Detection of *Paracoccidioides brasiliensis* gp70 Circulating Antigen and Follow-Up of Patients Undergoing Antimycotic Therapy

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Paracoccidioidomycosis (PCM), one of the most important systemic mycoses in Central and South America, is caused by the dimorphic fungus *Paracoccidioides brasiliensis* and has a high prevalence in Brazil. Glycoproteins of 43 and 70 kDa are the main antigenic compounds of *P. brasiliensis* and are recognized by Western blotting by 100 and 96% of PCM patient sera, respectively. In the present study, an inhibition enzyme-linked immunosorbent assay (ELISA) was used to detect gp70 in different biological samples from patients with PCM. gp70 was detected in 98.76% of 81 serum samples, with an average concentration of 8.19 μg/ml. The test was positive for 100% of the patients with the acute and chronic unifocal forms of PCM and 98.4% of the patients with the multifocal chronic form, with average concentrations of 11.86, 4.83, and 7.87 μg/ml, respectively. Bronchoalveolar lavage fluid from 23 patients with pulmonary unifocal PCM and 14 samples of cerebrospinal fluid of PCM patients with 95.1% sensitivity and 100% specificity, with mean gp70 concentrations of 7.5 and 6.78 μg/ml, respectively. To investigate the potential of gp70 detection by inhibition ELISA for the follow-up of PCM patients during antymycotic therapy with itraconazole (ITZ), the sera of 23 patients presenting with the chronic multifocal form of PCM were monitored at regular intervals of 1 month for 12 months. The results showed a decrease in circulating gp70 levels during treatment which paralleled the reduction in anti-*P. brasiliensis* antibody levels. The detection of *P. brasiliensis* gp70 from the biological fluids of patients suspected of having PCM proved to be a promising method for diagnosing infection and evaluating the efficacy of ITZ treatment.

Paracoccidioidomycosis (PCM), the most prevalent systemic fungal mycosis in Latin America, is caused by *Paracoccidioides brasiliensis*, a thermally dimorphic fungus (5, 34). Different clinical forms of the disease result from the interaction of different *P. brasiliensis* strains with the host, a phenomenon that may be related to the virulence of the infecting strain. Some investigators (21, 37, 38, 41) believe that the mechanism of virulence is determined by chemical components present on the fungal wall, but others contest this explanation (44). Diagnosis of PCM is usually based on the demonstration of multibudding yeast cells in different biological specimens or the detection of specific antibodies by histopathology or culture. During the past decade, substantial progress has been made in the development of innovative approaches and methods for the serological diagnosis of PCM. Clinically relevant antigens have been identified and adapted for use in immunoassays for the detection of specific antibodies (12). Some investigators have tried to detect circulating antigen in PCM patients using polyvalent antigens or antibodies in different assays such as competition enzyme-linked immunosorbent assay (ELISA) (16), immunoradiometric assay (13), immunoelectrophoresis-immunodiffusion (17), counterimmunoelectrophoresis (35), passive hemagglutination inhibition (24), inverted linear immunoelectrophoresis (23), and immunoblotting (28); but all of these assays presented low sensitivities. Gómez et al. (19) were the first to use monoclonal antibodies (MAbs) to detect the 87-kDa circulating antigen in PCM patients by inhibition ELISA (inh-ELISA), which had 80.4% sensitivity. More recently, the same technique was used to detect gp43 in serum, cerebrospinal fluid (CSF), and bronchoalveolar lavage (BAL) fluid of PCM patients with 95.1% sensitivity and 100% specificity (25) and to monitor PCM patients duringazole treatment (26).

The gp43 glycoprotein from *P. brasiliensis* is the main antigen used for the diagnosis of PCM (32, 33, 40), being recognized by virtually all sera from infected patients by different serological methods (25, 27). Another important glycoprotein expressed by the fungus is gp70, recognized by 96% of PCM patient sera (3). gp70 is predominantly composed of polysaccharides (27) and is able to induce proliferation of lymphocytes from PCM patients (2). gp70 is also detected in the urine of patients with the acute form of PCM (36). The treatment of mice by injection of anti-gp70 MAb abolishes lung infection, suggesting that gp70 may facilitate fungal installation and the progression of lesions during the primary infection (27).

Detection of circulating antigens in patients with PCM has proved to be useful not only for diagnostic purposes but also as a tool to evaluate clearance of the fungal burden during treatment. Gómez et al. (19) reported on the use of the inh-ELISA...
to detect gp87 circulating antigen in sera from patients with active disease and in patients receiving antimycotic therapy (20). A previous study that used the same methodology was able to detect gp43 in 96.29% of PCM patients, mainly in those with the acute form of the disease (100%) (25). Moreover, patients cleared this antigen during itraconazole (ITZ) therapy, suggesting that this molecule may be used to assess the host response to antifungal therapy.

When it is considered that, in addition to gp43, gp70 is an important molecule in the P. brasiliensis system, in the present study we investigated the application of gp70 antigen detection in different biological specimens to obtain a diagnosis of PCM as well as to assess antigen clearance during antifungal treatment for disseminated disease.

MATERIALS AND METHODS

Clinical samples for PCM diagnosis. Blood specimens from a total of 81 patients with active PCM were taken at the time of the primary diagnosis. The diagnosis of PCM was established for all patients by direct KOH examination, isolation or the fungus by culture, and/or conventional positive serological tests. Samples were collected from patients at different times between March 1990 and December 2001. The samples were from patients seen at Hospital Sao Paulo, Federal University of Sao Paulo, Sao Paulo, Brazil; Hospital das Clinicas, Faculdade de Medicina da Universidade de Parana, Curitiba, Brazil; and Hospital das Clinicas, University of Campinas, Campinas, Brazil. The 81 patients tested (73 males and 8 females) were classified by their clinical presentations, as follows: 11 had the acute form of PCM (mean age, 18.8 years) and 70 had the chronic form (mean age, 46.7 years), including 64 patients with multifocal disease and 6 with unifocal disease. Biological material from 14 patients with neurological PCM (neuro-PCM) were also tested, including 14 CSF samples and 11 serum samples, all of which were obtained before treatment. In addition, 13 BAL fluid samples from patients with pulmonary PCM were tested.

Serum samples from patients (n = 71) with fungal disease and 10 BAL fluid samples from patients with tuberculosis were included as negative controls.

Clinical samples for monitoring therapy. In order to evaluate the decrease in the antigen concentration in response to antifungal therapy, a total of 23 patients with active PCM were evaluated. Sequential serum samples were obtained from all selected patients, including one sample collected at the time of diagnosis (baseline) and at least seven others collected during 8 to 12 months of treatment. Samples were collected from patients attending the Infectious Diseases Division, Hospital das Clinicas, Federal University of Parana, between 2001 and 2002. The patients were males (mean age, 47.3 years) with the chronic multifocal form of the disease who were successfully treated with ITZ. The good clinical response to antifungal therapy exhibited by all selected patients was characterized by the cessation of all clinical signs and symptoms related to the infection, significant improvement in the chest X-ray pattern of pulmonary lesions, the absence of fungi in biological specimens, and negative serology or the presence of low titers of specific antibodies (as determined by the immunodiffusion [ID] test).

Fungal isolates, antigen preparations, and gp70 purification. P. brasiliensis 113 (which was originally from the Faculdade de Medicina da Universidade de Sao Paulo and which was a gift of C. S. Lacaz) was transformed to the yeast phase, and exoantigen was produced as described by Camargo et al. (7). P. brasiliensis 113 was selected, as previous studies have shown that it produces large amounts of gp70. The yeast cells were harvested from this growth, washed three times with lysing buffer (0.05 M Tris-HCI, 5 mM EDTA), and resuspended in 50 ml of the same buffer. Yeast cells were broken with glass beads (distance, 425 to 600 μm; Sigma) in a vortex mixer. A mixture of protease inhibitors containing 1 mM phenylmethylsulfonyl fluoride (Sigma) and 1 mM EDTA was added four times during the process. The homogenate was then centrifuged at 13,000 × g for 30 min at 4°C. The supernatant (which contained the cytoplasmic antigen) was recovered and used for gp70 purification. The cytoplasmic yeast antigen was fractionated by gel filtration chromatography with Sephadex G-50 resin (Pharmacia). The eluted fractions containing the gp70 molecule were concentrated and further purified by gel filtration on a HiTrap S-200 column (Pharmacia) to eliminate high-molecular-mass contaminants. The protein concentration was determined by the method of Bradford (4). To verify the real purification of gp70, the material was monitored by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), silver staining, and immunoblotting assays.

Mab production and immunization protocols. In the first schedule of immunization, 6-week-old BALB/c mice were injected intraperitoneally with a macerated polyacrylamide gel containing gp70 (gp70 was cut from the SDS-polyacrylamide gel) at 2-week intervals for 4 months. In a second schedule, mice were immunized intraperitoneally with 10 μg of gp70 purified in the first fusion) in phosphate-buffered saline (PBS) every 2 weeks. gp70 was incorporated in Freund's complete adjuvant in the first injection and in incomplete Freund's adjuvant in the subsequent ones. Injections were always made at four different sites in the axillary and inguinal regions with a final volume of 100 μl per site. Before each immunization, the mice were bled through the ocular plexus, and the serum was separated by centrifugation and stored at -20°C. The final immunization was made intravenously 2 days before cell fusion.

Fusion protocols. Cells of murine myeloma line sp2 were fused with spleen cells from immunized mice by the method of Lopes and Alves (22). For the first fusion, hybridomas were distributed on 48-well plates (Costar), and at 10 days postfusion the colonies were screened by immunoblotting, as described below. For the second fusion, hybridomas were distributed on 96-well plates (Costar), and screening was performed by an enzyme immunoassay, as described elsewhere (18). After the hybridomas were cloned by limiting dilution and the expression of the positive clones, large amounts of antibodies were obtained from the ascitic fluid induced in BALB/c mice injected with Pristane (Sigma) before inoculation of hybridoma cells.

Antibody screening by immunoblotting. Exoantigens were submitted to SDS-PAGE with 10% acrylamide under reducing conditions, and proteins were then transferred to nitrocellulose membranes (Sigma). Fragments of nitrocellulose membranes containing gp70 were cut into small pieces and distributed in 48-well plates (Costar). After the free sites were blocked with PBS containing 5% skim milk, 200 μl of the supernatants from the hybridomas cultures was added to each well. After 1 h of incubation at room temperature, the wells were washed five times for 5 min each time with PBS containing 0.1% Tween 20 (Sigma) (PBS-T) and treated with affinity-purified peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-Rad) for 1 h at room temperature. The wells were again washed with PBS-T five times. The presence of reactive immunoglobulin G (IgG) was tested for by the addition of 4-chloro-naphthol in 50 mM Tris buffer (pH 6.8)–methanol–H2O2, and the IgG was finally washed with distilled water.

Pretreatment of immune sera for use in in ELISA. Aliquots of immune serum, CSF, or BAL fluid (200 μl) were mixed with an equal volume of 0.1 M EDTA (pH 7.2; Sigma) and boiled at 100°C for 3 to 5 min. The tubes were cooled and centrifuged at 13,000 × g for 30 min, and the supernatant was used for the test.

In-h-ELISA. The in-h-ELISA was performed as described previously (19, 25). A standard inhibition curve was first prepared by adding known concentrations of gp70 to pooled normal human serum (NHS), CSF, or BAL fluid (a standard curve was prepared for each biological material) (25). The inhibition reaction occurred when constant aliquots of anti-gp70 Mab were mixed with the inhibition standards, PCM patient serum, CSF and BAL fluid samples, and NHS control samples. These samples were then plated on previously blocked microtiter plates (inhibition plates) and incubated overnight at 4°C. The reaction plates were coated with gp70, incubated overnight at 4°C, and blocked; and samples from each well in the inhibition plates (containing a mixture of Mab bound to circulating antigen and free Mab) were transferred to the respective wells in the reaction plates. The plates were washed and probed with goat anti-mouse IgG-peroxidase conjugate and developed with a chromogenic substrate, as described previously (19, 25). The optical density (OD) readings at 492 nm were then plotted on a standard curve constructed from the data derived from Mab titration with the inhibition standards. The antigen concentrations in standard, patient, and control fluids were calculated by using the regression model constructed with the reciprocal values of the fixed concentrations of gp70 and the OD values. All of the standards, samples, and controls were tested in duplicate. The cutoff point was established as the receiver operator characteristic (ROC) curve.

Statistical analysis. Data were analyzed statistically with Stata software (version 7.0 for Windows 98/95/NT, 2001; Stata Corporation, College Station, Tex.), and specific statistical tests were determined by use of the ROC curve model. The inhibition standard curves were constructed in duplicate for at least four independent assays. A regression model was constructed with the reciprocal values of
that the antigen concentrations and the OD values obtained. Comparisons were made by one-way analysis of variance.

RESULTS

MAb production. The anti-gp70 MAb belongs to the IgG1 subclass and recognizes an antigenic determinant of \textit{P. brasiliensis} with a relative molecular mass of 70 kDa. This MAb did not recognize antigens from \textit{Histoplasma capsulatum} or Cryptococcus neoformans.

Detection of \textit{P. brasiliensis} gp70 in serum samples. The standard curve was obtained by adding increasing concentrations of gp70 to a pool of control NHS samples. The sensitivity ranged from 0.001 to 30 \(\mu\)g/ml. The cutoff point was established from the ROC curve and was based on the antigen concentration in the sera of patients with PCM and NHS. Samples with antigen concentrations greater than 1.0 \(\mu\)g/ml were considered positive.

gp70 was detected in 80 of 81 patients (98.76%) with active PCM (mean concentration, 8.19 \(\mu\)g/ml) (Fig. 1). Among this group, 11 patients presented with the acute form, 6 presented with the chronic unifocal form, and 63 presented with the chronic multifocal form; and the mean antigen concentrations in these three groups were 11.86, 4.83, and 7.87 \(\mu\)g/ml, respectively (Fig. 2). Positive results were observed for all patients with the acute or chronic unifocal form and 98.43% of the patients with the chronic multifocal form; and the mean antigen concentrations in these three groups were 11.86, 4.83, and 7.87 \(\mu\)g/ml.

FIG. 1. Detection of circulating antigen in sera from patients with PCM or other mycoses and in NHS by inh-ELISA. Groups studied: 1, patients with PCM (\(n = 81\)); 2, patients with cryptococcosis (\(n = 20\)); 3, patients with histoplasmosis (\(n = 51\)); 4, NHS (\(n = 93\)). Bars represent the mean antigen concentration for each group. The long fine line represents the cutoff point (1.0 \(\mu\)g/ml).

FIG. 2. Detection of circulating antigen in sera from patients with PCM and other fungal infections and sera from healthy controls by inh-ELISA. Groups studied: 1, patients with acute form of PCM (\(n = 11\)); 2, patients with chronic unifocal form of PCM (\(n = 6\)); 3, patients with chronic multifocal form of PCM (\(n = 64\)). Bars represent the mean antigen concentration for each group. The long fine line represents the cutoff point (1.0 \(\mu\)g/ml).

Detection of \textit{P. brasiliensis} gp70 in CSF and sera from patients with neuro-PCM. A standard inhibition curve was constructed with known concentrations of gp70 diluted in BAL fluid samples from patients with no fungal diseases. The cutoff level for positivity was established to be 0.46 \(\mu\)g/ml. All BAL fluid samples were positive for gp70, and the mean antigen concentration for this group was 7.5 \(\mu\)g/ml. BAL fluid samples from individuals with no known infectious diseases were negative for antigen detection (Table 2; Fig. 3).

gp70 antigen clearance during treatment with ITZ. Table 3 shows the initial clinical manifestations of all 23 patients with PCM as well as the gp70 antigen concentrations obtained for serum samples collected at the baseline and at the end of treatment. All 23 serum samples from patients with PCM had levels of circulating gp70 antigen above the cutoff point at the time of diagnosis, with a mean antigen concentration of 8.23 \(\mu\)g/ml. Antigen levels in serum samples collected at the first visit after the start of therapy (30 days after the start of antifungal therapy) dropped to a mean concentration of 4.0 \(\mu\)g/ml and further decreased by the 12th month, when the mean antigen concentration was 0.30 \(\mu\)g/ml. Of interest, all but four

TABLE 1. Detection of \textit{P. brasiliensis} 70-kDa circulating antigen in sera from patients with PCM and other fungal infections and sera from healthy controls by inh-ELISA

<table>
<thead>
<tr>
<th>Serum group</th>
<th>No. of serum samples</th>
<th>Mean antigen concn ((\mu)g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Reactive</td>
</tr>
<tr>
<td>PCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Chronic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unifocal</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Multifocal</td>
<td>64</td>
<td>63</td>
</tr>
<tr>
<td>Histoplasmosis</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>Cryptococcosis</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>NHS</td>
<td>93</td>
<td>93</td>
</tr>
</tbody>
</table>

* A sample was considered positive (i.e., reactive) if the antigen concentration was >1.0 \(\mu\)g/ml.
serum samples collected at the end of antifungal treatment showed complete antigen clearance and exhibited negative results (Fig. 4).

Overall, the patients showed a significant reduction (P < 0.0001) in the levels of circulating gp70 by the third month after the initiation of therapy, and this reduction was maintained until the end of the follow-up period (Fig. 5). All patients improved clinically and mycologically with therapy. The antibody titers determined by the ID test at the time of diagnosis varied from 1:2 to 1:16, and in almost all patients the titers decreased during the course of treatment.

**DISCUSSION**

Since the demonstration of the antigenemia produced in animals experimentally infected with *Streptococcus pneumoniae* (9), there have been numerous reports on the detection of antigens in blood, urine, and CSF of patients and animals infected with bacteria, fungi, viruses, and parasites. In the case of fungi, with the exception of the capsular polysaccharide of *C. neoformans*, which has been detected since 1950 (10), advances have required sensitive immunoassay procedures that have become widely applied only recently. In immunocompromised patients with cryptococcosis, histoplasmosis, or aspergillosis, the detection of circulating antigens can provide a rapid diagnosis and prompt treatment in the first days of infection, when antibodies are not yet produced or are present at low levels (11, 12). Detection of circulating *H. capsulatum* antigen has proved to be useful for the diagnosis and follow-up of patients with histoplasmosis (42, 43).

Very few techniques have shown high sensitivities and specificities for antigen detection in biological specimens for the diagnosis and follow-up of PCM patients. In our previous studies, we successfully detected gp43 in sera, CSF, and BAL fluid samples from PCM patients by inh-ELISA, which proved to be suitable for the monitoring of patients receiving antimycotic therapy. Gómez et al. (19, 20) used the inh-ELISA to detect the 87-kDa molecule in the sera of patients with PCM for both diagnostic and follow-up purposes. Antibody detection systems have also been used to assay other molecules for the diagnosis of PCM, such as a 22- to 25-kDa protein (14), a 58-kDa glycoprotein (15), and an 87-kDa protein (8).

In the present study, the overall sensitivity of gp70 detection among the 81 PCM patients was 98.8% in the presence of a mean antigen concentration of 8.19 μg/ml and reached 100% in patients with the acute form of the disease, with a mean antigen concentration of 11.86 μg/ml. Among patients with the chronic form of PCM, antigenemia due to gp70 was observed in 98.43% of serum samples from patients with the multifocal form (mean antigen level, 7.87 μg/ml); on the other hand, the sera of 100% of the patients with the chronic unifocal form had gp70, with a mean antigen concentration of 4.83 μg/ml. gp70 was detected in 100% of the CSF and BAL fluid samples, with mean antigen concentrations of 6.78 and 7.5 μg/ml, respectively. No cross-reactions were observed when sera from patients with other mycoses were tested. The majority (90.9%) of serum samples from patients with neuro-PCM were positive for gp70, with a mean antigen concentration of 3.97 μg/ml. The inh-ELISA proved to be helpful in detecting *P. brasiliensis* gp70 in biological samples, including BAL fluid and CSF, with 100% sensitivity. Considering the limitations of the conventional methods for the diagnosis of neuro-PCM, our findings suggest that the gp70 antigen test may be useful for confirmation of the diagnosis in such patients. In addition, the inh-ELISA has the advantage of being able to process large numbers of samples at the same time and presents a high sensitivity and a high specificity.

For diagnostic purposes, tests based on the ID test are generally considered to have high specificities; however, false-

**TABLE 2. Detection of *P. brasiliensis* gp70 antigen by inh-ELISA in various specimens from patients with PCM and their respective controls**

<table>
<thead>
<tr>
<th>Specimen group</th>
<th>No. of serum samples</th>
<th>% Reactive samples</th>
<th>Mean antigen concn (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Reactive</td>
<td>Nonreactive</td>
</tr>
<tr>
<td>BAL fluid (from patients with PCM)</td>
<td>23</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>CSF (from patients with PCM)</td>
<td>14</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>BAL fluid (control)</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>CSF (control)</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Serum from patients with neuro-PCM</td>
<td>11</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

* A sample was considered positive (i.e., reactive) if the antigen concentration was >0.46 μg/ml.
* A sample was considered positive (i.e., reactive) if the antigen concentration was >0.21 μg/ml.
* A sample was considered positive (i.e., reactive) if the antigen concentration was >0.97 μg/ml.

**FIG. 3. Detection of gp70 antigen in CSF from patients with neuro-PCM and BAL fluid from patients with pulmonary PCM by inh-ELISA.** Groups studied: 1, CSF of patients with PCM (n = 14); 2, CSF of patients with no fungal disease (n = 6); 3, BAL fluid from patients with PCM (n = 23); 4, BAL fluid from patients with no fungal pulmonary disease (n = 10); 5, serum from patients with neuro-PCM (n = 11). Bars represent the average antigen concentration for each group.
negative results may also occur, and this may be related to the production of low-avidity IgG2 antibodies directed against carbohydrate epitopes (31). The sensitivities of ID test-based tests range from 65 to 100% (1, 6, 29), depending on the antigen preparation used. On the other hand, tests with higher sensitivities, such as immunoenzymatic assays, present problems associated with specificity due to cross-reactivity with heterogeneous sera (5, 19).

For many years, assessment of the clearance of the fungal burden during treatment for PCM has been based on the detection of antibodies against P. brasiliensis crude or purified antigens (5, 29, 30, 39). Although healing of apparent fungal lesions may occur within a short time after antifungal treatment is initiated, long therapeutic courses are desirable in order to prevent relapses. In this case, the status of the fungal infection and the host response are usually monitored by indirect methods, in which serology provides information about the prognosis (5).

Our experience with the follow-up of patients receiving treatment for PCM has shown that many times the antibody titers obtained by the ID test do not correlate with the clinical status of the patient. For example, in some patients elevated antibody titers (1:64) were observed until the end of treatment, when the patients were clinically cured. On the other hand, although for most patients low antibody titers are related to the absence of clinical symptoms (1:2 or 1:4), in some cases low titers are present for months and are present in concert with clinical symptoms. Such discrepancies between the clinical status of the host and specific antibody detection are probably related to the fact that the cell response and not the humoral response is the main immunologic mechanism able to contain P. brasiliensis in the infected organism. For these reasons, the use of serology as a single criterion of cure in patients with PCM is controversial. In clinical practice, clinical, radiological, mycological, and serological aspects must be evaluated over a long period of observation in order to assess the results of treatment.

Regarding the detection of antigen during treatment, Mendes-Giannini et al. (28), working with a pool of sera from patients with PCM using a Western blot assay, observed that...
gp43 started to disappear from the circulation after 10 months of treatment and was undetectable after 2 years of treatment. More recently, the same group of investigators (36) detected *P. brasiliensis* antigens in 75% of patient urine samples tested by an indirect competition enzyme immunoassay and by an immunoblot test for monitoring the response to therapy.

One of the main goals of the present study was to assess gp70 antigen clearance during treatment of PCM with ITZ by comparing the clearance from serum samples collected at the baseline and after 8 to 12 months of therapy. All 23 patients selected for the study showed a good clinical response to therapy, with no relapse of fungal disease documented over the follow-up period. Overall, decreasing levels of *P. brasiliensis* gp70 were detected in patients during successful antifungal therapy. In contrast, the ID test titers of these patients varied over the follow-up period (1:16 to 1:2). Table 3 shows the clinical manifestations of PCM in the patients and the correlation between the levels of gp70 antigenemia and the levels of anti- *P. brasiliensis* antibody at the time of diagnosis, 1 month after treatment, and the end of treatment. At the time of diagnosis, all except one of the patients had detectable gp70 antigen and anti-*P. brasiliensis* antibodies (one patient had a negative ID test result). A significant decrease in gp70 titers occurred during the first month of therapy in 22 patients; at the end of therapy, only 4 (17.39%) patients (patients 4, 8, 13, and 16) had detectable gp70 antigen (antigen levels, <2.5 µg/ml) and positive titers by the ID test.

By the end of the follow-up period, antibody titers were negative in three patients (patients 2, 3, and 12), and one patient (patient 9) had undetectable antibodies (by the ID test) from the time of diagnosis to the end of treatment. However, the antigen level was also negative until the end of follow-up. Once the levels of gp70 decreased, gp70 persisted at low levels in serum until the end of therapy. On the other hand, in various patients, the antibody titers seemed to decrease a little later, after about 6 months of therapy. Of particular note is the common persistence of antibodies for a considerable period of time even after the cessation of an apparently successful course of treatment.

Decreased levels of antigenemia were observed in two patients after 6 months of ITZ treatment, but their anti-*P. brasiliensis* antibody titers remained high (1:16) and did not change throughout the period of treatment (patient 20). On the other hand, patient 9 had undetectable antibodies from the time of diagnosis to month 11 of treatment but had a substantial decrease in antigenemia levels during treatment with ITZ. All PCM patients showed clinical improvement after the period of antifungal therapy (8 to 12 months).

In conclusion, our results indicate that the detection and quantification of *P. brasiliensis* gp70 antigen in serum by inhibition ELISA is a sensitive method for the diagnosis of PCM and may be used to evaluate patients for the clearance of the fungal burden during treatment. Moreover, the high sensitivity of this assay for the detection of gp70 in different biological fluids opens a new possibility for the diagnosis of PCM in patients for whom conventional methods present limitations.

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**REFERENCES**


