

Patients with Chronic-form Paracoccidioidomycosis Present High Serum Levels of IgE Anti-paracoccidioides brasiliensis Gp70

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Abstract Paracoccidioidomycosis (PCM) is a disease caused by the *Paracoccidioides* genus, which includes *P. brasiliensis* and the new phylogenetic species *P. lutzii*. Resistance to this infection has been correlated with a Th1 pattern of cellular immune response, while susceptibility is correlated to an intense humoral immune response with an increase in IgE levels. Serum levels of IgE and IgG anti-gp70 and anti-exoantigen in chronic PCM were analyzed by enzyme-linked immunosorbent assay. Results showed a higher gp70 concentration in somatic antigen (SA)

than in cell-free antigen (CFA) preparation and significantly higher levels of IgE and IgG anti-gp70 in chronic PCM patients' serum ($n = 12$) than in normal human serum ($n = 12$) ($p < 0.05$). Pearson's correlation analysis showed a strong correlation between IgG and IgE anti-gp70 ($r = 0.8424$). Additionally, IgE purified from a pool of acute and chronic PCM patient's serum was analyzed by immunoblotting. The patients with the acute form of the disease showed strong bands for gp43 and gp70 in SA but only for gp43 in CFA. In patients with the chronic form, solely the gp43 band was observed. In conclusion, we found that SA is a better source of gp70 than CFA is, and chronic PCM patients show high levels of IgE anti-gp70. This finding suggests that the Th2 immune response is potentially induced by gp70 in PCM disease, which calls for further study.

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Introduction

The thermo-dimorphic fungus *Paracoccidioides brasiliensis* (species S1, PS2, PS3) and *Paracoccidioides lutzii* are the causative agents of paracoccidioidomycosis (PCM), the most prevalent human systemic mycosis in Latin America and endemic in Brazil, Argentina, Venezuela, and Colombia [1–3].

Paracoccidioidomycosis is characterized by granulomatous inflammation, suppression of cellular immunity, and high serum levels of non-protective antibodies [4]. The chronic form is the most common clinical presentation and predominantly affects adult men, with frequent lung, mucosal, cutaneous, and adrenal impairment. Although the severity of the infection can be traced to several factors, it especially depends on the ability of the host's immune system to respond [5].

Decreased cellular immunity and increased production of Th2 cytokines, such as interleukin (IL)-4, IL-5, and IL-10, are commonly observed in PCM and correlate with acute and progressive disease states [6–8]. Resistance to infection by *P. brasiliensis* has been correlated with interferon-gamma (IFN- γ) and other Th1 cytokines [8].

The evaluation of humoral immune response is an important tool in the diagnosis and monitoring of PCM patients. The level of specific antibodies has been used to determine disease severity and to monitor response to treatment [5].

Gp70 is one of the main antigens expressed by the fungus *P. brasiliensis* and is recognized by 96 % of sera from PCM patients [9]. This glycoprotein is an important molecule, not only as a disease marker but also as an immunomodulatory agent in vitro [10]. For example, it can induce lymphoproliferative responses when tested with lymphocytes from PCM patients [11] and can decrease peritoneal macrophage function (phagocytosis, NO, and H₂O₂ production) [10]. Additionally, monoclonal anti-gp70 antibodies used in vivo have been found to confer immune protection when administered at the beginning of or during PCM infection in mice [10]. Recent work cloned gp70 and showed that the protein sequence has two N-glycosylation sites, belonging to the flavo-protein monooxygenase family; thus, gp70 may elicit immune cellular and humoral responses in the host while it also protects the fungus from oxidative stress generated by phagocytic cells [12]. Gp70 has been detected in the urine of patients with acute disease [13].

The current study analyzed IgE anti-gp70 in PCM patients and showed, for the first time, increased levels of IgE anti-gp70 in cases of chronic PCM, along with evidence for the presence of IgE antibodies specific to gp70 in the acute form of PCM.

Materials and Methods

Serum Samples

Twelve chronic PCM patients' serum samples (all from men with unifocal or multifocal disease, not yet treated or currently under treatment) and 12 samples of normal human sera (NHS) were used in this study, all from the biological samples bank (Applied Immunology Laboratory, CCB, State University of Londrina, Londrina, PR, Brazil). Current study was approved by the Internal Scientific Commission and the Research Bioethics Committee of the State University of Londrina.

P. brasiliensis Antigen Preparations and Gp70 Purification

Cell-free antigen (CFA) from *P. brasiliensis* (Pb339) was obtained according to the method described by Camargo et al. [14], modified by the addition of phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (2.5 mM) to the supernatant, which was subsequently frozen at -80°C . Somatic antigen (SA) was obtained according to the method described by Marquez et al. [15]. For gp70 purification, SA samples (3 mg/mL) were applied to a Sephadex G-150-120 column (2×48 cm) equilibrated with 0.15 M phosphate buffered saline (PBS), pH 7.2. Fractions were collected with an automatic fraction collector (2 mL/tube), diluted to 1:20, and read in a spectrophotometer (Ultrospec-2000 Pharmacia Biotech) at 280 nm; thereafter, each fraction was analyzed by immunoblotting, using a pool of PCM patients' sera. After gp70 fraction identification, the procedure was repeated, and the band corresponding to 70-kDa protein was selected and fractionated again using tube electrophoresis. Purity of gp70 was determined by immunoblotting, using a pool of PCM patients' sera or with anti-gp70 monoclonal antibody (MAbC5F11, gently donated by Dr. Jose Daniel Lopes, Universidade Federal de São Paulo, UNIFESP).

Immunoblotting

Immunoblotting was performed according to the method described by Marquez et al. [15], with some modifications. Pre-stained molecular weight protein

standard was used (Bio-Rad Precision Plus Protein™ Kaleidoscope cat. #161-0375, Bio-Rad Laboratories, Hercules, CA, USA). We used mercaptoethanol sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) to treat SA, CFA, fraction samples and purified gp70. SA and CFA were at the same concentration (2.13 mg/mL). Samples were examined in individual lanes of a 7.5 % polyacrylamide gel electrophoresis in tris-glycine buffer, pH 8.1, at 100 V. After nitrocellulose membrane transfer, each strip of SA, CFA, fractions samples or purified gp70 was incubated individually with a pool of PCM patients' serum samples (1:40) for 2 h at 37 °C, then with anti-human IgG-peroxidase-labeled antibody, diluted to 1:2000 (Sigma A8775, Sigma Chemical Co., St. Louis, MO, USA) for 2 h at 37 °C. Detection was performed using 3,3',5,5'-tetramethylbenzidine (TMB) (Zymed cat. #00-2019, Zymed, San Francisco, CA, USA).

Enzyme-linked Immunosorbent Assay for IgG Anti-gp70

The ELISA immunoplates were coated with gp70 at 10 µg/mL (100 µL/well) for 1 h at 37 °C and overnight at 4 °C. After blocking, serum samples diluted to 1:40 were added (100 µL/well) and incubated for 2 h at 37 °C. Anti-human IgG-peroxidase-labeled antibody (Sigma A8775 Sigma Chemical Co., St. Louis, MO, USA) was added at 1:4000 dilution (100 µL/well) and incubated for 1 h 30 min at 37 °C, followed by the addition of OPD substrate solution (100 µL/well) for 15 min. The reaction was stopped by the addition of 50 µL/well of 4 N H₂SO₄, and the absorbance was read at 492 nm in a Multiskan EX Reader (Labsystems, Helsinki, Finland).

ELISA for IgE Anti-gp70

For IgG adsorption, serum samples diluted to 1:10 (100 µL) were incubated (v/v) with protein A-Sepharose at 37 °C under agitation for 1 h. ELISA immunoplates coated with gp70 at 10 µg/mL (100 µL/well) were incubated for 2 h at 37 °C with the chronic PCM patients' serum samples previously adsorbed, diluted to 1:20 (100 µL/well). Thereafter, 30 µg/mL of anti-human IgE antibody (Sigma I0632, Sigma Chemical Co., St. Louis, MO, USA) (100 µL/well) was added and incubated for 1 h at 37 °C. Anti-IgG-peroxidase-labeled antibody diluted to 1:2000 (Sigma A3415, Sigma Chemical Co., St. Louis, MO, USA) (100 µL/

well) was added and incubated for 1 h 30 min at 37 °C, followed by the addition of OPD substrate solution (100 µL/well). The reaction was stopped by the addition of 50 µL/well of 4 N H₂SO₄, and the absorbance was read at 492 nm in a Multiskan EX Reader (Labsystems, Helsinki, Finland).

Chronic and Acute PCM Patients' IgE Purification and Gp70 Recognition Analysis

An Affi-Gel 10 column containing anti-human IgE (Sigma I0632) was prepared. Serum samples were individually diluted to 1:4 in an initial buffer of pH 7.4 and passed through the column previously equilibrated with initial buffer. Collecting tubes containing 70 µL of neutralizing buffer of pH 9.8 were prepared. The column was washed, placed in ice, and the purified IgE was eluted with elution buffer of pH 2.8. The fractions collected were read at 280 nm. CFA, SA, and protein marker (Invitrogen BenchMark™ Pre-stained Protein Ladder cat. #10748-010, Life Technologies Corporation, USA) were run in SDS-PAGE (10–20 %), and then transferred to a nitrocellulose membrane. After this, the strips were incubated with IgE purified from a pool of 3 acute or 3 chronic PCM patients' sera for 2 h at 37 °C, and then incubated for 2 h at 37 °C with anti-human IgE-alkaline phosphatase-labeled antibody diluted to 1:500 (Sigma A3525, Sigma Chemical Co., St. Louis, MO, USA). The immunological reaction was developed with 5-bromo-4-chloro-3-indolyl-phosphate plus nitro-blue tetrazolium (NBT/BCIP) (Boehringer Mannheim 1175041).

Statistical Analysis

To analyze variables between ELISA groups, the Shapiro–Wilk normality test was applied prior to Bartlett's and Tukey's tests. Significance was defined as $p \leq 0.050$. Pearson's correlation was defined as a strong correlation when $r \geq 0.75$. All statistical analyzes were performed using GraphPad Prism v. 5.00 for Windows (GraphPad Software, San Diego California, USA).

Results

P. brasiliensis CFA, SA, and Gp70 Analysis by Immunoblotting

Results from the immunoblotting of SA and CFA showed the presence of a gp70 band in both sources,

but with stronger evidence in SA (Fig. 1a). SA was fractionated by gel-filtration chromatography, and upon immunoblotting, higher concentrations of gp70 were observed in fractions 14 to 18 (Fig. 1b). To separate the gp70 from other contaminants, these gp70-rich fractions were submitted to SDS-PAGE analysis, and the gp70 band was eluted again. This resulted in recognition of only one 70-kDa band from the pool of PCM patients' sera. When analyzed with anti-gp70 monoclonal antibody (MAbC5F11), a 70-kDa and 2 other minor bands were observed by immunoblotting (Fig. 1c).

Serum Levels of IgE Anti-gp70, IgE/IgG Anti-gp70 and Correlation Analysis

Results expressed as OD at 492 nm showed a higher level of IgE anti-gp70 in the PCM patients' group (0.119 ± 0.049) than in the NHS (0.07 ± 0.01 ; $p < 0.05$), as well as a high level of IgG anti-gp70 in the PCM patients group ($n = 12$, 0.108 ± 0.053) when compared to the NHS group ($n = 12$, 0.071 ± 0.011 ; $p < 0.05$) (Fig. 2a, b).

We observed a strong correlation between IgG and IgE anti-gp70 levels ($r = 0.8424$) (Fig. 2c).

Chronic and Acute PCM Patients' IgE Analysis by Immunoblot

Purified IgE from a pool of acute PCM patients' sera recognized gp43 and gp70 from SA, but recognized only gp43 from CFA. IgE from a pool of chronic PCM patients' sera showed weak recognition of gp43 (Fig. 3).

Discussion

Correlation between IL-4 and IgE suggests that IgE production can be used as a marker for the existence of Th2 cell populations [16, 17].

The current study analyzed levels of IgE anti-gp70 by ELISA, which, unlike immunoblotting, requires purified antigens. Therefore, in the first step, the source of antigen was selected for purification. Although it might be difficult to compare amounts of a single protein within a mixture when using total amount of protein, Fig. 1a shows clearly that SA preparation had a higher concentration of gp70 recognized by IgG in immunoblotting than CFA preparation did, and so it was selected as the gp70 source. The gp70 was purified

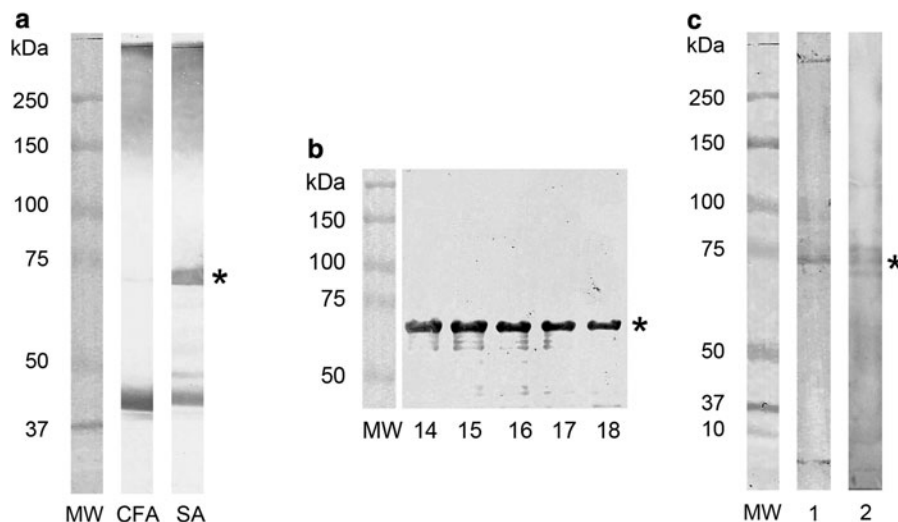


Fig. 1 Purification of gp70. **a** After SA and CFA separation in SDS-PAGE 75 %, the glycoproteins were transferred to a nitrocellulose membrane, and individual strips were incubated with a pool of chronic PCM patients' serum. A higher concentration of gp70 was observed in SA when compared to CFA. **b** After gel-filtration chromatography of SA in Sephadex G-150/120, fractions 14–18 showed the presence of gp70.

c After transferring a SDS-PAGE 5–20 % gel to a nitrocellulose membrane, western blotting showed the recognition of gp70 by a pool of PCM patient's sera (1) and by anti-gp70 monoclonal antibody (2) (MAbC5F11). (MW) molecular weight standard Bio-Rad Precision Plus Protein™ Kaleidoscope #161-0375. Asterisk gp70 region

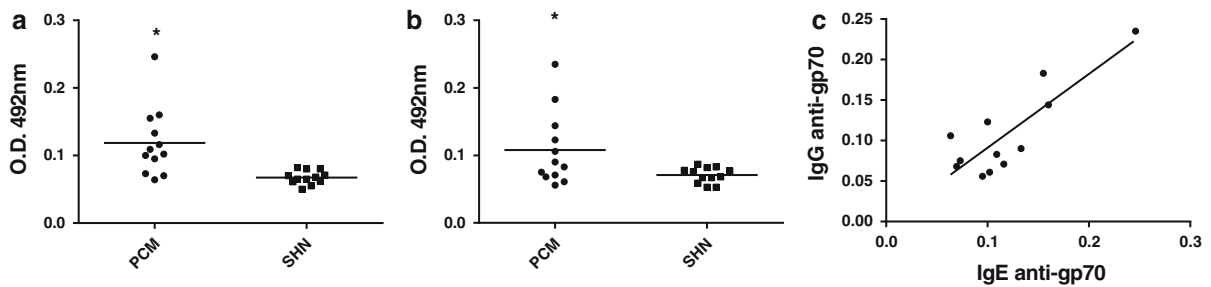


Fig. 2 Determination of anti-gp70 IgE (a) and IgG (b) levels by ELISA. The immunoglobulin levels against gp70 were determined in chronic PCM patients' serum ($n = 12$) and normal human serum (NHS; $n = 12$). Plates were coated with $10 \mu\text{g/mL}$ of gp70. For IgE detection, serum samples were previously

adsorbed in Sepharose-Protein A. Results are shown as optical density (O.D.) at 492 nm. PCM versus NHS, $p < 0.05$. c Pearson's correlation between serum IgG and IgE levels anti-gp70 showed a high correlation ($n = 12$; $r = 0.8424$)

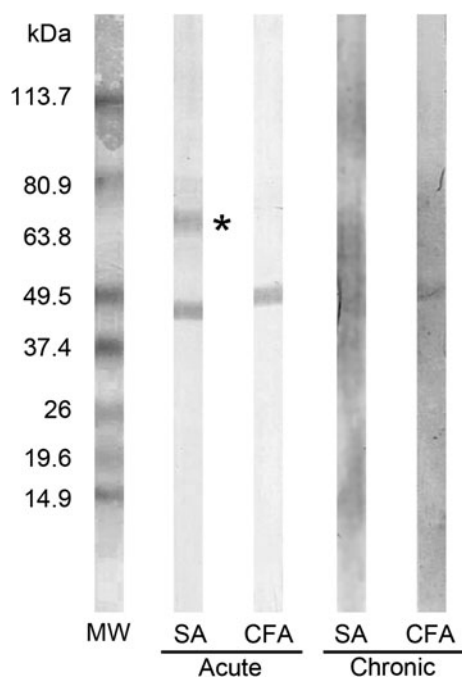


Fig. 3 Recognition of proteins from *P. brasiliensis* by IgE from acute or chronic PCM patients' sera in western blotting. After SA and CFA separation in 10–20 % polyacrylamide gradient gel, the glycoproteins were transferred to a nitrocellulose membrane, and individual strips were incubated with a pool of purified IgE obtained from acute or chronic PCM patients' sera. (MW) Molecular weight standard Invitrogen BenchMark™ Pre-stained Protein Ladder cat. #10748-010. Asterisk gp70 region

by gel-filtration chromatography associated with 2 steps of gel electrophoresis. Immunoblotting resulted in only 1 main band at 70-kDa recognized by IgG from a pool of the chronic PCM patients' sera. This was confirmed by the finding that the same migration

distance of the specific band was recognized by anti-gp70 monoclonal antibody (MAbC5F11). In addition to the 70-kDa band, other minor nearby bands were observed. Because the SA contains several internal components of the fungus, precursor molecules of gp70 that had not undergone complete glycosylation and that had lower molecular mass fragments could be recognized by monoclonal antibodies anti-gp70.

Detection of antigen-specific IgE antibodies in diseases associated with polyclonal activation of B cells and hypergammaglobulinemia is difficult, because IgG antibodies compete for the same epitopes as IgEs [18]. Therefore, for IgE analysis, serum samples were first adsorbed with Sepharose-protein A. Results showed higher levels of IgE anti-gp70 in the PCM patients' group serum than in NHS. To our knowledge, this is the first time the recognition of IgE anti-gp70 in serum samples from patients with PCM has been described. Other glycoproteins are recognized by the IgE from PCM patients' sera, especially gp43. A higher IgE level in response to this glycoprotein has been observed, particularly in those patients with the acute form [18, 19]. Moreover, chronic PCM patients express high levels of IgE anti-gp70, suggesting that these patients have the potential for a Th2 response.

Our analysis also showed IgG anti-gp70 levels to be higher in the PCM patients than in the NHS. These results agree with those obtained by Camargo et al. [20], which showed that antibodies in 96 % of sera from PCM patients reacted with gp70. Our study also showed a strong correlation between serum IgG and IgE anti-gp70. In this study, IgG isotypes were not investigated. Further investigation will be required to determine if the IgG4 isotype, regulated by IL-4, and

therefore associated with IgE, has some role in this strong correlation.

Considering the intensity of the gp70 band as shown by immunoblotting and the strong recognition of this antigen in the acute form, IgE anti-gp70 could serve as a stronger indicator of the acute form of PCM, as has already been described for anti-gp43 [21, 22]. However, this hypothesis needs further study to characterize the validity of the analysis in individual samples. The failure of IgE from patients with the acute form to recognize gp70 in CFA preparation is likely due to the low concentration of gp70 in this antigen preparation. This is in accordance with the results of confocal microscopy analysis of anti-rPbgp70 and MAbC5F11 that showed intense staining and cytoplasmic co-localization of gp70 [12]. These results are also in agreement with data showing irregular 72-kDa glycoprotein expression [23].

Similar to gp43, research on gp70 may become important because this antigen has the potential to act as a modulator of Th2 response and therefore may participate in PCM pathogenesis. The possibility to use synthetic peptides, such as P2 which mimics gp75 from *P. brasiliensis* [24], seems a promising alternative in PCM diagnosis which needs to be further studied, because cheaper and more sensitive/specific tests may be created.

In conclusion, SA is a better source of gp70 than CFA. Our preliminary results show firsthand increased levels of IgE anti-gp70 in chronic PCM and suggest higher levels of IgE anti-gp70 in acute than in chronic form of PCM, as demonstrated by immunoblotting.

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