Immunological Response to Cell-Free Antigens of *Paracoccidioides brasiliensis*: Relationship with Clinical Forms of Paracoccidioidomycosis

M. H. S. L. BLOTTA¹ AND Z. P. CAMARGO²*

Departamento de Patologia Clinica, Faculdade de Ciências Medicas, UNICAMP,¹ and Disciplina de Biologia Celular, Escola Paulista de Medicina, 04023-062 Rua Botucatu 862,² São Paulo, Brazil

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Sera from patients with the acute (AF) and chronic (CF) forms of paracoccidioidomycosis (PCM) were tested against *Paracoccidioides brasiliensis* cell-free antigens by Western blot (immunoblot). The CFA preparation contained components ranging in molecular mass from 18 to 102 kDa. The immunoglobulin G (IgG) reactivity profiles were similar for patients with both forms of the disease, and the 43-kDa component was recognized by 100% of the sera. IgM antibodies from the AF- and the CF-PCM sera recognized 21 and 20 components, respectively, the AF-PCM sera reacting preferentially with components with molecular masses above 50 kDa. None of the AF-PCM sera (IgM) reacted with the 43-kDa component, and only 10% of the CF-PCM sera recognized this molecule. The IgA response was more significant in the CF-PCM group than in the AF-PCM group, and the 43- and 74-kDa components were the most reactive ones (about 40% each). Our results showed that the cell-free antigen preparation is very appropriate for the immunoblotting analysis of PCM sera, and they also showed that the detection of IgG anti-gp43 is the best marker for the diagnosis and the following up of patients with the acute or the chronic form of the disease.

The systemic mycosis paracoccidioidomycosis (PCM) is caused by the dimorphic fungus *Paracoccidioides brasiliensis*, and it presents a wide spectrum of clinical manifestations. Over the years, it has been suggested that infection with *P. brasiliensis* occurs by inhalation of the airborne mycelial structures (not yet defined) of the saprobic phase of the fungus. Once in the lungs, the propagules convert themselves into the yeast phase, initiating the infection, and then they eventually disseminate themselves to the other parts of the body (9). Two main clinical forms of the disease are recognized by the International Committee of PCM (10), namely, the acute or subacute form (AF) and the unifocal or multifocal chronic form (CF). The pathogen is endemic to many regions of Latin America, mainly in Argentina, Brazil, Colombia, and Venezuela.

The diagnosis of PCM can be made by direct observation of the characteristic multiple-budding cells in clinical materials, by biopsy, or by culture. However, when these procedures are not available, the detection of antibodies to *P. brasiliensis* antigens is a valuable aid for diagnosing PCM, mainly by the immunodiffusion test (6).

In general, the antigenic preparations used in serological assays for *P. brasiliensis* antibodies have been undefined, and it is unknown if there is any relationship between the various antigens and the clinical forms of PCM. We have recently described a simple method for obtaining antigen from the surface of the fungus, which was designated "cell-free antigen" (CFA) (5).

In this study, the Western blot (immunoblot) assay was used to analyze the main components of *P. brasiliensis* CFA recognized by the individual immunoglobulin G (IgG), IgM, and IgA in sera from patients with the acute and the chronic forms of PCM, because of the fact that the CFA preparation is composed by the cell surface-associated antigens which might represent the first molecules to have contact with the host defense cells.

MATERIALS AND METHODS

Fungal strain. *P. brasiliensis* B-339 (kindly provided by A. Restrepo, Corporación para Investigaciones Biológicas, Medellin, Colombia) was maintained by frequent subculturing on Sabouraud glucose agar (Difco Laboratories). The fungus was converted into its yeast form by being grown on

![FIG. 1. Components of the CFA of *P. brasiliensis* after SDS-PAGE and silver staining. Molecular mass standards are indicated at the right.](http://jcm.asm.org/)

* Corresponding author.


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such medium at 35°C and was maintained in such form by being subcultured every third day.

**Preparation of antigen.** *P. brasiliensis* B-339 was grown on Sabouraud glucose agar at 35°C for 3 days. The fungal growth from three randomly selected tubes (about 300 mg, wet weight) was collected by gently scraping the surface. The cell mass was suspended in 1 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.4, mixed for 30 s in a Vortex mixer (Tecnal, São Paulo, Brazil), and immediately centrifuged at 10,000 × g in an Eppendorf tabletop centrifuge (Sorvall MC 12V; Dupont, Newtown, Conn.) for 60 s. The resulting supernatant fluid contained the antigen (CFA). The protein concentration in CFA was determined by the method of Bradford (4).

**Sera.** Serum samples were obtained from 60 PCM patients from the Hospital das Clínicas da UNICAMP, Campinas, Brazil. The patients were all Caucasians, grouped according to the clinical form of the disease as follows: 30 patients (30 serum samples) were 6 to 32 years of age with the acute form (PCM-AF), and 30 patients (30 serum samples) were 35 to 69 years of age with the chronic form (PCM-CF). Most of them were rural workers from the region of Campinas. These sera were taken before antimycotic therapy. Ten patients in each group were serologically monitored for 2 years during the course of therapy (adding up to 50 serum samples).

**SDS-PAGE.** CFA obtained as described above was mixed with the reducing sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10% 2-mercaptoethanol, and 0.05% bromophenol blue. The polypeptides were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (11) on 10% acrylamide gels. Protein standards with the following molecular masses were used: bovine serum albumin, 67,000 Da; ovalbumin, 43,000 Da; and concanavalin A, 29,000 Da (Pharmacia,

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**FIG. 2.** IgG, IgM, and IgA immunoblots of the CFA of *P. brasiliensis* B-339, revealed with 30 PCM-AF and 30 PCM-CF serum samples.
Uppsala, Sweden). Proteins were visualized by silver nitrate staining (1).

**Immunoblotting.** After electrophoresis, the CFA proteins were transferred overnight to nitrocellulose paper (NCP) at 100 mA in a transblotting chamber (Bio-Rad, Melville, N.Y.) (15, 16). The transfer buffer consisted of 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol, pH 8.3. Prior to the immunological staining, the free sites on the NCP were blocked with 2% bovine serum albumin (BSA; Sigma) in PBS and 0.2% Nonidet P-40 (Sigma), pH 7.4, for 4 h. The NCP was sliced vertically, and the strips were incubated individually for 60 min at room temperature with sera from patients with PCM diluted at 1:100 in PBS containing 0.02% Tween 20 and 0.25% gelatin (PBS-T-G). Then, each strip was washed four times for 15 min in PBS-T-G and afterwards incubated individually with peroxidase anti-human IgG, IgA, or IgM (Sigma) for 60 min. Afterwards, each strip was washed four times for 15 min in PBS-T-G and incubated with a freshly prepared mixture of 5 mg of DAB (3,3-diaminobenzidine-4HCl; Sigma) in 50 ml of 0.1 M Tris buffer, pH 7.5, plus 5 μl of 30% H₂O₂. After color development, the strips were rinsed in 10% acetic acid.

**Scanning densitometry.** After immunoblotting, each NCP strip incubated with the sera obtained from patients monitored during antimycotic therapy was analyzed by scanning densitometry (CS 9000; Shimadzu Corporation Spectrophotometric Instrument Plant, Kyoto, Japan) at 550 nm for quantitative evaluation of the band intensity.

**RESULTS**

The components of the CFA of *P. brasiliensis* B-339 were analyzed by SDS-PAGE. At least 42 components (18 to 102 kDa) were observed after silver staining (Fig. 1).

Fig. 2 shows IgG, IgM, and IgA blots of CFA components exposed to 30 individual AF-PCM serum samples and to 30 individual CF-PCM serum samples (all obtained before therapy). Several components of the following molecular masses were detected in IgG-positive patient serum samples: 18, 20, 22, 24, 28, 29, 33, 34, 39, 43, 50, 52, 54, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 86, 88, 90, 96, and 102 kDa. The number of components varied with the PCM serum. The AF-PCM sera (IgG) recognized 30 components, whereas the CF-PCM sera recognized 23 components. Patients with both forms of the disease had similar IgG reactivity profiles. The most frequent components that reacted with the IgG-AF serum group were 24 and 28 kDa (20%), 20, 22, and 78 kDa (25%), 76 kDa (28%), 80 kDa (30%), 70 kDa (45%), and 43 kDa (100%), whereas for the IgG-CF serum group, the components that reacted most frequently were 28 kDa (20%), 22 and 88 kDa (25%), 70 kDa (35%), and 43 kDa (100%).

IgM blots of the CFA exposed to 30 individual AF-PCM serum samples and to 30 individual CF-PCM serum samples recognized 21 and 19 components, respectively. Note that the components with molecular masses of 22, 24, 28, 29, 34, 35, 36, 39, 43, 47, 50, 52, 54, 58, 60, 62, 64, 66, 70, 72, 74, 76, 80, 82, 86, 88, and 90 kDa were recognized, but the number of antigens varied with the PCM serum. Sera from patients...
with AF-PCM reacted preferentially with components with molecular masses above 50 kDa. The most frequent reactive components for IgM-AF serum group were 50, 58, and 66 kDa (20%); 64, 82, and 88 kDa (40%); 90 kDa (55%); 80 kDa (60%); and 70 kDa (70%), whereas those for the IgM-CF group were 70 and 80 kDa (20%) and 88 and 90 kDa (30%).

IgA blots of CFA exposed to the same serum groups recognized 10 and 26 components, respectively. Various antigens with molecular masses of 20, 22, 24, 26, 28, 30, 32, 36, 38, 43, 46, 48, 50, 54, 56, 58, 60, 62, 64, 66, 70, 72, 74, 76, 80, and 82 kDa were detected. The number of antigens also varied with the individual PCM serum used. IgA antibodies were absent in 73% of the sera from patients with the AF-PCM, whereas the CF-PCM sera were able to recognize a more extensive set of antigens. The most frequent reactive components for the IgA-AF serum group were 24, 43, 70, and 76 kDa (10%), whereas those for the IgA-CF group were 46, 60, and 80 kDa (25%), 24 kDa (30%), and 43 and 74 kDa (40%).

Figure 3 presents the individual frequencies of recognition of CFA components by IgG, IgM, and IgA of AF-PCM and CF-PCM sera. The 43-kDa component was recognized by IgG in 100% of both groups of sera, followed by the 70-kDa component, which was recognized by 50% of the IgG sera. On the other hand, none of the IgM AF-PCM sera reacted with the 43-kDa component.

Profiles of immunoblots (IgG antibodies) obtained with sera from patients 8 (AF-PCM) and 9 (CF-PCM) undergoing treatment are shown in Fig. 4 and 5, respectively. The corresponding densitometries of IgG blots showed a decrease in antibodies against the gp43 molecule. Before therapy, patient 8 (AF) registered 18,408 mm² (IgG anti-gp43), whereas 1 year later, patient 8’s corresponding reading decreased to 11,851 mm²; enzyme-linked immunosorbent assay (ELISA) anti-gp43 titers were 1:102,000 and 1:12,800, respectively (data not shown), in agreement with the densitometric analysis. Patient 9 (CF) had antibody densitometries of 8,428 mm² before treatment and 5,108 mm² after 1 year of therapy and ELISA titers of 1:51,600 and 1:6,400, respectively (data not shown). Similar results were obtained with the other patients (data not shown), indicating that the response to gp43 is a good indication of clinical improvement.
DISCUSSION

Identification of antigens eliciting an antibody response to an infectious agent can lead to understanding of the immune response to a pathogenic organism at the level of individual molecules or epitopes. Such knowledge is the basis of current strategies for the development of simplified subunit vaccines to give protection against diseases (2). The *P. brasiliensis* CFA is composed of various molecules loosely bound to the yeast cell surface which are easily detached by being suspended in an aqueous solution. These molecules are probably more protected against endogenous proteases than those excreted into liquid culture medium, in which there are proteases released from dead cells during cell growth. These presumably better-preserved CFA molecules may mimic the antigens released in vivo from the cells in the site of the infection or released into the circulation, where they become available for interaction with the cells of the immune system.

Various investigators have used the immunoblot approach to study the humoral response to PCM. However, the antigenic preparations used for this purpose were exoantigens from aged filtrate cultures (7, 8, 12). The CFA, a young growth preparation, is constituted of 42 components ranging from 18 to 102 kDa, the 43-kDa component being the major glycoprotein excreted by the fungus (5).

Our results indicate that despite the clinical form of the disease, the IgG antibodies were able to recognize quite similar components of the CFA. In both forms, the 43- and the 70-kDa major components were recognized by 100 and 50% of the sera studied, respectively. Molecules 70, 74, 76, 78, and 80 kDa in size were more reactive in the acute form than in the chronic form.

Vaz et al. (17) showed with a murine experimental PCM that a diffuse 43- to 47-kDa band was recognized by all sera tested, and the IgG against it was present at a high frequency during the whole period of infection. In a previous study, Camargo et al. (7) found 100% reactivity of IgG anti-gp43 in PCM sera. Casotto et al. (8) showed that the IgG sera from 92% of the PCM patients recognized the 43-kDa component from *P. brasiliensis* B-339.

We found that IgM response to the CFA antigens was more intense against components with molecular masses greater than 50 kDa, mainly 54, 58, 60, 70, 80, 82, 86, 88, and 90 kDa, in the acute form of PCM than in the chronic form. No response against the 43-kDa component in the AF-PCM sera was verified, and fewer than 10% of the CF-PCM sera recognized the 43-kDa component. On the other hand, Mendes-Giannini et al. (12) found 100% reactivity with IgM anti-gp43 in both clinical forms of the disease. Vaz et al. (17) in their murine experimental PCM study found great reactivity of IgM against the diffuse 43- to 47-kDa band during the period of infection (16 weeks). The differences between our results and those obtained by others may be due to differences in the glycosylation process of the gp43 molecule, which seems to be dependent on the strain and on the substrate on which the fungi were cultured. In this way, carbohydrate epitopes might be inaccessible to IgM antibodies in this kind of *P. brasiliensis* CFA preparation.

The IgA response was significant in the CF-PCM group, and was directed mainly against the components with molecular masses of 20, 24, 26, 43, 46, 50, 58, 60, 70, 76, and 80 kDa, whereas a poor IgA response was obtained in the AF-PCM group. The 43- and 74-kDa components were the most reactive components in chronic PCM (about 40% reactivity each).

Our study showed that the CFA preparation is very useful in immunoblot analysis of humoral responses in PCM. Regarding the Ig class, the IgG response has apparently been the best one to be studied once it recognizes quite similar antigenic components in both clinical forms of the disease. Regarding the antigenic component to be selected for analysis of serum reactivity, the 43-kDa glycoprotein seems to represent the most important antigenic component of the PCM system.

Antigenic variability in the murine PCM model and even in the human PCM, with respect to the specific molecules involved in the *P. brasiliensis* system, was observed (8, 17). Casotto et al. (8) showed that this variability may be observed not only among different isolates of *P. brasiliensis* but also among antigens with the same molecular mass. In their study, the 44-kDa molecule (probably similar to our gp43) of three different strains of *P. brasiliensis* was recognized by the same serum only in two strains (B339 and 688) and not in strain 1789.88. Vaz et al. (17), working with resistant and susceptible mice, found significant differences in the IgM responses to many components. The susceptible mice recognized fewer components than the resistant mice. These findings may explain the different results obtained by various researchers.

For serological studies, we suggest the use of the *P. brasiliensis* B-339 isolate, which has been investigated by various groups during the last 20 years (3, 6, 13) and which contains specific antigen recognized by 96% (14) to 100% (7, 12) of the PCM sera. Casotto et al. (8) verified that freshly isolated virulent strain 1789.88 did not appear to be useful for diagnostic purposes, whereas isolates B339 and 688, which had been subcultured for many years, appear to be better.

On the basis of the present findings, as well as previous reports, and because the gp43 component is recognized by all or almost all of the PCM sera (IgG), we propose that measurement or titration of IgG anti-gp43 be used for the following up of patients under antymycotic therapy. This may be done by the reading of IgG blots by densitometry or by titration of sera by ELISA.

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