Detection of Circulating Paracoccidioides brasiliensis Antigen in Urine of Paracoccidioidomycosis Patients before and during Treatment

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For the diagnosis and follow-up of paracoccidioidomycosis patients undergoing therapy, we evaluated two methods (immunoblotting and competition enzyme immunoassay) for the detection of circulating antigen in urine samples. A complex pattern of reactivity was observed in the immunoblot test. Bands of 70 and 43 kDa were detected more often in urine samples from patients before treatment. The immunoblot method detected gp43 and gp70 separately or concurrently in 11 (91.7%) of 12 patients, whereas the competition enzyme immunoassay detected antigenuria in 9 (75%) of 12 patients. Both tests appeared to be highly specific (100%), considering that neither fraction detectable by immunoblotting was present in urine samples from the control group. gp43 remained present in the urine samples collected during the treatment period, with a significant decrease in reactivity in samples collected during clinical recovery and increased reactivity in samples collected during relapses. Reactivity of some bands was also detected in urine specimens from patients with “apparent cure.” The detection of Paracoccidioides brasiliensis antigens in urine appears to be a promising method for diagnosing infection, for evaluating the efficacy of treatment, and for detecting relapse.

Paracoccidioides brasiliensis is the causal agent of paracoccidioidomycosis, a systemic disease that presents a marked tendency toward dissemination with involvement of any organ or system. The clinical presentation mimics those of other serious disease entities and also could be associated with immunosuppression, AIDS, and other diseases (6, 14). The constant movement of people from rural to urban areas and the increase in the average life span will certainly contribute to a higher frequency of patients with immunosuppressive diseases or conditions for endogenous reinfection of quiescent paracoccidioidomycosis foci. A definitive diagnosis is usually made by visualization or isolation of the fungus from the lesions, which is time-consuming and lacking in sensitivity. Detection of specific antibodies in serum has also been one of the main tools in diagnosing this disease and may be useful in monitoring the evolution of the disease and its response to treatment (19). Serum antibodies are long lasting; some diagnosed patients have low levels of specific antibodies for a long time, and it is doubtful whether they are ever cured (16). Eventual remission frequently occurs. Thus, studies are still under way to design a test that would permit a more accurate characterization of cure in patients with paracoccidioidomycosis. For that, the detection of antigen, not antibody, may be such a test.

The detection of circulating antigen represents a useful approach in the serodiagnosis of invasive fungal disease (4, 5, 11, 15). In cases of paracoccidioidomycosis, attempts to identify antigenemia have been made by using various tests. Most studies have employed methods with low sensitivities (7, 8, 23).
tion, we used these samples for our ensuing experiments. All the urine samples were filtered, dialyzed overnight against distilled water, concentrated against polyethylene glycol 20000 (Sigma) in dialysis tubing with a molecular weight cutoff of 6000 to 8000 (Sigma) and stored at −80°C.

**Fungal strains and antigen preparation.** *P. brasiliensis* 113, *Histoplasma capsulatum* 58, *Aspergillus fumigatus* 354, *Candida albicans* 461, and *Cryptococcus neoformans* 35 were obtained from the culture collection of Instituto Medicina Tropical de São Paulo.

Two extracts of *P. brasiliensis* were used: (i) the total antigen prepared after mixing a cell-free antigen, culture filtrate, and crude cellular extract and (ii) a culture filtrate. The cell-free antigen and culture filtrate were prepared as described elsewhere (1, 18). The crude extract was prepared from a suspension of fungal cells containing 1 mM phenylmethylsulfonyl fluoride (Sigma). The cells were frozen and broken with glass powder and liquid nitrogen. The mixture was centrifuged, and the supernatant was stored at −70°C. Culture filtrates of *H. capsulatum* and *A. fumigatus* and somatic antigens of *Candida albicans* and *Cryptococcus neoformans* were prepared in accordance with Centers for Disease Control procedures. The protein content was measured by the method of Lowry et al. (13), and the electrophoretic pattern was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12) and immunoblotting (18).

**Preparation of antiserum.** Two antisera were prepared, namely, anti-culture filtrate and anti-total antigen of *P. brasiliensis*. Rabbit sera were initially immunized by intradermal injections of 1 ml of *P. brasiliensis* extract mixed with 1 ml of Freund’s complete adjuvant. Subsequent injections of antigen in Freund incomplete adjuvant were given weekly for a period of 4 weeks and then monthly for a period of 3 months. The rabbits were bled 7 days after the last dose. The immunoglobulin G (IgG) fractions of these antisera were obtained by precipitation with ammonium sulfate followed by protein A-Sepharose chromatography. These antisera gave strong reactions in response to the culture filtrate antigen and the total antigen by immunoblotting, respectively.

**ELA-c.** The ELA-c was developed from a modification of the method proposed by Freitas da Silva and Roque-Barreira (7). A reference standard urine sample produced by adding the total *P. brasiliensis* antigen to a pool of urine from healthy volunteers to a concentration of 1 mg/ml was used for ELA-c standardization. The standard was used to prepare dilutions in buffer containing 0.05% Tween 20 and 3% defatted powdered milk, providing final antigen concentrations of 100 to 0.0004 μg/ml. Previous studies were done by using the culture filtrate antigen and total antigen for detection of urine-soluble antigens. Since the best results were obtained with the total antigen, it was used in all experiments.

Polystyrene plates (Nunc) were sensitized with 0.1 ml of *P. brasiliensis* total antigen, diluted in 0.2 M sodium carbonate buffer (pH 9.6) at a final protein concentration of 1.5 μg/ml. The plates were incubated at 37°C in a humid chamber for 2 h and then at 4°C for an additional 18 h and were washed three times (5 min each) with 0.01 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20.

The urine specimens of the patients were diluted from 1:2 to 1:8 by serial twofold dilution in buffer. The same procedure was used for the negative control. A volume of 0.1 ml of each dilution was incubated with an equal volume of anti-*P. brasiliensis* rabbit IgG incubated with buffer only. The result was considered positive only if the percent inhibition observed with increasing dilutions of the reference standard antigen to a pool of urine plus antigens (positive controls), and a pool of normal urine (negative control). The reagents chosen were the ones that distinguished the best and largest number of bands in the positive control. To determine the sensitivity of the test described above, serial dilutions of positive and negative controls were also tested in the same way.

In the immunoblot test, urine samples that demonstrated gp70 or gp43 either alone or concurrently were considered positive for a diagnosis of paracoccidioidomycosis.

**Statistical analysis.** A comparison of the sensitivities of the methods was performed by the binomial test described in the specifications of Siegel (24). The *P* values reported are two-sided, and results were considered significant if the *P* value was lower than 0.05.

**RESULTS**

The analysis of two antigens of *P. brasiliensis* by SDS-PAGE revealed a distinct electrophoretic pattern of proteins. The total *P. brasiliensis* antigen had at least 26 bands, ranging from 160 to 6 kDa, and the culture filtrate was less complex, with major bands of 70 and 43 kDa.

The intensity of the immunoblot was proportional to the amount of the antigen of the standard curve. The data demonstrate that immunoblotting performed with artificially added antigen detected the 70- and 43-kDa bands at concentrations up to 2 to 5 ng. The anti-culture filtrate serum was used to develop the immunoblot test for detection of urine-soluble antigens; it was chosen because it best discriminated between *P. brasiliensis* fractions. Bands in the 160-, 100-, 70-, 75-, and 43-kDa areas were observed in the positive control and the culture filtrate antigen. Principal bands of 70, 55, and 43 kDa were seen in the positive control (data not shown).

Nineteen bands were present in the urine samples from the paracoccidioidomycosis patients (group 1) in the pretreatment phase, three bands were present in individuals with albuminuria, and four bands were present in normal subjects. The antiserum did not recognize any band in the samples from the tuberculosis patients.

We observed a complex pattern of reactivity in regard to the urine samples from paracoccidioidomycosis patients, with bands ranging from 110 to 24 kDa being recognized. Bands of 70, 64, 55, and 43 kDa were recognized in 66.7, 41.7, 50.0, and 66.7% of the patient samples, respectively. Weak bands in the 94-, 90-, and 60-kDa areas were seen in 10, 10, and 20%, respectively, of the samples from patients with albuminuria. Of the urine samples from healthy individuals, weak bands were observed in the 82 (10%), 72 (30%), 67 (10%), and 48 (10%)-kDa areas.

gp43 and gp70 were detected by immunoblotting in the urine samples from 11 (91.7%) of 12 patients with paracoccidioidomycosis (Table 1). The specificity of this immunoblot was 100%, since gp43 and gp70 fractions were not present in urine samples from the control group nor in the extracts of other fungi.

When we compared samples from the first group of patients before and during treatment by the immunoblot test, we observed a distinct reactivity pattern. Some of the patient urine samples presented mainly a decrease in reactivity to the 43-kDa antigen in some periods (Fig. 1A and B), which correlated with clinical improvement. In other patients, constant decay in reactivity was not observed following treatment, while in others, an increase in reactivity of gp43 was found, as shown in Fig. 1C. This result preceded and/or coincided with a worsening of the clinical state of the patients, who showed weight loss, weakness, and enlarged lymphatics.

Of the two individuals (group 2) with signs and symptoms...
suggesting paracoccidioidomycosis but without a confirmed diagnosis, immunoblot analysis showed the presence of the 43-kDa fraction in urine samples from these patients.

In the urine samples from group 3, we observed reactivity of some bands, such as those at 70, 67, 64, 43, 28, and 24 kDa. Fractions of 70, 43, and 28 kDa were detected more frequently in these samples, and the 67-kDa band appeared in the urine specimens of healthy individuals (Fig. 2).

The limit of antigen detection by the EIA-c was found to be 2.3 ng of protein per ml of urine. The assay had a sensitivity of 75% and a specificity of 100% when a 35% cutoff point was used. The antigen levels detected ranged from 0.08 to 4.8 μg/ml, with a median of 0.79 μg/ml for the 12 patients analyzed (Table 1).

There were no statistically significant differences between the results of the EIA-c and immunoblot tests ($P < 0.05$). Discrepant results were observed only in two patients with paracoccidioidomycosis, both of which were positive by the immunoblot test but falsely negative by the EIA-c. On the other hand, antigen was not detected by immunoblotting nor by EIA-c in the urine sample from only one patient of group 1. This patient presented a mild chronic form of the disease with involvement of the oral mucous membranes and a regional lymph node.

Regarding therapy follow-up for the first group, a decrease in urinary antigen correlated well with clinical improvement whereas an increase correlated with worsening. A representative four cases of paracoccidioidomycosis were chosen to demonstrate different behaviors during the follow-up treatment (Fig. 3). If disease reactivation occurred with active lesions, the highest levels of urinary antigen were detected. In some cases, an increase in this antigen was demonstrated before clinical manifestation.

One of the two patients with paracoccidioidomycosis in group 2 had antigen levels in urine of 0.46 μg/ml, and the other

### Table 1. Detection of antigens in urine samples from paracoccidioidomycosis patients before treatment by immunoblot test and EIA-c

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Band size(s) detected by immunoblotting (kDa)</th>
<th>Antigen concn (μg/ml) detected by EIA-c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>1.86</td>
</tr>
<tr>
<td>3</td>
<td>70, 43</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>43</td>
<td>2.09</td>
</tr>
<tr>
<td>5</td>
<td>70, 43</td>
<td>0.46</td>
</tr>
<tr>
<td>6</td>
<td>70, 43</td>
<td>3.89</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>2.01</td>
</tr>
<tr>
<td>8</td>
<td>70, 43</td>
<td>0.08</td>
</tr>
<tr>
<td>9</td>
<td>70, 43</td>
<td>1.12</td>
</tr>
<tr>
<td>10</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>—a</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>70, 43</td>
<td>4.79</td>
</tr>
</tbody>
</table>

*a*—, negative or no reagent.
had negative results. Of the patients in group 3, which consisted of treated patients, only one patient had 0.2 mg of antigen per ml and the others were negative.

**DISCUSSION**

Several studies have shown that assays for the detection of antigens in biological specimens are powerful tools for quantitative and qualitative diagnoses and for monitoring chemotherapy. Antigen detection tests may be more effective than antibody tests for diagnosing paracoccidioidomycosis mainly in immunocompromised patients. These patients possibly have diminished humoral antibody responses (10, 14). On the other hand, the greatest challenges with paracoccidioidomycosis patients are treatment control and the more accurate characterization of cure. The newly developed immunoblot and EIA-c tests were capable of detecting antigens in urine specimens, and the potential of these are evident.

The antigenic mosaic of *P. brasiliensis* comprises at least 60 distinct components which may or may not exhibit enzymatic activity (1, 3, 20, 21, 27, 29). Of this group of soluble antigens, the 43-kDa antigen is the most frequently recognized by patient sera (2, 18, 22), followed by the 70-kDa antigen (2).

The 43-kDa antigen has already been detected in the serum and urine of patients with paracoccidioidomycosis (17, 19). The presence of this antigen in urine was demonstrated earlier in a patient with the acute form of the disease (19). Recently, Gómez et al. (9), using an inhibition enzyme-linked immunosorbent assay, reported the presence of an 87-kDa *P. brasiliensis* antigen in urine samples. However, the sensitivity obtained was low.

The two fractions gp43 and gp70 were detected separately or concurrently with 91.7% sensitivity by the immunoblot test. These antigens were detected exclusively in paracoccidioidomycosis patients, not in patients with other diseases, in healthy individuals, or in other controls. Our results indicate that these antigens have potential for the diagnosis of paracoccidioidomycosis.

The EIA-c assay used in the present study allowed the detection of antigen concentrations in urine as low as 2.3 ng/ml. Despite the high sensitivity of the EIA-c used in this study, the presence of *P. brasiliensis* antigens could not be detected in 25% of the urine samples tested. The particular cutoff chosen optimized the true-positive rate while maintaining a low false-positive rate.

When we compared the two methods, the EIA-c demonstrated lower sensitivity than that of the immunoblot test. Sev-
eral factors may have contributed to the false-negative determinations in the EIA-c in our study, such as the low concentration of the \textit{P. brasiliensis} antigen detected by this test, the infrequency of sampling, and the use of urine samples from patients without previous treatment (unlike the immunoblot test, which used urine samples from patients that had received reduced treatment). On the other hand, antigenic presentation in the EIA-c (plastic plates) and in the immunoblot test (nitrocellulose paper) may also involve different conformations.

We noticed that only one patient was negative by both tests. This patient presented low levels of antigen in serum (unpublished results) and was also negative for specific antibodies by routine test. The failure to detect specific antigen in urine in this case is probably related to the urinary antigen load. The evaluation of only one sample of urine could also have contributed to this negative result.

The sensitivities of both methods in detecting urinary \textit{P. brasiliensis} antigens were similar despite successful identification in these two cases by immunoblotting alone. Further refinements in the EIA-c procedure may improve the sensitivity of this test.

At present, the immunoblot technique should be the preferred test to be introduced into a laboratory routine until future comparative analysis could be performed with a larger number of patients. The diagnosis of this disease based on antigen detection in urine could then be made earlier and the number of patients. The diagnosis of this disease based on future comparative analysis could be performed with a larger number of this test.

The demonstration of various antigens in the urine samples of patients with paracoccidioidomycosis and the correlation between antigenuria levels and the clinical evolution of the patients suggest the clinical applicability of the methods described here, especially with respect to the evaluation of disease activity. The detection of \textit{P. brasiliensis} antigens in the urine thus appears to be a promising method for the diagnosis of paracoccidioidomycosis, for monitoring the effects of treatment, and for reducing the incidence of relapsing infection.

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REFERENCES


