Increased Immunoglobulin G Anti-Paracoccidioides brasiliensis Serum Antibody Avidity as a Predictor of Favorable Posttherapeutic Evolution in Paracoccidioidomycosis

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Paracoccidioidomycosis is endemic in Latin America, and ca. 80% of all cases occur in Brazil. Little is known about antibody avidity or the evolution of such avidity in the posttherapeutic period for the different clinical presentations of the disease. In the present study, we evaluated 53 patients with paracoccidioidomycosis and calculated the avidity index. Medium- and high-avidity antibodies were found in 79.5% of patients with chronic presentation (n = 39). Among patients with the acute form (n = 14), 57.1% of the antibodies presented low avidity. In the posttherapeutic period, there was a significant increase in antibody avidity in patients presenting with the chronic multifocal form. In our preliminary study, which needs to be confirmed using a larger number of samples, the optimized method for studying antibody avidity detected differences among the clinical presentations of the mycosis and indicated the value of the avidity index as a marker of posttherapeutic evolution of patients with a multifocal chronic form of the disease.

Materials and Methods

Patients and blood samples. The patients evaluated in the present study were undergoing treatment at the Hospital das Clínicas, Division of Clinical Infectious and Parasitic Diseases, School of Medicine, São Paulo University. Blood samples were collected from 53 patients diagnosed with paracoccidioidomycosis, confirmed by mycology testing (culture and/or direct microscopy) and histopathology. Patients were classified according to the clinical forms of the disease. A total of 53 patients were included: 14 patients with the acute form and 39 with the chronic form (34 with the multifocal chronic form and 5 with the unifocal chronic form). For the chronic unifocal form, only patients with lesions in the upper airways or oral mucosa without visceral involvement or lung involvement and normal lung X-ray images were included. For the study of the posttherapeutic period, 17 patients with the chronic multifocal form were included, each of whom consented to the collection of two to four samples (at least the first and the fourth samples). One sample was collected prior to the initiation of the antifungal therapy (T0), and the other three samples were collected after the antifungal treatment: T1, 5 to 9 months (mean = 6.5, standard deviation = 1.1); T2, 11 to 14 months (mean = 12.6, standard deviation = 0.8); and T3, 23 to 39 months (mean = 27.1, standard deviation = 5.4). The Ethics Committee of the Hospital das Clínicas approved the study design. Informed consent was obtained from all patients, and the Brazilian Ministry of Health guidelines for human experimentation were strictly followed.

Preparation of the cytoplasmic culture and filtrate antigens. The antigens were obtained from the P. brasiliensis 113 isolate selected on the basis of previous standardized production of high amounts of cytoplasmic antigen, as previously described (12). After 7 days of culture, glass beads and liquid nitrogen were added to the suspension of yeastlike cells, which was then macerated and centrifuged, and the supernatant was recovered. For culture filtrate antigen, the fungus was cultivated in a liquid medium of neopeptone, glucose, thiamine, and asparagine, after which the culture supernatant was filtered, concentrated, and dialyzed against distilled water for 48 h, and then lyophilized (3). Protein levels were determined by the Lowry method (9).

ELISA for the study of IgG anti-P. brasiliensis antibody avidity. The dissociation method was used with urea as a denaturing agent (6). High-binding Costar plates (Corning-Costar, Cambridge, MA) were sensitized with 5 μl of cytoplasmic antigen and 5 μl of culture filtrate antigen mixture. The free sites of the plates were blocked with 5% skim milk. Serum samples were diluted in duplicate from 1/100 to 1/1,600 (serial twofold dilutions), and 100 μl was added to each well. After a 30-min incubation, one of the duplicates was washed with 8 M urea (Sigma, St. Louis, MO) and the other with 100 μl of phosphate-buffered saline (PBS) for 5 min at room temperature. Peroxidase-conjugated human anti-IgG antibody was used at a dilution of 1:20,000, and o-phenylenediamine-hydrogen peroxide was used as the chromogenic solution.

AI determination. The avidity index (AI) was calculated for each sample as the logarithm of the ratio between the absorbance of PBS (P) and urea (U) curves, to the level of 50% of the maximum absorbance of urea (Fig. 1). According to a logarithmic scale of concentrations, the 50% point of urea (point U in Fig. 1) is given by $U = -\log P$. Therefore, the AI is given by the formula: $AI = 10^{\log P - \log U}$, where $P$ and $U$ are the absorbances at the 50% point of the urea and PBS curves, respectively.

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maximum absorbance of \( U \) Abs

is 50% of the sample, treated \((U)\) or not treated \((P)\) with urea, corresponding to 1/100 to 1/1,600 (serial twofold dilutions) are shown.

### Designation of avidity levels

Levels of avidity were assigned from lowest to highest and divided into three groups: low (AI < 0.35), medium (AI ranging from 0.35 to 0.50) and high avidity (AI > 0.50), according to a previous study (7).

### Statistical analysis

Friedman repeated measures analysis of variance on ranks and the Student-Newman-Keuls method were used to analyze differences between groups. Comparisons between medians were made by using the Mann-Whitney rank sum test, comparisons between proportions were made by using the Fisher exact test, and comparisons between medians were made by using the Mann-Whitney rank sum test. Correlations between the AI and the duration of disease were made by linear regression.

### RESULTS

#### Avidity and clinical forms

The clinical forms of paracoccidioidomycosis were assessed according to the level of avidity: low \((<0.35)\) and high \((>0.50)\). Among patients with chronic presentation, 79.5% had a predominance of medium- and high-avidity antibodies (Fisher exact test, \(P < 0.0001\)); 57.1% of patients with the acute form had a predominance of low-avidity antibodies \((P = 0.706)\). On the other hand, the frequency of low-avidity antibodies was significantly higher in the acute form \((57.1\%)\) than in the chronic form \((20.5\%)\) \((P = 0.0173)\). The difference in the median \((m)\) values between the acute \((m = 0.33)\) and chronic \((m = 0.41)\) forms was significant \((P = 0.041, \text{Mann-Whitney rank sum test})\) (Table 1).

#### Avidity and duration of disease

The disease duration was defined as the time since the onset of symptoms as reported by the patients at the first consultation. In the assessment of correlation between AI and the duration of disease among 26 patients with the chronic form and 9 with the acute form, a positive linear correlation was seen only for patients with the acute form (linear regression, \(r = 0.788, P = 0.0116\)); the mean AI \((95\% \text{ confidence interval})\) in the acute form was 0.40 \((0.28 \text{ to } 0.52)\). One patient with the acute form was excluded from the analysis because the duration of disease was 108 months; for the other nine patients with the acute form the duration of disease ranged between 1 and 8 months.

#### Avidity and the posttherapeutic period

In the assessment of the posttherapeutic evolution of antibodies in 17 patients with the chronic multifocal form, we found that antibody avidity was significantly higher in the final period \(T3\) than in the first period \((t \text{ test}, P = 0.0008)\) (Fig. 2). Most patients were prescribed sulfamide derivates for periods of 6 to 36 months. Although the treatment was the same for the two clinical forms, its duration varied according to the severity of disease. When treatment was interrupted, the following criteria were established: absence of signs and symptoms, improvement of thorax X ray, negative or scar antibody levels established by counterimmunoelectrophoresis, as standardized by the Brazilian Guidelines on Paracoccidioidomycosis (13). Unfavorable evolution was considered when persistence of antibodies or

### TABLE 1. Distribution of the AIs for IgG anti-

P. brasiliensis antibodies of low, medium, and high avidity according to clinical presentation

| Clinical presentation | No. of subjects with indicated AI (%) |
|-----------------------|--------------------------------------|
|                       | Low (<0.35) | Medium (0.35 to 0.50) | High (>0.50) |
| Chronic               | 39          | 8/39 (20.5)            | 20/39 (51.3) | 11/39 (28.2) |
| Acute                 | 14          | 8/14 (57.1)            | 3/14 (21.4)  | 3/14 (21.4)  |
| Total                 | 53          | 16/53 (30.2)           | 23/53 (43.4) | 14/53 (26.4) |

* \(n\), Total number of subjects.
Of the 17 patients, 14 presented a favorable clinical course. The disease was clinically inactive, serology was negative in the final assessment period (T3), and most evolved to higher avidity levels (Fig. 2). The other three patients presented unfavorable evolution due to nonadherence to the treatment regimen: one patient continued to present medium-avidity antibodies during the posttherapeutic period; two patients, although also nonadherent, continued to present high-avidity antibodies during the follow-up period (Fig. 2). These two patients had been diagnosed with paracoccidioidomycosis 3 and 8 years previously and were therefore considered cases of recurrence.

**DISCUSSION**

In the present study, we evaluated a method of studying the AI of IgG anti-*P. brasiliensis* serum antibodies and the role of avidity as a predictor in the posttherapeutic outcome of mycosis.

We observed different distributions of antibody avidity according to the clinical presentation of the mycosis. Patients with the chronic form presented a predominance of medium- and high-avidity antibodies; in contrast, patients with the acute form presented a predominance of low-avidity antibodies. Patients with the chronic form presented benign and more insidious evolution than the patients with the acute form, with more severe disease characterized by fungus dissemination and involvement of the lymph nodes, liver, spleen, and bone marrow (13). These severe forms of the disease evolve to antigen-specific cellular immunosuppression (1) and humoral hyperactivity response by B lymphocytes (10).

The predominance of low-avidity antibodies against *P. brasiliensis*, observed in the acute form, is similar to that seen in primary toxoplasmosis (6, 8) and dengue infection (4) in contrast to the high-avidity levels found in chronic forms of the mycosis and in secondary toxoplasmosis and dengue.

In the posttherapeutic period, a significant increase in antibody avidity was seen during the follow-up of 17 patients with the chronic multifocal form and may be attributed to the long duration of the disease (up to 96 months), and to the long period of treatment in these cases (6 to 36 months). In contrast, we did not detect a significant increase in the AI in the posttherapeutic period of the acute form and chronic unifocal form, perhaps due to the short duration of the disease or the small number of samples analyzed. It is important to emphasize that the two clinical forms of the paracoccidioidomycosis occur with different frequencies (acute form in 5 to 10% of cases). Since this was a prospective study, we evaluated only cases that occurred during the study period, so it was not possible to include similar numbers of patients with the different clinical forms. In the posttreatment period, there was a reduction in the number of patients because some patients did not attend for the collection of some samples.

In our study, we found a relationship between increased avidity and favorable clinical response to treatment in chronic cases of the disease. However, in patients with an unfavorable clinical course, due to nonadherence to treatment, the AIs remained unchanged at 23 to 39 months after the beginning of treatment. The persistence of high antibody avidity in two cases of unfavorable clinical course may be explained by the longer disease duration related to the recurrence of mycosis initiated several years previously.

Although it was not an objective of the present study to evaluate mechanisms to guide the evolution of antibody avidity, fully understanding such mechanisms presents new challenges. In 2003, Neves et al. (11) studied patients with paracoccidioidomycosis and found that the lack of serum reactivity in immunodiffusion tests was attributable to the presence of low-avidity IgG2 antibodies against carbohydrate epitopes. In addition, it is extremely important to verify the relationship between the maturation of antibody avidity and the clinical profile after treatment in patients with unfavorable clinical course, as observed in a limited number of cases in our study.

Although this is a preliminary study that needs to be confirmed using a larger number of samples, the assessment of antibody avidity of paracoccidioidomycosis in our study not only showed the relationship between avidity and clinical presentation, relating it to severity, but also demonstrated the value of the AI as an indicator of the control of the disease in patients with the chronic form of the mycosis.

**FIG. 2.** AIs of IgG *P. brasiliensis* antibodies from 17 patients with chronic multifocal form. There were significant differences in avidity between T0 and T3 (Student *t* test, *P* = 0.0008). T0, time point 0 (prior to treatment); T3, time point 3 (after 23 to 39 months of treatment). ue, unfavorable evolution.
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