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Immunohistochemical Detection of a Novel 22- to 25-Kilodalton Glycoprotein of *Paracoccidioides brasiliensis* in Biopsy Material and Partial Characterization by Using Species-Specific Monoclonal Antibodies

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Two murine monoclonal antibodies (MAbs) specific to *Paracoccidioides brasiliensis* (as determined by enzyme-linked immunosorbent assay [ELISA] and Western blot [immunoblot]) were produced by using a modification of standard hybridization protocols, with cyclophosphamide included as an immunomodulator to abolish responses to highly cross-reactive immunodominant epitopes. MAbs PS14 and PS15 are two different clones which exhibit similar characteristics by ELISA and Western blot. They are directed against a 22- to 25-kDa antigen which is present in *P. brasiliensis* and which could not be identified in other dimorphic fungi by ELISA or Western blot. Partial purification of the antigen was accomplished by isoelectric focusing, and deglycosylation studies suggested that the 22- to 25-kDa antigen is a glycoprotein with a pI of between 4.5 and 5 and that O-linked sugars may be part of the recognized epitope. The MAbs stained the cytoplasm of *P. brasiliensis* yeast and hyphal cells in cryostat sections of fresh cultures of the fungus. In addition, the MAbs stained the wall of paracoccidioidomycotic granulomas, as well as the cytoplasm of the fungus, as determined by the use of immunofluorescence, immunoperoxidase, and immuno-alkaline phosphatase staining techniques in paraffin-embedded sections of human biopsy material, and they failed to stain granulomas resulting from other clinical conditions. These findings suggest that these MAbs have potential use in the immunohistochemical identification of *P. brasiliensis*.

Paracoccidioidomycosis, a disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*, is one of the most prevalent deep mycoses in Latin America (9, 16). There are multiple clinical manifestations of this disease, and it can present in either a disseminated or a localized form and as a chronic or a subacute disease (1, 6, 7, 19).

As a result of the wide variety of clinical presentations and despite the fact that serological tests give indirect evidence of infection with *P. brasiliensis* (3, 17), the definitive diagnosis of the disease requires the microscopic identification of the fungus, in clinical material when possible (5). More often diagnosis is delayed until the isolation of the fungus by culture at the laboratory.

The histopathological features of the tissue response in paracoccidioidomycosis are not specific, being similar to those of blastomycosis and coccidioidomycosis. Typical *P. brasiliensis* multiple budding yeast forms are not always found. If small intracellular yeast forms are present or if the tissue contains only *P. brasiliensis* blastospores, which are often intracellular, it is difficult to differentiate them from *Histoplasma capsulatum*. When old fibrogranulomatous or calcified lesions occur, especially in the lungs, typical multiple budding cells are almost never seen; *P. brasiliensis* yeast forms are usually distorted and fragmented and appear as empty shells of various shapes and sizes which stain poorly even when special fungal staining procedures (such as methenamine silver stain) are used. These fungal structures can be confused with small empty spherules of *Coccidioides immitis* or with poorly stained yeast forms of *Blastomyces dermatitidis* (8, 20).

There are very few data in the literature on the use of immunohistochemistry in the histopathological diagnosis of paracoccidioidomycosis. Kaplan (13) reported the use of immunofluorescence for the identification of the fungus in tissue sections. By using two different batches of fluorescein-conjugated rabbit polyclonal antisera, good fluorescent labelling was achieved in that work, but without specificity because of high degrees of cross-reactivity, mainly with *C. immitis*, *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii*, *Sporothrix schenckii*, and *B. dermatitidis*. The degrees of cross-reactivity varied with the different fungi and the batch of polyclonal sera used. Conjugates had to be sequentially adsorbed with different fungal antigens and tested to ensure specificity.

There are no published data on the use of monoclonal antibodies (MAbs) in the identification of *P. brasiliensis* in tissue sections. The production of MAbs against fungi has been hampered by the high degree of cross-reactivity between species. The main components of the *P. brasiliensis* cell wall, in either the mycelial or yeast form, are glucans (15). Carbohydrates are highly immunogenic and are often shared among different fungi. Our group has already described a modified MAb production technique (14, 21) in which cyclophosphamide is used to suppress mouse B-cell responses to epitopes shared among similar fungal antigens. This has made it possible to produce species-specific MAbs (4, 10, 11), allowing the partial characterization of specific fungal glycoproteins (12).

In this paper we describe the production of two MAbs specific for *P. brasiliensis*, MAbs PS14 and PS15, which exhibited similar characteristics when tested by enzyme-linked immunosorbent assay (ELISA) and Western blot (immunoblot).

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These MAbs have been used successfully in the immunohistochemical detection of the pathogen in biopsy material and in the partial characterization of a previously undescribed 22- to 25-kDa antigen of *P. brasiliensis.*

MATERIALS AND METHODS

Fungal strains. Three *P. brasiliensis* isolates (NCPF 4110, NCPF 4115, and NCPF 4095) were obtained from the National Collection of Pathogenic Fungi, Mycological Reference Laboratory, Colindale, London, United Kingdom. *P. brasiliensis* CIB 339 and five other recently obtained human isolates were supplied by A. Restrepo (CIB, Medellin, Colombia). Isolates of other dimorphic fungi were obtained from the National Collection of Pathogenic Fungi, as follows: *H. capsulatum* var. *capsulatum* (NCPF 4100 and NCPF 4088), *H. capsulatum* var. *duboisii* (NCPF 4094 and NCPF 4077), *B. dermatitidis* (NCPF 4076), and *S. schenckii* (NCPF 3268 and NCPF 3181).

Culture conditions. *P. brasiliensis* isolates were grown on peptone-glucose-yeast extract medium agar slopes. Yeast forms were grown at 35.5°C and subcultured every 4th day; mycelial forms were incubated at 25°C and subcultured monthly. Other dimorphic fungi were grown on brain heart infusion agar slants (Difco Laboratories, Detroit, Mich.) supplemented with L-cysteine (0.2 mM) (Sigma Chemical Co., St. Louis, Mo.) at 35.5°C and were subcultured weekly.

Antigen preparation. The total yeast growth on a 3-day-old slant was precultured in a 1-liter Erlenmeyer flask containing 200 ml of peptone-glucose-yeast extract liquid broth and incubated at 35.5°C on a gyratory shaker incubator (Gallenkamp, Fisons, Loughborough, Leics., United Kingdom); after 3 days of incubation, cells were counted with a hemocytometer, and a volume containing 10^7 yeast cells was subcultured into 200 ml of fresh peptone-glucose-yeast extract medium and incubated under the same conditions for 7 days. Cultures were harvested by centrifugation (3,000 $\times$ *g* for 20 min) and filtered through Whatman paper no. 1; the cells were washed twice in 0.01 M phosphate-buffered saline (PBS), pH 7.4. Yeast cytoplasmic antigen (YCA) was prepared as described previously (4), and the protein content was determined by the Coomassie blue method (18). Culture filtrate (CF) was concentrated to 1/10 of its original volume by using Microsep 1-kDa centrifugal concentrators (Filtron Technology Co., Northborough, Mass.) spun at 7,500 $\times$ *g*. Three-day-old cultures of other dimorphic fungi were subcultured onto brain heart infusion liquid broth and incubated under the same conditions as for *P. brasiliensis*; antigens were prepared as described for *P. brasiliensis.*

Blotta and Camargo's cell-free antigen (CFA) was prepared by a modification of their technique (2). The total growth of three 3-day-old *P. brasiliensis* cultures in agar slopes was washed with 1 ml of sterile PBS, collected in an Eppendorf tube, gently vortexed for 30 s, and spun at 13,000 $\times$ *g* for 5 min. The supernatant was recovered, and the protein content was determined by the Coomassie blue method.

Hybridoma production. (i) Immunization protocol. *S. schenckii* (NCPF 3268) YCA (50 μg of total antigen per mouse given intraperitoneally in Freund's incomplete adjuvant) was used as the primary antigen and was followed by cyclophosphamide (Sigma) at a dose of 50 mg/kg per mouse intraperitoneally as previously described (4). *P. brasiliensis* (NCPF 4110) YCA, at the same dose, was used as the secondary antigen (4).

(ii) Fusion protocol. Sp 2/0 murine myeloma cells were fused with mouse spleen cells by a modification of the protocol of Zola and Brooks (23). Hybridomas were grown in 96-well plates (Nunc, Roskilde, Denmark) and screened by ELISA against *P. brasiliensis* and *S. schenckii* antigens as described previously (4, 10) 10 days after the fusion. Briefly, 96-well microtiter plates coated with either antigen in 0.06 M carbonate buffer (pH 9.6) by overnight incubation at 4°C were washed and blocked (1% bovine serum albumin in PBS–Tween 20). After 1 h of incubation with neat culture supernatants, plates were washed and incubated for a further hour at 37°C with peroxidase-conjugated goat anti-mouse immunoglobulin diluted 1:5,000 in PBS–Tween 20 (Jackson Immuno-Research). The substrate *o*-phenylenediamine (Sigma) (0.2 mg/ml) with 0.005% H_2O_2 in 0.01 M sodium citric buffer was used to visualize positive reactions. The A_{492} was measured. Colonies producing supernatants reactive to *P. brasiliensis* antigen were subcloned twice by limiting dilution. Pristane-primed BALB/C mice were injected intraperitoneally with 10^4 cells from each hybridoma line; ascitic fluid was collected 1 week to 10 days later. Subclassing of MAbs was performed by using the Serotec kit (Serotec, Kidlington, Oxford, England) as previously described (10).

Polyacrylamide gel electrophoresis, Western blotting, and immunoenzyme development. YCA, CFA, and CF antigen of the different strains of *P. brasiliensis* and YCA of other fungal species (*H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, *B. dermatitidis*, and *S. schenckii*) were electrophoresed simultaneously on a 10% polyacrylamide gel and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) by using a semidry multigel electroblotter at 200 mA for 55 min. The entire blot was reacted with ascitic fluid diluted 1:1,000 to 1:2,000 in PBS–Tween 20–1% casein as previously described (5, 10).

Isoelectric fractionation of antigen. After exhaustive dialysis against hypergrade water at 4°C, 20 mg of crude *P. brasiliensis* YCA was made up to 50 ml with deionized water and 2% ampholytes (pI range, 3 to 10) (Biolyte 3/10; Bio-Rad Laboratories, Hercules, Calif.) and loaded onto a Rotofor isoelectric focusing system (Bio-Rad). Electrophoretic separation was performed at a constant power of 12 W until the voltage stabilized (6 to 8 h). The pHs of the fractions were measured by using a pH meter, and protein content was determined by the Coomassie blue method. The reactivities of the MAbs against each of the 20 Rotofor fractions were determined by ELISA and Western blot.

Periodate oxidation and alkaline degradation of the antigen. Periodate oxidation of the antigen was performed by mixing 1 ml of *P. brasiliensis* NCPF 4110 YCA (1.2 mg/ml) with 1 ml of 5 mM $NaIO_4$ in 0.2 mM sodium acetate buffer (pH 4.7); after 30 min of incubation at 4°C in the dark, the mixture was desalted on a Bio-Gel P6 column (Bio-Rad) equilibrated with PBS–0.5% Triton X-100. The exclusion peak was collected for epitope analysis.

For alkaline degradation, equal volumes of the same *P. brasiliensis* antigen preparation and of 0.2 M potassium hydroxide were mixed and sealed under N_2. After overnight incubation at 37°C, the mixture was acidified to pH 6.5 with 2 M acetic acid, and the sample was extensively dialyzed against PBS at 4°C in the dark.

Enzymatic deglycosylation of the antigen. *P. brasiliensis* NCPF 4110 YCA (1.2 mg/ml) aliquots were treated with different enzymes as follows (some of the antigen was treated with sodium dodecyl sulfate [SDS] at a final concentration of 0.1% at 100°C for 3 min, prior to enzymatic treatment): (i) 10 μg of non-SDS-treated antigen in a volume of 8 μl was incubated with 0.05 U of neuraminidase (BDH, Merck Ltd., Leicestershire, United Kingdom) in 0.1 M potassium acetate buffer (pH 4.5, supplemented with 2 mM CaCl) at 37°C for 24 h; (ii) 200 μg of SDS-treated and non-SDS-treated antigen in

200-μl aliquots was incubated with either 0.2 μg of trypsin or 0.2 mg of elastase (Sigma) at 37°C in a water bath for 15-min intervals up to 1 h; (iii) 20 μg of SDS-treated protein was incubated with 0.3 U of recombinant peptide *N*-glycanase F (Genzyme Corp., Cambridge, Mass.) in a final volume of 30 μl at pH 7.5 and 37°C for 24 h; and (iv) 100 μg of SDS-denatured and nondenatured protein previously treated with neuraminidase (BDH) in 10 mM calcium acetate for 1 h at 37°C was incubated with 4 mU of *O*-glycanase enzyme (Genzyme) in 20 mM sodium cacodylate buffer (pH 7.0) in a final volume of 50 μl for 24 h at 37°C. After treatment, 10 μl of 1% SDS–1.6% β-mercaptoethanol was added to each sample, and the samples were then boiled for 3 min prior to being loaded onto an SDS–10% polyacrylamide gel. Gels were processed for electroblotting and immunodeveloped with the different MAbs as previously described.

Cryostat sections. Seven-day-old yeast cultures were harvested as previously described; fungal samples were immediately placed in optimal-cutting-temperature medium (Lab-TEK; Miles Scientific) and frozen in liquid nitrogen. Sections (6 μm thick) were cut and mounted onto 3-aminopropyltriethoxysilane (Sigma)-coated slides, fixed by immersion in cold acetone for 10 min, air dried, and stored at −70°C. Prior to being stained, sections were transferred to Tris-acetate buffer, pH 7.5 (TAB) (0.5 M, 0.05% Brij 35, 0.05% Tween 20) and left for 10 min.

Paraffin-embedded specimens. Formalin-fixed paraffin-embedded samples of human lung, adrenal gland, and myocardium infected with *P. brasiliensis* were obtained from M. Franco (UNESP, Botucatu, Brazil). Paraffin-embedded sections from keloid, lung infected with *Mycobacterium tuberculosis*, liver infected with *Candida albicans*, and brain infected with *Cryptococcus neoformans* were obtained from the Histopathology Department, Guy's Hospital, London, United Kingdom. Sections of tissue (6 μm thick) were mounted onto 3-aminopropyltriethoxysilane-coated slides, dried at 37°C for 24 h, and stored at room temperature. Paraffin sections were dewaxed by immersion in fresh xylene and rehydrated by immersion in a graded ethanol series. Sections were then treated with pronase (0.5 mg/ml, pH 8.0) (Sigma) at 37°C for 35 min, washed in tap water, and transferred to TAB prior to being immunostained. Skin biopsies of sarcoid, amyloid, and fibroadenoma were obtained from consenting patients. Tissue was cryoprotected and snap frozen by immersion in melting isopentane (BDH) and was stored under liquid nitrogen. Sections (6 μm thick) were mounted onto 3-aminopropyltriethoxysilane-coated slides, air dried, and stored at −20°C. Sections were treated with pronase as described above and transferred to TAB prior to being stained.

Immunohistochemistry. Indirect immunofluorescence, immunoperoxidase, and immuno-alkaline phosphatase techniques were used to stain cryostat and paraffin-embedded sections as described below.

(i) Indirect immunofluorescence. After transfer to TAB, slides were incubated for from 1 h to overnight at 37°C with each MAb; the concentration of MAb used was determined by serial dilutions in TAB (1:100 to 1:10,000). Slides were then washed with three changes of fresh TAB and incubated for 30 min at room temperature with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (Ortho Diagnostic Systems Inc., Raritan, N.J.) diluted 1:20 in TAB. After further washes with fresh TAB, slides were mounted in a 50% glycerol–PBS (pH 7.5) buffer.

(ii) Immunoperoxidase. After TAB rehydration, slides were transferred to normal swine serum diluted 1:5 in TAB and left for 10 min at room temperature, washed in TAB, and incubated for from 1 h to overnight at 37°C with different dilutions of MAbs in TAB. After three washes in TAB, slides were incubated for 30 min with peroxidase-conjugated anti-mouse immunoglobulin G (Dakopatts, Glostrup, Denmark) diluted 1:10 in normal swine serum as the secondary antibody. The slides were then washed again with three changes of fresh TAB and incubated with mouse peroxidase-antiperoxidase complex (Dakopatts) diluted 1:20 in TAB for 30 min at room temperature. After further washes in TAB, positive staining was determined by further incubation with 3,3′-diaminobenzidine substrate (1 mg/ml) in 0.1 M phosphate buffer containing 0.3% hydrogen peroxide for 15 min. Slides were counterstained with Mayers haemalum for 90 s after the final TAB washes, dehydrated by immersion in an ascending ethanol series prior to clearing in three changes of xylene, and mounted with De PeX mounting medium (Gurr; BDH Ltd.).

(iii) Immuno-alkaline phosphatase. The immuno-alkaline phosphatase technique was performed like the immunoperoxidase technique, but alkaline phosphatase-labelled antimouse antibody (Dakopatts) diluted 1:25 in TAB was used as the secondary antibody, and mouse alkaline phosphatase–anti-alkaline phosphatase complex (Dakopatts) diluted 1:50 in TAB was used instead of peroxidase-antiperoxidase. Positive staining was determined by incubating the slides with freshly made 0.1% fast red TR salt (Sigma) in 0.1 M Tris buffer (pH 8.2)–0.2% naphthol AS-Mx (Sigma)–2% dimethylformamide–1 mM levamisole for 20 min at room temperature. Slides were counterstained with hematoxylin and mounted in Uvinert mounting medium (Gurr).

RESULTS

Hybridoma production. Cyclophosphamide-treated mouse sera showed a differential response, with a high ELISA titer against *P. brasiliensis* YCA and a low titer against *S. schenckii* YCA. The mouse showing the greatest difference in the response was selected for the fusion protocol. Control mouse sera, which were not cyclophosphamide treated, showed similar ELISA titers with both fungal antigens (data not shown).

After successive subcloning by limiting dilution, 14 different clones were selected. Two immunoglobulin G1 hybridoma lines (MAb PS14 and MAb PS15) which showed high specificities against *P. brasiliensis* antigens by ELISA and Western blot and which behaved similarly when used as labels in immunohistochemical procedures were grouped as the PS15 group. The remaining hybridoma lines showed different degrees of specificity, and they will be further characterized in future papers. Figure 1 illustrates the differential reactivities by ELISA of MAb PS14 and MAb PS15 to various dimorphic fungal antigens. As seen in Fig. 1A, MAb PS14 showed specificity against *P. brasiliensis* YCA and CF antigen. Figure 1B demonstrates the specific reaction of PS15 against *P. brasiliensis* CF antigen, with less recognition of YCA. Different dilutions of ascitic fluid were tested, and PS15 was reactive at dilutions as high as 1:64,000.

Immunoenzyme development. Figure 2A and B illustrates the patterns of recognition of MAb PS14 and PS15 by Western blot, at dilutions of 1:2,500 and 1:2,000, respectively, against a range of dimorphic fungal antigens. The two MAbs are specific to *P. brasiliensis* antigens by Western blot and recognized an antigenic determinant with a molecular mass of 22 to 25 kDa which is present in *P. brasiliensis* YCA, CFA, and CF. There was no evidence of recognition of this molecule in antigen preparations from other dimorphic fungi. When reacted against *P. brasiliensis* CFA by Western blot, PS14 recognized a 22- to 25-kDa antigen and had low reactivity against a 55-kDa

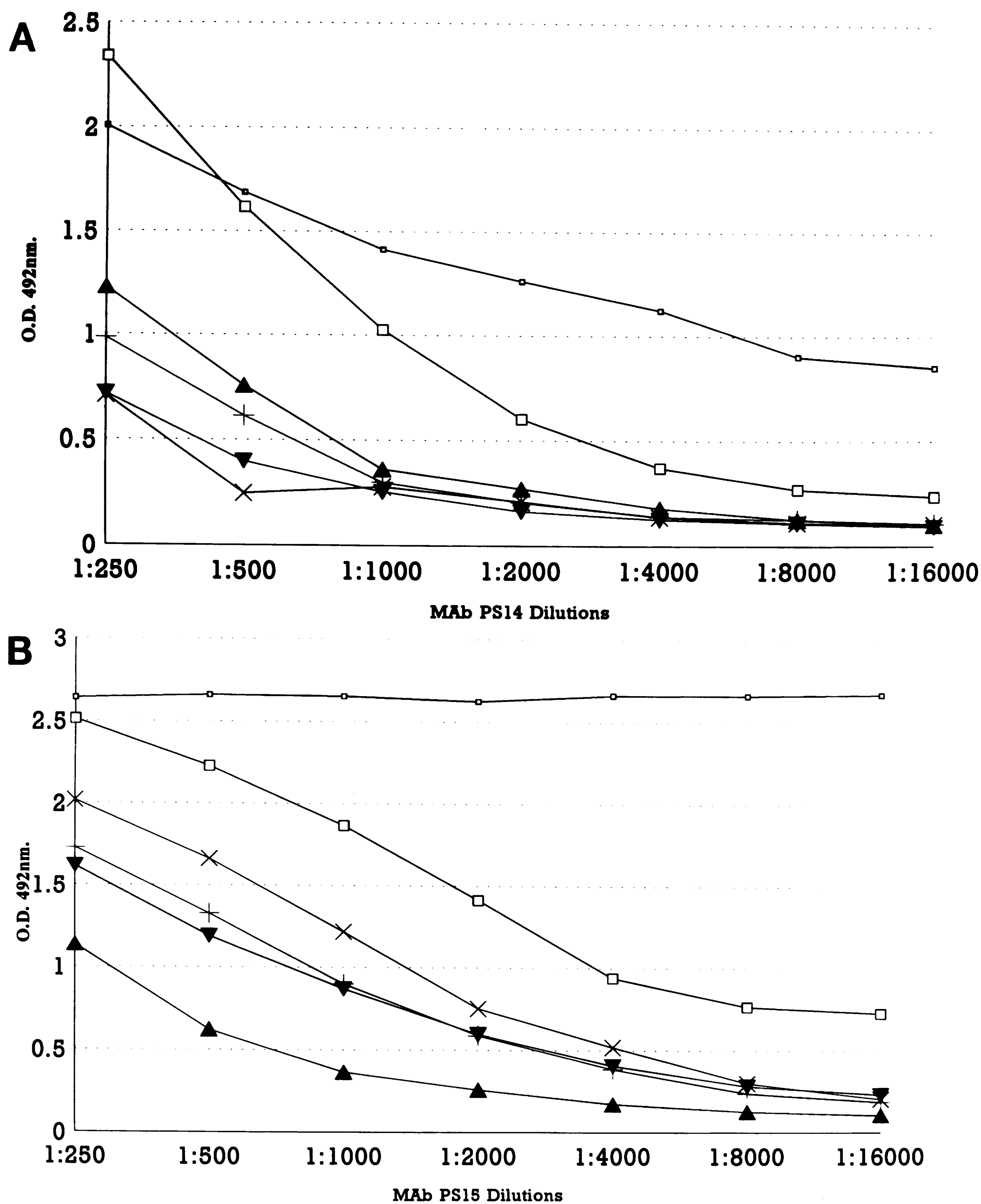

FIG. 1. Differential ELISA reactivities of MAb PS14 (A) and MAb PS15 (B) ascitic fluids against dimorphic fungal antigens. □, *P. brasiliensis* YCA; ▫, *P. brasiliensis* CF, ▲, *H. capsulatum* var. *capsulatum* YCA; ▼, *H. capsulatum* var. *duboisii* YCA; ×, *B. dermatitidis* YCA; +, *S. schenckii* YCA. Antigen concentration, 1 μg per well. O.D., optical density.

molecule (Fig. 2A); PS15 recognized the same antigenic determinants as PS14 but in addition reacted against a 38-kDa species present in CFA (Fig. 2B). It is important to note that some *P. brasiliensis* YCA preparations were not recognized by these MAbs; in particular, old antigen preparations which had undergone long-term storage at −70°C or preparations subject to repeated freeze-thaw cycles were not recognized (data not shown).

Partial purification of the 22-kDa antigen. Total *P. brasiliensis* CIB 339 YCA and CF antigen were fractionated according to their isoelectric point by liquid isoelectric focusing with a Rotofor system. Figure 3 shows the pH, protein concentra-

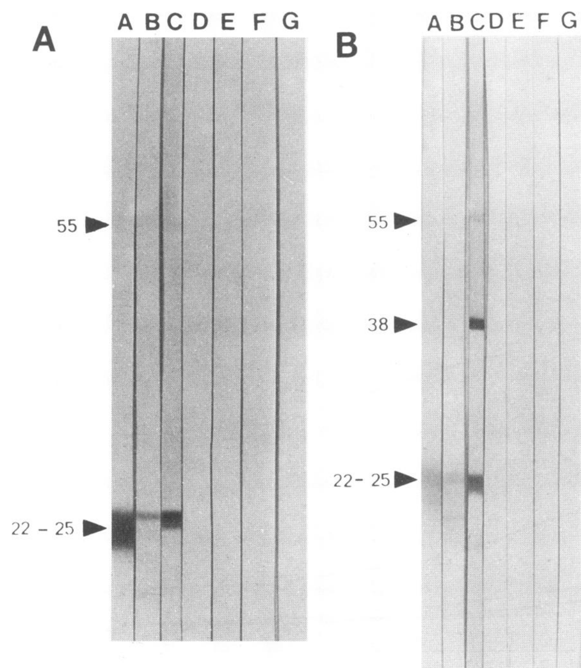

FIG. 2. Immunoblots showing the differential recognitions by MAb PS14 ascitic fluid at a dilution of 1:2,500 (A) and MAb PS15 ascitic fluid at a dilution of 1:2,000 (B) with *P. brasiliensis* and other dimorphic fungal antigens. Lanes: A, *P. brasiliensis* YCA; B, *P. brasiliensis* CF; C, *P. brasiliensis* CFA; D, *H. capsulatum* var. *capsulatum* YCA; E, *H. capsulatum* var. *duboisii* YCA; F, *B. dermatitidis* YCA; and G, *S. schenckii* YCA. Molecular masses are indicated in kilodaltons.

tion, and reactivity, when tested by ELISA, of MAb PS14 against the 20 different Rotofor fractions derived from YCA. As seen, there is a higher peak of reactivity against fraction 6, indicating a pI range for the antigen of 4.5 to 5.0. Further purification procedures were attempted, such as gel filtration by fast protein liquid chromatography on Superose 12 columns, but these proved unsuccessful since the reactivities of both MAbs against all eluent fractions were lost (data not shown).

Epitope disruption. Figure 4 shows the effects of alkaline degradation, periodate oxidation, and enzymatic treatment of *P. brasiliensis* YCA on the ability of MAb PS14 to recognize the 22- to 25-kDa antigen (PS15 showed similar results [data not shown]). Alkaline degradation and periodate oxidation destroyed the recognition of the 22- to 25-kDa antigenic determinant (Fig. 4, lanes B and C). Treatment with neuraminidase did not inhibit the immunorecognition of the epitope by PS14, whereas *O*-glycanase treatment of reduced and nonreduced antigen appeared to partially inhibit the recognition of the epitope by both MAbs, but only after pretreatment with neuraminidase (lanes F and G). Treatment with different concentrations of *N*-glycanase F had no obvious effect on the recognition of the antigen by the antibodies (lane H). Treatment of the antigen with trypsin or elastase resulted in a loss of recognition by the antibodies (lanes J and K). The immunoblot staining intensity was generally reduced for both MAbs in these epitope disruption studies; overnight incubation at 37°C alone for the negative control substantially reduced MAb recognition.

Immunostaining. On cryostat sections MAbs PS14 and PS15 reacted with the cytoplasm of *P. brasiliensis* yeast forms (isolates CIB 339, CIB 4, and CIB 7) as demonstrated by indirect immunofluorescence. The staining pattern of speci-

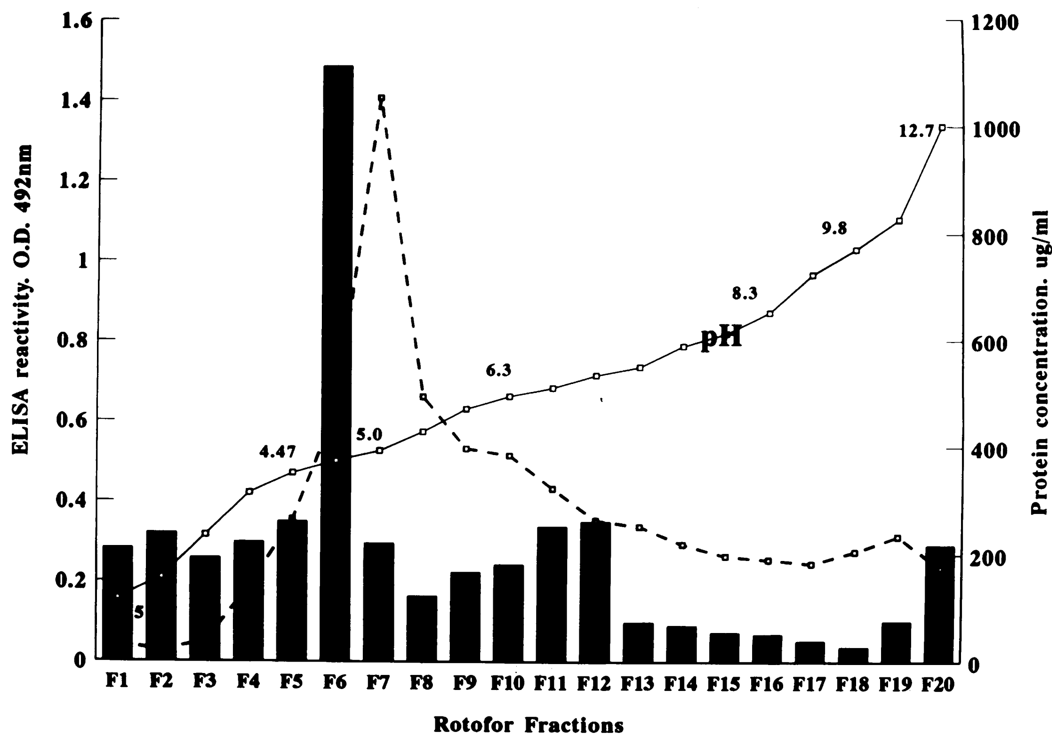

FIG. 3. Isoelectric point fractionation of *P. brasiliensis* YCA by the Rotofor system. Bars represent ELISA reactivities of MAb PS14 ascitic fluid (1:2,000). - - -, protein concentrations; ——, pH. O.D., optical density.

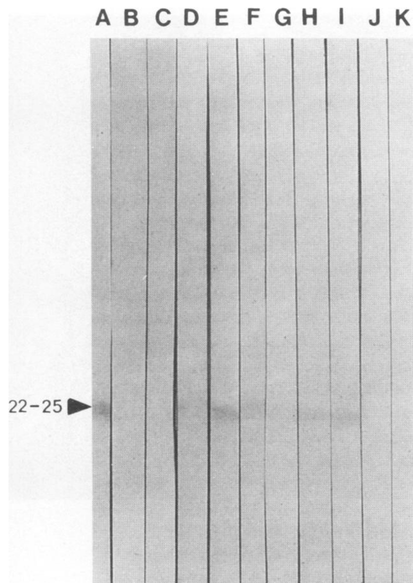

FIG. 4. Immunoblot showing the effect of chemical and enzymatic deglycosylation of *P. brasiliensis* YCA on the recognition by MAb PS14 ascitic fluid (1:2,000); PS15 had a similar pattern of recognition. Lanes: A, *P. brasiliensis* YCA control incubated overnight at 37°C without enzyme treatment; B, YCA treated with KOH (0.2 M); C, YCA treated with $NaIO_4$ (0.2 nM); D, neuraminidase-treated YCA; E, untreated YCA control; F, YCA treated with *O*-glycanase F; G, YCA treated with *O*-glycanase F after treatment with neuraminidase; H, YCA treated with *N*-glycanase; I, untreated YCA control; J, elastase-treated YCA; K, trypsin-treated YCA. Molecular mass is given in kilodaltons.

mens was variable (Fig. 5A), although it was similar for the two MAbs, from an irregular speckled appearance to an even cytoplasmic distribution. Both budding cell and parent cell cytoplasm showed this pattern of labelling. A very small number of cells did not react under these conditions. In some cells there was evidence of cell wall reactivity (Fig. 5A, arrow). Mycelial forms demonstrated a similar pattern of staining on cryostat sections (data not shown).

PS14 and PS15 were used separately to label by indirect immunofluorescence paraffin-embedded biopsy material from cases of paracoccidioidomycosis, tuberculosis, candidiasis, and cryptococcosis and frozen skin sections of material from cases of other noninfectious granulomatous and fibrotic diseases. The two antibodies demonstrated a similar pattern of labelling, although it was necessary to treat paraffin-embedded tissue with pronase prior to staining. Of particular note was the intense fluorescence demonstrated by the granuloma walls in cases of paracoccidioidomycosis (Fig. 5B). Within the granuloma there were numbers of yeast cells which demonstrated a pattern of variable cytoplasmic staining of lower intensity than that seen in cryostat sections. However, many of these yeast cells appeared to be ghost cells, resulting possibly from old calcified lesions or from the loss of cytoplasmic content during the fixation process, and in these cases only the cell wall was fluorescent, together with what remained of the cytoplasmic content (Fig. 5C, arrow).

Biopsy material containing granulomas resulting from causes other than paracoccidioidomycosis showed no fluorescent labelling with these MAbs, with the exception of a high-level background staining in amyloid specimens stained with MAb PS14 at a high concentration (1:10 dilution).

Each MAb was used individually as a label in the immunoperoxidase and immuno-alkaline phosphatase staining of paraffin-embedded human biopsy material at dilutions of 1:10 to 1:1,000. Generally, the immuno-alkaline phosphatase staining was less intense (Fig. 5D), although again in ghost cells within granulomas, there was evidence of cell wall and residual cytoplasm labelling. The immunoperoxidase staining demonstrated clear cytoplasmic reactivity in intact cells (Fig. 5E). In a number of cases, there was evidence of extracellular deposition of antigen (Fig. 5F, arrow).

DISCUSSION

We report the first successful use of species-specific MAbs to label yeast forms of *P. brasiliensis* in both frozen and wax-embedded histological sections. Previously, the only published report of an attempt to use immunospecific antisera was that by Kaplan (13), who described the use of polyclonal rabbit sera raised against *P. brasiliensis*. In that study significant cross-reactivity was observed, resulting in the need to cross-adsorb the sera before use. In addition, Kaplan noted that the cross-adsorption needed for each new batch of rabbit sera was different, because of variability in the cross-reactivity pattern from batch to batch.

The direct identification of *P. brasiliensis* in histological sections can be very difficult. The production of two species-specific MAbs and their successful use as labels in immunohistochemical procedures constitute a significant advance in the histological diagnosis of this disease. The generation of these two MAbs is further evidence of the successful application of in vivo cyclophosphamide modulation of murine immune responses to generate useful hybridomas.

Both MAbs are directed principally against a glycoprotein present in YCA and CF; this protein has a molecular mass of 22 to 25 kDa and an isoelectric point of 4.5 to 5.0 and was identified only in *P. brasiliensis* antigens by Western blot. In addition, PS14 and PS15 are reactive to a 55-kDa band present in CFA preparations, while PS15 also recognizes a 38-kDa molecule present in *P. brasiliensis* CFA. This indicates that the MAbs are not directed against identical epitopes. This was borne out by the observation that while PS14 and PS15 gave a similar pattern of immunohistochemical labelling, PS14 was capable of nonspecifically recognizing amyloid biopsies, whereas PS15 was not. The relationship of the 22- to 25-kDa antigen to the 55- and 38-kDa molecules in CFA is unclear at this time.

The results of the disruption studies with the 22- to 25-kDa antigen suggested that the epitope recognized by MAbs PS14 and PS15 is glycosylated, as illustrated by the abrogation of recognition by MAbs when the antigen was subjected to alkaline degradation or periodate treatment. Both of these reactions are directed against carbohydrate targets, through either destruction of hexose rings or oxidation of O-linked sugars. *O*-Glycanase F treatment partially reduced the recognition of the antigen by the MAbs, which is again suggestive of the presence of a carbohydrate moiety in the epitope. Both trypsin and elastase treatments of the antigen resulted in the abolition of the reactivities of PS14 and PS15, confirming the molecule to be a glycoprotein.

The immunohistochemical observation that the 22- to 25-kDa antigen was also present in the extracellular space, in close proximity to yeast cells, corroborated the observation that *P. brasiliensis* CF was highly reactive to both MAbs. Although the antigenic leakage observed in tissue sections may be the result of fixation procedures, the high reactivity of the granuloma periphery suggests that this is a genuine extracellular product

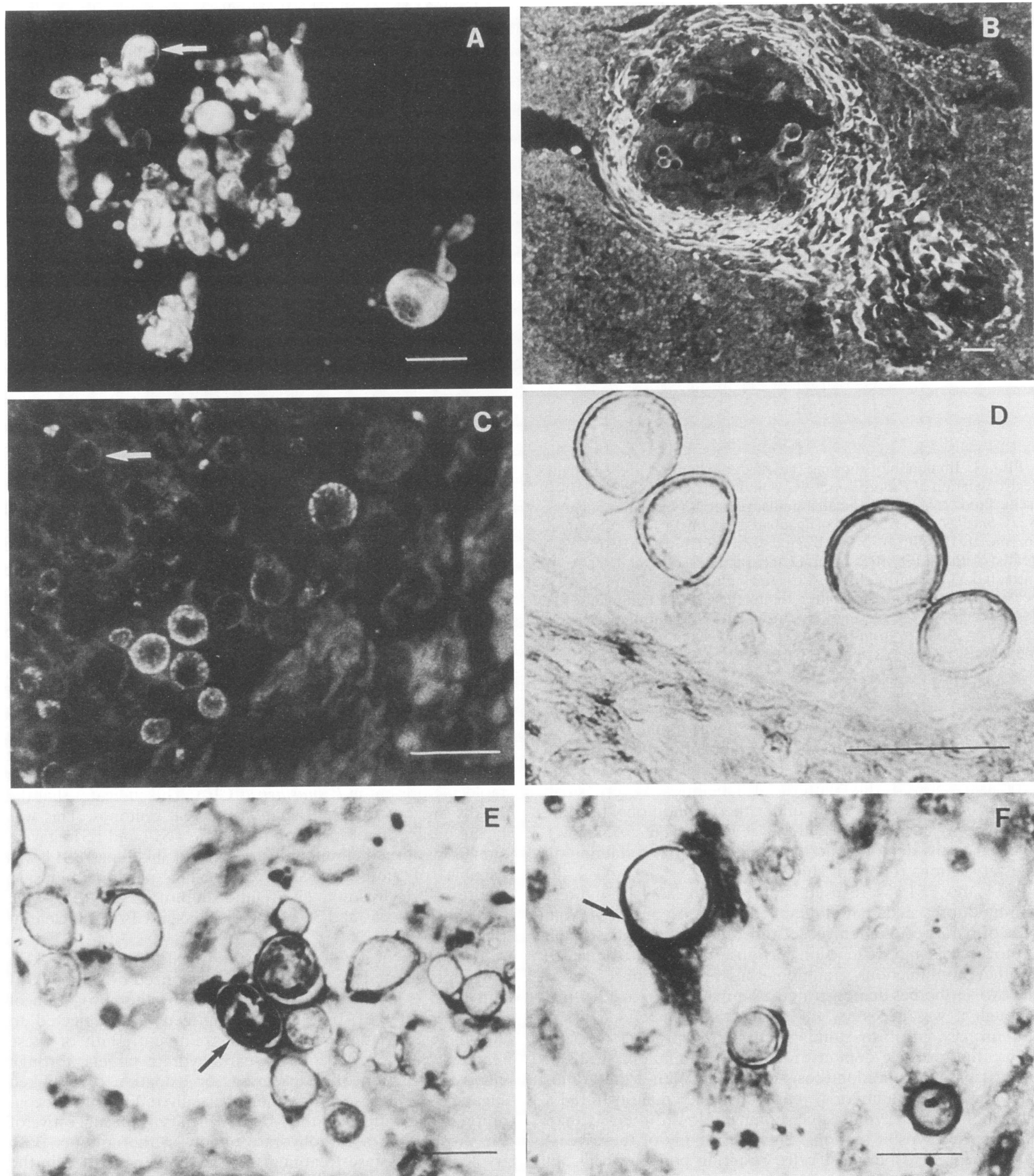

FIG. 5. Immunofluorescence, immuno-alkaline phosphatase, and immunoperoxidase staining using MAbs PS14 and PS15. (A) Cryostat section of fresh *P. brasiliensis* yeast forms stained by indirect immunofluorescence with MAb PS15. The arrow indicates evidence of cell wall labelling. (B) Paraffin-embedded myocardium section stained with MAb PS15 by the indirect immunofluorescence technique, showing a paracoccidioidomycotic granuloma. Note the fluorescent labelling of the fungus inside the granuloma as well as of the granuloma walls. (C) Paraffin-embedded section of an infected adrenal gland stained with MAb PS15 by immunofluorescence. Ghost cells which had lost cytoplasmic content are reactive (arrow). (D) Lung section stained with MAb PS14 by the alkaline phosphatase technique, demonstrating the presence of ghost cells. (E) Immunoperoxidase staining, using MAb PS15, of a paraffin-embedded lymph node biopsy specimen, showing positive cytoplasmic labelling of the fungus (arrow). (F) Immunoperoxidase staining, using MAb PS14, of *P. brasiliensis* yeasts present in a lymph node biopsy specimen. Note the apparent leakage of cytoplasmic content into the extracellular space (arrow). Bars, 75 μm.

which then adheres to, or is trapped by, the cell membrane of polymorphonuclear cells or macrophages making up the walls of the granuloma. The fact that other granulomas arising from other pathological processes, including those induced by other fungal species, are not reactive strongly suggests that this labelling is specific to *P. brasiliensis* and is not a result of cross-reactivity. The one exception to this observation would appear to be the background reactivity of PS14 when used in high concentrations against sections containing amyloid. In practice, in terms of immunohistochemical diagnosis, this would not pose a problem, since yeast cells are clearly reactive and the clinical pictures of these diseases are significantly different.

It is of note that in paraffin-embedded sections not all the yeast cytoplasm was reactive. This could be caused by variability in the expression of this antigen by yeast cells or by the presence of ghost cells which have lost cytoplasmic content during the fixation processes. It might also have resulted from the labelling of old, calcified lesions in which the fungus usually appears as an empty shell, or, alternatively, it could be due to epitope destruction by pronase. Multiple conformational changes in the antigens present in a tissue section are induced by fixation processes, and pronase is often used in immunohistochemistry to unmask epitopes. While the recognition of the epitope by MAbs PS14 and PS15 was abolished by incubation with trypsin and elastase, the short incubation period and low concentration of pronase on paraffin-embedded sections used here did not affect recognition. In fact, it was noted that longer periods of incubation with pronase greatly diminished the degree of labelling.

There is some degree of variability in the expression of the 22- to 25-kDa antigen in *P. brasiliensis*, since in cryostat sections of fresh cultures there are also differences in the labelling of the cytoplasmic content, even within yeast cells from the same isolate. It is possible that frequent subculturing may result in genetic variation, which may have contributed to this observation. However, isolates recently obtained by our laboratory showed the same degree of variability in the expression of the antigen as isolates that we have had for a number of years. Since we have no data on the exact nature and function of this 22- to 25-kDa antigen, the question of differential expression remains unclear. While we have stressed the usefulness of these MAbs in identifying *P. brasiliensis* infection in biopsy material, they may also be of use in the identification of yeasts in samples obtained by less invasive techniques, such as sputum samples or bronchoalveolar lavage specimens. Work in assessing the suitability of the MAbs in this context is under way.

The complete purification of the 22- to 25-kDa antigen for further analysis has proved difficult. The initial isoelectric focusing appears to be a useful step, but subsequent purification procedures have faltered because MAb reactivity to fractions appears to be rapidly lost. This may be a reflection of the lability of the antigen in vitro. In support of this contention is the observation that the antigenic reactivities of our different *P. brasiliensis* antigen preparations diminish in direct relation to the length of time that they are stored at −20°C. This apparent lability may also be reflected in the fact that the antigen which is most easily and rapidly prepared (CFA) is the most readily recognized by the MAbs. This does not seem to be a problem in relation to the recognition of the antigen by the MAbs in fixed sections by immunohistochemical techniques.

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