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Detection of the 43,000-Molecular-Weight Glycoprotein in Sera of Patients with Paracoccidioidomycosis

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The 43,000-molecular-weight (43K) soluble glycoprotein was detected in sera of patients with paracoccidioidomycosis by the immunoblot technique by using as the probe rabbit monospecific antisera to this fraction. The 43K antigen was present before treatment in sera of patients with the acute (juvenile) form; it started to disappear from circulation after 10 months of chemotherapy, and it was undetectable after 2 years of treatment. In the chronic cases, the 43K antigen was detected in patients without treatment, and it was absent in the healed cases. The detection of the 43K protein specific to *Paracoccidioides brasiliensis* may be important for its diagnostic value as well as for modulation of the host immune response.

Paracoccidioidomycosis is a disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The main components of the cell wall, in either the mycelial or the yeast phase of the fungus, are glucans. In the former, there is predominance of α-1,3-glucan, and in the latter, the β-1,3-glucan is the most abundant substance. Also, galactomannan is present in the surface of both forms of the fungus. It has been shown that this substance cross-reacts with similar structures of other dimorphic fungi (1, 7, 18). Soluble antigens produced by the mycelial and yeast forms comprise up to 60 components, including glycoproteins and proteins with enzymatic activity (26). Among this group of soluble antigens, two components (E1 and E2) with cathodic migration were demonstrated by immunoelectrophoresis. The E2 fraction was proposed to be an antigen with diagnostic value (27, 28).

More recently, biochemical identification was performed in a yeast culture filtrate and three main glycoproteins of 72,000, 55,000, and 43,000 molecular weight (72K, 55K, and 43K, respectively) were analyzed. Moreover, the immunoprecipitation of this preparation with anti-E rabbit antiserum recognized the 43K glycoprotein (14).

Circulating antigens were identified by the immunoprecipitin test in sera of paracoccidioidomycosis patients (4, 17; S. W. Magaldi and D. W. R. Mackenzie, Abstr. Col. Intern. Paracoccidioidomycosis, p. 41, 1986). However, the characterization of the antigenic fraction has not been established. Soluble antigens are being detected in other mycoses, and it has been postulated that they can be used as a tool for the diagnosis of disease or as a possible way to obtain a direct correlation between the presence of the antigen and the active disease (5, 11, 16, 19, 24).

We analyzed the humoral immune response of patients with paracoccidioidomycosis by using the immunoblot technique with the culture filtrate of the yeast form as the antigen, and it was found that 100% of the patients without treatment produced antibody to the 43K component. Moreover, the decay of immunoglobulin G (IgG) antibody response to this protein seemed to correlate with clinical improvement (12).

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In view of these results, we searched for the presence of the 43K antigen in sera of these patients in order to verify whether this antigen, which is easily found in the culture supernatant, is also liberated from the fungus in the active disease.

MATERIALS AND METHODS

Sera. A total of 28 serum specimens obtained from 15 patients with proven paracoccidioidomycosis and 5 healthy donors were separated into the following groups. Group 1 included 12 serum specimens from four patients with the acute (juvenile) form, and they were analyzed at three different periods (before treatment and after 10 and 24 months of treatment). Group 2 included five serum specimens from patients with the chronic (adult) form, taken during active disease. They were not under treatment at the time serum specimens were collected. Group 3 included six serum specimens from individuals considered healed, by clinical criteria, for more than a year. Group 4 included five serum specimens from clinically healthy individuals (controls).

Preparation of antisera. The antigen extract was prepared as described elsewhere (13). Briefly, *P. brasiliensis* was maintained in Casal's medium for 90 days at 35°C. After the filtrate was concentrated, it was dialyzed in phosphate-buffered saline, 0.15 M, pH 7.2. The protein content was determined by the Lowry method (8). Then it was purified by gel filtration and affinity chromatography by using a concanavalin A (ConA)-Sepharose 4B column (ConA fraction) (2, 14) (Pharmacia Fine Chemicals). This preparation was heated with sample buffer (2% sodium dodecyl sulfate [SDS], 0.5 M dithiothreitol, 0.002% bromophenol blue, 62.5 mM Tris hydrochloride, pH 6.8) at 100°C for 2 min before being loaded onto a 10% SDS-polyacrylamide gel.

After electrophoresis, the horizontal band containing the 43K antigen was excised and emulsified with Freund complete adjuvant. The antigen-adjuvant mixture was injected into the dermis of rabbits in biweekly intervals for a total of seven times.

The antiserum was absorbed with *Histoplasma capsulatum* and was tested by immunodiffusion and immunoblot.

Immunoblot. The partially purified fraction obtained from

the ConA-Sepharose 4B column or the culture filtrate of *P. brasiliensis* was electrophoresed in 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore Corp.) in a transblotting chamber containing 25 mM Tris and 192 mM glycine in 20% methanol at pH 8.3 (20). The membrane was cut into vertical strips, and free protein-binding sites were blocked with 5% skim milk in phosphate-buffered saline, 0.15 M, pH 7.2, at 4°C for 4 h. Each strip was incubated overnight at room temperature with patient sera diluted 1/100 in 5% phosphate-buffered saline–skim milk. After being washed, the strips were incubated for 2 h with goat anti-human IgG labeled with peroxidase (horseradish peroxidase; Sigma Chemical Co.). The enzymatic reaction was developed with diaminobenzidine–H_2O_2 as substrate.

Detection of the 43K antigen in serum. The IgG fraction of rabbit antiserum to the 43K antigen of *P. brasiliensis*, purified by protein A-Sepharose chromatography, was coupled to 5 ml of a CNBr-activated Sepharose 4B column (Pharmacia Fine Chemicals).

Each group of sera was pooled in a total volume of 2 ml and passed through the column. The proteins bound to it were eluted with glycine, 0.2 M, pH 2.8. After the pH was adjusted to 7.2, they were concentrated 10 times and dialyzed. Then the samples were electrophoresed in 10% SDS-polyacrylamide gel. Part of each sample was analyzed by silver staining, and the other part was transferred to a nitrocellulose membrane and probed with the anti-43K rabbit antisera (1:600); it was also probed with patient sera containing these antibodies. The reaction was developed with goat anti-rabbit IgG peroxidase (1:3,000) and was developed as described above.

RESULTS

A representative experiment showing an immunoblot done with a pool of sera obtained from patients with the acute form who were followed up during 2 years of treatment is shown in Fig. 1. It was observed that after 10 months of treatment, a decrease of antibody reactivity to the 43K protein found in the culture filtrate of the fungus began to occur (lane 2), and at 2 years, antibodies to this protein were hardly detected (lane 3). This phenomenon was also observed with some cases of the chronic form. Also, antibodies could not be detected in individuals considered healed (data not shown). These same pools of sera were used to determine the possible presence of the 43K antigen.

In order to detect the 43K antigen in sera of patients, an antibody to this antigen was raised by immunization of rabbits. These antisera reacted specifically with the 43K protein found in the ConA fraction of the culture filtrate of the fungus at a titer of 1/600 by immunoblot. The IgG fraction of the anti-43K rabbit antisera was coupled to CNBr-activated Sepharose 4B to perform affinity chromatography by using the different sera to concentrate the 43K antigen. The SDS-polyacrylamide gel electrophoresis of the eluates obtained from the column which was silver stained is shown in Fig. 2. We observed that sera from healthy individuals (negative control) showed various bands, but not the 43K antigen (lane 1). On the other hand, the ConA column eluate (positive control) presented the 43K band (lane 2). The eluate of a pool of sera obtained from patients with the acute form also stained a band with 43K (lane 3). The eluate of sera obtained from patients with the acute form treated for 10 months showed a faint 43K band (lane 5), and after 2 years of treatment, this band could not be detected (lane 6).

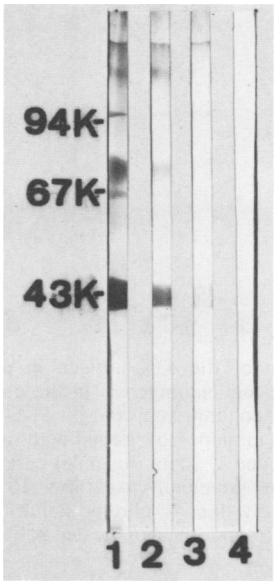

FIG. 1. Antibody response to the 43K glycoprotein of patients with the acute (juvenile) form of paracoccidioidomycosis at different periods of treatment as analyzed by immunoblot. Each determination was obtained with a pool of sera. Lane 1, Before treatment; lane 2, 10 months after treatment; lane 3, 24 months after treatment; lane 4, controls.

To demonstrate specifically that the 43K band seen in the silver-stained gel is, in fact, the antigen which is also present in the culture filtrate, the eluates obtained from sera of patients with acute or chronic forms and sera obtained at different periods of treatment were electrophoresed and transferred to the nitrocellulose membrane and probed with the rabbit anti-43K antisera (Fig. 3). The 43K band was developed with sera obtained from acute cases before (lane 1) and after (lane 2) 10 months of treatment. After 2 years of treatment, the 43K antigen could not be detected (lane 3). In the chronic cases without treatment, this band was present. In contrast, sera from the healed patients did not show this specific band (lane 6).

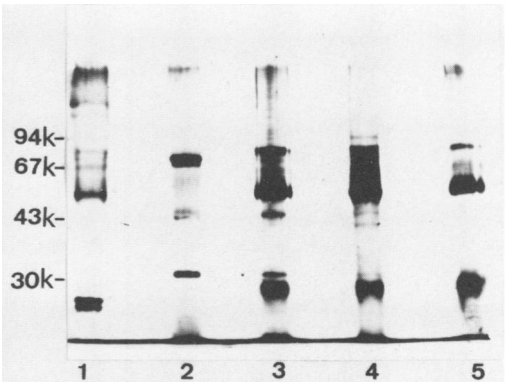

FIG. 2. Detection of the 43K antigen in pools of sera from patients with paracoccidioidomycosis in the eluate obtained from the anti-43K affinity column, analyzed by SDS-polyacrylamide gel electrophoresis and silver stained. Lane 1, Healthy individuals; lane 2, ConA fraction; lane 3, acute (juvenile) cases before treatment; lanes 4 and 5, acute (juvenile) cases after 10 and 24 months of treatment, respectively.

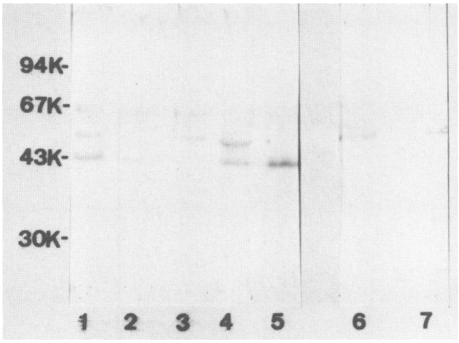

FIG. 3. Detection of the 43K antigen in pools of sera from patients with paracoccidioidomycosis in the eluate obtained from the anti-43K affinity column, analyzed by SDS-polyacrylamide gel electrophoresis, and immunoblot probed with rabbit antisera to the 43K glycoprotein. Lane 1, acute (juvenile) cases before treatment; lanes 2 and 3, acute (juvenile) cases after 10 and 24 months of treatment, respectively; lane 4, chronic (adult) cases before treatment; lane 5, ConA fraction; lane 6, healed patients; lane 7, healthy individuals.

The immunoblot of the eluates obtained from sera of patients with acute cases probed with their own sera also recognized the 43K antigen (Fig. 4).

DISCUSSION

The detection of circulating antigen represents a useful approach for the serodiagnosis of invasive fungal disease. It has been shown that the presence of a specific antigen in serum is indicative of infection with the corresponding pathogen in many mycotic diseases caused by fungi such as *Candida albicans* (9, 10, 21), *Aspergillus fumigatus* (15, 23, 24), *Histoplasma capsulatum* (25), *Cryptococcus neoformans* (5, 19), *Coccidioides immitis* (3, 4, 22), and *P. brasiliensis* (4, 17; Magaldi and Mackenzie, Abstr. Col. Intern. Paracoccidioidomycosis, 1986). In the last case, the antigen was detected by immunoelectrophoresis and immunodiffusion; however, the chemical nature of the antigen remained to be determined.

We describe here the detection of circulating antigen in sera of patients with the acute and chronic forms of paracoccidioidomycosis by the use of rabbit monospecific antisera to the 43K antigen obtained from the culture filtrate of the fungus. At 10 months after the establishment of chemotherapy, the antigen was still present in sera of patients with the acute form, but none was demonstrated after 2 years of treatment or in individuals clinically healed. These data suggest that this antigen is liberated from the fungus in a large quantity, therefore sustaining a persistent stimulus for antibody production. Our previous work demonstrating the decay in antibody titer to the 43K antigen obtained from culture filtrates of the fungus, apparently correlating with clinical improvement, led us to consider that the anti-43K antibody might be playing a role in the pathogenesis of the paracoccidioidomycosis, rather than playing a protective role as pointed out by Matthews et al. (10) for the 47K antigen of *C. albicans*, which demonstrated an increase in antibody level to the 47K antigen in patients who recovered from the disease.

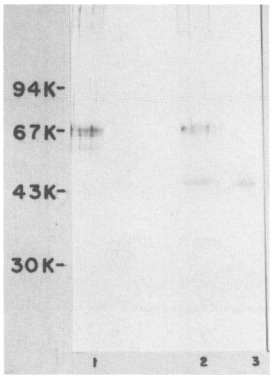

FIG. 4. Detection of the 43K antigen in pooled sera of patients with paracoccidioidomycosis in the eluate obtained from the anti-43K affinity column, analyzed by SDS-polyacrylamide gel electrophoresis, and immunoblot probed with their own sera. Lane 1, Healthy individuals (negative control); lane 2, acute (juvenile) cases before treatment; lane 3, ConA fraction (positive control).

Although the 43K protein cross-reacts with the histoplasmosis antiserum , a fact which has been pointed out to be a disadvantage for the use of this antigen in diagnosis, we think that the detection of the 43K protein may have important diagnostic value once the rabbit antiserum to the 43K antigen is extensively absorbed with *H. capsulatum* antigens, making it specifically reactive to *P. brasiliensis* and avoiding the necessity of isolating the fungus from the patients.

Another interesting topic concerning soluble antigen is its relationship with the mechanism of suppression of the cellular immune response in paracoccidioidomycosis, as described by Jimenez-Finkel and Murphy (6) in a mouse model. The presence of 43K soluble antigen in patients may, therefore, be an important factor in the modulation of the host immune response.

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