Antigenemia in Paracoccidioidomycosis

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Severe forms of paracoccidioidomycosis (Pcm) are accompanied by intense immunological involvement characterized by depression of the cell-mediated immune response and by high levels of antibodies in serum with no protective function. These changes can be reversed by antifungal treatment. It has been suggested that antigens of *Paracoccidioides brasiliensis* released into the circulation during the active phase of the disease may be involved in the genesis of the changes in the immune response. In the present study, we evaluated the antigenemia of patients with Pcm using a competitive enzyme-linked immunosorbent assay (ELISA-c) capable of detecting 6 ng of antigen per ml of serum. Twenty-seven of 88 serum samples tested gave positive results, with the highest frequency of positivity being detected in patients with the severe acute form of the disease; these patients had the highest antigen levels (0.03 to 3.4 μg/ml). Follow-up of one case showed a correlation between antigen levels in serum and evolution of the disease. False-positive reactions were observed in sera from patients with histoplasmosis, aspergillosis, and cryptococcosis. The results indicate that the described method has potential for clinical application, especially with respect to the evaluation of disease activity. Quantification of fungal antigens in the serum of patients with active Pcm represents an objective parameter for the study of the physiopathology of the disease.

Paracoccidioidomycosis (Pcm) is a chronic granulomatous disease caused by the thermodimorphic fungus *Paracoccidioides brasiliensis*. The infection is endemic in Latin America, with high incidences in Brazil, Colombia, Venezuela, and Argentina (13). Humans acquire the disease by inhaling the infecting forms (conidia), which are present in the environment. The lungs are initially affected (12), and the fungi then spread to multiple organs through the lymph nodes and bloodstream (8).

Different clinical forms of the disease result from the interaction of different *P. brasiliensis* strains with the host, a phenomenon that is related to the virulence of the infecting strain, which is determined by chemical components present on its wall. For example, strains in which α-1,3-glucan is predominant are more virulent than strains in which β-1, 3-glucan is predominant, although other components must also be involved (23, 29). There is a wide variety of clinical forms of the disease that can be classified into two major groups: the acute form, which is of abrupt onset, with intense involvement of the phagocytic mononuclear system and deterioration of the general condition of the host, and which attacks young individuals of both sexes, and the chronic form, which is of slow evolution, with lesions restricted to a few organs and preservation of the general condition of the host, and which usually attacks adult men (8).

The diagnosis is usually made on the basis of serological tests for the detection of specific antibodies or by isolating the fungus from the secretions of the lesions (25).

Increased levels of specific antibodies without protective properties (1) and a depressed cell-mediated immune response (19, 20) with reduced T lymphocytes and a predominance of T supressor cells (18) are detected in patients with the disseminated form of the disease, in which granulomatous lesions and a great proliferation of *P. brasiliensis* are also observed. These alterations are reversible when specific

treatment is applied (20), and soluble factors present in the plasma of patients with Pcm have been found to depress the lymphocyte proliferation response to mitogenic stimuli (5) and to lead to morphological lymphocyte changes (4).

As suggested experimentally, *P. brasiliensis* antigens may be the main factors responsible for the alterations of the immune responses observed in patients with Pcm (14); immune complexes may also contribute to such changes (3). Although the presence of circulating soluble antigens has been observed in human systemic mycoses (9, 22, 24), it has proved to be difficult to demonstrate in patients with Pcm. Attempts have been made by using immunodiffusion (21), immunoelectroosmophoresis-immunodiffusion (10), inverted linear rocket immunoelectrophoresis (15a), and passive hemagglutination inhibition (15b). The presence of the 43-kDa glycoprotein antigen fraction was detected in a pool of sera from patients with Pcm by using the immunoblot reaction (17).

In the present investigation, we determined the presence of *P. brasiliensis* antigens in the sera of patients with the acute and chronic forms of the disease by using a competitive enzyme-linked immunosorbent assay (ELISA-c). Antigens were predominantly detected in patients with the disseminated forms of the disease at levels ranging from 0.03 to 3.4 μg/ml of serum.

MATERIALS AND METHODS

Patients with Pcm. The study was conducted on 88 serum samples obtained from 80 patients with active Pcm (9 samples were from one patient), all of them exhibiting clinical and laboratory signs of the disease, such as pneumonia, polyadenopathy, mucosal or mucocutaneous lesions, and increased specific antibody levels. The diagnosis was also confirmed by histopathological examination or culture.

The patients were divided into groups corresponding to those with the acute and the chronic forms of the disease. The group with the acute form of the disease consisted of 51 patients subdivided into patients with the moderate form (*n*

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= 20) and patients with the severe form (n = 31), according to the extent of dissemination of the disease. Males predominated over females (40 versus 11, respectively), and the age range was 8 to 55 years, with most patients being 10 to 43 years old.

The group with the chronic form of the disease consisted of 29 patients subdivided into patients with the unifocal form (n = 7) and patients with the multifocal form (n = 22). Again, males predominated over females (27 versus 2, respectively), and the age range was 28 to 83 years, with most patients being 38 to 63 years old.

Other patients. Thirty-one serum samples from patients with other types of mycoses were also studied. Diagnosis had been confirmed by secretion culture, hemoculture, or histopathological examination of biopsy fragments. The patients were distributed as follows: histoplasmosis, 8 cases (3 of which were associated with AIDS); cryptococcosis, 6 cases (2 of which were associated with AIDS); aspergillosis, 14 cases (11 of which had aspergillomas); and candidiasis, 3 cases.

Serum samples from patients with pulmonary tuberculosis (four cases) and multiple myeloma (two cases) were also studied.

Healthy controls. Fifty-three serum samples from healthy individuals, most of them employees in the administrative sector of the University Hospital of Ribeirão Preto, University of São Paulo (UH-FMRP-USP), with no history of pulmonary disease, were also studied. These sera were tested separately and were used as negative controls; these sera were denoted normal human sera (NHS).

P. brasiliensis **antigen.** Yeast-like *P. brasiliensis* colonies (strains isolated from patients admitted to UH-FMRP-USP) were cultured at 37°C in enriched medium (7) and ruptured by maceration in a mill in the presence of glass beads in three consecutive phases of 10 min each. The material was resuspended in 0.01 M phosphate-buffered saline (PBS; pH 7.2), left to stand for 48 h at 4°C, and centrifuged at 1,760 × g for 15 min. The supernatant was stored at −20°C in the presence of 0.01% thimerosal until use and was used as the antigen (total antigen).

Protein (15) and sugar (6) concentrations were 5.56 and 5.3 mg/ml, respectively.

Other antigens. The following antigens were used: 43-kDa glycoprotein fraction prepared from the filtrate of a *P. brasiliensis* culture kindly provided by Zoilo Pires de Camargo (Escola Paulista de Medicina), metabolic antigens of *Histoplasma capsulatum* and *Aspergillus fumigatus*, and metabolic and somatic antigens of *Cryptococcus neoformans* kindly provided by Roberto Martinez (UH-FMRP-USP).

Anti-*P. brasiliensis* immunoglobulin G (IgG). Male rabbits were immunized with 1.0 ml of total *P. brasiliensis* antigen emulsified in complete Freund's adjuvant and injected into each leg (0.5 ml per leg). Total antigen boosters (0.5 ml) were applied monthly up to the fifth month. The sera that formed precipitation lines against total antigen when they were submitted to counterimmunoelectrophoresis were stored at −20°C.

The IgG fraction of the antiserum was obtained by precipitation with ammonium sulfate followed by ion-exchange chromatography on DEAE–Sephadex-A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). Immunoelectrophoresis of rabbit total antiserum showed only one precipitation line corresponding to IgG. The final protein content was 12 mg/ml (15).

Anti-rabbit IgG-peroxidase conjugate. Anti-rabbit IgG serum was obtained by immunizing a male sheep with rabbit

IgG. The IgG fraction of sheep serum was purified as described above and was tested by immunoelectrophoresis in the presence of total sheep antiserum, which showed only one precipitation line corresponding to IgG. The protein content was 24.6 mg/ml (15). This sheep IgG was conjugated with peroxidase (Sigma Chemical Co., St. Louis, Mo.) by the optimized $NaIO_4$ method as described by Tijssen and Kurstak (27). The conjugate was purified on Sephacryl-S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), concentrated, and stored at −20°C in 50-μl aliquots in the presence of an equal volume of glycerol. The protein content was 4.2 mg/ml (15).

ELISA-c. A reference standard serum (RSS) that was produced by adding the total *P. brasiliensis* antigen to an NHS pool to a concentration of 556 μg/ml was used for ELISA-c standardization. The standard was used to prepare dilutions in diluent buffer (NHS diluted 1:10 in 0.01 M PBS [pH 7.2] containing 0.05% Tween 20, 5% defatted powdered milk, and 20 mM $MgCl_2$), and the following final antigen concentrations were obtained: 55.6, 13.9, 3.48, 0.87, 0.22, 0.054, 0.013 and 0.003 μg/ml.

Polystyrene plates (Immulon type II, Dynatech) were sensitized with 0.1 ml of total *P. brasiliensis* antigen diluted in 0.2 M sodium carbonate buffer (pH 9.6) containing 20 mM $MgCl_2$ at a final total protein concentration of 1.1 μg/ml. The plates were incubated at 37°C in a humid chamber for 1 h and then at 4°C for an additional 17 h and were then washed three times with 0.01 M PBS (pH 7.2) containing 0.05% Tween 20 and $MgCl_2$ for 3 min each time.

The patient sera to be tested were diluted from 1:2 to 1:32 by serial twofold dilution in diluent buffer. The same procedure was followed for the negative control (NHS pool); i.e., 0.1 ml of each RSS dilution of the patient sera and of the negative control was incubated with an equal volume of anti-*P. brasiliensis* rabbit IgG diluted 1:8,000 in diluent buffer. After 12 h of incubation (1 h at room temperature with shaking and 11 h at rest at 4°C), 0.1 ml of each mixture was added in duplicate to the previously sensitized wells and the plate was left to stand for 2 h at 37°C in a humid chamber. After washing as described above, 0.1 ml of the conjugate diluted 1:1,000 in diluent buffer was added to each well and the plate was incubated for 2 h without shaking in a humid chamber at 37°C. The plate was washed again, and the reaction was developed by the addition of 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 2 N sulfuric acid. The A_{492} was measured with a Bio-Tek-100 instrument (EMBRABIO; Empresa Brasileira de Biotecnologia Ltda., São Paulo, Brazil).

The mean value of the readings obtained at the 1:2 dilution was calculated in relation to the mean value of the inhibition control (anti-*P. brasiliensis* rabbit IgG incubated with diluent buffer only), and if the value was higher than the cutoff point for the plate, the result was considered positive.

The cutoff was established by using the Student t test for one sample, with a 95% confidence limit according to the following equation: cutoff point = AB + [SD · t (n − 1)/$\sqrt{n}$], where AB is the mean absorbance of the negative control, SD is the standard deviation of the negative control, t corresponds to degrees of freedom, and n is the number of samples in the negative control.

The percent inhibition observed with increasing RSS dilutions in relation to the inhibition control was used to construct the standard curve for each plate.

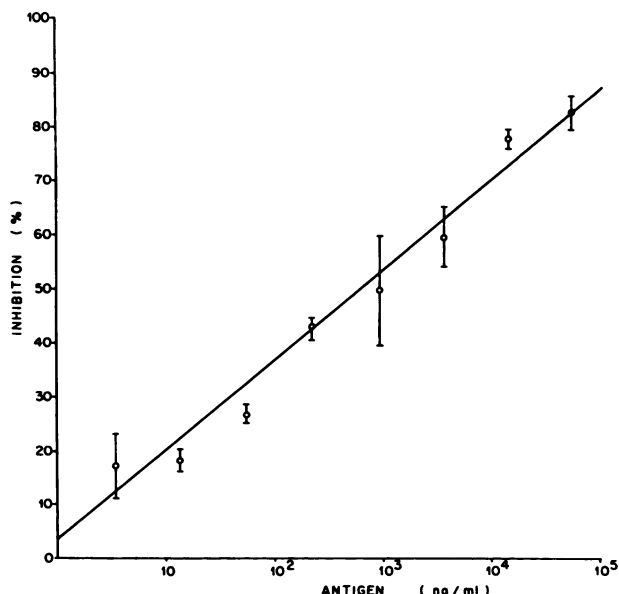Antigen concentration was calculated by using the linear

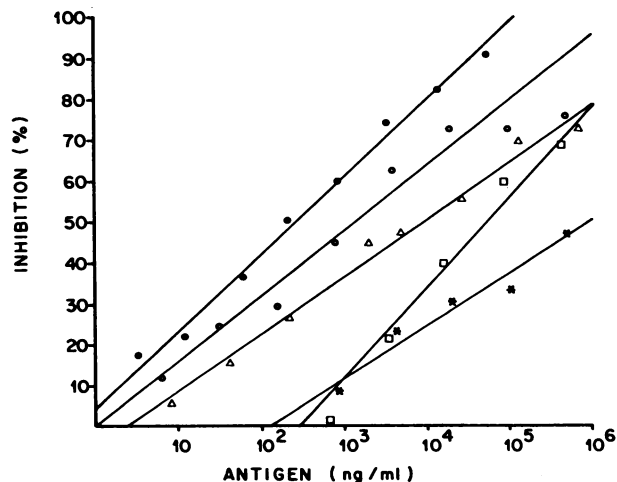FIG. 1. Standard curve of *P. brasiliensis* antigen detection by ELISA-c.

FIG. 2. Inhibition curves obtained by ELISA-c by using a standard serum sample containing known concentrations of total *P. brasiliensis* antigen (●), the 43-kDa fraction (O), metabolic *H. capsulatum* antigens (△), metabolic *A. fumigatus* antigens (□), and metabolic and somatic *C. neoformans* antigens (*).

regression equation of the standard curve from the percent inhibition obtained with the 1:2 dilution of the test serum with a positive reaction.

Statistical analysis. Data were analyzed statistically by the exact test of Fisher, the chi-square test, and the nonparametric Mann-Whitney U test, with the level of significance set at $P < 0.05$.

RESULTS

P. brasiliensis **antigen detection.** The standard curves that permitted antigen quantification in patient sera were obtained by adding increasing concentrations of total antigen to an RSS pool (Fig. 1). The sensitivity ranged from 0.006 to 60 μg of protein per ml of serum.

The total *P. brasiliensis* antigen was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was submitted in parallel to silver staining (2) and to staining with the Schiff reagent (11). A subsequent immunoblot developed with anti-*P. brasiliensis* IgG indicated that the glycoprotein and polysaccharide fractions of the fungus are recognized by the anti-*P. brasiliensis* IgG used in the ELISA-c (data not shown).

Assay of other antigens. To evaluate the specificity of the method, the inhibition curves caused by different antigen preparations in the ELISA-c were analyzed comparatively. Taking the curve obtained with the total *P. brasiliensis* antigen as a reference, we noted that 50% inhibition was determined by 10 times higher concentrations of the 43-kDa glycoprotein fraction of *P. brasiliensis* and by 100 to 10,000 times higher concentrations of the antigen preparations of *H. capsulatum*, *A. fumigatus*, and *C. neoformans* (Fig. 2).

Serum study. In a total of 88 serum samples studied, antigens were detected in 27 patients with active Pcm, 20 of whom had the acute form and 7 of whom had the chronic form of the disease. The largest proportion of positive results was observed in patients with the severe acute form (48.4%) when compared with the results observed in patients with other forms of the disease, i.e., acute moderate (25%),

chronic unifocal (28.6%), and chronic multifocal (22.5%). The antigen levels detected ranged from 0.03 to 3.4 μg/ml and were significantly higher in patients with the severe acute form of the disease than in patients with any of the other forms (Fig. 3).

Among the 31 serum samples from patients with other mycoses, cross-reactions were observed in sera from two patients with disseminated histoplasmosis and AIDS, one patient with cryptococcosis associated with AIDS, and one patient with aspergillosis (a form of aspergilloma) at a level
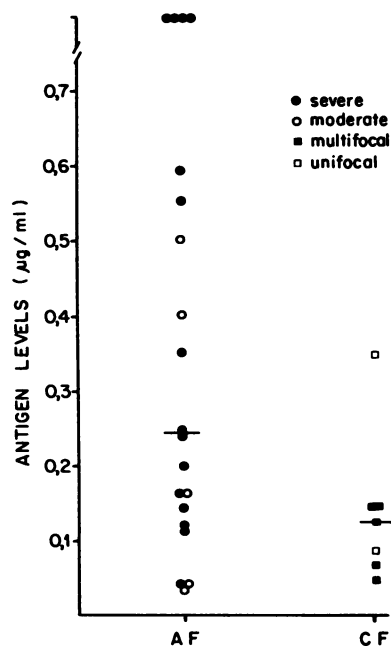

FIG. 3. Distribution of fungal antigen levels detected by ELISA-c in the serum of patients with the acute (AF) and chronic (CF) forms of Pcm. Antigenemia was significantly higher in patients with the severe acute form of disease than it was in all other patients ($P = 0.04$). The horizontal lines represent the median.
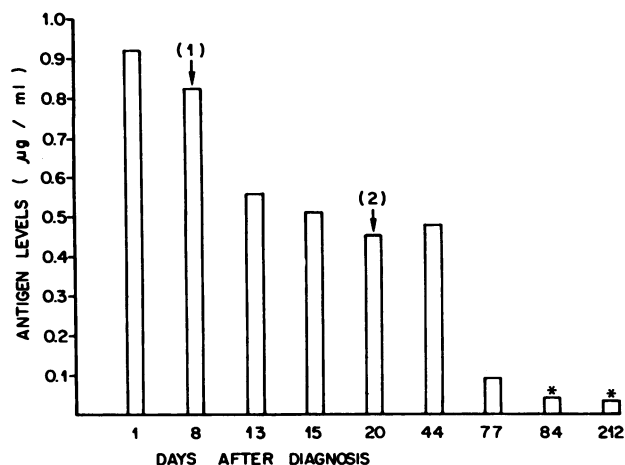
FIG. 4. Fungal antigen levels in serum samples from one patient with the acute severe form of Pcm over a period of 9 months after diagnosis. The institution of antifungal treatment is shown: (1) 2 g of sulfadiazine per day (2), 4 g of sulfadiazine per day. *, values considered negative because they were below the cutoff point.

ranging from 0.05 to 0.16 μg/ml. No positive reactions were observed in the sera of patients with candidiasis, tuberculosis, or multiple myeloma or the sera of healthy controls.

A 9-month follow-up of a patient with the severe acute form of Pcm showed that a progressive fall in antigen levels in serum occurred after treatment (sulfadiazine), with clinical improvement (Fig. 4).

The frequency of positivity was not correlated with the number or type of structures involved, specific antibody titers, or the time of disease manifestation.

DISCUSSION

The ELISA-c used in the present study offered the possibility of detecting antigen concentrations in serum of as low as 6 ng/ml. Its application to the study of antigenemia in 80 patients with the acute and chronic forms of Pcm showed a positive reaction in 33.7% of cases, with a higher frequency noted among patients with the acute severe form of the disease, who also presented with the highest antigen levels. This is in agreement with the extensive dissemination of the disease in such patients.

Previous attempts to detect circulating antigens in patients with Pcm have not been successful. Most studies have used methods with low sensitivities. The more sensitive immunoblot technique has yielded positive results; these results, however, are limited because they are not quantitative and refer to the detection of the antigen in a pool of sera from patients with Pcm (17).

Despite the high sensitivity of the ELISA-c used in this study, we did not detect the presence of P. brasiliensis antigens in 66.3% of the tested sera. This fact may be explained by (i) antigenemia levels below the sensitivity of the methods caused by individual characteristics of the disease or by the treatment instituted and/or (ii) by the formation of circulating immune complexes with no free epitopes that would permit antigen detection. The drastic effect of treatment on the decrease in antigenemia is illustrated by the 9-month follow-up of a patient with the severe acute form of Pcm (Fig. 4).

The anti-P. brasiliensis antibody used in the ELISA-c was obtained by immunization of rabbits with a mixture of somatic and metabolic fungus antigens in order to permit the detection of a wide range of antigens of the species which are present in the serum of patients with Pcm. The observation by electrophoresis and immunoblot that the antiserum recognized glycoprotein and polysaccharide components of P. brasiliensis led us to optimize the ELISA-c by adding 20 mM magnesium chloride to the reaction buffers. This procedure has been applied successfully to the detection of antigens in the sera of patients with coccidioidomycosis (9) and led to a 20-fold increase in the sensitivity of our ELISA-c in relation to that of the assay carried out in the absence of magnesium chloride (data not shown).

Many of the antigenic determinants of P. brasiliensis detectable by ELISA-c are shared by other fungal species (28), a fact that impairs the specificity of the method. When 31 serum samples from patients with other mycoses were submitted to our ELISA-c, cross-reactions with samples from patients with histoplasmosis, cryptococcosis, and aspergillosis were observed. This observation led us to evaluate the specificity of the method by constructing inhibition curves with antigen preparations from different fungal species. We noted that 50% inhibition levels are caused by these preparations only at concentrations much higher than the concentration of total P. brasiliensis antigen, suggesting that false-positive ELISA-c results would be possible only with sera from patients with mycoses and high levels of circulating fungal antigens.

The demonstration of circulating antigens in 34% of patients with Pcm and the correlation between antigenemia levels and the clinical evolution of one patient with the severe acute form of the disease suggest the clinical applicability of the method described here, especially with respect to the evaluation of disease activity. Maximal performance in terms of sensitivity could be reached by previous sample processing for the dissociation of immune complexes in serum and, in terms of specificity, by use in the assay of antibodies that recognize only epitopes that belong exclusively to P. brasiliensis.

Once maximal assay performance is achieved, it is hoped that full advantage will be taken by using it to measure circulating antigens rather than using standard serological tests, as reported for other mycoses (16, 24, 26). The expected advantages include confirmatory or early diagnosis of the disease, especially for immunodeficient patients. Another perspective experimentally supported by the observed induction of T supressor cells by soluble P. brasiliensis antigens (14) is the possibility of evaluating the role of antigenemia in the alterations of the immune response associated with Pcm.

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