Abstract. Paracoccidioidomycosis (PCM) is the most frequent systemic mycosis in South America. The disease is characterized by a polyclonal activation of B cells, resulting in hyperimmunoglobulinemia. The production of immunoglobulin (Ig) E in deep mycosis has been related to the severity of the disease. However, the detection of specific IgE in sera of patients is difficult because of the competition with the IgG. We compared a capture and an indirect enzyme-linked immunosorbent assay (ELISA) technique to detect Paracoccidioides brasiliensis IgE. We found that the capture ELISA presented higher performance and lower background values than the indirect assay, resulting in a significant quantitative discrimination between sera from patients with the 2 major clinical forms of PCM. Patients with the juvenile form presented significantly higher levels of *P. brasiliensis* IgE, as compared with patients with the adult form. The capture ELISA was used in the follow-up of patients receiving treatment, showing that the levels of specific IgE decreased as the patient’s clinical conditions improved.

INTRODUCTION

Helmint infections such as atopic disorders show characteristic elevations of serum immunoglobulin (Ig) E. This condition has been associated with predominance of T-helper 2 (Th2) cells and interleukin (IL)-4 production over T-helper 1 (Th1) cells and interferon gamma secretion. Elevated serum IgE has also been found in patients with fungal infection such as blastomycosis, coccidioidomycosis, and paracoccidioidomycosis (PCM). Experimental evidence strongly suggests that in humans, PCM resistance and susceptibility may be associated with a Th1 and Th2 pattern of cytokines, respectively. Considering that the isotypes of the immunoglobulins are determined by the patterns of cytokines present in microenvironment where B cells are being activated, the detection of particular isotypes may be considered as indicators of the corresponding pattern of inducive cytokines. The excellent correlation between IL-4 and help for IgE suggests that IgE production can be used as a good marker for the existence of a strong Th2-like response.

Paracoccidioidomycosis is characterized by a polyclonal activation of B cells resulting in high levels of serum IgE. The detection of antigen-specific IgE in diseases associated with polyclonal B-cell activation and hypergammaglobulinemia is difficult because of IgG antibody competition for the same epitopes.

In the indirect enzyme-linked immunosorbent assay (ELISA), the test antigen adsorbed in the solid phase binds specific antibodies of all isotypes in amounts proportional to their concentration in the test sample. Bound, specific IgE is subsequently detected with a secondary antibody. The main limitations of indirect ELISA are falsely negative or falsely low IgE results due to competition with specific IgG for antigenic sites. This limitation may be overcome by the capture principle because the selective binding of patient IgE eliminates IgG competition.

Here we describe a simple and specific capture ELISA to detect IgE anti-Paracoccidioides brasiliensis gp43 and compare it with indirect ELISA by use of serum specimens from patients with the juvenile and adult forms of PCM.

MATERIAL AND METHODS

**Paracoccidioides brasiliensis gp43 antigen.** Purified gp43 was obtained by affinity chromatography of the crude extract of *P. brasiliensis* B-339 on Sepharose 4B-IgG (monoclonal antibody anti-gp43 IgG). The gp43 was eluted from this column with 0.1 M glycine-HCl, pH 2.8, immediately neutralized with 2 M Tris, pH 9.0, concentrated in an Amicon (Danvers, MA) 10K apparatus and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein content was measured by the method of Bradford.

**Serum specimens.** Patients with proven PCM cared for at the University Clinical Hospital of the State University of Campinas, Campinas, Brazil, were classified into 2 groups according to the clinical forms: the acute or juvenile form (JF), and the chronic or adult form (AF). Sera from 16 patients with the JF and from 16 patients with the AF of PCM that had been obtained before treatment were analyzed. The diagnosis was confirmed by histopathologic examination of biopsy, tegumentary, ganglionary, or pulmonary samples and also by the finding of fungus in scrapings of tegumentary lesions or of lymph node secretion, or in sputum (direct examination). Serological tests were positive in all patients, with titers ranging from 1/2 to 1/4,096 for immunodiffusion and 1/800 to 1/409,000 for ELISA (IgG). Fifteen sera samples from healthy patients were used as controls. For some experiments, we used consecutive sera from 6 patients of each clinical form obtained before, during, and after treatment.

**Indirect ELISA.** We coated 96-well polystyrene flat-bottomed plates (Greiner, Kremsmünster, Austria) with *P. brasiliensis* gp43 at 2 μg/mL in 0.1% carbonate buffer and incubated them for 2 hr at room temperature and overnight at 4°C. The plates were washed once with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T), and the remaining sites were blocked with PBS-T containing 10% fetal calf serum for 1 hr at room temperature. After 2 washings with PBS-T, the serum samples diluted 1/20 in PBS-T containing 0.25% gelatin (PBS-T-G) were added in duplicate: the plates were incubated for 2 hr at 37°C. The wells were washed as described above, and mouse anti-human anti-IgE (Sigma Chemical Co., St. Louis, MO) was added at 2
μg/mL in PBS-T-G. The plates were incubated 1 hr at 37°C and then washed again before receiving the conjugate (anti-mouse IgG-peroxidase; Sigma) at 1:1000. After a 1-hr incubation at 37°C, the plates were washed 3 times, and then 100 μL of substrate solution (5 mg of O-phenylenediamine in 10 mL of 0.1 M citrate-phosphate buffer, pH 5.0, plus 10 μL of 30% H2O2) was added to each well. After color development, the reaction was interrupted by the addition of 50 μL of 4 N H2SO4. The optical densities were read in an ELISA reader (SLT Spectra, SLT Labinstruments, Salzburg, Austria) at 492 nm.

**Indirect ELISA with RF (rheumatoid-factors)-Absorbent–treated sera.** The same protocol above was used except that the serum samples were previously treated with RF-Absorbent (Behring Diagnostic, Marburg, Germany). Serum samples were diluted 1:10 in PBS-T-G, combined 1:1 to the RF-Absorbent, mixed well, and incubated for 10 min at room temperature. The mixture was then added directly in the ELISA plate (100 μL/well).

**Anti-gp43 monoclonal antibody conjugate.** Monoclonal antibody anti-gp43 was conjugated with peroxidase (Sigma) and used at 1:350.17

**Capture ELISA.** The 96-well plates were coated with 100 μL of mouse anti-human IgE in 0.1 M carbonate buffer (pH 9.6) at 2 μg/mL overnight at 4°C. The plates were washed with PBS-T, and the remaining binding sites were blocked with PBS 10% fetal calf serum for 1 hr at room temperature. After washing 3 times with PBS-T, serum samples, diluted at 1:20, were added in duplicate and incubated for 2 hr at 37°C plates were then washed. The *P. brasiliensis* gp43 (2 μg/mL) was added to the wells for 1 hr at room temperature. The wells were washed again, and anti-gp43 monoclonal antibody-peroxidase (1:350 dilution) was added for 1 hr at room temperature, then washed again. Optical path difference substrate was added, and after color development, optical density (OD) readings were taken as described above.

**Statistical analysis.** To compare the variables among the ELISA tests and the phases of treatment for each clinical form, the Wilcoxon nonparametric test was used. Patients with the JF and AF of PCM were compared with the Mann-Whitney nonparametric test. Significance was defined as *P* ≤ 0.05.

**RESULTS**

To detect specific IgE antibodies to *P. brasiliensis* gp43 in sera from patients with PCM, we compared 2 types of ELISAs: indirect and capture. Initially, a dose-response test was performed by examining a serial 2-fold dilution of a pool consisting of 4 sera from patients with proven PCM, without considering the clinical form. One negative serum from a healthy donor was used as the control. The indirect ELISA was performed with unabsorbed sera (ELISA) and serum samples treated with RF-Absorbent (RF-ELISA). The RF-Absorbent is currently used to remove IgM rheumatoid factors from samples of serum or plasma before they are used in a test for specific IgM antibodies by the indirect method (ELISA or immunofluorescence). It is an anti-human IgG antibody preparation that is directed against IgG, forming immune complexes to which the IgM rheumatoid factors are bound and thus removed. Because any pathogen-specific IgG present is also removed by the action of RF-Absorbent, competition between specific IgG and IgM (or other isotypes), which may be of importance in some tests, is also minimized.

As can be seen in Figure 1, when we used the indirect assay (ELISA and RF-ELISA) and the capture assay (Cap-ELISA), the absorbances decreased with the increasing serum dilution. The capture technique yielded much higher OD signals than indirect ELISA, even when RF-Absorbent–treated serum samples were used. For the following experiments, we chose the 1/20 dilution because at this point, the ELISA techniques showed the highest differences in the absorbance values.

Next, we compared sera from patients with the JF and AF of PCM. The indirect ELISA resulted in high background (OD, 0.326) and only low levels of IgE were detected for both clinical forms (Figure 2, FA and FJ). The background of the indirect assay using RF-treated serum samples was also high (OD, 0.353), and although higher levels were detected as compared with the test performed with nontreated samples, no major improvement was observed. The capture ELISA, in which plates were coated with anti-IgE selecting only this isotype, enabled the detection of higher levels of specific IgE, mainly in the patients with the JF of PCM. By means of this technique, the background was very low (OD, 0.076). The capture ELISA produced higher OD signals than did indirect ELISA, resulting in a significant quantitative discrimination between samples from patients with the JF and AF of PCM.

Figure 3 shows the results of the specific IgE detection in consecutive serum samples from 6 patients with the JF and 6 patients with the AF of PCM, followed for 2 years while receiving antifungal therapy. The patients with the JF produced higher levels of specific IgE when compared with the AF, showing significant differences in sera 1 and 2. The IgE levels decreased significantly for patients with the JF, while no differences were observed for patients with the AF, be-

![Figure 1](https://via.placeholder.com/150)
DETECTION OF P. BRASILIENSI S BY ELISA

A major problem in most studies of the IgE response in infections is a lack of a simple, sensitive technique to detect specific IgE antibodies. Many times, the presence of excessive amounts of specific antibodies of the IgG class lead to competitive inhibition of IgE binding to antigenic determinants in indirect type of assays, such as indirect ELISA. This would greatly limit the sensitivity of the technique. The use of an antibody capture technique solves this problem because antibodies of the IgE class are separated from other classes of antibodies before the incubation with the antigen.

The RF-Absorbent is frequently used to remove the IgM rheumatoid factors from samples of serum before they are used in a test for specific IgM antibodies detection. Additionally, it removes the excess of IgG present in the samples. Souza-Atta and others noted that the use of RF-Absorbent permitted the detection of specific IgE in sera from patients with visceral leishmaniasis and schistosomiasis. In the present study, the treatment of the serum samples with RF-Absorbent was not enough to improve the IgE detection.

Since the introduction of the µ-capture ELISA in 1978, this technique has shown to be useful for the detection of specific IgM. More recently, the capture principle has been applied for the detection of other immunoglobulin isotypes such as IgE and IgA. The binding of a representative part of the total IgE and subsequent detection of anti-P. brasiliensis IgE reflects the relative amount of specific versus total IgE, and not the absolute concentration of specific IgE as measured by indirect ELISA. An increase in the relative amount of specific IgE seemed to be a more sensitive parameter than an increase in the absolute concentration of specific IgE in serum measured by indirect ELISA.

Another relevant point is the antigen used in the immunoassay. The P. brasiliensis gp43 is the immunodominant antigen, recognized by the majority of sera (IgG) from patients with PCM. This molecule, isolated from the supernatant fluid of yeast cultures by affinity chromatography, specifically binds to the extracellular matrix protein laminin, leading to enhancement of fungal pathogenesis.

Pathogenesis in PCM is associated with depressed cellular immunity and significant elevation of antibodies titer. In general, JF PCM is more severe and disseminated than the AF. Accordingly, these patients are characterized by severe immunosuppression as evidenced by the failure to respond.

FIGURE 2. Detection of immunoglobulin E antibodies to Paracoccidioides brasiliensis gp43 in sera from 16 patients with the adult form (AF) and 16 patients with the juvenile form (JF) of paracoccidioidomycosis, analyzed by an indirect enzyme-linked immunosorbent assay ELISA (ELISA), indirect ELISA with RF-Absorbent–treated (Behring Diagnostic, Marburg, Germany) serum samples (RF-ELISA), and capture ELISA (Cap-ELISA).

FIGURE 3. Detection of immunoglobulin E antibodies to Paracoccidioides brasiliensis gp43 in consecutive sera from patients with the adult form (AF) and juvenile form (JF) of paracoccidioidomycosis, analyzed by capture enzyme-linked immunosorbent assay. Sera were obtained before, during, and after treatment (S1, S2, and S3, respectively). The average time between the collection of serum 1 and serum 2 was 11 months and 13 months between serum 2 and serum 3.
to *P. brasiliensis* antigens in terms of delayed-type hypersensitivity and lymphoproliferative response in vitro.\(^{26,29}\) Baida and others\(^{30}\) investigated the antibody response to the major *P. brasiliensis* antigen (gp43-kDa) in juvenile and adult clinical forms of PCM. The IgG4 isotype was detected in all patients with the JF of PCM but only in 12% of the patients with the AF of PCM. In the present study, we showed that JF patients present significantly higher titers of IgE anti-gp43. These results are important evidences of a dominant Th2 response in the JF of PCM because IgG4 and IgE are the 2 isotypes characteristically induced by IL-4.\(^{31}\)

The antibody titters in PCM have been exploited for specific diagnosis, and little attention has been paid to the analysis of the isotypes levels in relation to their role in resolution of the disease and protective immunity. The IgE capture ELISA we describe here was suitable for monitoring the specific IgE in consecutive samples. Follow-up of the patients showed that IgE antibodies consistently dropped in patients with the JF during the treatment. On the other hand, for the AF, patients had IgE levels that were low and constant during the whole treatment. These results are in agreement with the existence of a relationship between the onset and the magnitude of the anti-fungus IgE and the severity of disease, as previously suggested.\(^{32,33}\)

It is tempting to speculate on the significance of the *P. brasiliensis* IgE response and its possible role in immunological defense or fungus pathogenesis. However, further studies are required to more clearly define the IgE response in patients with different clinical manifestations of PCM. The method described here is simple and easy to perform and permits carrying out these investigations.

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