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Detection of Antibodies against *Paracoccidioides brasiliensis* Melanin in *In Vitro* and *In Vivo* Studies during Infection▽

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Several cell wall constituents, including melanins or melanin-like compounds, have been implicated in the pathogenesis of a wide variety of microbial diseases caused by diverse species of pathogenic bacteria, fungi, and helminthes. Among these microorganisms, the dimorphic fungal pathogen *Paracoccidioides brasiliensis* produces melanin in its conidial and yeast forms. In the present study, melanin particles from *P. brasiliensis* were injected into BALB/c mice in order to produce monoclonal antibodies (MAbs). We identified five immunoglobulin G1 (IgG1) κ-chain and four IgM melanin-binding MAbs. The five IgG1 κ-chain isotypes are the first melanin-binding IgG MAbs ever reported. The nine MAbs labeled *P. brasiliensis* conidia and yeast cells both *in vitro* and in pulmonary tissues. The MAbs cross-reacted with melanin-like purified particles from other fungi and also with commercial melanins, such as synthetic and *Sepia officinalis* melanin. Melanization during paracoccidioidomycosis (PCM) was also further supported by the detection of IgG antibodies reactive to melanin from *P. brasiliensis* conidia and yeast in sera and bronchoalveolar lavage fluids from *P. brasiliensis*-infected mice, as well as in sera from human patients with PCM. Serum specimens from patients with other mycoses were also tested for melanin-binding antibodies by enzyme-linked immunosorbent assay, and cross-reactivities were detected for melanin particles from different fungal sources. These results suggest that melanin from *P. brasiliensis* is an immunologically active fungal structure that activates a strong IgG humoral response in humans and mice.

Melanins, or melanin-like compounds, are cell wall constituents in a wide variety of microorganisms, including several species of pathogenic bacteria, fungi and helminthes. These polymeric pigments are implicated in the pathogenesis of diverse microbial diseases (31, 40). Notably, melanin production has been demonstrated in a wide range of human pathogenic fungi, including *Cryptococcus neoformans* (30, 57), *Lacazia loboi* (52), *Scedosporium prolificans* (43), *Histoplasma capsulatum* (32), *Sporothrix schenckii* (26), *Pneumocystis carinii* (*Pneumocystis jiroveccii*) (14), *Fonsecaea pedrosoi* (1, 2), *Blastomyces dermatitidis* (36), *Aspergillus fumigatus* (59), *Exophiala dermatitidis* (50), *Penicillium marneffei* (58), *Candida albicans* (25), and *Coccidioides posadasii* (37). Importantly, *P. brasiliensis* also produces melanin (11). In *P. brasiliensis*, conidia and yeast cells produce melanin or melanin-like compounds *in vitro* and *in vivo*. Notably, *P. brasiliensis* melanization protects the fungus from phagocytosis and increases its resistance to antifungal drugs (9). In the present study we focus on *P. brasiliensis* melanin in order to determine its capacity to induce antibodies (Abs) in murine immunization, murine infection, and human disease.

(The data presented in this study are from a master's thesis of M. E. Urán, approved by the Faculty of Health Sciences, Universidad Pontificia Bolivariana, Medellín, Colombia.)

MATERIALS AND METHODS

Fungal strain. *P. brasiliensis* strain ATCC 60855, originally isolated from a Colombian patient, was obtained from the American Type Culture Collection (Manassas, VA) and used for all of the experiments.

***P. brasiliensis* yeast growth with or without L-DOPA.** *P. brasiliensis* ATCC 60855 was converted from the mycelium to the yeast form in solid Difco Sabouraud dextrose (Becton Dickinson, Co., Le Pont de Claix, France) with 10% L-asparagine (Sigma Chemical Co., St. Louis, MO) and 10% thiamine hydrochloride (Sigma). To obtain melanized yeast cells, the fungus was grown for 15 days in the synthetic defined liquid minimal McVeigh-Morton medium (41) at pH 5.5 with or without 1.0 mM L-DOPA (Sigma) at 37°C in a rotary shaker at 150 rpm. All cultures were kept in the dark to prevent photopolymerization, as reported previously (53). Cells were collected by centrifugation at 3,000 rpm for 30 min at 4°C (refrigerated centrifuge, IEC Centra, GP8R; Thermo Fisher Scientific, Inc.), autoclaved, washed with 1× phosphate-buffered saline (PBS), and stored at 4°C until used (11, 46).

***P. brasiliensis* mycelial growth and conidium production.** *P. brasiliensis* ATCC 60855, known to sporulate on special culture media, was used for the production of conidia (42). Standard techniques were used to grow the mycelial form, and to collect and dislodge conidia (12, 42). Conidial melanization does not require the addition of exogenous phenolic or other compounds (11).

Conidia used to infect mice for the *in vivo* model were obtained by the traditional glass wool method previously described (12). Conidia were counted in a hemacytometer, and their viability was evaluated by the fluorescein diacetate and ethidium bromide staining procedure, as described previously (6).

Melanin particles: isolation and purification from *P. brasiliensis* conidia and yeast cells. Melanin particles were isolated from wild-type conidia and yeast cells induced with L-DOPA using published methodologies (26, 49). Briefly, conidia

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and yeast cells were collected by centrifugation, autoclaved, and treated with lysing enzymes (of *Aspergillus* spp. [Sigma]) to generate protoplasts that were then collected by centrifugation, washed, and incubated overnight in denaturant solution (26, 49). Cell debris was collected by centrifugation, washed, and treated with recombinant proteinase K (PCR grade from Roche Applied Science, Indianapolis, IN); the resultant materials were washed and then boiled in 6 M HCl. The materials remaining after acid digestion were collected by centrifugation, washed extensively with PBS, dialyzed against distilled water for 10 days at 4°C, and then lyophilized (26). The melanin particles (ghosts) from yeast cells were used to generate monoclonal Abs (MAbs), and particles from both conidia and yeast cells were used as antigens for the coating of the enzyme-linked immunosorbent assay (ELISA) plates for detection of melanin-binding Abs (see below).

Pulmonary paracoccidioidomycosis (PCM) experimental model. We utilized the mouse model previously described by our group (12, 24). All murine experiments were approved by the animal use committees of the Corporación para Investigaciones Biológicas (CIB) and the Guy's, Kings, and St. Thomas Medical Schools. The animals were obtained from the CIB breeding colony and used in all experiments. They were divided into two groups depending on the inoculum: (i) the infected group ($n = 7$ for each time interval assessed), consisting of 6-week-old male BALB/c mice that were inoculated intranasally with 4×10^6 conidia/60 μl of 1× sterile PBS buffer, and (ii) the control group ($n = 5$ for each time point), 6-week-old male BALB/c mice inoculated intranasally with 60 μl of 1× sterile PBS buffer. Mice were sacrificed at 0, 48, and 96 h and 1, 2, 3, 4, 6, 8, 10, 12, and 16 weeks after infection. After sacrifice, blood and bronchoalveolar lavage fluid (BAL) samples were collected. Samples were kept individually, and also a pool for each group was made. Serum was separated from blood by centrifugation at 1,500 rpm for 5 min and then frozen at −20°C. For BAL samples, the trachea was exposed and a blunt-tipped needle inserted. One milliliter of cold RPMI 1640 medium was instilled into the lungs, aspirated, and centrifuged at 1,500 rpm for 5 min at 4°C; the cells were discarded, and the supernatants were frozen at −20°C.

Production of MAbs against *P. brasiliensis* yeast cells' melanin particles. BALB/c mice (6- to 8-week-old females) from Harlam Olac (United Kingdom) were maintained at Guy's and King's Campus. Mice were bled from the tail vein prior to immunizations to define baseline antibody concentrations. At 1-week intervals, animals received five intraperitoneal injections of 50 μg or 100 μl of melanin particles extracted from *P. brasiliensis* L-DOPA-induced yeast cells suspended in a 1:1 (vol/vol) emulsion of incomplete Freund adjuvant (Difco, East Molesey, United Kingdom) in PBS. A week before sacrifice, the mice were bled to measure polyclonal antibody responses against melanin by an ELISA (protocol 1, see below); the spleen from the most responsive mouse was used to generate hybridomas using the sp2/0 myeloma cells fusion partner as described previously (13, 60). Supernatants from each hybridoma obtained were screened for the presence and classification of melanin-binding MAbs by ELISA (protocol 1, see below). The MAbs were subclassified using a mouse MAb isotyping test kit (MMT1; Serotec, Kidlington, United Kingdom).

ELISAs (protocols 1, 2, and 3) for melanin-binding Ab determination: coating of plates. Techniques used to develop ELISAs (protocols 1, 2, and 3, see below) were as follows. The plates were coated with a suspension of 50 μg of melanin derived from *P. brasiliensis* L-DOPA yeast or wild-type conidia. Plates were also generated using melanins from *Sepia officinalis* (Sigma M-2649), *S. schenckii* conidia (26), *P. marneffei* conidia (58), *A. fumigatus* conidia (59), *Aspergillus niger* conidia (59), *C. neoformans* yeast cells (35), or *C. albicans* yeast cells (25). In addition, plates were made using synthetic melanin (Sigma M-8631). Each melanin was mixed with distilled water, and 50 μg of the polymer was plated in individual wells of a polystyrene 96-well ELISA plate (catalog no. 3591; Costar, Corning, NY). Control wells were not coated with melanin particles. The plates were kept at room temperature till dry and then heat fixed by incubating the plates at 60°C for 1 h as described previously (48). The plates were subsequently used for the different ELISA protocols.

ELISA protocol 1: ELISA to detect melanin-binding polyclonal and MAbs and to determine the sensitivity and specificity of MAbs. The plates coated with different melanins were blocked overnight with SuperBlock (Pierce Biotechnology, Rockford, IL) blocking buffer in Tris-buffered saline (TBS) at 4°C to prevent nonspecific binding. After each step in the ELISA, plates were washed three times with 0.1% Tween 20 in TBS. Sera from mice inoculated with *P. brasiliensis* yeast cell melanin or hybridoma supernatants were diluted 1:10 in SuperBlock blocking buffer in PBS, and 100 μl of each sample was incubated in triplicate for 1.5 h at 37°C. After washes, 100 μl of a 1:1,000 peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) or goat anti-mouse IgM (Jackson Immunoresearch, West Grove, PA) diluted in SuperBlock was added for 1.5 h at 37°C. The plates were washed, and *o*-phenylenediamine in 0.01 M sodium citrate buffer (pH 5.0) was used as the enzyme substrate. The plates were incubated for 10 min in the dark, and the reaction was stopped with 0.01 M H_2SO_4. The solutions were transferred to clear ELISA plates, and their optical densities (OD) were measured at 490 nm with an ELISA plate reader (Microplate Reader 450/550; Bio-Rad, Hercules, CA) (26). The transfer of solutions was performed as the adherent melanin particles interfered with OD determinations. As controls, we used melanized wells without primary antibody, melanized wells in which the primary antibody had been replaced by the culture medium used for hybridoma growth (histone acetyltransferase [HAT], supplemented RPMI 1640 [Gibco-Invitrogen, Carlsbad, CA]), or wells without melanin (secondary antibody alone). OD readings higher than that of the controls (0.1 OD) were considered positive.

ELISA protocol 2: determining melanin-binding Ab titers during experimental infection. ELISA plates were blocked and washed as detailed above. To test for Ab production, mouse sera and BALs from the experimental pulmonary PCM model were diluted 1:10 in SuperBlock blocking buffer in PBS, and 100-μl portions were added to triplicate wells and processed as described for ELISA protocol 1, except that the enzyme substrate was *p*-nitrophenylphosphate at 1 mg/ml (Sigma Chemical Co.) in 98% diethanolamine 0.5 mM $MgCl_2$ buffer. The enzymatic reaction was stopped with 3 N NaOH and read spectrophotometrically at 405 nm. As controls, we used melanized wells incubated with serum or BAL samples from uninfected animals, melanized wells without sera or BALs (secondary Ab only), or wells without melanin (secondary Ab only). OD readings higher than those of controls (0.5 OD) were considered positive. One of the generated *P. brasiliensis* melanin-binding MAbs, 8C1-IgG, was used as a positive control.

ELISA protocol 3: determining melanin-binding Ab titers in human sera. A total of 27 serum specimens from patients with different clinical forms of PCM (14 with the chronic form and 13 with the subacute form) were tested individually. As a positive control, we utilized a pool consisting of 64 sera from newly diagnosed PCM patients (i.e., untreated) from the CIB's Biobank collection. As negative controls, we used pooled sera from six tuberculosis patients, and four pools from healthy subjects provided by two local blood bank laboratories, each pool consisting of more than 40 samples from healthy subjects without histories of pulmonary diseases. The use of the sera was approved by the institutional review board of the CIB.

To measure human melanin-binding Abs, wells were blocked and washed as described for ELISA protocol 1. Sera were diluted 1:500 in SuperBlock blocking buffer in PBS, and 100 μl of each sample was incubated in triplicate for 1.5 h at 37°C. After washes, 100 μl of peroxidase-conjugated goat anti-human IgG (Jackson Immunoresearch) (2) diluted in SuperBlock at 1:5,000 was added for 1.5 h at 37°C. After washes, the reaction was developed with *o*-phenylenediamine in 0.01 M sodium citrate buffer (pH 5.0) as the enzyme substrate. Plates were incubated for 10 min in the dark, and the reaction was stopped with 0.01 M H_2SO_4. The solutions were transferred to clear the ELISA plates, and their OD were measured at 490 nm with an ELISA plate reader (iMark microplate absorbance reader; Bio-Rad, Hercules, CA). In addition to the non-PCM sera, negative controls included uncoated wells and coated wells from which the primary antibody had been omitted.

Immunofluorescence assays from cultured samples. Reactivity of *P. brasiliensis* MAbs was assessed by immunofluorescence according to methods described in Youngchim et al. (59). Briefly, *P. brasiliensis* conidia or melanized yeasts (cultured with L-DOPA) were heat fixed and then blocked overnight with SuperBlock blocking buffer in PBS at 4°C. MAbs generated from the immunized mouse were diluted in SuperBlock (10 μg/ml) and applied for 1 h at 37°C. After washing, a 1:100 dilution of fluorescein isothiocyanate-conjugated (FITC) Affinipure goat anti-mouse IgG or IgM (Jackson Immunoresearch), was used as a secondary Ab. The slides were mounted using 50% glycerol–50% PBS, coverslips were applied, and the samples were examined by using a Zeiss immunofluorescence microscope. As a negative control, slides and particles were incubated with PBS instead of the primary Ab, followed by incubation with FITC-conjugated goat anti-mouse as described above. The IgM melanin-binding MAb, 6D2, generated in response to *C. neoformans* melanin (49), was used as a positive control.

Immuno-transmission electron microscopy (immuno-TEM). Samples of pulmonary tissues from *P. brasiliensis*-infected mice 24 weeks postinfection and melanin ghosts obtained from conidia or yeast cells (as described above) were fixed by incubation with formaldehyde (4%) and glutaraldehyde (0.1%) solutions overnight. The paraffin-embedded pulmonary tissues were passed through gradients of decreasing xylol and alcohol (100 to 50%) solutions before initiating the process. Frozen samples were then transferred to a Leica EM AFS freeze substitution unit (Leica Microsystems, Vienna, Austria), and ultrathin lung tissue sections were placed on nickel grids and processed as described previously (29).

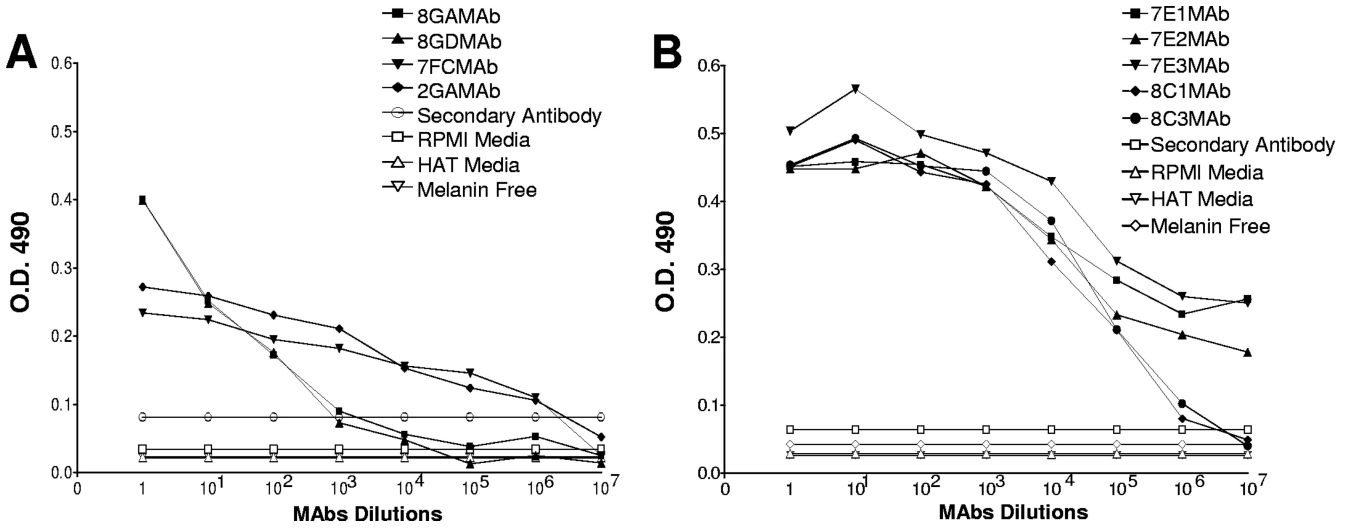

FIG. 1. ELISA reactivity of the nine *P. brasiliensis* melanin-binding MAbs produced (four IgMs and five IgG1s) against *P. brasiliensis* yeast melanin (50 μg/well), supernatants were diluted 1:10. The negative controls were wells incubated with secondary antibody, RPMI, and HAT media, and wells without melanin were incubated with secondary antibody. This panel show a representative reaction of IgM MAbs (A) and IgG MAbs (B) against *P. brasiliensis* yeast melanin. The experiment was performed twice with similar results.

The samples were then fixed in 2% glutaraldehyde in 0.1 M cacodylate at room temperature overnight. *P. brasiliensis* melanin-binding 8C3-IgG or 2GA-IgM MAbs (10 μg/ml) diluted in SuperBlock were incubated overnight at room temperature. The grids were washed, and 2.5 μg of biotin-conjugated goat anti-mouse IgG or IgM (5 μg/ml, conjugated with streptavidin and 10-nm gold; Goldmark Biologicals, Phillipsburg, NJ) in SuperBlock was added for 2 h at room temperature. The grids were fixed in 2% glutaraldehyde. Samples were viewed on a JEOL (Tokyo, Japan) 1200EX transmission electron microscope at 80 kV.

Statistical analyses. Results were collected in a database and analyzed by one-way analysis of variance with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA). Methods such as analysis of variance and the Student *t* test were applied to the data, and the results are expressed as the mean standard error of the mean of two or three independent experiments, with significance defined as $P < 0.05$. To analyze microscopic procedures such as fluorescence, TEM, or histopathology photographic studies, percentage comparisons were done.

RESULTS

Generation of MAbs against *P. brasiliensis* yeast cells melanin particles. A total of nine *P. brasiliensis* melanin-binding MAbs were produced (Fig. 1). The four IgM isotype MAbs were considered reactive (>0.1 OD greater than the controls) at dilutions to 10^2 for MAbs 8GA and 8GD and 10^3 for MAbs 2GA and 7FC (Fig. 1A). The five IgG1 κ-chain MAbs were significantly more reactive than the IgMs, since the reactivities for MAbs 8C1 and 8C3 occurred to 10^5 dilutions and for MAbs 7E1, 7E2, and 7E3 to 10^7 dilutions (Fig. 1B).

Table 1 shows the reactivities of the MAbs using the different melanin ELISAs. In each ELISA, the IgG MAbs produced

TABLE 1. ELISA reactivities for *P. brasiliensis* melanin-binding MAbs that reacted with different sources of melanin[a]

MAb against *P. brasiliensis* melanin	Mean OD observed with various melanin sources								
	Synthetic	*S. officinalis*	*P. brasiliensis* yeast cell	*P. brasiliensis* conidia	*A. fumigatus* conidia	*A. niger* conidia	*S. schenckii* conidia	*P. marneffei* conidia	*C. neoformans* yeast cell
IgG									
7E1	0.412	0.414	0.422	0.401	0.380	0.369	0.369	0.371	0.418
7E2	0.394	0.390	0.422	0.389	0.398	0.361	0.429	0.384	0.422
7E3	**0.437**	0.435	**0.471**	0.436	0.405	0.381	0.431	0.403	**0.435**
8C1	0.441	0.444	0.425	**0.459**	0.454	0.384	0.469	0.419	0.434
8C3	0.441	**0.477**	0.444	0.438	**0.459**	**0.417**	**0.503**	**0.480**	0.408
Bk-IgG	0.190	0.043	0.015	0.090	0.105	0.167	0.152	0.105	0.166
IgM									
8GA	0.149	0.074	0.172	0.207	0.018	0.311	0.225	0.105	0.062
8GD	0.145	**0.081**	0.176	**0.210**	0.043	0.269	0.249	0.090	0.062
7FC	0.215	0.038	0.195	0.205	0.093	**0.421**	0.303	**0.186**	0.275
2GA	**0.239**	0.064	**0.231**	0.184	**0.110**	**0.447**	**0.361**	0.184	**0.281**
Bk-IgM	0.047	0.001	0.005	0.111	0.024	0.173	0.102	0.026	0.130

[a] The fungal melanin was included at 50 μg per well. Numbers in boldface indicate the highest mean OD against each type of melanin. Negative controls were wells with melanin incubated with the secondary antibody or without melanin (blank reactions were subtracted from each value). The ELISAs were repeated at least twice with similar results. Supernatants were diluted 1:10.

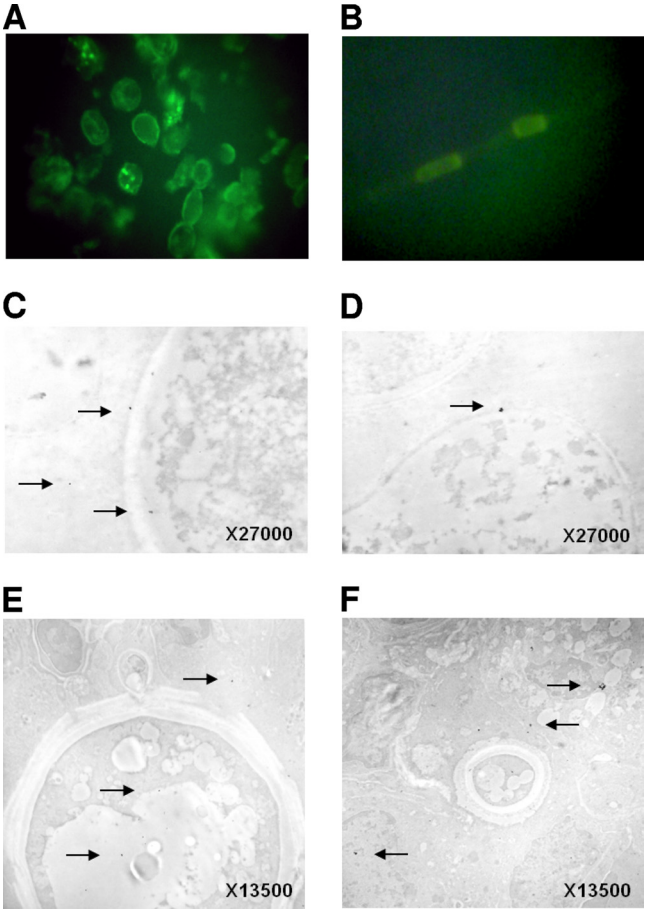

FIG. 2. Immunomicroscopy using *P. brasiliensis* melanin-binding MAbs. Representative immunofluorescence and immune-TEM images of the reactivity of melanin-binding IgG 8C3 MAb with *P. brasiliensis* melanized yeast cells (with L-DOPA induction) (A and C) and wild-type conidia (B and D). The reactivity was similar for MAbs IgG 8C1 and IgM 2GA (data not shown). MAbs 8C3 (E) and 2GA (F) labeled pigmented granules present in the lungs of mice infected with *P. brasiliensis* for 24 weeks. Arrows indicate the presence of a gold particle demonstrating immunoreactivity with melanin. Magnifications: A and B, ×1,000; C and D, ×27,000; E and F, ×13,500.

higher OD than the IgM MAbs, except for IgMs 2GA and 7FC, which produced the highest OD in response to the *A. niger* melanin. Wells without melanin or with melanin but incubated with either the growth media (supplemented RPMI) or the secondary Ab were nonreactive. Based on the reactivities observed in these experiments, we used IgG MAbs 8C1 and 8C3 and IgM MAb 2GA for further studies.

Binding of melanin *in vitro* and *in vivo*. The melanin-binding MAbs (IgG and IgM) reacted with *P. brasiliensis* yeast only after cultures had been induced with L-DOPA. Reactivity predominantly occurred along the yeast cell wall (Fig. 2A). When these yeast cells were analyzed by immuno-TEM, MAb labeling occurred on the interior and exterior aspects of the cell wall (Fig. 2C). The conidia from mycelial cultures grown in water agar without L-DOPA induction were also labeled by both MAb isotypes, as demonstrated by fluorescence and immuno-TEM (Fig. 2B and D). Controls incubated only with the fluorescein-conjugated secondary Ab or with the conjugated control Ab were nonreactive (data not shown). In addition, reactivity was demonstrated in the lungs of the *P. brasiliensis* conidium-infected mice using immuno-TEM with IgG (Fig. 2E) and IgM MAbs (Fig. 2F).

Melanin-binding Abs *in vivo*. The pooled sera or BALs from infected mice were reactive by ELISA (Fig. 3). Polyclonal IgG Abs to melanin were detected in serum within 4 weeks after infection (Fig. 3A). Interestingly, the reactivity fell between weeks 10 and 12 but rose again at week 16. In BAL samples, significant amounts of melanin-binding IgG Abs were detected by 8 weeks postinfection (Fig. 3B). Notably, for both sera and BAL, the results were similar between ELISAs performed using yeast or conidium-derived melanin. ELISAs performed with different melanins also demonstrated strong reactivity with IgG Abs in the PCM sera and BALs (Table 2).

In contrast to the IgG responses, no significant differences in concentrations of IgM Abs to either yeast or conidium-derived melanin were measured in serum or BAL samples between infected and control mice (data not shown). Similarly, ELISAs using different melanin sources also failed to detect an IgM response in the sera or BALs.

Melanin-binding Abs in patients with PCM and patients with other mycoses. The sera from PCM patients revealed significantly increased titers ($P < 0.05$) compared to healthy controls of IgG Ab to the yeast cell-derived melanin. Interestingly, there were no differences in the reactivity according to the clinical form (Fig. 4). Sera from PCM patients recognized other melanin sources as well, including synthetic melanin and melanin from *S. officinalis*, *S. schenckii* conidia, and *C. neoformans* yeast cells, with significant differences, as indicated in Table 3. Reactivity with different melanin sources was also demonstrated using pooled sera from patients with different mycoses. The boldface values in Table 3 indicate statistical significance between infected and healthy human sera ($P \leq 0.05$). Interestingly, synthetic and *S. officinalis* melanins were only recognized by sera from patients with PCM, histoplasmosis and aspergillosis. The reactivity of the sera from the patients with tuberculosis to the melanins was nearly identical to that of healthy controls.

DISCUSSION

Work from several laboratories has demonstrated that most of the human pathogenic fungi, including several dimorphic fungi such as *P. brasiliensis*, produce melanin. The relation between melanin and virulence has been studied extensively (31). Melanin can confer protection against extracellular redox buffering via the exporting of electrons, and this antioxidant function has been observed in *C. neoformans* (16, 38, 55), *A. niger* (22, 23), *E. dermatitidis* (17, 50), *S. schenckii* (44), and *A. fumigatus* (18, 59). Melanin can also impact host-fungal cell interactions, since melanized cells are more resistant to phagocytosis than nonmelanized cells in *P. brasiliensis* (9, 51) and other fungi (19, 44, 54). The pigment can enhance antifungal resistance. For example, melanization protects *P. brasiliensis* yeast against amphotericin B, ketoconazole, fluconazole, itraconazole, and sulfamethoxazole (9, 51). Additional beneficial effects of melanin in fungi include resistance to enzymatic degradation (3, 5, 10, 15, 20), UV light, heat, and desiccation (34, 44, 45).

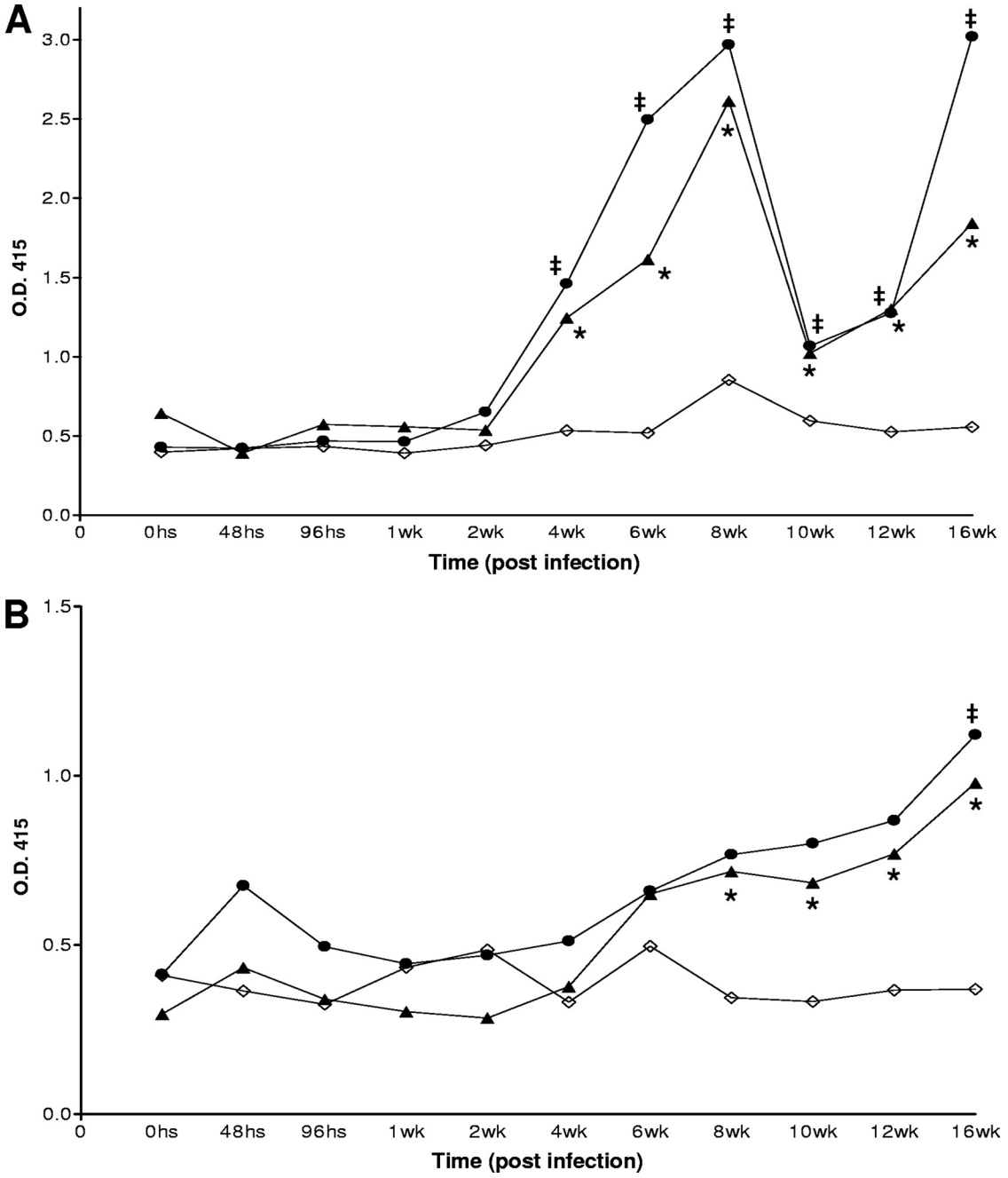

FIG. 3. IgG melanin-binding Abs from serum (A) and BAL (B) samples. Mice were infected with *P. brasiliensis* conidia, and samples were obtained at different intervals. Uninfected mice were used as controls. The reactivity with conidial melanin (▲) and with yeast cell melanin (●) using samples from infected mice was determined. The reactivity to both conidial and yeast melanin using samples from control animals is also indicated (◇). The statistical significance ($P < 0.05$) compared to controls that reacted with conidial melanin (∗) and yeast cell melanin (‡) was determined. Samples were diluted 1:10.

Fungal melanins are immunologically active compounds capable of inducing specific Ab production *in vivo*, as demonstrated in humans (2, 26) and in experimental animal models (35, 58). The humoral immune response to melanin appears to be T cell independent (33). The capacity of melanin to induce Abs has led to the generation of MAbs to melanins from different fungi, such as *C. neoformans* (MAb 6D2-IgM) (49), *S. schenckii* yeast cells (MAb 8B5-IgM) (26), and *A. fumigatus* (MAb 8F5-IgM) (58).

Our group has demonstrated that *in vitro* the parasitic form of *P. brasiliensis* (the yeast cell) synthesizes melanin using at least two different substrates, L-DOPA and L-epinephrine, thus generating melanized yeast cells (53). We have also demonstrated that *in vitro* mycelia and mycelium-derived conidia pro-

duce melanin on minimal growth medium, in the absence of phenolic compounds, suggesting that melanization is likely to occur in nature (11). Moreover, melanin pigments from *P. brasiliensis* yeast cells are synthesized during mammalian infection, and melanin ghosts can be isolated from *P. brasiliensis*-infected tissues (11). The ability of conidia to produce melanin-like compounds in the absence of substrates such as L-DOPA differentiates the melanization process seen in *P. brasiliensis* mycelia from that seen in *C. neoformans*, a fungus that relies on the presence of an exogenous substrate to drive the process (29, 35).

The present work has focused on the generation of MAbs to *P. brasiliensis* melanin. The long-term goal of our studies is to utilize melanin-binding MAbs to better understand how melanization of *P. brasiliensis* may contribute to the pathogenesis of paracoccidioidomycosis. We have shown that *P. brasiliensis* melanin is able to induce a strong immune response in a murine model, and we also found a strong humoral immune response to the pigment in patients with PCM. We successfully generated the first MAbs to *P. brasiliensis* melanin, including the first reported melanin-binding IgG MAb. Nine hybridomas in total were identified producing two isotypes, IgM and IgG1κ MAbs. Interestingly, the reactivity of the IgG MAbs was much stronger than that of the IgM isotype. The techniques used in the present study did not allow us to determine affinity or avidity of the MAbs for the melanin substrate. However, previous studies from our group and other authors have demonstrated differences in the stable free radical population of melanin particles isolated from different fungi as measured by electron spin resonance spectroscopy (8, 11, 27, 32, 36, 39, 56, 59). There are also pronounced differences in the sizes and shapes of melanin particles obtained from diverse fungi. Hence, the differences in the immune responses to melanin in PCM and the isotypes of MAbs achieved upon immunization in the present study compared to prior work with other fungi may be due to differences in the steric conformation of the *P. brasiliensis* melanin.

The results presented here further support our prior work showing that *P. brasiliensis* polymerizes melanin during experimental infection and that *in vivo* this compound is immunogenic, inducing IgG isotype Abs that react with melanin particles obtained from *P. brasiliensis* conidia and yeast cells. These IgG Abs were detected in serum and BAL samples from *P. brasiliensis*-infected mice by the week 4 postinfection, and significant Ab levels were present during the chronic period of infection. We noted a decrease in serum IgG Ab levels between weeks 10 and 12 postinfection, which may correspond to a decrease of the antigenic stimulus, as suggested by the reduction in the number of CFU in lung observed after the week 8 postinfection (26). In addition, we have previously demonstrated a similar decrease in the percentage of mononuclear cells (28) and a reduction of interleukin-5 (IL-5), IL-13, granulocyte-macrophage colony-stimulating factor, gamma interferon, macrophage inflammatory protein 1β (MIP1β), tumor necrosis factor alpha (TNF-α), IL-2, IL-3, IL-9 and IL-15 in the lungs of infected mice at this time period (21). Our group has also observed that during the early stages of experimental *P. brasiliensis* the highest levels of proinflammatory cytokines (TNF-α, IL-6, IL-1β, and MIP2) are found in BAL fluids and lung homogenates and not in sera, indicating that these mol-

TABLE 2. Reactivity of sera and BAL from *P. brasiliensis*-infected animals tested by ELISAs using 10 different melanin sources[a]

Sample	Mean OD ± SEM observed with various melanin sources								
	Synthetic	*S. officinalis*	*P. brasiliensis* yeast cells	*P. brasiliensis* conidia	*A. fumigatus* conidia	*A. niger* conidia	*S. schenckii* conidia	*P. marneffei* conidia	*C. neoformans* yeast cells	*C. albicans* yeast cells
Serum	1.154 ± 0.078	**0.468 ± 0.052**	**1.538 ± 0.030**	**1.684 ± 0.037**	**0.706 ± 0.015**	**2.266 ± 0.035**	**2.515 ± 0.033**	**0.625 ± 0.035**	**0.963 ± 0.040**	**2.891 ± 0.185**
BAL	0.749 ± 0.008	0.245 ± 0.008	0.475 ± 0.005	0.733 ± 0.003	0.277 ± 0.018	1.051 ± 0.006	1.186 ± 0.004	0.317 ± 0.036	0.306 ± 0.018	1.687 ± 0.028
MAb 8C1	**2.289 ± 0.000**	**0.557 ± 0.000**	**1.016 ± 0.000**	**1.244 ± 0.000**	0.606 ± 0.000	**2.075 ± 0.000**	**2.071 ± 0.000**	0.526 ± 0.000	**0.843 ± 0.000**	**2.427 ± 0.000**
Control noninfected mice sera	0.891 ± 0.438	0.231 ± 0.010	0.387 ± 0.091	0.578 ± 0.223	0.275 ± 0.029	0.815 ± 0.053	1.091 ± 0.388	0.272 ± 0.022	0.344 ± 0.091	1.366 ± 0.035
Control secondary Ab	0.272 ± 0.000	0.146 ± 0.000	0.202 ± 0.000	0.749 ± 0.000	0.131 ± 0.000	0.436 ± 0.000	0.738 ± 0.000	0.143 ± 0.000	0.151 ± 0.001	0.286 ± 0.000

[a] The reactivities were determined using melanin 50 μg/well. IgG antibodies to a broad range of melanin were detected in pooled sera or BAL. The melanin-binding *P. brasiliensis* IgG 8C1 MAb was used as a positive control; sera from noninfected animals and secondary Ab alone were used as negative controls. Boldface values represent significant increases of IgG compared to controls. The ELISAs were repeated three times with similar results. Samples were diluted 1:10.

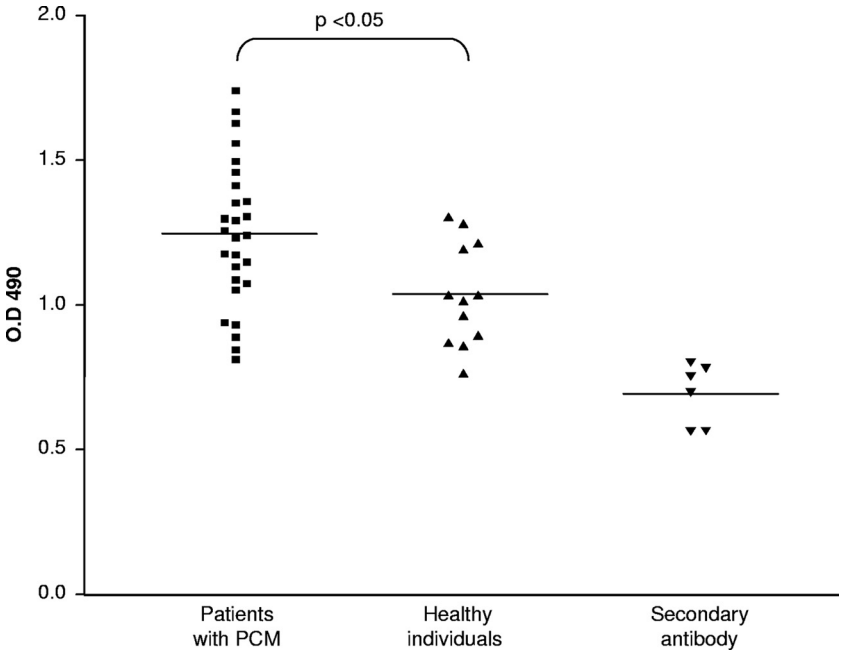

FIG. 4. IgG anti-melanin Abs against *P. brasiliensis* yeast melanin detected in sera from humans infected with *P. brasiliensis* (■) and in healthy individuals (▲) diluted 1:500 and reactivity of a secondary control antibody (▼) diluted 1:10,000. The statistical significance between infected and healthy human sera is indicated.

ecules predominate in pulmonary compartments (12). In the present study, we found that the IgG Ab levels in sera from *P. brasiliensis*-infected mice were statistically significantly higher by week 4 postinfection compared to the Ab levels in BAL. Hence, there appears to be a discordant immunological response between the locally affected lung and the whole host.

We have also demonstrated that sera from PCM patients contained IgG Abs that recognized *P. brasiliensis* melanin particles. PCM sera and sera from patients with different mycoses displayed significant cross-reactivities against a wide spectrum of fungal melanin types. We similarly observed significant cross-reactivity among different melanin types with sera from mice infected with *P. brasiliensis*. The promiscuity of melanin-binding Abs has been well described. For example, MAb 6D2, which was raised against *C. neoformans* melanin (48), binds melanin produced by *H. capsulatum* (32), *S. schenckii* (26), *C. albicans* (25), *Scytalidium dimidiatum* (27), *B. dermatitidis* (36), *A. fumigatus* (59), and *C. posadasii* (37). Similarly, MAbs produced against DHN or DOPA melanins from different fungi have been able to recognize different melanins, including those from *Aspergillus* spp. (DHN melanin), *C. neoformans* (DOPA melanin), and *S. officinalis*, as well as synthetic DOPA melanin (48, 59). These results support the hypothesis that melanin may represent a "common" or "universal" immunological or therapeutic target (7) for pathogenic fungi. Antibodies to fungal melanin have been shown to be protective against *C. neoformans* (47) and *F. pedrosoi* (2). The therapeutic potential of these melanin-binding MAbs could be significantly augmented if a radioimmunotherapeutic approach is taken, in which an antifungal MAb is linked to a radionuclide, as dramatically

TABLE 3. ELISA reactivities from pooled human sera of different mycoses that reacted with eight sources of melanin[a]

Pooled serum type	Mean OD ± SEM observed with various melanin sources							
	Synthetic	*S. officinalis*	*P. brasiliensis* yeast cells	*A. fumigatus* conidia	*A. niger* conidia	*S. schenckii* conidia	*P. marneffei* conidia	*C. neoformans* yeast cells
Paracoccidioidomycosis	**3.259 ± 0.055**	**1.638 ± 0.086**	**1.505 ± 0.013**	1.416 ± 0.002	1.977 ± 0.127	**2.352 ± 0.050**	1.153 ± 0.055	**1.525 ± 0.007**
Chromoblastomycosis	1.431 ± 0.052	0.709 ± 0.014	1.440 ± 0.082	0.989 ± 0.182	1.639 ± 0.125	2.087 ± 0.055	0.994 ± 0.038	1.049 ± 0.022
Coccidioidomycosis	1.898 ± 0.116	1.080 ± 0.157	1.235 ± 0.164	1.487 ± 0.008	1.727 ± 0.056	2.149 ± 0.040	**1.511 ± 0.080**	1.273 ± 0.009
Cryptococcosis	2.190 ± 0.057	1.090 ± 0.006	1.090 ± 0.052	0.748 ± 0.030	1.885 ± 0.155	2.158 ± 0.023	1.030 ± 0.008	1.221 ± 0.174
Histoplasmosis	**3.162 ± 0.052**	**1.658 ± 0.077**	**1.810 ± 0.098**	1.649 ± 0.021	**2.424 ± 0.213**	**2.432 ± 0.056**	**1.653 ± 0.081**	**1.878 ± 0.086**
Aspergillosis	**3.178 ± 0.095**	**1.537 ± 0.072**	**1.572 ± 0.083**	1.347 ± 0.017	2.056 ± 0.169	**2.405 ± 0.068**	**1.489 ± 0.051**	**1.719 ± 0.122**
Sporotrichosis	2.379 ± 0.382	1.141 ± 0.045	1.237 ± 0.096	0.959 ± 0.040	1.973 ± 0.015	**2.269 ± 0.010**	1.105 ± 0.009	**1.480 ± 0.042**
Candidiasis	2.406 ± 0.071	1.241 ± 0.091	1.299 ± 0.001	**1.768 ± 0.545**	1.976 ± 0.067	**2.296 ± 0.092**	**1.500 ± 0.001**	**1.856 ± 0.160**
Tuberculosis	1.302 ± 0.033	0.736 ± 0.030	0.991 ± 0.000	0.681 ± 0.017	1.512 ± 0.044	2.081 ± 0.112	1.024 ± 0.114	1.029 ± 0.067
Healthy individuals	1.606 ± 0.034	0.913 ± 0.002	1.035 ± 0.009	0.785 ± 0.067	1.551 ± 0.027	1.986 ± 0.086	1.064 ± 0.074	0.945 ± 0.031
Secondary Ab	0.842 ± 0.031	0.195 ± 0.002	0.740 ± 0.042	0.313 ± 0.028	1.262 ± 0.006	1.341 ± 0.079	0.775 ± 0.039	0.707 ± 0.011
Blank reaction	0.256 ± 0.067	0.079 ± 0.001	0.062 ± 0.000	0.067 ± 0.008	0.160 ± 0.001	0.224 ± 0.015	0.053 ± 0.000	0.070 ± 0.001

[a] The samples were tested at 50 μg per well. Boldface numbers indicate statistical significance ($P \leq 0.05$) between infected and healthy human sera for each type of melanin. The ELISAs were repeated twice, and similar results were achieved each time. Samples were diluted 1:500.

shown for *C. neoformans* where radioimmunotherapy outperformed amphotericin B in experimental murine cryptococcosis (4).

In summary, our results confirmed that *P. brasiliensis* is able to synthesize melanin *in vitro* and *in vivo* and support the view that this ability could be related to virulence. Melanin from *P. brasiliensis* L-DOPA-induced yeast cells was shown to be an immunogenic particle that allowed melanin-binding MAbs to be produced for this pathogenic fungus. Moreover, five of the MAbs were of the IgG type. The polymerized melanin formed during the *in vivo* conidium-yeast transition was also immunogenic, as demonstrated in serum and BAL samples from infected animals with Abs that were able to bind to isolated melanin particles from conidia and yeast cells when tested by ELISA. In addition, PCM patients have circulating Abs that react with *P. brasiliensis* melanin and also melanins from different sources. We also characterized the antibody response to melanin induced during other infectious diseases and found that the melanin-binding Abs cross-reacted with the additional melanins tested. These results support the hypothesis of "common" or "universal" targets for different fungal species that induce Abs. Future studies will explore the effects of the melanin-binding *P. brasiliensis* MAbs on the pathogenesis of PCM.

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