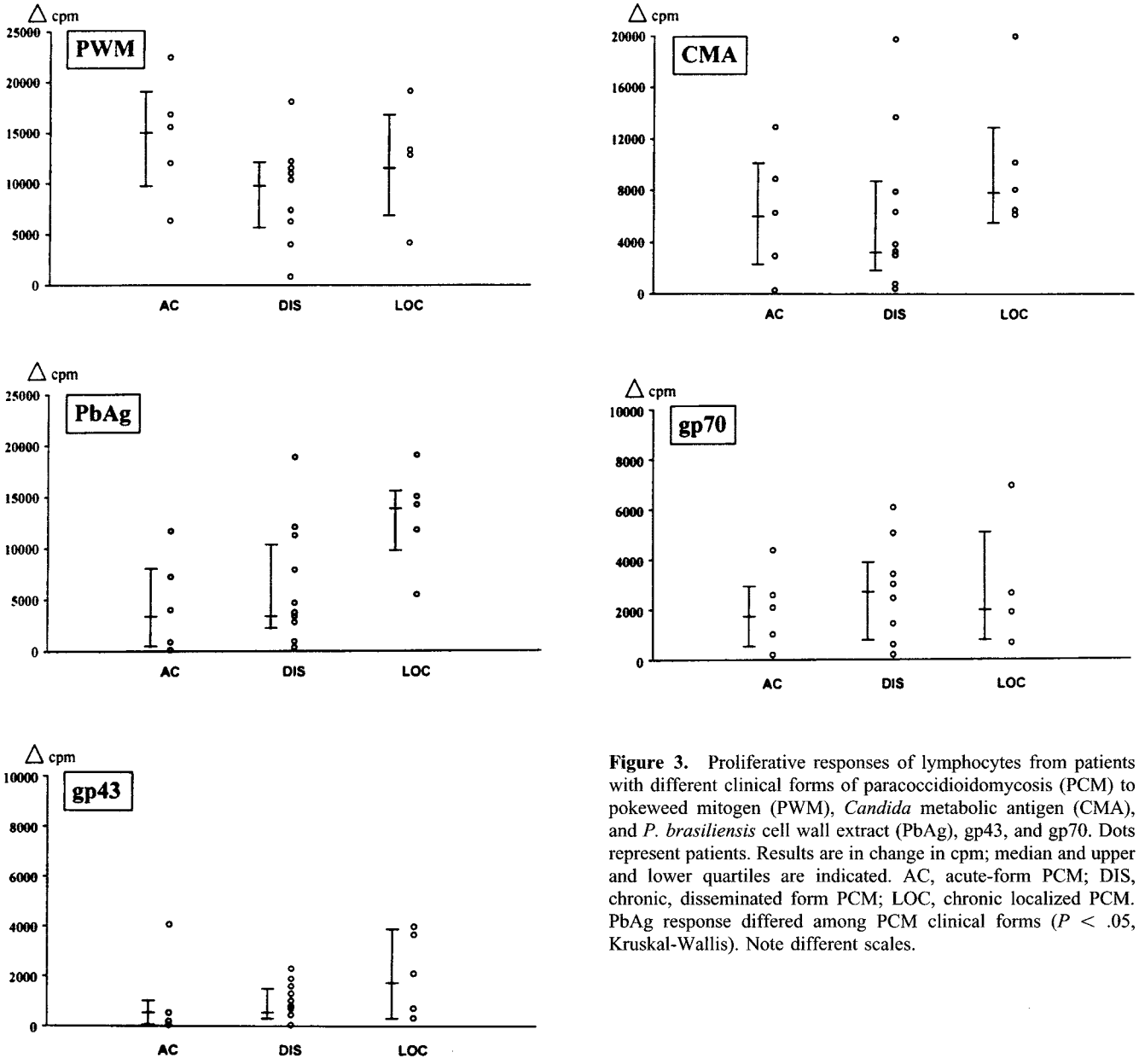

Figure 3. Proliferative responses of lymphocytes from patients with different clinical forms of paracoccidioidomycosis (PCM) to pokeweed mitogen (PWM), *Candida* metabolic antigen (CMA), and *P. brasiliensis* cell wall extract (PbAg), gp43, and gp70. Dots represent patients. Results are in change in cpm; median and upper and lower quartiles are indicated. AC, acute-form PCM; DIS, chronic, disseminated form PCM; LOC, chronic localized PCM. PbAg response differed among PCM clinical forms ($P < .05$, Kruskal-Wallis). Note different scales.

lished observations). Therefore, the use of gp43 to assess cellular immunity of PCM patients (e.g., to determine the proper time to change from initial to maintenance treatment) warrants further study. Furthermore, virtually nothing is known about the factors related to the susceptibility or resistance of persons from areas in which PCM is endemic who have been exposed to the fungus. Since most patients were previously healthy and can be cured with prolonged treatment, the nature of the immunologic failure seems predominantly antigen-specific.

The PCM patients studied showed significant cellular immune hyporesponsiveness to gp43 and gp70; their lymphocyte responses to gp43 were even more depressed than to gp70. Although some patients responded to the crude cell wall extract (PbAg), mean responses of patients were significantly lower than those of healthy Pb-positive donors. In contrast, mean responses to the other *P. brasiliensis*–nonrelated stimuli, PWM and CMA, were comparable to those of the healthy Pb-positive group, similarly to results in previous studies [3]. The role of these low responses in the immunopathology of PCM is not known. Nonetheless, our data emphasize the dissociation between *P. brasiliensis*–specific humoral and cellular immune responses: Virtually all patients with active disease present with high levels of anti-gp70 and anti-gp43 antibodies [5, 6, 9]. This fits well with the current view of the immune response as two nonoverlapping patterns of cytokine secretion: Th1,

which favors T cell–mediated responses, and Th2, which favors humoral responses. According to this hypothesis, PCM patients with active disease would develop a predominant Th2 immune response to fungal antigens. Indeed, PCM patients usually present with eosinophilia, hypergammaglobulinemia, and high levels of specific IgE antibodies, characteristic features of a Th2 immune response. On the other hand, it was recently suggested that proinflammatory cytokines may also participate in the immunoregulatory disturbances of PCM patients [11].

Alternatively, it is also possible that most gp43- or gp70-reactive T cell clones are not present in the peripheral blood of PCM patients when the disease is active, instead being recruited to the inflammatory sites and resulting in peripheral hyporesponsiveness. This mechanism has been described in cutaneous leishmaniasis [12], but this is less likely in PCM, because high levels of antigenemia are detected in patient sera [13], enabling the local generation of T cell–reactive clones. In fact, we frequently observe blast cells in peripheral blood smears of patients.

In conclusion, PCM patients' immune reactivity to 70- and 43-kDa *P. brasiliensis* glycoproteins shows a strong, but nonprotective, antibody response and an inadequate T cell response, possibly indicating the mechanisms underlying the immunopathogenesis of PCM.

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