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Reactivity of Antibodies From Patients With Acute and Chronic Paracoccidioidomycosis to a High Molecular Mass Antigen From *Paracoccidioides brasiliensis*

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Yeast forms of *Paracoccidioides brasiliensis* produce polydispersed high molecular mass (h-MM) antigens. We investigated the antibodies to an h-MM antigen from *P. brasiliensis* by immunoblotting and ELISA in sera from paracoccidioidomycosis (PCM) patients. IgG from the sera of chronic PCM patients was able to recognize the h-MM antigen at a higher frequency in the cell-free antigen (CFA) (8/13) than in the somatic antigen (SA) (2/13), as assessed by immunoblotting. The CFA was fractionated by Sephadex G-200 chromatography, and fraction 17 (F17) with the h-MM antigen of approximately 366 kDa was used in ELISA to analyze specific levels of IgG and IgE. Patients with the chronic form showed significantly higher levels of IgG ($P < 0.05$) but not IgE ($P > 0.05$) to F17 by ELISA, compared to patients with the acute form or to healthy donors. In conclusion, CFA is better than SA as a source of the *P. brasiliensis* h-MM antigen. This study reveals a new characteristic to differentiate between the acute and chronic forms of PCM, by demonstrating a higher level of seric IgG to h-MM antigen in chronic compared to acute PCM patients. J. Clin. Lab. Anal. 19:199–204, 2005.
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INTRODUCTION

Paracoccidioidomycosis (PCM), which is caused by the dimorphic fungus *Paracoccodioides brasiliensis*, is one of the most important systemic mycoses in Latin America (1,2). Airborne fungal propagules consisting of conidia or hyphal fragments begin the infection. They then convert to the yeast phase (the infective stage of *P. brasiliensis*) in the lungs, and progress to hematogenic or lymphatic dissemination to the liver, spleen, skin, and mucosa (3). Both acute (AF) and chronic (CF) forms of the disease are distinguished (4). The AF is rarer and more severe. It is characterized by a rapid course and involvement of the reticuloendothelial system that leads to lymph node enlargement and hepatosplenomegaly. The CF occurs more frequently in adult males who are involved in agricultural work (4,5). This form of the disease may be developed in multiple forms, ranging from benign and localized (unifocal) to severe and disseminated (multifocal), depending on the depression degree of cellular immunity (4–6).

Epidemiological studies using paraccocidioidin skin tests (7) or ELISA to detect IgG antibodies (8) have shown that exposure resulting in transient subclinical infection is high in adult populations, mainly in rural areas. The asymptomatic infection may persist in

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quiescent foci for decades, as observed in imported cases such as in patients that lived in or visited the endemic countries and developed the disease after returning to the non-endemic area (4,9). This indicates that during such periods, patients are able to control the fungal dissemination.

The cellular immune response is considered the most important mechanism for host defense (10). Patients with disseminated infection show a negative paracoccidioidin skin test, as well as poorly defined granulomatous reactions (11). Depression of cellular immunity in PCM may be associated with a Th2 immune response based on the IgE and IgG class or subclass present, and the pattern of development of the cytokines. In disseminated or severe PCM, high levels of total IgE or specific IgE to 43 kDa glycoprotein (gp43), IL-4, and IL-5 may be observed (11–14).

Puccia et al. (15) showed that the glycoproteins of 43 kDa and 55 kDa and a polydispersed high molecular mass (h-MM) with heterogeneous electrophoresis migration are more regularly expressed in crude *P. brasiliensis* antigen. The authors considered gp43 to be an important factor for diagnosis, and discussed the potential importance of gp55 and polydispersed h-MM glycoprotein, which on being excreted into the exocellular environment in vivo could interfere with the host defense mechanisms and eventually influence the course of infection. Considering that gp43 (14,16,17) induces IgE immune response, and gp55 is not immunogenic in humans (15), we chose the h-MM glycoprotein to study the pattern of humoral immune response that it induces.

It is well established that induction of the IgE isotype indicates a Th2 immune response and is not important for intracellular pathogens. Moreover, the Th2 cytokines may depress the Th1 response (17). The components of *P. brasiliensis* that modulate the Th1 or Th2 immune response may be important subjects for investigation. One simple way to indicate the presence of Th2 modulation is to determine the specific IgE isotype (18), which may be helpful in determining the evolution of the disease.

Initially we were interested in evaluating the reactivity of the IgG to the *P. brasiliensis* h-MM antigen by using two main types of antigen preparations: the somatic antigen (SA) (19) and the more recently introduced cell-free antigen (CFA) (20). We expected better results with CFA, considering the possibility of degradation of the h-MM antigen by natural proteases (15) liberated as a result of rupture of *P. brasiliensis* in SA. We also analyzed the anti-h-MM antigen IgE as a marker for the Th2 immune response, and determined the IgG and IgE isotypes in both forms of disease in order to distinguish between CF and AF PCM disease.

MATERIALS AND METHODS

Serum Samples

Serum samples from 35 chronic PCM patients (unifocal and multifocal) from Londrina State University Clinical Hospital (Londrina, Brazil), 12 acute PCM patients from the Mycosis Immunodiagnostic Laboratory, Immunology Section, Adolfo Lutz Institute (São Paulo, Brazil), and 16 healthy blood donors (previously selected) were used. Informed consent was obtained from all subjects who participated in this study, which was approved by the Internal Scientific Commission and the Londrina State University Bioethics in Research Committee.

CFA and SA Preparation

CFA was obtained according to the method of Camargo et al. (20), modified by the addition of phenylmethylsulfonyl fluoride (PMSF; Sigma, St. Louis, MO) at 2.5 mM to the supernatant, which was subsequently frozen at –80°C. For SA, 5-day *P. brasiliensis* B-339 subcultivation (approximately 10.38 g wet weight) was suspended in 15 mL phosphate-buffered saline (PBS), pH 7.4, treated with liquid nitrogen, and ground three times with a mortar and pestle. The homogenate was centrifuged (Hitachi Himac CR21, Tokyo, Japan) (14.000 *g*, 4°C, 10 min) and PMSF at 2.5 mM was added to the supernatant, which was subsequently frozen at –80°C. PMSF was used to reduce the activity of *P. brasiliensis* natural proteases (21), which may be liberated during antigen preparation.

CFA and SA Analysis by Immunoblotting

CFA (1 mg/mL) or SA (1 mg/mL) samples in reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10% β-mercaptoethanol, and 0.05% bromphenol blue) were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10–20% gradient acrylamide gels, in tris-glycine buffer, pH 8.2, at 125 v, and transferred to a nitrocellulose membrane (NCM). The NCM was incubated with serum samples (1:40), followed by the addition of goat anti-human IgG-peroxidase conjugate diluted 1:2.000 (Sigma A-8775; Sigma Chemical Co., St. Louis, MO) and then of DAB (3,3-diaminobenzydine-4HCl; Sigma D-5637). Protein standards with the following MMs were used: 180, 116, 84, 58, 48.5, and 36.5 kDa α2-macroglobulin, β-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, fumarase, and lactic dehydrogenase, respectively (Biolabs 7707, USA).

Sephadex G-200 Chromatography

Two milliliters of *P. brasiliensis* CFA (1 mg/mL) or SA (1 mg/mL) were applied to a Sephadex G-200

column (2 × 48 cm) buffered with 0.15 M PBS, pH 7.2. Fractions of 1 mL each were collected in an automatic fraction collector and read in a spectrophotometer (UV/visible, Ultrospec-2000; Pharmacia Biotech, Auckland, Northland) at 280 nm. Fraction 17, corresponding to a first peak, was submitted to SDS-PAGE and stained with Coomassie brilliant blue, and was also analyzed by immunoblotting (performed as described above), using a pool of sera from five CF PCM patients that were reactive with the h-MM antigen (numbers 1, 4, 7, 8, and 12, shown in Fig. 1). The approximate MMs were calculated by the Curve Expert 1.3 program. Fraction 17, reduced and nonreduced, was analyzed by 5–20% acrylamide gradient gel in parallel to the following MMs of protein standards: 176.5 kDa, 113.7 kDa, 80.9 kDa, 63.8 kDa, 49.5 kDa, 37.4 kDa, 26.0 kDa, 19.6 kDa, 14.9 kDa, and 8.4 kDa (Invitrogen-Bench Mark prestained protein ladder catalog #10748-010; Invitrogen, Carlsbad, CA).

IgG and IgE to h-MM *P. brasiliensis* Antigen by ELISA

For IgG, ELISA immunoplates sensitized with F17 (100 µL/well) at 4 µg/mL in carbonate buffer, pH 9.6, were incubated with serum samples (1:100) and goat anti-human IgG peroxidase conjugate (Sigma A-8775) 1:4.000, followed by the addition of 10 mg ortho-phenylenediamine (OPD). The absorbance was read at 492 nm in a Multiskan EX reader (Labsystems, Helsinki, Finland). For IgE, immunoplates sensitized with goat immunoglobulin fraction anti-human IgE (Sigma I-0632, St. Louis, MO) at 16 µg/mL in carbonate buffer, pH 9.6, were incubated with serum samples (1:40) and then with F17 at 6.5 µg/mL, and with IgG reactive to F17 (purified from serum of PCM patients in a Sepharose-G protein column) at 40 µg/mL used as the reagent substance. After the addition of goat anti-human IgG peroxidase conjugate (Sigma A-8775, St. Louis, MO) diluted 1:4.000 (100 µL/well) and OPD in 25 mL phosphate-citrate buffer, pH 5.0, plus 10 µL H_2O_2 30% (100 µL/well), the absorbance was read at 492 nm.

IgE Purification and Immunoblotting Analysis of Sera From PCM Patients

IgE was purified by an Afigel-10 (no. 153-6046; Bio-Rad) column, prepared with anti-human IgE (I-0632; Sigma) from serum samples from three chronic and three acute PCM patients. The IgE fractions eluted by glycine-HCl 0.1 M, pH 2.7, were collected in tubes containing 70 µL of Tris-HCl 1M, pH 9.0, and read at 280 nm. The purified IgE was incubated with NCM strips containing CFA and SA antigens. After incubation with goat anti-human IgE-alkaline phosphatase conjugate diluted 1:500 (Sigma 2765, St. Louis, MO) for 2 hr at 37°C, the reaction was developed by 60 µL of nitro-blue tetrazolium plus 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, no. 1175041; Boehringer Mannheim Indianapolis, IN).

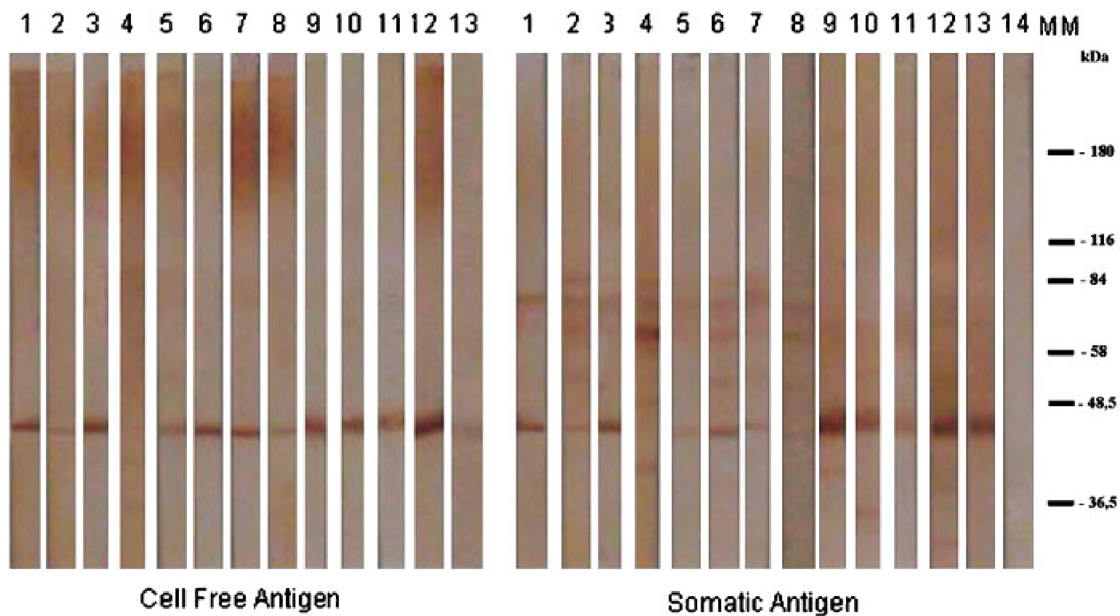

Fig. 1. Results of CFA and SA immunoblotting with serum from chronic PCM patients and mouse anti-human IgG conjugated with peroxidase. CFA and SA SDS-PAGE was performed in 10–20% gradient acrylamide gel. Samples 1–13: serum from chronic PCM patients, 14 = normal human serum (NHS) and MM protein standards: α2-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), and lactic dehydrogenase (36.5 kDa).

Statistical Analysis

To analyze the variables among the ELISA test groups for each clinical form, Tukey's *t*-test was applied. The significance level was set at $P \leq 0.05$.

RESULTS

IgG to CFA and SA H-MM Antigen by Immunoblotting

The polydispersed h-MM band was observed in eight of 13 serum samples from chronic PCM patients when the CFA antigen was used. A weak reaction for the h-MM band was observed in serum samples from two of 13 chronic PCM patients when SA was performed (as the first step of the study, 13 of 35 samples were randomly selected). All of the serum samples showed stronger or weaker reactivity to the main antigen from *P. brasiliensis*, gp43 (Fig. 1).

CFA and SA Chromatography Fractions Analysis

The elution profiles in optical density (OD) at 280 nm of CFA and SA samples from a Sephadex G-200 column are shown in Figs. 2 (CFA) and 3 (SA). The first peak, which included the h-MM antigen corresponding to the void volume (determined by dextran blue), with over 250 kDa (I), was termed F17. The gp43 was detected by dot blotting in a base, and comprised part of the second peak (data not shown). F17 showed a higher protein concentration in preparations from CFA compared to SA. Immunoblotting analysis of F17 revealed a broad band ranging from approximately 278 kDa to 466 kDa (mean = 366 kDa). This band was recognized by IgG purified from the sera of chronic PCM patients. At this step, patient sera that were reactive to the h-MM antigen (pooled serum numbers 1, 4, 7, 8, and 12; Fig. 1) were used (Fig. 4).

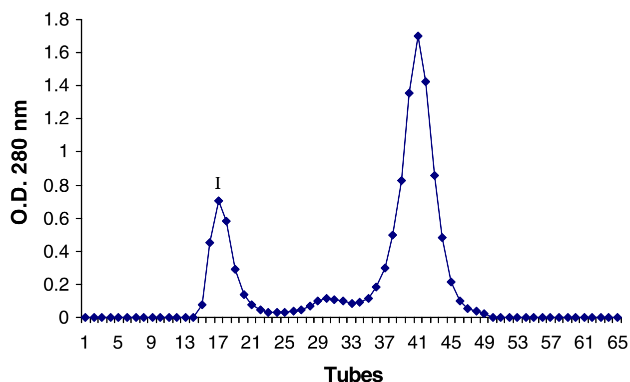

Fig. 2. Spectrophotometric profile at 280 nm of CFA fraction chromatography in Sephadex G-200. The fractions (1.0 mL) collected with an automatic fraction collector were read in a spectrophotometer at 280 nm. The first peak (I), corresponding to the void volume, with over 250 kDa, the h-MM antigen, was termed F17.

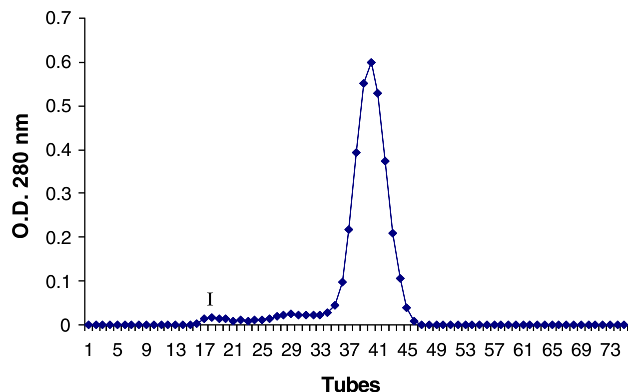

Fig. 3. Spectrophotometric profile at 280 nm of SA fraction chromatography in Sephadex G-200. The fractions (1.0 mL), collected with an automatic fraction collector, were read in a spectrophotometer at 280 nm. The first peak (I), corresponding to the void volume, with over 250 kDa, the h-MM antigen, was termed F17.

IgG and IgE to H-MM Antigen by ELISA

The results of the ELISA for IgG to the h-MM antigen, expressed as OD units, were significantly higher in sera from CF PCM patients (n = 35; 0.584 ± 0.350) than in sera from AF PCM patients (n = 12; 0.211 ± 0.071; $P < 0.05$) or in normal human serum (NHS; n = 16; 0.104 ± 0.044; $P < 0.05$; Table 1). For IgE, similar results were observed amongst these groups. The capture IgE ELISA background was relatively high (0.193), which may be due to the number of ELISA steps used in this analysis, although it did not interfere with the statistical analysis. The results of IgE to h-MM antigen by capture ELISA (in OD) were as follows: serum from chronic PCM patients (0.247 ± 0.110), serum from acute PCM patients (0.227 ± 0.075), and NHS (0.166 ± 0.054), $P > 0.05$ (Table 1). Purified IgE from pooled sera from AF or CF PCM patients showed no recognition of the h-MM antigen by immunoblotting for both CFA and SA preparations, nor did it recognize the NHS used as a negative control (data not shown).

DISCUSSION

In the first step of our study, the presence of a polydispersed h-MM antigen was demonstrated by immunoblotting in serum samples from eight of 13 PCM patients when CFA antigen was used. A weak reaction was observed in two of 13 samples when SA was performed. This difference may be due to higher concentrations of the h-MM antigen in CFA than AS, as observed in the spectrophotometric profiles in Figs. 2 and 4, respectively. When cells of *P. brasiliensis* are ruptured during SA preparation, many natural proteases

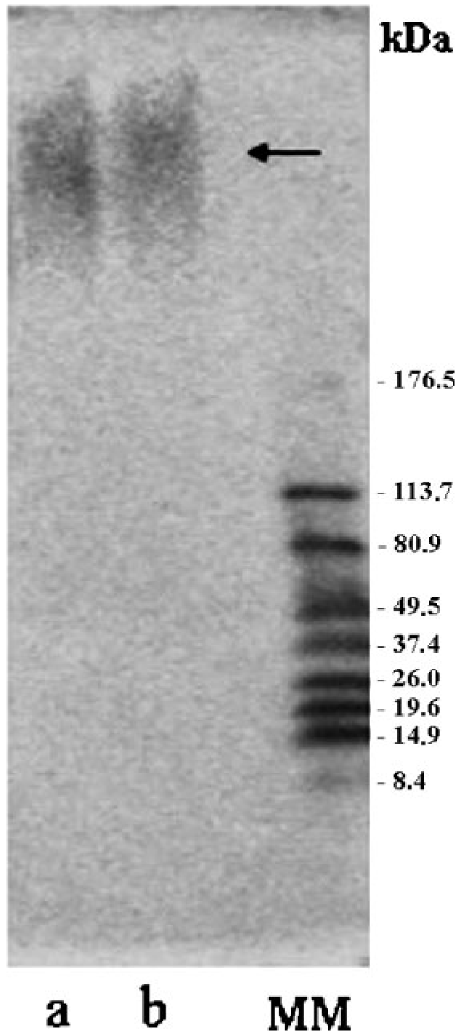

Fig. 4. Immunoblotting of fraction F17 from Sephadex G-200 chromatography, showing a polydispersed h-MM antigen ranging from approximately 278 kDa to 466 kDa (mean = 366 kDa) recognized by sera from chronic PCM patients (pool of serum samples 1, 4, 7, 8, and 12; see Fig. 1). The fraction sample nonreduced (**a**), and fraction sample reduced (**b**) protein standards (MM) were separated in 5–20% gradient acrylamide gel by SDS-PAGE. MM protein standards (Invitrogen-Bench Mark prestained protein ladder, catalog #10748-010): 176.5 kDa, 113.7 kDa, 80.9 kDa, 63.8 kDa, 49.5 kDa, 37.4 kDa, 26.0 kDa, 19.6 kDa, 14.9 kDa, and 8.4 kDa. ← Mean MM value of the h-MM antigen (366 kDa).

may be liberated, which may decrease the h-MM antigen. PMSF protease inhibitor (21) was used in both CFA and SA, and the entire process was performed under low-temperature conditions. However, even in these circumstances the h-MM fraction and also the second peak (with part of the gp43 and possibly other degradation components) were lower in the somatic than the cell-free antigen, perhaps because of the action of proteases that were not inhibited by PMSF.

In the second step, to determine the specific IgG and IgE levels, Sephadex CFA F17, corresponding to the void volume (> 250 kDa), was used. It was composed of a polydispersed component ranging from 278 kDa to 466 kDa (mean = ~366 kDa) according to immunoblotting analysis.

Table 1. Levels of IgG and IgE to high-MM *P. brasiliensis* antigen by ELISA in optical density (OD)*

Group	Number of samples (n)	IgG anti-F17	IgE anti-F17
Chronic	35	0.584 ± 0.350^{a}	0.247 ± 0.110
Acute	12	0.211 ± 0.071^{a}	0.227 ± 0.075
NHS	16	0.104 ± 0.044	0.166 ± 0.054

*For IgG, plates coated with the h-MM antigen of *P. brasiliensis* were incubated with serum samples (chronic PCM patients, acute PCM patients and normal donors), then with peroxidase-conjugated anti-human IgG and OPD solution, and read at 492 nm. For IgE, ELISA plates coated with goat anti-human IgE were incubated with serum samples and then with the h-MM antigen, purified IgG from PCM patients, and anti-human IgG-peroxidase conjugate in sequential incubations. The results are shown in optical density units at 492 nm.
[a]$P < 0.050$; IgG chronic × NHS, IgG acute × NHS, IgG chronic × acute, $P < 0.05$, IgE chronic × NHS, IgE acute × NHS, IgE chronic × acute, $P < 0.05$.
[b]Values represent mean ± SD in optical density (OD) at 492 nm.
NHS, normal human serum.

The results demonstrated significantly higher levels of IgG, but not of IgE, to F17 in the group of sera from CF patients compared to the AF and NHS groups. No reactivity of IgE to F17 was confirmed by immunoblotting. These results suggest that F17 induces a Th1 (but no Th2) immune response. If h-MM antigens of *P. brasiliensis* modulate the Th1 immune response, they may influence the course of infection in a manner that is favorable to the host. The study of components that induce protection is important for many reasons. The development of alternative treatment strategies, such as immunotherapeutic procedures, is desirable in view of the toxicity of the current antifungal agents (22). It may also be important to determine IgG and IgE anti-F17 serum levels to develop a prognosis of multiple forms of CF PCM, to monitor treatment, and to better predict dissemination following asymptomatic infection.

Apart from the Th1 immune response, the potential efficacy of humoral immunity in protecting the host against certain pathogenic fungi unrelated to the IgE isotype, such as antibodies to *Cryptococcus neoformans* polysaccharides or to *Candida albicans* cell wall components, has been discussed (23). In this context, investigations of the direct action of the antibodies may also important.

In conclusion, CFA is a better source of *P. brasiliensis* h-MM antigen than SA. This study reveals a new characteristic to differentiate between AF and CF PCM, and provides the first evidence that the *P. brasiliensis* h-MM antigen modulates the Th1 immune response.

This effect may be confirmed by further investigations of cytokines.

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