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Production of *Paracoccidioides brasiliensis* Exoantigens for Immunodiffusion Tests

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Growth curves of the yeast form of *Paracoccidioides brasiliensis* B-339 based on total and viable cell counts were determined. Crude culture filtrate antigens were obtained after 7, 10, 15, 20, 25, and 30 days of incubation. Different patterns of proteins were obtained by affinity chromatography on Sepharose 4B-immunoglobulin G complex made with immunoglobulin G from patients with paracoccidioidomycosis, with subsequent analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and scanning densitometry. Three major proteins were excreted during the time course of a 30-day culture: a doublet at 20 to 21 kilodaltons (kDa) and molecules of 43 and 52 kDa. The 43-kDa antigen was present throughout the growth period, and its level reached a peak on days 15 to 20 and then decreased considerably toward day 30. The antigenic preparations collected on days 7, 10, 15, and 20 gave better reactions in immunodiffusion tests than those collected on days 25 and 30. The 7-day exoantigen gave a sensitivity of 97.1% and specificity of 100% on immunodiffusion. The main line of precipitation had a very high intensity, showing a total identity with that of a previously purified glycoprotein of 43 kDa. A 7-day crude exoantigen displayed a high level of sensitivity and specificity, being reproducible from batch to batch and retaining its activity for years when kept lyophilized. A protocol is recommended for the production of a stable diagnostic antigen to be used in immunodiffusion tests for paracoccidioidomycosis.

Paracoccidioidomycosis has long been recognized as a public health problem in many areas of Latin America. Its clinical diagnosis has been a challenge to physicians and laboratory technicians, since certain clinical features and lung X-ray films give variable results and frequently mimic the features of other types of respiratory diseases. Since this infection is not classified as a notifiable disease, its prevalence in Latin America is not known. It is also unknown how the infection is transmitted to humans, but there is a general belief that soil is a reservoir for the fungus (13). A definitive diagnosis of paracoccidioidomycosis includes direct observation of the characteristic multiple-budding cells in biologic fluids and tissue sections or isolation of the fungus from human specimens. Serological tests generally provide results earlier than culture and histopathology and can be of great assistance in the diagnosis of disease. Good antigenic preparations are thus necessary for this purpose, but so far, only crude antigenic preparations have been used. The immunodiffusion test for paracoccidioidomycosis has a sensitivity of approximately 95.6% (7, 16) and is rather specific when used with a reference serum. The formation of a precipitation line with a clinically obtained serum sample that interacts with a selected antigen has a diagnostic value if the precipitation line is continuous (identical) with that obtained with a positive control serum. The difficulty with precipitation serodiagnosis is in preparing *Paracoccidioides brasiliensis* antigens for immunodiffusion tests. Many investigators agree that these antigens vary in activity from batch to batch and that some are unacceptable for routine use (2, 14).

During the last 6 years, we have been preparing *P. brasiliensis* antigens for immunodiffusion tests with very consistent results. In this report we relate our experience in preparing active and reproducible exocellular antigen preparations from *P. brasiliensis* yeast forms in an attempt at a possible standardization of methodology.

MATERIALS AND METHODS

Fungal strain. *P. brasiliensis* B-339 was obtained from A. Restrepo, Corporation Investigaciones Biológicas, Medellin, Colombia, and has been maintained by frequent subculture (every third day) on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) in our laboratory for several years. The fungus was converted to the yeast form on modified Sabouraud dextrose agar containing 0.01% thiamine (Difco) and 0.14% asparagine (Difco) (Sab-T-A) at 35°C and has since been maintained in this form on the same medium.

Culture medium for growth curve determinations. The medium used for these experiments (medium S) was modified from that of Negroni (11) and was made as follows. Neopeptone (60 g; Difco) was dissolved in 100 ml of distilled water, boiled, dialyzed overnight against distilled water at 4°C, and filtered through filter paper (medium fast, Qualitative 1; Whatman Ltd., Maisdstone, England). Then 36 g of glucose, 0.18 mg of thiamine (Difco), and 2.5 mg of asparagine (Difco) were added, and the volume was made up to 1,800 ml.

Growth curves. Slants containing the initial inoculum were previously subcultured for 3-day periods on Sab-T-A at 35°C. Fungus collected from the slants of at least 10 tubes (approximately 2×10^6 cells) was inoculated into 500-ml Erlenmeyer flasks each containing 100 ml of Neopeptone medium to be used as preinoculum. These cultures were incubated for 8 days at 35°C on a gyratory shaker at 50 rpm (ETICA, S. A., São Paulo, Brazil). From the preinoculum culture, 1.6×10^6 cells were transferred to 500-ml Erlenmeyer flasks containing 100 ml of the same medium and further incubated as above for 30 days at 35°C. A_{550} values were determined by UV-visible spectrophotometry (model 139 spectrophotometer; The Perkin-Elmer Corp., Norwalk,

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Conn.). Data were obtained at 24-h intervals for 30 days and expressed as the mean A_{550} for three samples. The number of living cells in a preparation was determined daily for 30 days by vital staining, and cells were counted by using a Neubauer (Weber Scientific International, Lancing, Sussex, England) chamber (3). Cell aggregates were counted as single cells, with total counts also expressed as the mean of three independent determinations.

A_{550} data were correlated with dry-weight measurements of *P. brasiliensis* B-339 in culture. Cells were grown in 500-ml Erlenmeyer flasks and killed with merthiolate (0.2 g/liter), and aliquots of 25 ml were transferred into four 100-ml Erlenmeyer flasks and diluted with distilled water as follows: neat, 1:1, 1:2, and 1:3. A_{550} values were recorded for each dilution, and five aliquots of 5 ml each were then filtered through membrane filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) and washed three times with distilled water. The fungus mass collected in the filter was oven dried at 105°C for 12 h and weighed; the weight of the filter was discounted.

Yield of exocellular antigen production. Material for exoantigens was prepared in parallel with the material for the growth curves; it was isolated after 7, 10, 15, 20, 25, and 30 days of incubation. Samples taken at these intervals were killed with merthiolate (0.2 g/liter), left at 4°C overnight, and filtered through filter paper. Crude culture filtrates were concentrated in vacuo at 45°C, dialyzed for 48 h against distilled water, and lyophilized. Solutions of lyophilized crude antigens at 200 mg/ml (previously determined) were used for immunodiffusion tests with serum samples from patients with active paracoccidioidomycosis. Protein and total carbohydrate were determined by the methods of Lowry et al. (9) and Dubois et al. (4), respectively.

Pooled antigens. When the ideal incubation time for the production of reactive antigenic preparations had been determined, the next batches of antigens were produced in 2,800-ml Fernbach flasks containing 500 ml of medium, inoculated with 8×10^6 cells, under the same conditions described above. Each antigenic preparation consisted of lyophilized filtrates from three 2,800-ml Fernbach flasks containing 550 ml of medium each. The pooled antigen was the product of six lots and was compared by immunodiffusion with a specific purified *P. brasiliensis* glycoprotein of 43 kilodaltons (kDa) (12) produced in our laboratory.

Serum samples. Serum samples were obtained from the Hospital São Paulo Mycological Serum Bank, Escola Paulista de Medicina. The samples were isolated from individuals with proven cases of active paracoccidioidomycosis (70 cases), aspergillosis (10 cases), candidiasis (10 cases), histoplasmosis (19 cases), Jorge Lobo's disease (20 cases), and tuberculosis (20 cases). In addition, 50 samples were obtained from healthy individuals within an endemic area, which served as normal controls. All samples were aliquoted and kept frozen at −20°C before use.

Immunodiffusion tests. A 3-ml portion of a 1% solution of agarose (A-6877; Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline was poured onto a glass slide (75 by 25 mm). The pattern for this microimmunodiffusion test consisted of a central well surrounded by six wells, each 3 mm in diameter. The central well was located 6 mm away from the surrounding wells. Each well was filled with 10 μl of reagent (antigen or serum). Slides were incubated overnight in a moist chamber at room temperature (20 to 25°C) and washed for 1 h in 5% sodium citrate and then for 24 to 48 h in saline. They were dried, stained for 3 to 5 min in 0.15% Coomassie brilliant blue (Sigma) in ethanol-acetic acid-water (4:2:4), and destained in the solvent mixture whenever necessary. Bands of precipitation were then recorded.

When the efficacy of the antigens produced at different incubation times was being tested, the central well was filled with the antigen and the peripheral wells contained various serum samples and control sera (see Fig. 5). When the minimal concentration of detectable antigen was being determined, the peripheral wells contained various dilutions of the antigen and the positive control serum was located in the central well.

The positive control system consisted of the pooled yeast antigen and a rabbit reference serum which demonstrated precipitin bands. The middle band was the most prominent, sharing a common antigen with the 43-kDa purified glycoprotein (identical with the E antigen of Yarzabal et al. [18]). It was also continuous with the band obtained with a human paracoccidioidomycosis serum sample in a mixed immunodiffusion pattern (see Fig. 6). Thus, this middle band is a specific one in this system.

Fractionation of *P. brasiliensis* exocellular antigen by affinity chromatography. Solutions of 100 mg of each lyophilized crude antigen in 50 ml of phosphate-buffered saline, obtained after 7, 10, 15, 20, 25, and 30 days of culture, were individually passed through affinity columns of immobilized human paracoccidioidomycosis immunoglobulin G antibodies (7.2 mg of immunoglobulin G was purified on protein A-Sepharose 4B [Pharmacia, Uppsala, Sweden] columns and subsequently coupled to cyanogen bromide-activated Sepharose 4B [Pharmacia]). Bound antigens were eluted with 0.1 M HCl–glycine buffer (pH 2.8). Collected fractions were immediately neutralized with 2 M Tris (pH 9.0; 60 μl of Tris per ml of eluate), freeze-dried, redissolved in sodium dodecyl sulfate (SDS) sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (8).

Electrophoresis of glycoproteins. Antigens eluted by affinity chromatography were electrophoresed in 10% acrylamide slab gels in the presence of SDS buffer as previously described (8). Glycoproteins were located by silver nitrate staining (1). Protein standards of molecular masses 67, 43, and 24.5 kDa were used.

Scanning densitometry. After SDS-PAGE and silver nitrate staining, each lane of the slab gel was analyzed by scanning densitometry (Quick Scan, Fluor-Vis; Helena Laboratories, Beaumont, Tex.) at 550 nm for quantitative determination of separated polypeptides.

RESULTS

A typical growth curve for the yeastlike form of *P. brasiliensis* B-339 based on A_{550} is shown in Fig. 1 and was found to correlate well with the dry weight of the organism (Fig. 2). A linear plot relating the A_{550} and the recorded weights of the heat-dried cells (in milligrams per milliliter) is shown in Fig. 2. Maximum growth and the beginning of the stationary phase occurred around day 13. Growth curves based on total cell counts paralleled these observations (Fig. 3). Determination of the number of viable cells showed that the lag phase lasted 1 day. The exponential phase started on day 2 and reached its maximum on day 5. On day 8 there was a decrease in the number of viable cells, heralding the stationary phase, which extended to day 20, when a rapid decrease in the number of viable cells was observed such that by day 30 virtually all the cells were dead.

The protein content of the extracellular material or crude antigen produced after 7, 10, 15, 20, 25, and 30 days of incubation was 878, 851, 821, 731, 634, and 926 μg/ml,

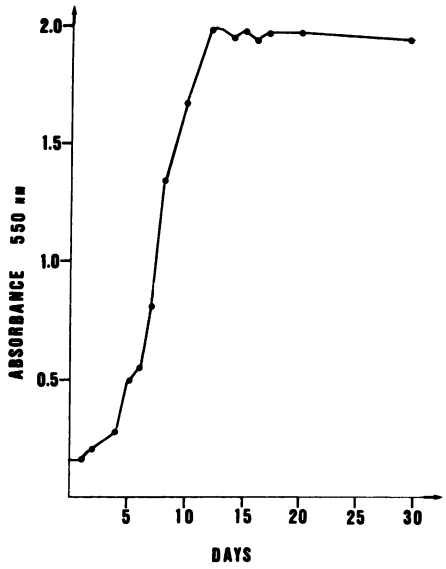

FIG. 1. Growth curve of the yeast form of *P. brasiliensis* B-339 in medium S.

respectively. Each antigen was purified by affinity chromatography and analyzed by SDS-PAGE (Fig. 4A). Following silver nitrate staining, each lane was examined by scanning densitometry to obtain a better resolution of the separated antigenic material. The main component, the 43-kDa protein, appeared early in the filtrate on day 7, reached its maximum level between days 15 and 20, and decreased considerably by the day 30.

Further analysis of antigens by immunodiffusion showed that exoantigens were present within 7 days of incubation (Fig. 5). The intensity of precipitation and the number of bands varied in the aging cultures, peaking at day 15. This was followed by a progressive reduction of visible reactions through day 30.

The pool of 7-day antigenic preparations was chosen for all immunodiffusion experiments. A comparison between the reactivity of this antigen and that of the gp43-specific fraction with an anti-gp43 serum is shown in Fig. 6.

Of 70 paracoccidioidomycosis serum samples tested, 68 (97.1%) gave strong precipitation lines when the day 7 antigen was used, whereas 2 (2.9%) gave weak reactions.

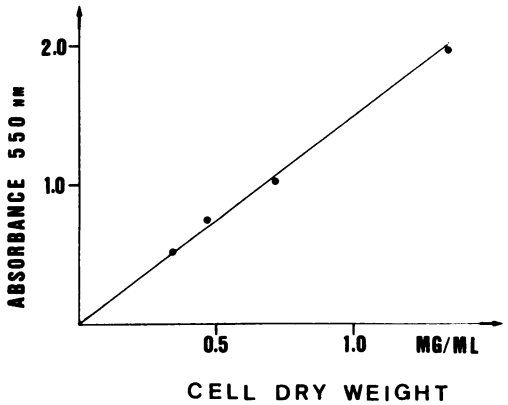

FIG. 2. Correlation between A_{550} and dry weight of cell suspensions of *P. brasiliensis*.

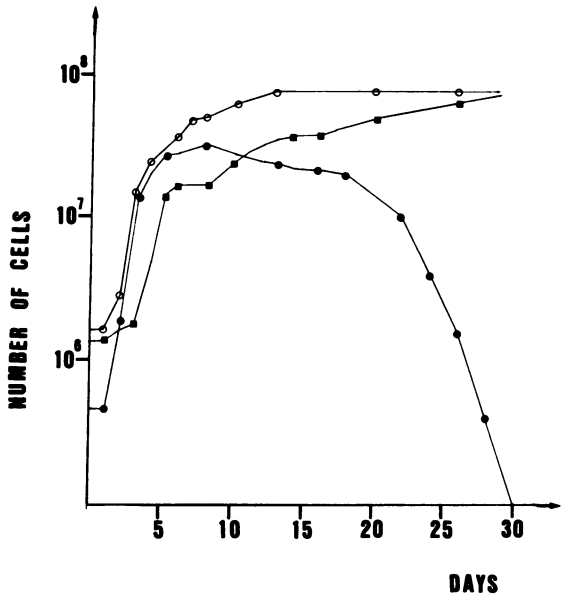

FIG. 3. Growth curves (in medium S) of the yeast form for *P. brasiliensis* B-339. Symbols: ○, total cells; ●, viable cells; ■, dead cells.

However, strong precipitation lines were obtained when these two samples were tested with the day 15 antigen. Of 19 histoplasmosis serum samples tested, only 1 gave a precipitation line with the day 7 antigen, but showed no identity with the bands obtained with the reference *Paracoccidioides* system. None of the other heterologous serum samples gave reactions with the day 7 *P. brasiliensis* antigen (these were 10 candidiasis serum samples, 10 aspergillosis serum samples, and 20 Jorge Lobo's disease serum samples).

DISCUSSION

For several years, many different antigenic preparations have been used for the serodiagnosis of paracoccidioidomycosis by immunodiffusion. These various antigens lacked a standardized preparation from one laboratory to another and included sonic extracts from the yeast forms, concentrated filtrates, and lyophilized filtrates to cite just a few approaches. Moreover, each antigen was prepared from cells grown in different culture media and under different growth conditions (such as incubation time, growth temperature, size of initial inoculum, and shaken or stationary cultures). It is possible for different antigenic preparations to vary considerably in activity and quality as a result of lack of proper lot-to-lot production standards even within the same laboratory. With this variety of problems in producing a proper *P. brasiliensis* antigen, it is not surprising that there is considerable disagreement regarding the sensitivity of the immunodiffusion test for the serodiagnosis of paracoccidioidomycosis (2, 10, 11, 14, 15, 17, 18).

P. brasiliensis is a fungus that grows very well in a variety of different media. During the past 6 years, we have obtained good antigenic preparations from yeast cultures of *P. brasiliensis* grown in either Sabouraud dextrose broth or brain heart infusion broth. These preparations have demonstrated a high degree of sensitivity, specificity, and reproducibility in the serodiagnosis of paracoccidioidomycosis by immunodiffusion. They also compare favorably with preparations obtained from totally different media by Restrepo (13) and Negroni (11).

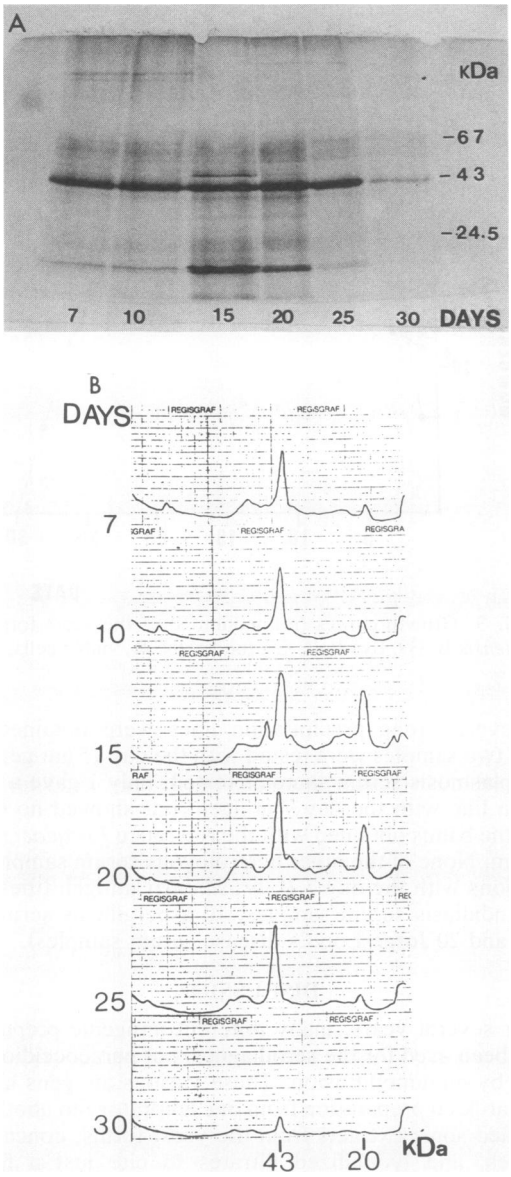

FIG. 4. (A) SDS-PAGE of *P. brasiliensis* antigens eluted from affinity columns of Sepharose 4B-immunoglobulin G complex with immunoglobulin G from paracoccidioidomycosis patients as related to culture incubation time. The gel was stained with silver nitrate stain. (B) Scanning densitometry of the different lanes from the gel in panel A.

After numerous preliminary studies, we were able to establish the most favorable growth conditions for rapid production of the exoantigen from the yeast form of *P. brasiliensis* B-339. Cultures were developed in 2,800-ml Fernbach flasks containing 550 ml of Neopeptone medium plus asparagine and thiamine inoculated with 8×10^6 cells of actively growing cultures. The flasks were incubated at 35°C in a gyratory shaker at 50 rpm, with an abundant mass of yeasts forming after 7 days. Gilardi and Laffer (5) also observed that optimum growth of *P. brasiliensis* was obtained in shaken cultures when sufficient oxygen was available to the fungus. For this reason, it is important that the medium occupies only one-fifth of the volume of the culture flask.

By analyzing the growth kinetics of the yeast form of *P. brasiliensis* B-339, we observed that exoantigens were already being produced during the exponential phase (after 3 to 8 days) of growth). A 200-mg/ml concentration of the lyophilized antigen appeared the most active, having at least a 97.1% sensitivity with serum samples from patients known to have paracoccidioidomycosis. The main line of precipitation was very intense and showed a total identity with that of the 43-kDa purified glycoprotein, previously identified by Puccia et al. (12). Of all heterologous serum samples tested, only one sample from a histoplasmosis patient gave a precipitation line, but without identity with the bands obtained with the homologous *Paracoccidioides* system, thus confirming a specificity of 100%. We recommend the production of a pool of six batches of antigen so that a large, carefully standardized quantity can be obtained and used for a long time. The very few serum samples that showed a weak reactivity with this antigen reacted better with the day 15 exoantigen. These exoantigens showed two components on SDS-PAGE, in addition to the 43-kDa protein, that could well be responsible for the differential reactivity observed (Fig. 4A). This aspect was, however, not further explored.

In analyzing the major polypeptides excreted during the time course of a 30-day culture, we observed that there was a consistent variation in the molecular masses and amounts

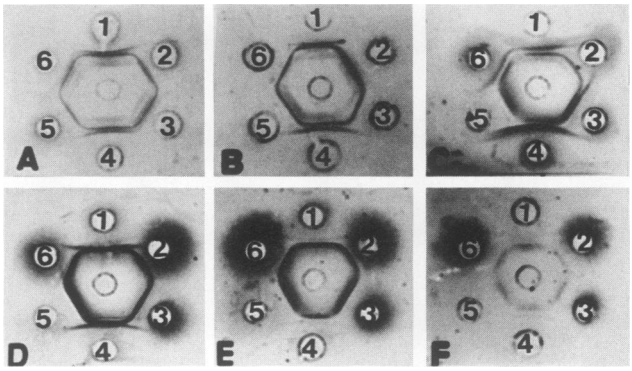

FIG. 5. Comparison of the reactivity by immunodiffusion tests of *P. brasiliensis* antigens prepared after different growth incubation times (A, 7 days; B, 10 days; C, 15 days; D, 20 days; E, 25 days; F, 30 days). Wells 1 and 4, positive serum controls; wells 2, 3, 5, and 6, serum samples from individuals with paracoccidioidomycosis. Antigens are in the central wells.

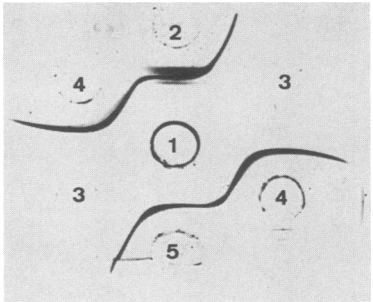

FIG. 6. Immunodiffusion tests with purified and crude antigens in the presence of hyperimmune rabbit serum, anti-gp43 rabbit serum, and serum samples from patients. Wells: 1, crude antigen of *P. brasiliensis* after 7 days of incubation; 2, hyperimmune rabbit serum raised against the crude antigen; 3, purified antigen of *P. brasiliensis* (gp43); 4, serum sample from patient with paracoccidioidomycosis; 5, anti-gp43 rabbit serum.

of secreted proteins. The major antigen present throughout the growth period was the characteristic 43-kDa glycoprotein, which showed a concentration peak at days 15 to 20, followed by a considerable decrease toward day 30. High-molecular-mass proteins of 94 and 100 kDa were expressed on days 7 and 10 but were not detectable on subsequent days. Also, on days 7 and 10 a doublet of 20 to 21 kDa was also detected, with a concentration peak on day 15. The maximal appearance of the 20- to 21-kDa doublet coincided with the appearance of a 52-kDa band, and both disappeared by day 20. After 30 days of incubation, the overall pattern of detectable proteins was very much reduced, with only small amounts of the 43-kDa glycoprotein still present.

The present data are consistent with an active proteolysis occurring over the culture incubation period. This observation might explain the fact that antigens prepared from cultures after 25 to 30 days of incubation are less reactive than those prepared from cultures after 7 to 15 days as tested by immunodiffusion. An intense proteolytic effect has already been reported by several researchers (6, 14). Apparently, the gp43 is more stable to proteolysis, but with 30 days of incubation its concentration is also reduced. With day 7 preparations the gp43 seems to be quite stable, and the crude antigen can be stored and conveniently lyophilized without addition of protease inhibitors. The increase in proteolysis could be associated with the increase in the number of dead cells (Fig. 3).

When we tested serum samples against the six different antigens, the preparations collected on days 7, 10, 15, and 20 gave better reactions than those collected on days 25 and 30. Differences could not be ascribed to simple variations in total protein and carbohydrate contents of the antigens. They could also reflect culture-age-dependent liberation of proteases.

It is clear that the ideal reference antigen for the immunodiffusion test is the purified specific 43-kDa glycoprotein previously isolated in our laboratory by Puccia et al. (12) from *P. brasiliensis* culture filtrates. However, it is produced in small amounts, and the methodology involved may not be generally adaptable for routine laboratory use. However, we have shown here that a feasible alternative may be a day 7 crude exoantigen which displayed a high level of sensitivity, specificity, and reproducibility from batch to batch and retained its activity for years when lyophilized. We therefore recommend the protocol described in the present work for the production of a stable diagnostic antigen to be used in immunodiffusion tests for paracoccidioidomycosis.

Limited amounts of antigen for diagnosis of paracoccidioidomycosis can be provided by our laboratory to serve as standards for those willing to prepare it in their own laboratories. Scaling up of antigen production will depend on the number of requests addressed to our department and on the costs involved.

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