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Coccidioides posadasii produces melanin *in vitro* and during infection

Joshua D. Nosanchuk [a,b,*], Jieh-Juen Yu [c,d], Chiung-Yu Hung [c,d], Arturo Casadevall [a,b], Garry T. Cole [c,d]

[a] *Department of Medicine (Division of Infectious Diseases), Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[b] *Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[c] *South Texas Center of Emerging Infectious Diseases, University of Texas at San Antonio, San Antonio, TX 78249, USA*
[d] *Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249, USA*

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Abstract

Using techniques developed to study melanization in other fungi, we demonstrate that *Coccidioides posadasii* arthroconidia, spherules, and endospores produce melanin or melanin-like compounds *in vitro* and tissue forms synthesize pigment *in vivo*. Since melanin is an important virulence factor in other pathogenic fungi, it may affect the pathogenesis of coccidioidomycosis.

Keywords: *Coccidioides*; Fungi; Melanin; Virulence factor

Coccidioides posadasii is a dimorphic fungus endemic in the United States, Mexico, and Central and South America (Fisher et al., 2002). The fungus grows as a filamentous saprobe in desert and semiarid soils. Disturbances of soil contaminated with the organism results in the dispersal of spores (arthroconidia), which are the infectious propagules. The arthroconidia are inhaled and the spores convert to the parasitic spherule/endospore phase in tissue. Infection with this fungus may be asymptomatic, but approximately 50% of immunologically competent individuals develop an atypical pneumonia characterized by a cough, fever, and pleuritic chest pain often accompanied by rashes, sore throat, headache, arthralgia, myalgia, or anorexia (Cole et al., 2004).

Melanins are multifunctional polymers that are found in species from all biological kingdoms (Hill, 1992). These polymers are negatively charged, hydrophobic pigments of high molecular weight that are formed by the oxidative polymerization of phenolic and/or indolic compounds (Wheeler and Bell, 1988). Melanin synthesis is associated with virulence for the human pathogenic fungi *Cryptococcus neoformans*, *Aspergillus* species, *Exophiala (Wangiella) dermatitidis*, and *Sporothrix schenckii* [reviewed in (Nosanchuk and Casadevall, 2003)]. In *C. neoformans*, pigment production protects the fungus against diverse insults, including oxidants, extremes in temperature, ultraviolet light, antifungal drugs, microbicidal peptides, enzymatic degradation, and macrophages *in vitro* [reviewed in (Nosanchuk and Casadevall (2003))]. Melanins may play an important role in virulence in diverse fungal species. Since other important endemic dimorphic fungi such as *Blastomyces dermatitidis* (Nosanchuk et al., 2004), *Paracoccidioides brasiliensis* (Gómez et al., 2001), *Sporothrix schenckii* (Morris-Jones et al., 2003), *Penicillium marneffei* (Youngchim et al., 2005), and *Histoplasma capsulatum* (Nosanchuk et al., 2002) have been shown to produce melanin, we investigated whether *C. posadasii* could synthesize melanin or melanin-like compounds. Utilizing techniques developed to study and isolate melanin *in vitro* and *in vivo* for other pathogenic fungi, we demonstrated that the environmental and tissue forms of *C. posadasii* produce melanin or melanin-like compounds.

The saprobic phase of *C. posadasii* (isolate C735) was grown *in vitro* for 20 days under conditions described previously (Hung et al., 2000). Melanin containing cell ghosts

* Corresponding author. Fax: +1 718 430 8701.
E-mail address: nosanchu@aecom.yu.edu (J.D. Nosanchuk).

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were isolated after treatment of arthroconidia as described for other melanotic fungi (Rosas et al., 2000a,b). Briefly, cells grown in media were collected by centrifugation and washed with PBS. The cells were autoclaved, washed with PBS and suspended in 1.0 M sorbitol, 0.1 M sodium citrate (pH 5.5). Cell wall lysing enzymes (from *Trichoderma harzianum*; Sigma) were added at 10 mg ml^{-1} and the resulting suspensions were incubated at 30 °C overnight. The cell preparations were collected by centrifugation, washed with PBS, and incubated with 4 M guanidine thiocyanate (Sigma) for 12 h. The samples were then treated with 1.0 mg ml^{-1} proteinase K (Roche Laboratories; Indianapolis, IN) in a reaction buffer (10.0 mM Tris, 1.0 mM $CaCl_2$, and 0.5% SDS; pH 7.8) at 37 °C overnight and then extracted three times with chloroform. The debris was collected, washed with PBS, and boiled in 6.0 M HCl for 1 h. The remaining cell ghosts were collected, washed in PBS, and dialyzed extensively against distilled water. This procedure solubilizes non-melanized fungal cells (Rosas et al., 2000a,b). Chitin is also solubilized by this treatment (Gómez et al., 2001). Arthroconidial ghosts appeared grayish by brightfield microscopy and maintained the morphology of the cells from which they were derived (Fig. 1A and C).

Ten μl suspensions containing approximately 10^6 arthroconidial ghosts isolated as described above or equal numbers of intact arthroconidia killed by incubation at 95 °C for 2 h were air-dried on separate poly-L-lysine-coated slides (Sigma) (Nosanchuk et al., 1998) (Figs. 1B, D, and 2 A, B, respectively). The slides coated with the cell ghosts were washed in PBS, incubated in Superblock® (Pierce, Rockford, IL) blocking buffer for 1 h at 37 °C followed by incubation with 10 μg ml^{-1} of the melanin-binding monoclonal antibody (mAb) 6D2 for 1 h at 37°C. MAb 6D2 [IgM] was generated against melanin derived from *C. neoformans* that also binds other types of melanins, but does not bind *Candida albicans*, *Saccharomyces cerevisiae*, or a laccase deficient albino mutant of *C. neoformans* (Rosas et al., 2000a,b). After washing, the slides were incubated with a 1:1000 dilution of tetramethyl rhodamine isothiocyanate (TRITC) fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (Southern Biotechnologies Associates, Inc., Birmingham, AL) for 1 h at 37 °C. The slides were washed, mounted using a 50% glycerol, 50% PBS, and 0.1 M *N*-propyl gallate solution, and viewed with an Olympus AX70 microscope (Melville, NY) equipped with fluorescent filters. The melanin-binding antibody

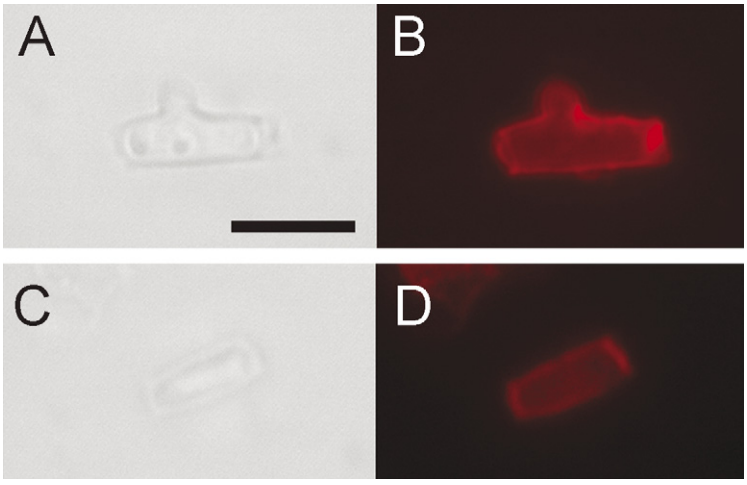

Fig. 1. (A,C) Arthroconidial ghosts obtained after treatment with enzymes, denaturant, and hot HCl and examined by brightfield microscopy. (B,D) Arthroconidial ghosts reacted with mAb raised against melanin followed by fluorescent secondary antibody. Bar in (A) represents 5 μM.

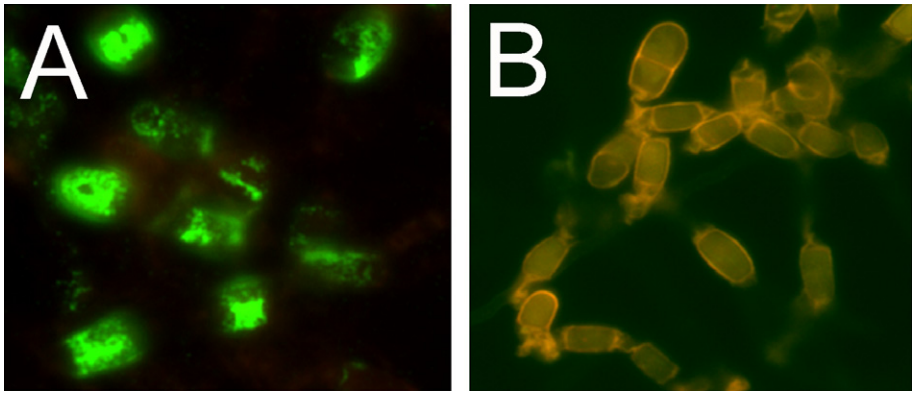

Fig. 2. (A) Immunoflourescence labeling of intact, heat-killed arthroconidia with melanin-binding antibody 6D2. (B) No binding was observed after cells were incubated with control antibody 2D10 followed by FITC-conjugated secondary antibody.

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reacted with the arthroconidial ghosts, which indicates the presence of melanin or melanin-like deposits in the residual cell wall material (Fig. 1 B, D). Additionally, intact heat-killed arthroconidia were incubated with blocking buffer and then fluorescein isothiocyanate (FITC)-conjugated with goat anti-mouse IgM (Southern Biotechnologies Associates, Inc., Brimingham, AL) for 1 h at 37 °C. The slides were washed, mounted and examined as described above. The intact arthroconidia were labeled by the melanin-binding mAb (Fig. 2A). Negative controls consisted of slides incubated with primary antibody that consisted of either isotype matched mAb [5C11] that binds mycobacterial lipoarabinomannan (Glatman-Freedman et al., 1996) or 2D10 that binds to glucuronoxylomannan on *C. neoformans* (Mukherjee et al., 1994)]. Alternative control preparations were incubated with fluorescently labeled secondary Ab alone. In all cases, no binding occurred with the control antibodies (Fig. 2B).

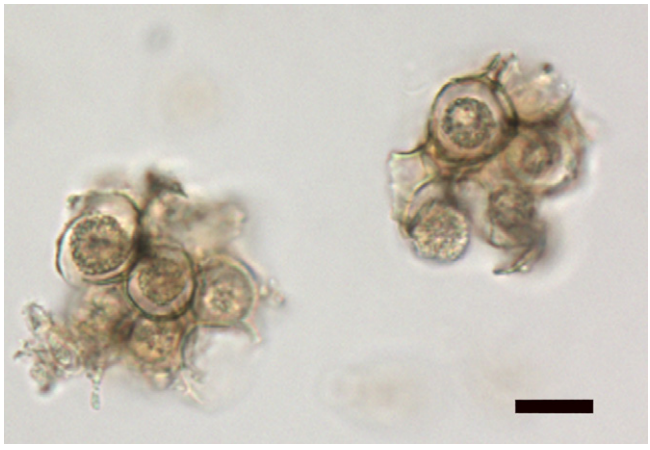

Fig. 3. Spherule ghosts isolated from infected murine lung tissue after treatment with enzymes, denaturant and hot acid. Bar represents 20 μm.

The parasitic phase of *C. posadasii* was initially evaluated in the lungs of C57BL/6 mice (females, 8 weeks old, National Cancer Institute Bethesda, MD) that were infected intranasally with 80 viable arthroconidia and sacrificed after 12 days. The lungs were homogenized and subjected to serial treatment with enzymes, denaturant, and hot HCl as above to isolate parasitic cell (spherule) ghosts. The resultant particles were brownish and similar in appearance to the intact spherule propagules (Fig. 3). Additionally, infected murine lung tissue was embedded in paraffin and sectioned for immunofluorescent analysis with melanin-binding antibody. The sections were deparaffinized in xylene, rehydrated in an ethanol series and then incubated with melanin-binding mAb or control antibody. Microscopy revealed that only the endospores within the spherules in the tissue sections reacted with the melanin-binding mAb (Fig. 4A and B). The wall of the spherules appeared to be poorly labeled. *In vitro*-grown spherules (48 h cultures) were isolated, heat-killed as above, and incubated with the anti-melanin mAb followed by secondary antibody conjugated with TRITC. The intact spherules were also not labeled (data not shown). We hypothesized that the lipid-rich, membranous spherule outer wall (SOW), which has been reported (Cole et al., 1988; Hung et al., 2000),

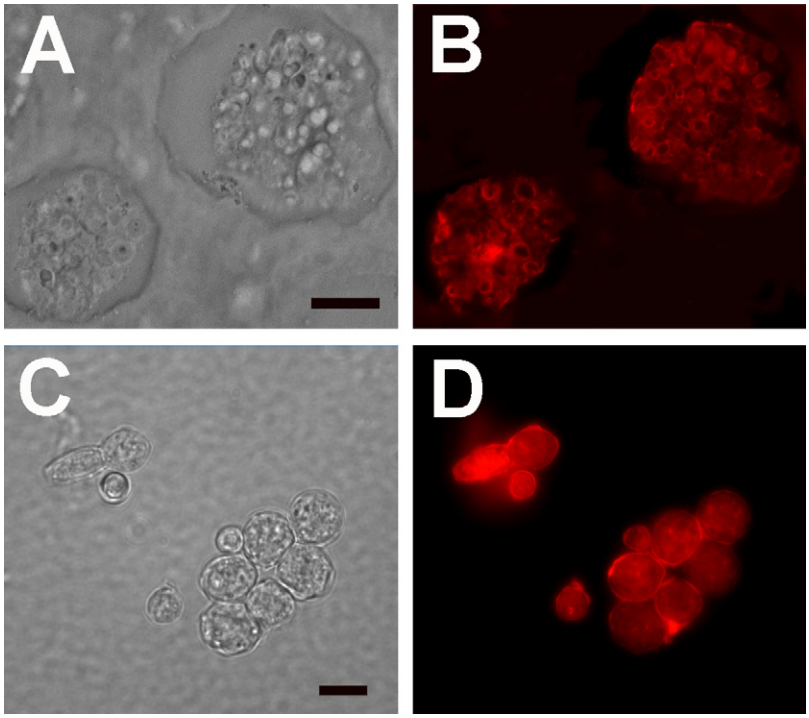

Fig. 4. (A,B) Paraffin-embedded and sectioned endosporulating spherules in infected lung tissue examined by brightfield microscopy (A) and immunofluorescence microscopy (B) after reaction with anti-melanin mAb followed by TRITC-conjugated secondary antibody. (C,D) *In vitro*-grown, heat-killed spherules which had been incubated with Triton X-114 to remove the spherule outer wall (SOW) layer and then examined by bright field microscopy (C) or immunofluorescence microscopy (D) as above. Bars in (A) and (C) represent 10 μM.

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may have interfered with the binding of the mAb to melanin-like compounds in the spherule wall. To generate cells largely devoid of SOW, we first incubated viable arthroconidia in RPMI 1640 (American Type Culture Collection, Rockville, MD; Cat. # 30-2001) with heat-inactivated calf serum (ATCC, Cat. #30-2030) for two days at 39 °C. The arthroconidia germinated to produce spherules, which were harvested by centrifugation, washed with PBS, and then incubated with 1% Triton X-114 (Sigma) in PBS for 1 h with vigorous shaking. Triton X-114 treatment of *in vitro*-grown spherules has been shown to solubilize the SOW layer at the cell surface (Hung et al., 2000). The treated spherules were then washed three times with PBS and heat-killed as above. When incubated with melanin-binding mAb followed by fluorescently-labeled secondary antibody, the intact spherules reacted with the mAb to melanin (Fig. 4C and D), whereas no binding occurred with control antibody (data not shown). Hence, the SOW layer may interfere with host cell interactions with *C. posadasii* pigments.

Our results show that *C. posadasii* produces melanin or melanin-like compounds. The evidence supporting the formation of melanin by *C. posadasii in vitro* and *in vivo* is as follows: (i) treatment of saprobic and parasitic forms of *C. posadasii* with enzymes and chemicals produced acid-resistant, insoluble grayish or brownish ghosts, similar in size and shape to their respective propagules, and (ii) both the cell ghosts and intact, heat-killed saprobic, and parasitic cells (the latter after Triton X-114 treatment) were labeled with melanin-binding mAb. As with *Aspergillus* spp., *Alternaria* spp., and *Fonsecaea pedrosoi*, *C. posadasii* arthroconidia and spherules are able to generate the pigment without the addition of exogenous phenolic compounds. The type of melanin and the enzymatic pathway responsible for the production of *C. posadasii* pigment have not been determined. Interestingly, analysis of the C. *posadasii* genome database (www.tigr.org) reveals a putative gene with 80% similarity to a laccase (UniProtKB Entry: Q12570) of *Botrytis cinerea* (Cantone and Staples, 1993). Additionally, a sequence homologous (hypothetical protein CIMG_08002.2; score = 159, *E* value = 4E−38) to the Lac2 of *C. neoformans* that has been associated with L-dopa melanin synthesis (Missall et al., 2005; Pukkila-Worley et al., 2005) is found in the *C. immitis* database (www.broad.mit.edu). Our findings will be followed with further studies exploring the role of melanin in the pathogenesis of *C. posadasii* infection and disease. Since the production of melanin has been linked with virulence in several pathogenic fungi, formation of melanin or melanin-like compounds could be an additional reason for the difficulties in treating coccidioidomycosis, particularly in immunocompromised individuals.

With this report we complete the survey of the major fungal pathogens and add *C. posadasii* to the list of melanotic fungi. Although the capacity for melanization is found in many fungal species, we find it noteworthy that practically all the major human pathogenic fungi synthesize melanin-like pigments. Given that not all fungi are melanotic, the association of melanization with the human pathogenic fungi and the availability of a large body of literature associating melanization with virulence suggest that this pigment has a broad role in fungal virulence.

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