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

MARGARETE APARECIDA SALINA,[1] MARIA APARECIDA SHIKANAI-YASUDA,[2] RINALDO PONCIO MENDES,[3] BENEDITO BARRAVIERA,[3] AND MARIA JOSÉ SOARES MENDES GIANNINI[1]*

Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas, UNESP, Araraquara,[1] Departamento de Doenças Infecciosas e Parasitárias, Faculdade de Medicina, USP, São Paulo,[2] and Departamento de Moléstias Infecciosas, Faculdade de Medicina, UNESP, Botucatu,[3] Brazil

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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). gp43, the most important antigen of *P. brasiliensis* (22), has been demonstrated in the sera of patients with the chronic and acute forms of paracoccidioidomycosis. Prior to treatment and after commencement of antifungal therapy, the antigen starts to disappear from the circulation (17). More recently, antigen detection in urine has been proposed as an alternative method for diagnosis of systemic mycoses (28). Recently, our group detected the presence of gp43 and other antigens in the urine of patients exhibiting the acute form of paracoccidioidomycosis (19).

In the present investigation, we determined the presence of *P. brasiliensis* antigens in the urine of patients by an indirect competition enzyme immunoassay (EIA-c) and an immunoblot test for diagnosing infection and for monitoring the response to therapy.

MATERIALS AND METHODS

Biological specimens. Forty-eight urine samples were collected from 12 patients with paracoccidioidomycosis. The diagnosis was established by histological examination of biopsy samples and by visualization and isolation of the fungus in culture. This group of patients (group 1) was monitored during treatment, and the samples were collected in different periods. Group 2 consisted of two individuals with active disease but without a confirmed diagnosis. One of these patients had experienced a paracoccidioidomycosis relapse, and the other one was a patient under diagnostic investigation. However, neither the typical *P. brasiliensis* yeast forms nor seroconversion could be demonstrated to confirm the diagnosis. Group 3 consisted of nine patients who had previously documented *P. brasiliensis* infection but with an "apparent cure." The apparent cure was defined in accordance with Mendes et al. (16) by the following criteria: (i) absence of symptoms for 2 years after the end of the maintenance therapy; (ii) stabilization of the chest X-ray findings, which present only fibrotic scar sequelae; and (iii) negative serology (immunodiffusion test) or presence of insignificant titers (complement fixation test, for example). Group 4 consisted of a variety of subjects who contributed 31 urine samples, 10 from clinically healthy individuals, 10 from patients with albuminuria, and 10 from patients with tuberculosis and a patient with aspergillosis.

Samples. Previous studies were done with urine concentrated 10- and 30-fold. Since all the bands had appeared in the urine samples upon the first concentra-

* Corresponding author. Mailing address: Laboratório de Micologia Clínica, Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas—UNESP, Rua Expedicionários do Brasil, 1621, Cx. Postal 502, Araraquara SP, Brasil, CEP 14801-902. Phone: (016) 232-1233, ext. 149. Fax: 5516-232-0880. E-mail: giannini@fcfar.unesp.br.

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 6,000 to 8,000 (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 cellular 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 antisera. Two antisera were prepared, namely, anti-culture filtrate and anti-total antigen of *P. brasiliensis*. Rabbits were initially immunized by intradermal injections of 1 ml of *P. brasiliensis* extract mixed with 1 ml of Freund 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.

EIA-c. The EIA-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 EIA-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 diluted 1:1,000 in buffer. After 4 h of incubation with shaking at room temperature, 0.1 ml of each mixture was added in duplicate to the previously sensitized wells and the plate was left standing for 1 h at 37°C in a humid chamber. After washing as described above, 0.1 ml of sheep anti-rabbit IgG antiserum conjugated with peroxidase (Sigma) in the appropriate dilution was added to each well and the plate was incubated for 1 h in a humid chamber at 37°C. The plate was washed again, and the reaction was developed by adding a solution containing peroxide and *ortho*-phenylenediamine (Sigma). After 30 min of incubation in the dark at room temperature, the enzyme-substrate reaction was stopped by the addition of 50 μl of 3 N HCl. The absorbance was measured at 450 to 630 nm with a Boehringer spectrophotometer.

The mean value for the readings obtained at the 1:2 dilution was calculated in relation to the mean value for the inhibition control (anti-*P. brasiliensis* rabbit IgG incubated with buffer only). The result was considered positive only if the value was higher than the cutoff point for the plate. The mean ± 1.25 standard deviation of the absorbance obtained with the urine samples from healthy subjects and individuals with albuminuria was established as the cutoff point. The percent inhibition observed with increasing dilutions of the reference standard urine sample in relation to that of the control was used to construct the standard curve for each plate. The antigen concentration was calculated from a linear regression equation of the standard curve by using the percent inhibition obtained with the 1:2 dilution of the test urine sample with a positive reaction.

The cross-reactivity of the EIA-c was also tested with antigens of *H. capsulatum*, *A. fumigatus*, *Cryptococcus neoformans*, and *Candida albicans*. Dilutions of these antigens were made in pooled normal human urine and were tested by EIA-c.

Immunoblotting. The protein components of the biological specimens were fractionated by electrophoresis on an SDS–10% PAGE gel under reducing conditions by use of a discontinuous buffer system (12). After electrophoresis, the gel was transferred by electrophoresis to nitrocellulose membranes. The immobilized antigens were revealed by treating the blots with a specific serum for paracoccidioidomycosis. The nitrocellulose membranes were processed by the methods of Towbin et al. (25) and Mendes-Giannini et al. (18).

In a previous experiment, both antisera were assayed separately against the following preparations: culture filtrate or somatic antigens of *P. brasiliensis*, a pool of normal 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

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

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

[a] —, negative or no reagent.

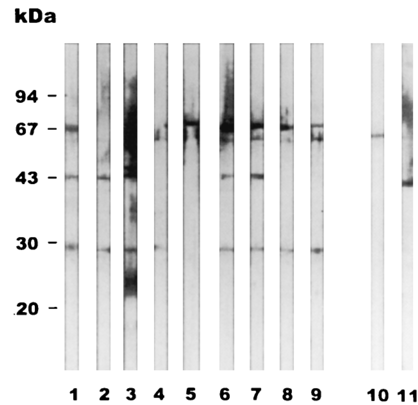

FIG. 2. Immunoblot of urine samples from paracoccidioidomycosis patients probed with anti-culture filtrate *P. brasiliensis* serum. Lanes: 1 to 9, urine samples from apparently cured patients; 10, negative control; 11, positive control.

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

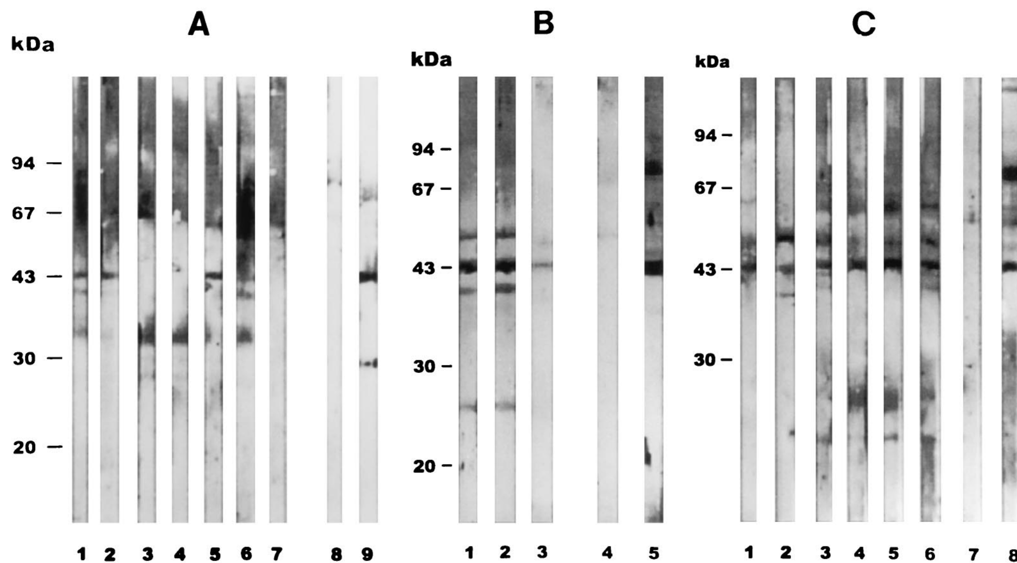

FIG. 1. Immunoblot of urine samples from paracoccidioidomycosis patients probed with immune anti-*P. brasiliensis* serum; samples were taken before and during treatment. (A) Patient 12 (with acute form of the disease). Lanes: 1, before treatment; 2 to 7, during treatment; 8, negative control; 9, positive control. (B) Patient 5 (chronic form). Lanes: 1, before treatment; 2 and 3, during treatment; 4, negative control; 5, positive control. (C) Patient 2 (acute form with clinical worsening). Lanes: 1, before treatment; 2 to 6, during treatment; 7, negative control; 8, positive control.

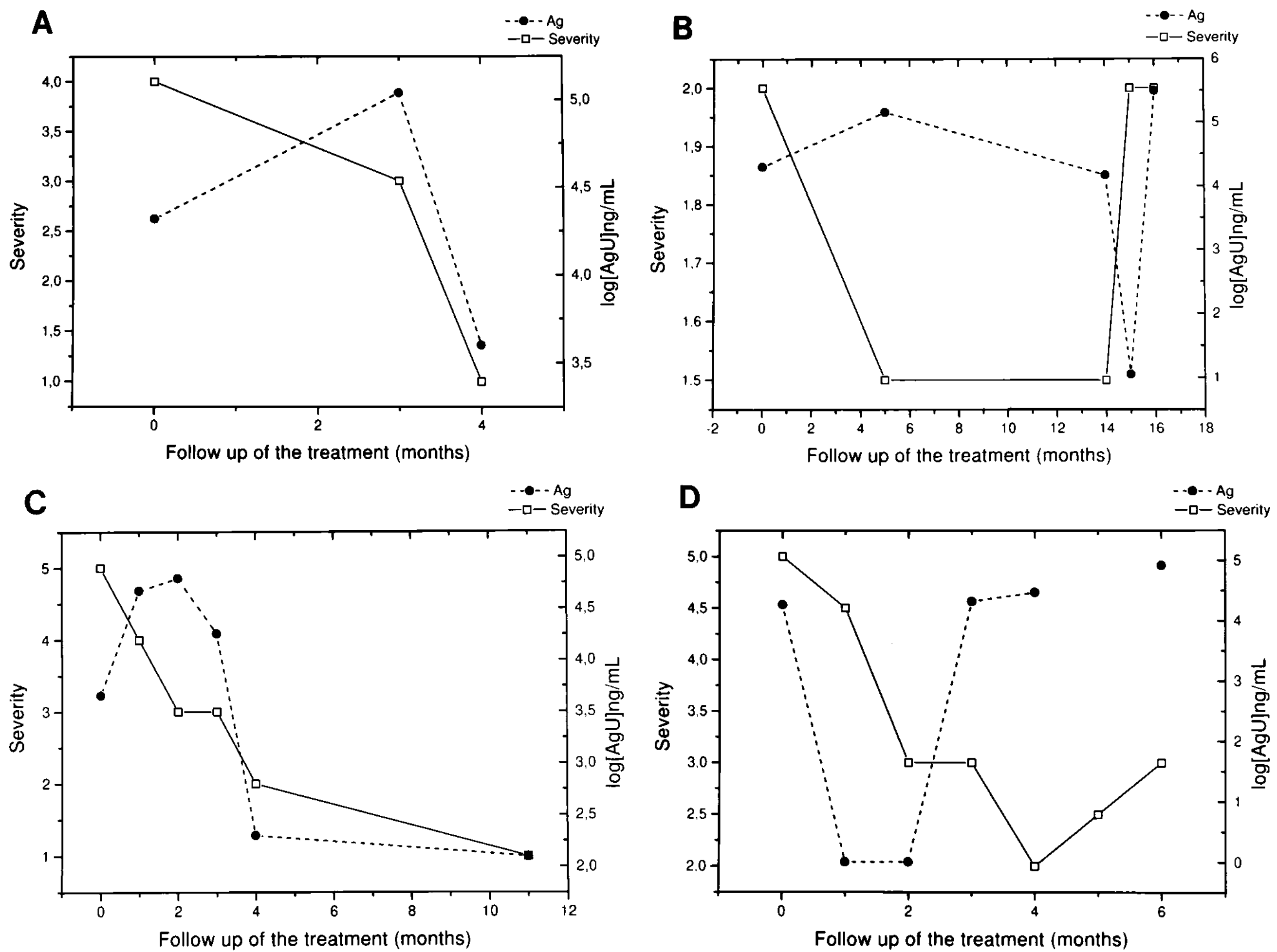

FIG. 3. Paracoccidioides antigenuria in four patients during follow-up treatment correlated with severity degrees. (A and C) Chronic and acute cases demonstrated good response to treatment and correspondingly low levels of antigen. (B and D) Chronic and severe acute cases with worsening of clinical aspects corresponding with raised antigen levels. AgU, units of antigen.

had negative results. Of the patients in group 3, which consisted of treated patients, only one patient had 0.2 μg 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, 23, 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 *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 *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 application of therapy and clinical improvement would be more effective and, finally, less expensive.

During the follow-up of patients undergoing therapy, monitoring the circulating antigen instead of antibodies may also be important mainly as a criterion of cure. Verification of *P. brasiliensis* antigen levels offers the reassurance that increases or decreases are valid and not attributable to inaccuracies of the test. The differential diagnosis between a paracoccidioidomycosis relapse and another disease was performed by the identification of the specific urinary antigen. The reintroduction of treatment led to improvement in the patient and consequently may become a valuable adjunct in the diagnosis of this fungal disorder.

The frequent relapse observed in patients who discontinue treatment early has stimulated the study of criteria of cure. Apparent cure is an expression that was introduced to characterize the situation when all other criteria of cure (clinical, mycological, radiological, and serological) had been observed for at least 3 years, the first year during which the patient was still under maintenance treatment and the other two during which the patient was without antifungal administration. One of the objectives of this research is also to evaluate the detection of specific antigen in patients with apparent cure.

In this work, gp43 was present in the urine samples of patients during the treatment period and diminished reactivity occurred during clinical recovery. Increased reactivity occurred in samples during relapses. This antigen was also detected, although faintly, in patients considered healed, revealing the presence of this antigen over long periods, even after treatment.

These data suggested one feasible explanation for the frequency of relapses in patients with this disease. The fungus probably remains quiescent in some part of the human body and liberates antigens such as gp43 and gp70 for prolonged periods of time. In the present work, one patient from group 3 showed clinical and laboratory signs indicating pulmonary lesion sequelae due to cigarette smoking and to paracoccidioidomycosis. Serum antibodies were not detected, but specific antigen was detected in the urine by EIA-c. Tuder et al. (26) performed a careful study of lungs at autopsy from paracoccidioidomycosis patients and demonstrated the presence of areas with fibrosis interspersed with areas of emphysema and foci of active disease. The antigen revealed in the present study could have spread from foci of active disease to the bloodstream and, finally, the urine. Considering the specificity of the method for the detection of *P. brasiliensis* antigen, these findings suggest the introduction of this procedure as a criterion of cure.

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 *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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