Available online at www.sciencedirect.com

SCIENCE DIRECT

Fungal Genetics and Biology 42 (2005) 989–998

FUNGAL GENETICS AND BIOLOGY

www.elsevier.com/locate/yfgbi

Comparative analysis of *Cryptococcus neoformans* acid-resistant particles generated from pigmented cells grown in different laccase substrates

Javier Garcia-Rivera [a,1], Helene C. Eisenman [b], Joshua D. Nosanchuk [b], Philip Aisen [c], Oscar Zaragoza [a], Tiffany Moadel [d], Ekaterina Dadachova [d], Arturo Casadevall [a,b,*]

[a] *Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[b] *Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[c] *Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[d] *Department of Nuclear Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, USA*

Received 1 August 2005; accepted 14 September 2005
Available online 11 November 2005

Abstract

Cryptococcus neoformans produces pigments in vitro in the presence of exogenous substrate. We characterized acid-resistant particles isolated from pigmented cells grown in L-dopa, methyl-dopa, (−)-epinephrine or (−)-norepinephrine. The goals of this study were to determine whether pigments made from each of these substrates were melanins and the consequences of pigmentation on related cell characteristics. The greatest yield of acid-resistant particles occurred with methyl-dopa followed by L-dopa. Electron microscopy indicated that L-dopa and methyl-dopa produced particles with thicker shells. The mAb 6D2 reacted with all particles, but a lower reactivity was observed with epinephrine-derived particles. ESR analysis revealed that epinephrine-derived particles failed to produce a stable free radical signal typical of melanins. Growth of *C. neoformans* in different substrates affected cell and capsule size but not capsule induction. Hence, the type of pigment produced by *C. neoformans* is dependent on the substrate and not all pigments meet the criteria for melanins.

Keywords: *Cryptococcus neoformans*; Melanin

1. Introduction

Melanins are complex biopolymers to which a wide range of biological functions have been attributed including camouflage, radical scavenging, photo and radiosensitization, energy transduction, and protection against sunlight (Hill, 1992). Melanin synthesis is associated with virulence in several microbes (Brush and Money, 1999; Jacobson, 2000; Money et al., 1998; Nosanchuk and Casadevall, 2003b). One such organism is the pathogenic fungus, *Cryptococcus neoformans*. Despite the fact that melanins are widely distributed in nature, the structures of these amorphous pigments are poorly understood. However, there is a general consensus that they are insoluble, hydrophobic, and negatively charged polymers of large molecular weight (Casadevall et al., 2000).

Melanins are classified into two broad categories depending on their chemical composition: eumelanins and pheomelanins (Prota, 1988). Eumelanins are dark-brown to black pigments with 6–9% nitrogen and 0–1% sulfur, composed of 5,6-dihydroxyindole (DHI)[2] and 5,6-dihydroxyindole-2-car-

* Corresponding author. Fax: +1 718 430 8701.
E-mail address: casadeva@aecom.yu.edu (A. Casadevall).
[1] Present address: Department of Microbiology, Escuela De Medicina San Juan Bautista, P.O. Box 4968, Caguas, PR 00726-4968, USA.

[2] *Abbreviations used:* DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; ESR, electron spin resonance; L-dopa, L-3,4-dihyroxyphenyl-alanine; methyl-dopa,−)-3-(3,4-dihydroxyphenyl-2-methyl)-L-alanine; PDCA, pyrrole-2,3-dicarboxylic acid; PTCA, pyrrole-2,3,5-tricarboxylic acid; TDCA, 1,3-thiazole-4,5-dicarboxylic acid; TEM, transmission electron microscopy; TTCA, 1,3-thiazole-2,4,5-tricarboxylic acid.

1087-1845/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2005.09.003

boxylic acid (DHICA) monomer units (Ito and Fujita, 1985; Wakamatsu and Ito, 2002). In contrast, pheomelanins are reddish-brown pigments with 8–11% nitrogen and 9–12% sulfur, composed of benzothiazine monomer units (Ito and Fujita, 1985; Wakamatsu and Ito, 2002). Acidic permanganate oxidation yields pyrrole-2,3,5-tricarboxylic acid (PTCA) from DHICA-derived structures, pyrrole-2,3-dicarboxylic acid (PDCA) from DHI subunits, and 1,3-thiazole-4,5-dicarboxylic acid (TDCA) and 1,3-thiazole-2,4,5-tricarboxylic acid (TTCA) from benzothiazole subunits (Ito and Fujita, 1985; Wakamatsu and Ito, 2002). Hence, the presence of PTCA and PDCA in oxidation products indicates eumelanin and the presence of TDCA and TTCA indicates pheomelanin.

Cryptococcus neoformans produces pigments in vitro in the presence of exogenous substrate, including several catecholamines. The pigment produced with L-dopa substrate has been shown to be a melanin (Wang et al., 1995). Melanin is also produced in vivo (Nosanchuk et al., 2000; Rosas et al., 2000a). This is relevant during infection since melanized cells are more resistant to several types of stress (Garcia-Rivera and Casadevall, 2001; Wang and Casadevall, 1994a,b). Although the dark pigments produced in vitro are often assumed to be melanin-like, this has not been formally proven. In *C. neoformans*, melanin is deposited in the cell wall (Nosanchuk and Casadevall, 2003a). When pigmented cells are subjected to stepwise treatment with fungal cell wall digesting enzymes, denaturants, proteinase, chloroform extraction, and boiling with hydrochloric acid (Rosas et al., 2000b), the net result is the recovery of "ghosts". These are hollow shells with proportions similar to cells that are amenable to biophysical analysis (Wang et al., 1995).

Catecholamine-derived melanins are known as dopamelanins (Polacheck et al., 1982). Dopa-melanins, such as those in *C. neoformans*, are produced by the oxidative polymerization of dihydroxyphenols and/or indolic compounds, a process facilitated by phenoloxidases, specifically laccases. Laccases catalyze the removal of a hydrogen atom from phenolic hydroxyl or aromatic amino groups to form free radicals and amino radicals, which can then autopolymerize to melanins (Leonowicz et al., 2001). The association of melanin with other cell components makes its isolation difficult (Casadevall et al., 2000). Synthetic melanin, prepared enzymatically or chemically from L-dopa contains more carboxyl functions than natural melanins (Knorle et al., 1998), suggesting chemical differences between synthetic and natural melanins. Heterogeneity, insolubility, and the likely presence of non-tyrosine or L-dopa derived compounds originating from the cell wall have severely limited the analysis of melanins and prompted the search for alternative approaches to study this pigment.

Recently, the dependence of *C. neoformans* melanization on exogenous substrate was exploited to study the molecular structure of fungal melanins by generating biosynthetically labeled [^{13}C] melanin suitable for study with solid-state NMR techniques (Tian et al., 2003). The success of that approach suggested the need for detailed analysis of melanin ghosts generated with various precursors. In this paper, we investigate the efficiency of pigment synthesis with four commercially available substrates and report on the physical properties of the ghosts recovered.

Since melanin is found in the cell wall of *C. neoformans*, we analyzed the effect of the various substrates on related growth characteristics and melanin formation. *C. neoformans* produces a polysaccharide capsule located outside the cell wall that is composed mainly of glucuronoxylomannan (McFadden and Casadevall, 2001). The polysaccharide confers some of the fungi's most peculiar characteristics (Buchanan and Murphy, 1998; Vecchiarelli et al., 1994). The polysaccharide capsule can manifest great variations in size, which could affect pathogenesis (Zaragoza and Casadevall, 2004). Thus, the effects of melanin substrate on cell and capsule were measured.

2. Materials and methods

2.1. Strains

Cryptococcus neoformans serotype D strain 24067 and *C. neoformans* serotype A strain H99 were obtained from the American Type Culture Collection (ATCC 208821) (Perfect et al., 1993). *C. neoformans* serotype D strain Cap67, containing a mutation in the *CAP59* gene, was generously provided by J. Kwon-Chung (Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA) (Chang and Kwon-Chung, 1994).

2.2. Preparation of "ghosts" and yields

Cryptococcus neoformans strain 24067 was grown in chemically defined medium (15.0 mM glucose, 10.0 mM $MgSO_4$, 29.4 mM K_2HPO_4, 13.0 mM glycine, and 3.0 mM thiamine, pH 5.5) with 1.0 mM of either L-3,4-Dihyroxyphenyl-alanine (L-dopa) (Sigma), (−)-3-(3,4-dihydroxyphenyl-2-methyl)-L-alanine (methyl-dopa) (Sigma), (−)-Epinephrine (Sigma) or (−)-Norepinephrine (Sigma) for 21 days at 30 °C in a rotatory shaker at 150 rpm. Melanin particles were isolated as described (Rosas et al., 2000b). Briefly, cells were incubated with lysing enzymes (Sigma) overnight at 30 °C, then treated with 4 M guanidine thyocyanate, 1 mg mL^{-1} proteinase K (Sigma), extracted with chloroform, and boiled for 1 h with 6 M HCl. The resulting particles or "ghosts" were dialyzed against water for 10 days. Ghosts were weighed after lyophilization to determine yield.

2.3. Immunofluorescence

Melanin particles (10^5) were fixed to poly-lysine treated slides and blocked with Superblock PBS (Pierce) overnight at 4 °C. The slides were then incubated with

10 μg mL^{-1} of mAb 6D2 (μκ) for 2 h at 37 °C. MAb 6D2 is a melanin-binding antibody generated using *C. neoformans* L-dopa derived melanin ghosts (Rosas et al., 2000a). MAb 6D2 binding was then detected with a goat anti-mouse IgM conjugated to fluorescein isothiocyanate (FITC) (1:1000). The slide was sealed with a solution of *n*-propyl gallate in PBS-glycerol to preserve the immunofluorescence signal (1:1). The mAb 5C11 to mycobacterial polysaccharide was used as an isotype matched negative control (Glatman-Freedman et al., 1996). Pictures were taken with an Olympus AX70 microscope equipped with a FITC filter and the figures prepared with Photoshop 7.0 (Adobe Systems).

2.4. Zeta potential and electron spin resonance spectroscopy

The *C. neoformans* encapsulated strain 24067 and the acapsular strain Cap67 were grown in minimal medium with the respective pigment substrate for 10 days. Cells from *C. neoformans* 24067 were measured to determine cell size, capsule percentage, and capsule thickness. Also, the zeta potentials of 24067 and Cap67 strains grown under the different conditions were measured with a Zeta Potential Plus machine (Brookhaven Instruments). The zeta potential, or surface charge, of the particles is determined by applying an electric field to the particles in suspension, and determining the direction and velocity of the particle movement by measuring light scattering of a laser beam passed through the sample. Samples were prepared at concentration of 10^7 cells or melanin ghosts per milliliter in 1 mM KCl. Each reading represents the average of twenty measurements. Also, melanin ghosts were examined for the presence of free radicals by using electron spin resonance (ESR), as previously described, except with a Gunn diode as the microwave source (Enochs et al., 1993; Wang et al., 1995).

2.5. Oxidation of melanins and HPLC of oxidized melanins

The ghosts produced by growing *C. neoformans* cells in the presence of various pigment precursors underwent acidic permanganate oxidation using the procedure described by Ito and Fujita (1985). Pyrrole-2,3,5-tricarboxylic acid (PTCA), pyrrole-2,3-dicarboxylic acid (PDCA), 1,3-thiazole-2,4,5-tricarboxylic acid (TTCA), and 1,3-thiazole-4,5-dicarboxylic acid (TDCA) were used as standard compounds (gift from Dr. K. Wakamatsu of Fujita Health University of the Health Sciences, Toyoake, Japan). The oxidation products were analyzed by HPLC using a Shimadzu LC-600 liquid chromatograph, Hamilton PRP-1 C_{18} column (250 × 4.1 mm dimensions, 7 μm particle size), and Shimadzu SPD-6AV UV detector. The mobile phase was 0.1% v/v trifluoroacetic acid in water (solvent A) and 0.1% v/v trifluoroacetic acid in acetonitrile (solvent B). At 1.0 mL min^{-1}, the elution gradient was (min, %B): 0, 0; 1, 0; 12, 25; 14, 25; and 16, 0. The UV detector was set at a 255 nm absorbance.

2.6. Transmission electron microscopy

Ghosts were fixed in 2% v/v glutaraldehyde in 0.1 M cacodylate at room temperature for 2 h, followed by overnight incubation in 4% v/v formaldehyde, 1% v/v glutaraldehyde, and 0.1% v/v PBS. The samples were subjected to 90 min post-fixation in 2% w/v osmium tetroxide, serially dehydrated in ethanol, and embedded in Spurrs epoxy resin. Sections (70–80 nm) were cut on a Reichert Ultracut UCT and stained with 0.5% w/v uranyl acetate and 0.5% w/v lead citrate. Samples were viewed in a JEOL 1200EX transmission electron microscope at 80 kV. At least 30 ghosts were measured for L-dopa, methyl-dopa, and norepinephrine. Four epinephrine ghosts were measured.

2.7. Induction of capsule growth in melanized cells

Cells from H99 and 24067 strains were melanized in the presence of L-dopa, epinephrine, norepinephrine, and methyl-dopa as indicated above for 11 days. Capsule growth was then induced in 50 mM Mops, pH 7.3, containing 10% v/v Sabouraud medium as described (Zaragoza and Casadevall, 2004). After overnight induction, the cells were suspended in India ink, and pictures were taken with an Olympus AX70 microscope. The images were analyzed in Photoshop 7.0 (Adobe Systems). To calculate the capsule size, the diameters of the whole cell (D_{wc}) and the cell body (D_{cb}) were measured. The percentage of capsule in the whole cell was determined as follows: $[(D_{wc} - D_{cb}) \times 100]/D_{wc}$ (Zaragoza et al., 2003). Between 40 and 70, cells were measured.

2.8. Statistical analysis

Cell size and zeta potential result measurements were analyzed by Kruskal–Wallis statistic using Prism version 4 (GraphPad Software). Capsule induction results were compared by *t* test.

3. Results

3.1. Different melanization substrates are not equally incorporated into pigments

We first analyzed the color of the pigment produced from each substrate. Cap67 cells, lacking the polysaccharide capsule, grown in methyl-dopa or L-dopa were black, whereas cells grown in norepinephrine and epinephrine were dark and light brown, respectively (Fig. 1). Similar results were observed in cultures of the encapsulated strain 24067 (data not shown). Culture supernatants were light brown for L-dopa and methyl-dopa, dark brown for norepinephrine, and red for epinephrine (Fig. 1). When viewed under light microscope, particles derived from epinephrine had a transparent appearance whereas the other particles were brown and opaque (Fig. 2).

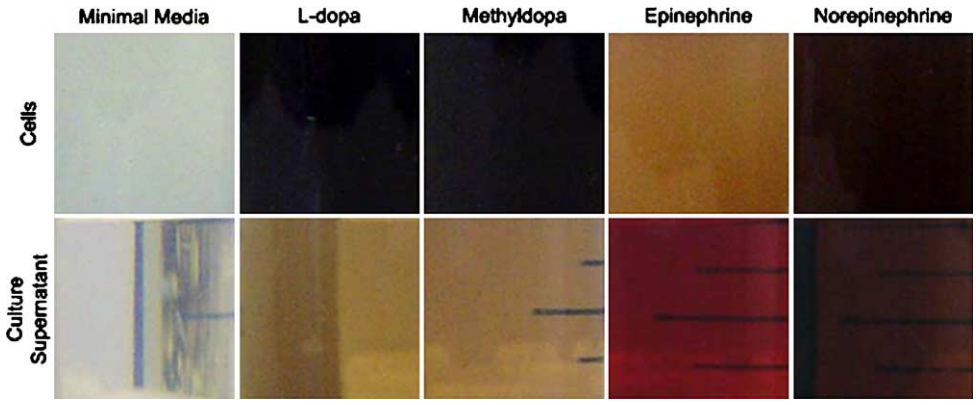

Fig. 1. *Cryptococcus neoformans* strain Cap67 was grown in minimal medium with different substrates. Cells and culture supernatants were separated by centrifugation. Cell suspensions were placed in tubes and photographed (upper panels). The culture supernatants were placed in separate tubes and photographed (bottom panels).

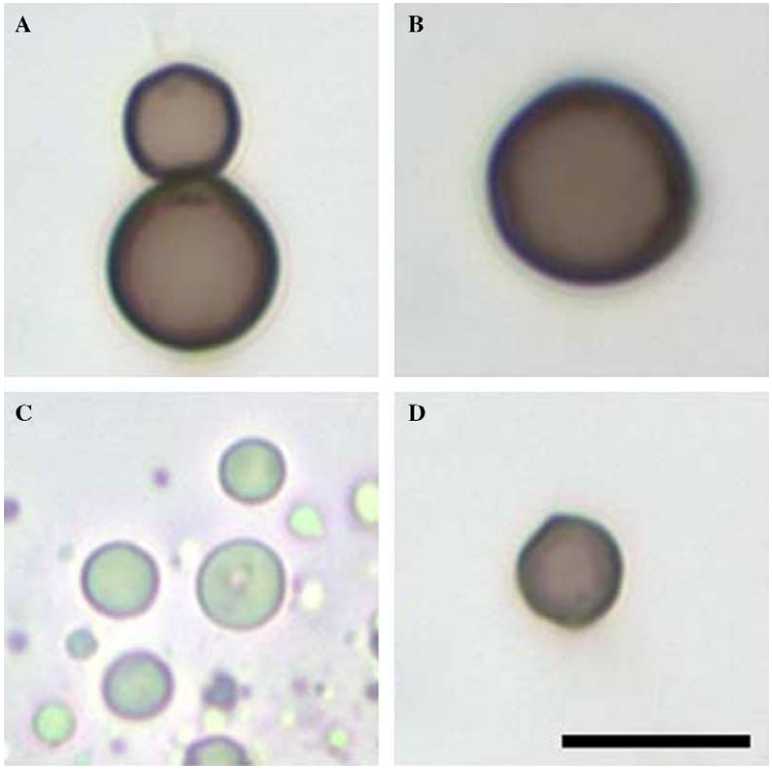

Fig. 2. Acid-resistant particles isolated from *C. neoformans* strain 24067 grown in minimal medium with (A) L-dopa, (B) methyl-dopa, (C) epinephrine, and (D) norepinephrine as seen by light microscopy. Original magnification, 100× scale bar, 5 μm.

Since the color analysis suggested differences in the production of melanin from each substrate, we subjected the products to further analysis. We tested the efficiency of incorporation into pigment by each of the substrates by measuring the weight of the recovered acid-resistant pigment, which we tentatively assumed, was a melanin-like substance based on insolubility, acid resistance and color. Since the molecular weight of polymerized melanin is unknown, we expressed synthetic yields of this pigment on a mass percentage basis relative to the mass of the precursor. The amount of particulate material recovered from cells varied with the substrate used. A yield of 60.7% was obtained for methyl-dopa, 46.6% for L-dopa, 26.5% for norepinephrine, and 1.8% for epinephrine (Table 1). The differences in yield were not the result of growth differences in the various substrates since growth rates were similar in all media as determined by colony forming units (data not shown).

Particles made from the different substrates were sectioned and viewed by transmission electron microscopy (Fig. 3). As observed previously for L-dopa generated ghosts, the particles recovered from L-dopa, methyl-dopa, and norepinephrine pigmented cells had a multi-layered structure (Eisenman et al., 2005). However, the microscopic

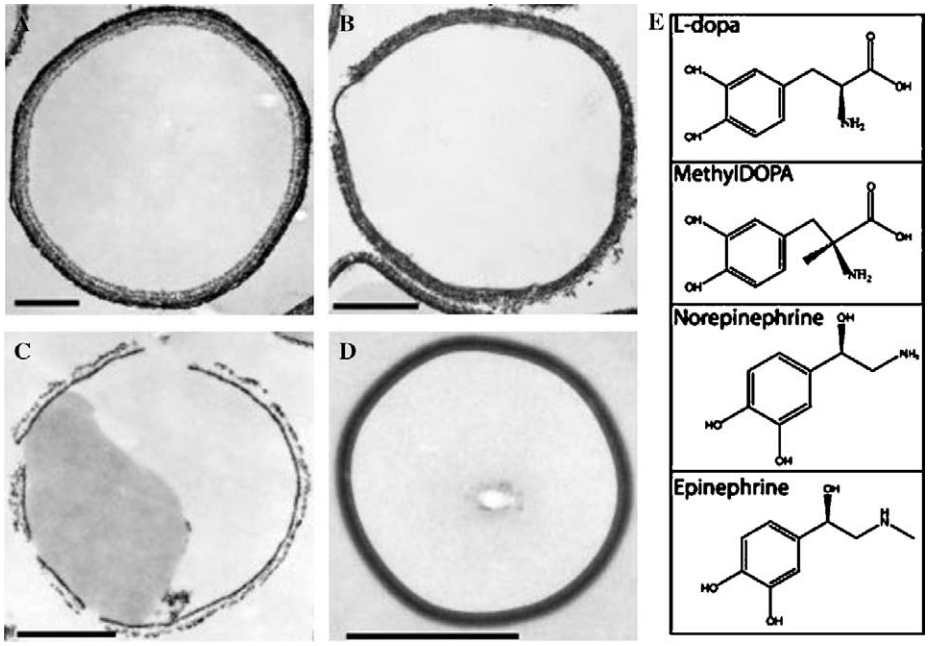

Fig. 3. Transmission electron micrographs of representative acid-resistant particles derived from (A) L-dopa, (B) methyl-dopa, (C) norepinephrine or (D) epinephrine, scale bars, 1 μm, and (E) chemical structure of each substrate.

analysis also revealed structural differences in the particles. Ghost particles derived from L-dopa or methyl-dopa had dimensions similar to their progenitor cells. However, ghosts derived from norepinephrine appeared fragmentary and often had large spaces, possibly indicating that they are less able to withstand the TEM sample preparation. Very few epinephrine-derived ghosts were detected by TEM and they appeared smoother compared to the texture of particles made from other substrates. The thickness of the ghost shell was measured for each substrate and the average thickness was determined (Table 1). L-Dopa-derived melanin ghosts had significantly thicker shells than other substrates ($P < 0.05$). Methyl-dopa-derived particles exhibited an intermediate thickness, significantly different from L-dopa and norepinephrine ($P < 0.05$). Norepinephrine-derived particles were significantly thinner than both L-dopa and methyl-dopa ghosts ($P < 0.05$). The average thickness of the epinephrine ghosts was similar to that of norepinephrine.

All the particles generated from L-dopa-, norepinephrine-, and methyl-dopa-pigmented cells bound the melanin binding mAb 6D2, unlike most particles derived from epinephrine (Fig. 4). No fluorescence was observed with the isotype matched negative IgM negative control mAb 5C11.

3.2. Pigments produced from different substrates have different biochemical properties

A pigment can be defined as melanin based on ESR spectral characteristics (Enochs et al., 1993). Since only the L-dopa derived pigment has been shown to be a melanin, we evaluated the particles derived from each of the different substrates by ESR. The acid resistant particles derived form L-dopa, methyl-dopa, and norepinephrine produced a signal indicative of a stable free-radical population (Fig. 5). In contrast, the particles derived from cells grown in epinephrine did not have a signal characteristic of a stable free radical population, such as that found in melanins. On the basis of the spectroscopic findings, we identify the particles from L-dopa, methyl-dopa, and norepinephrine as melanin-like ghosts.

Particles from pigmented cells generated with the various substrates were oxidized with acidic permanganate and the amount of PTCA, PDCA, TTCA, and TDCA was determined by HPLC. Chromatograms of PDCA, TDCA, TTCA, and PTCA standards yielded peaks at 5.1, 6.1, 7.1, and 11.0 min, respectively (results not shown). The chromatograms of oxidized particles generated from cells grown with L-dopa, methyl-dopa, and norepinephrine ghosts revealed only a PTCA peak at

Table 1
Generation of melanin using different catecholamines as substrate

Substrates	1.0 mM (g)	Particle amount (g)	% of yield	Thickness of "ghost" shell (nm)[a]
L-Dopa	0.1973	0.092	46.6	281 ± 109
Methyl-dopa	0.2382	0.1447	60.7	169 ± 68
Epinephrine	0.1832	0.00334	1.8	101 ± 57
Norepinephrine	0.1692	0.0448	26.5	110 ± 6.9

[a] Values are mean thicknesses ± SD.

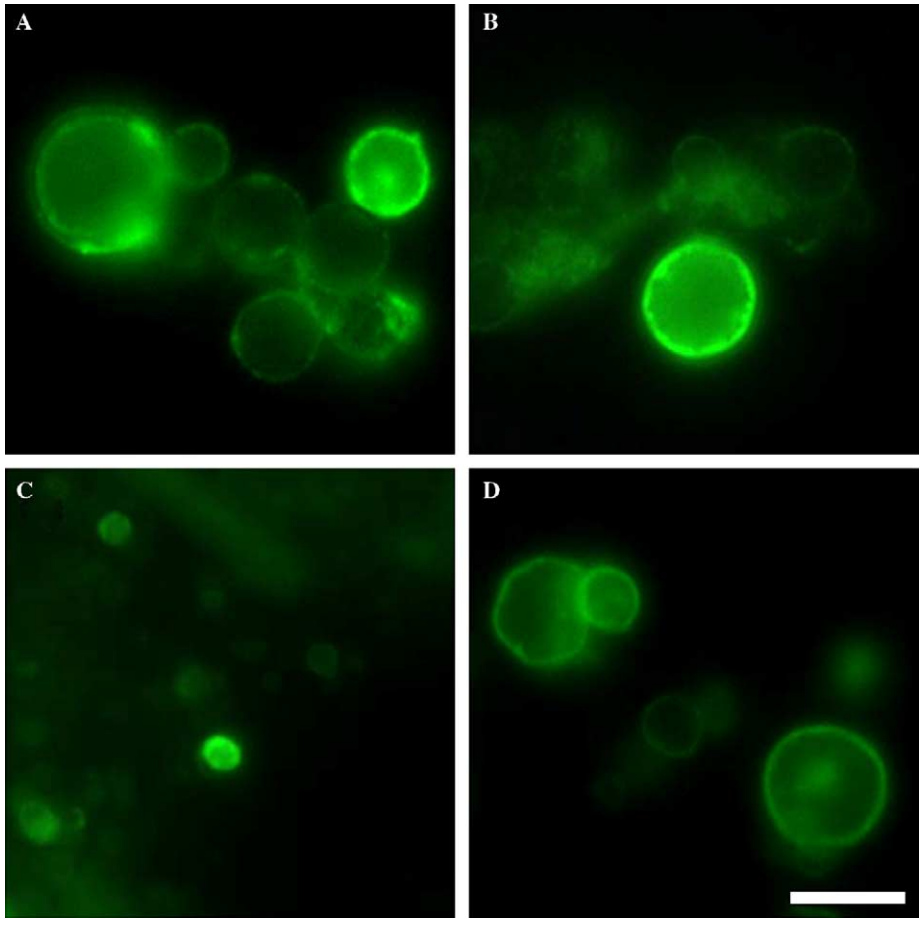

Fig. 4. Immunoreactivity of acid-resistant particles isolated from *C. neoformans* strain 24067 grown in minimal medium with (A) L-dopa, (B) methyl-dopa, (C) epinephrine, and (D) norepinephrine with mAb 6D2 as seen by immunofluorescence.

11.0 (Figs. 6B–D). The absence of TDCA/TTCA content in these melanins is consistent with very low levels of aminohydroxyphenylalanine in *C. neoformans*, which is a specific indicator of cysteinyl-dopa (Liu et al., 1999). The chromatogram of particles generated from pigmented cells grown with epinephrine revealed a very small peak at 11.0 min which can be assigned to PTCA and no detectable TDCA or TTCA (Fig. 6E). The absence of pheomelanin-related peaks in the oxidation products of particles from cells grown with epinephrine indicates that the brown color is not due to presence of pheomelanin.

Zeta potential reflects the charge of particles and is an important characteristic dispersion of particles in suspension. The zeta potentials of *C. neoformans* 24067 (−39.62 mV) and CAP67 (−14.53 mV) grown in norepinephrine were significantly more negative than L-dopa, methyl-dopa and epinephrine ($P < 0.001$) (Table 2). The polysaccharide capsule was responsible for the majority of the negative charge associated with strain 24067. Epinephrine-derived particles had significantly less negative zeta potential than those derived from the other substrates.

3.3. Effect of melanization substrate on cell growth characteristics

The effects of the different substrates on cell size were determined (Table 3). Since the polysaccharide capsule contributes substantially to total cell size, we measured the diameter of both the yeast cell size and the total cell size under conditions that do not induce large capsules. Cells grown in L-dopa were significantly larger with relatively thinner capsules compared to cells grown with other substrates. In addition, cells grown in norepinephrine had significantly smaller radii than the other cells due to reduced capsule thickness.

Since melanin ghosts are rigid, and there is evidence that some components of the capsule are synthesized in the cytoplasm and are transported to the extracellular space (Garcia-Rivera et al., 2004), we investigated whether melanization interfered with capsule growth and induction. It has been reported that capsule growth is an heterogeneous phenomenon (Feldmesser et al., 2001; Zaragoza et al., 2003). For this reason, we used two different strains for this experiment, H99 and 24067. H99 and 24067 yeast strains were grown in minimal medium supplemented with

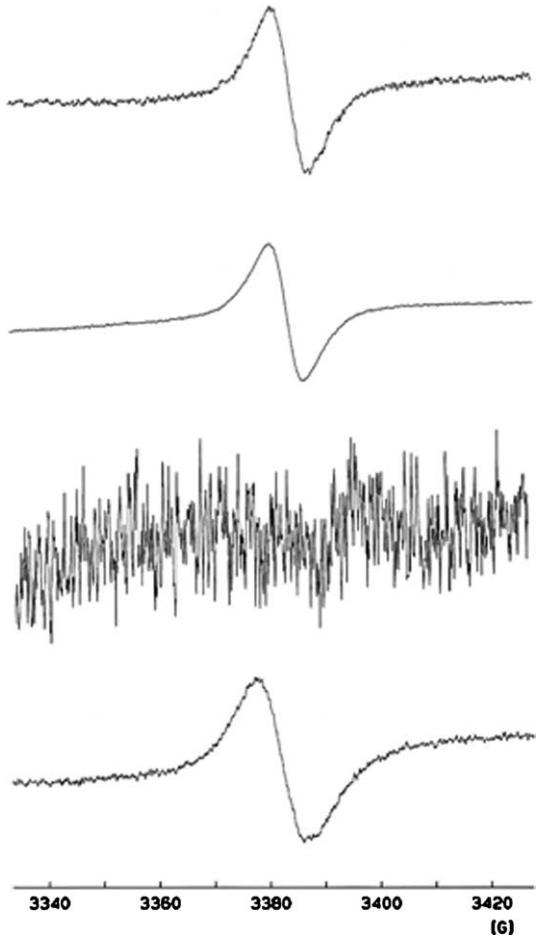

Fig. 5. Electron spin resonance spectra acid-resistant particles isolated from *C. neoformans* strain 24067 grown in minimal medium with (A) L-dopa, (B) methyl-dopa, (C) epinephrine, and (D) norepinephrine. Note that the epinephrine spectrum (C) was recorded at maximum gain in an effort to identify a signal and hence has more background noise than with the others.

L-dopa, epinephrine, norepinephrine, or methyl-dopa for 11 days. In contrast to the above experiments, cells were melanized for a shorter time to avoid negative results due to loss of viability or metabolic activity required for capsule growth. Capsule measurements were made and compared to non-pigmented controls. After pigmentation of the cells, we observed different relative capsule sizes according to the melanization substrate and *C. neoformans* strains used. In strain H99, non-pigmented cells had larger capsules than pigmented cells ($P < 0.01$ for all substrates when compared to minimal medium). In contrast, in 24067, cells pigmented in the presence of norepinephine, methyl-dopa or L-dopa had larger capsules than cells grown in minimal medium ($P < 0.01$ when these substrates were compared to minimal medium). However, when pigmented and non-pigmented cells were placed in capsule inducing medium (Zaragoza and Casadevall, 2004), both types of cells (pigmented and non-pigmented) induced capsule growth (Fig. 7). For strain H99, no significant differences were found when capsules were induced in cells grown in L-dopa or norepinephrine when compared to minimal medium ($P > 0.05$ compared to minimal medium). Slight, but significant differences were observed for epinephrine and methyl-dopa compared to minimal medium ($P < 0.01$ for epinephrine and methyl-dopa compared to minimal medium). For strain 24067, no significant differences were found when capsules were induced in cells grown in the various substrates when compared to minimal medium ($P > 0.05$ for all substrates compared to minimal medium). Overall, these data indicate that pigmentation does not interfere with capsule induction.

4. Discussion

In this study, we analyzed the effect of substrate on pigment production by *C. neoformans* as well as on general growth characteristics of the organism. A goal of this study was to determine whether the pigments made with substrates other than L-dopa are melanin-like. The color of the ghosts pellet (data not shown), cells suspended in PBS, and the cell supernatant varied depending on the substrate used for pigment production (Fig. 1). L-Dopa and methyl-dopa produced black cells and ghosts, whereas epinephrine and norepinephrine produced lighter pigmentation with brown cells and ghosts. Culture supernatants from medium containing epinephrine and norepinephrine substrate were heavily colored, consistent with the accumulation of soluble pigments. Differences in ghost color reflect differences in light wavelength absorption that imply differences in molecular structure. This can be attributed to the particular way the substrates are polymerized to form pigments and the displacement of electrons in the phenol groups. *o*-Diphenols with hydroxyl groups in the 2,3- or 3,4-position produce cell associated pigments whereas *p*-diphenols (1,4- or 2,5-hydroxyl groups) produce large amounts of soluble pigments (Chaskes and Tyndall, 1975). In this regard, L-dopa and methyl-dopa are 3,4-diphenols, whereas epinephrine and norepinephrine are 2,3-diphenols. However, we observed differences in polymerization of these substrates into yeast-associated particles.

The highest yields of pigmented cell-like particles (ghosts) on a weight basis were observed with methyl-dopa, followed by L-dopa which form cell-wall-associated pigments. For epinephrine and norepinephrine, the yields were 26 and 1%, respectively, consistent with diffusion of certain products into the culture supernatants. On the basis of acid resistance, insolubility, pigmentation, and origin from phenolic precursors, we tentatively concluded that these particles were melanin ghosts that were derived from melanized cells. The melanin layer was thinner for epinephrine and norepinephrine ghosts compared to methyl-dopa and L-dopa. One explanation for these findings is that for epinephrine most of the substrate is oxidized or autopolymerized in the supernatant. Alternatively, oxidized products may fail to polymerize in the cell wall. Another factor that could have contributed to polymerization of these substrates in the supernatant is secreted laccase (Garcia-Rivera et al., 2004). In this regard,

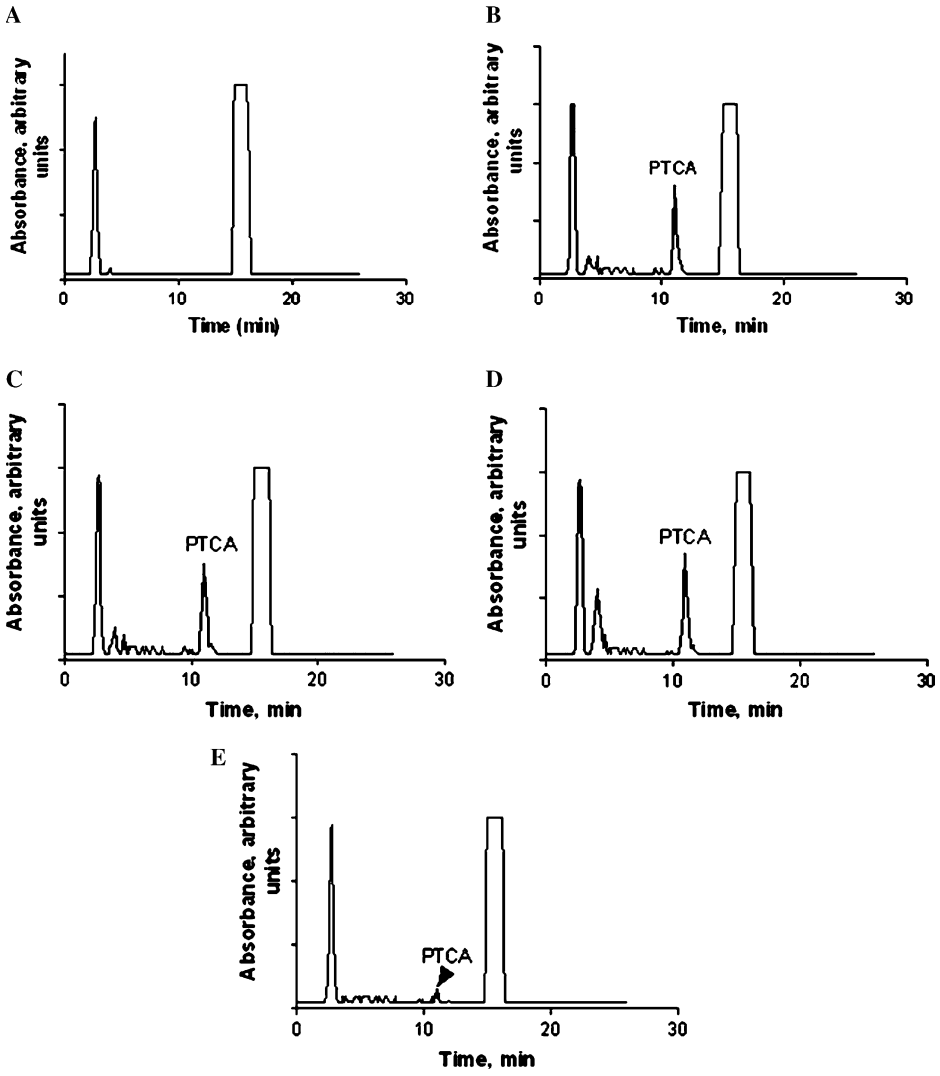

Fig. 6. HPLC of permanganate-oxidized pigmented acid resistant particles. (A) Background solution, (B) dopa, (C) methyl-dopa, (D) norepinephrine, and (E) epinephrine. The oxidation products were analyzed by HPLC using a C_{18} column. The mobile phase was 0.1% v/v trifluoroacetic acid in water (solvent A) and 0.1% v/v trifluoroacetic acid in acetonitrile (solvent B). At 1.0 mL/min, the elution gradient was (min, %B): 0, 0; 1, 0; 12, 25; 14, 25; and 16, 0. The UV detector was set at a 255 nm absorbance.

laccase has higher activity in catalyzing the oxidation of epinephrine and norepinephrine than with L-dopa and methyl-dopa (Polacheck et al., 1982).

Table 2
Zeta Potential measurement *C. neoformans* 24067 and CAP67 grown in different catecholamines as substrates

Medium conditions	Zeta potential (mV)		
	24067	CAP67	"Ghost" particles
Minimal medium	-32.21 ± 16.93	-6.14 ± 4.94	N/A
L-Dopa	-31.64 ± 8.17	-6.73 ± 5.55	-26.97 ± 9.08
Methyl-dopa	-30.95 ± 8.23	-6.90 ± 5.05	-29.80 ± 3.08
Epinephrine	-29.32 ± 10.73	-6.96 ± 5.41	$-19.02 \pm 13.92^{***}$
Norepinephrine	$-39.62 \pm 3.71^{*}$	$-14.53 \pm 6.06^{**}$	-35.87 ± 6.56

Values are means ± SD. N/A, not applicable.

* $P < 0.001$ significantly higher compared to L-dopa, methyl-dopa and epinephrine.

** $P < 0.001$ significantly higher compared to minimal medium and $P < 0.01$ compared to L-dopa, methyl-dopa and epinephrine.

*** $P < 0.001$ significantly lower value compared to methyl-dopa and norepinephrine and $P < 0.05$ compared to L-dopa.

The zeta potential of melanized cells generated with the various substrates was measured to gain insight into the effects of different substrates on cell charge. A prior study using a different instrument had documented that melanization with L-dopa resulted in an increase in the zeta potential of *C. neoformans* cells (Nosanchuk and Casadevall, 1997). Since capsule makes a dominant contribution to the large zeta potential of *C. neoformans*, we measured the effect of different substrates on the charge of acapsular cells. As expected, all cells were negatively charged and significant increases in the charge of melanized cells relative to non-melanized cells were observed only for cells grown with L-dopa and methyl-dopa. This is consistent with the results that these substrates are more efficiently incorporated into melanin.

Consistent with the findings of our prior study, most of the large negative charge of the encapsulated cryptococcal cells was generated primarily by the polysaccharide capsule, with melanin making a relatively small contribution to the

Table 3
Measurements of cell size, cell body, percentage of capsule, and capsule thickness from *C. neoformans* cells grown in different catecholamines as substrates

Medium conditions	Cell body radius (μm)	Radius of cells with capsule (μm)	% of capsule	Capsule thickness (μm)
Minimal medium	3.52 ± 0.58	8.93 ± 1.91	59.8 ± 5.6	5.41 ± 1.51
L-Dopa	4.37 ± 0.69*	9.08 ± 1.28	51.5 ± 7.1***	4.71 ± 1.08
Methyl-dopa	3.77 ± 0.59	9.47 ± 1.58	59.6 ± 6.5	5.70 ± 1.39
Epinephrine	3.69 ± 0.60	8.51 ± 1.31	56.2 ± 6.5	4.82 ± 1.12
Norepinephrine	3.43 ± 0.57	7.60 ± 0.83**	54.9 ± 5.7	4.18 ± 0.62†

Values are means ± SD.
* $P < 0.001$ cell body radius is bigger when compared to the other conditions.
** $P < 0.001$ radius of cells including capsule is smaller when compared to the other conditions.
*** $P < 0.001$ percent of capsule is smaller when compared with minimal medium, methyl-dopa and epinephrine.
† $P < 0.001$ capsule thickness in smaller when compared to minimal medium, methyl-dopa and epinephrine and $P < 0.01$ compared to L-dopa.

total charge of the encapsulated cells. The zeta potential values measured in this study were significantly more negative than measured in our previous study using a different instrument. For example, our prior study reported that the charge of melanized and non-melanized strain 24067 cells were −27.5 and −25.4 mV, respectively (Nosanchuk and Casadevall, 1997). In contrast, the zeta potentials measured in this study for these cells were −32.2 and −31.6 mV, respectively. The differences likely represent different experimental conditions as well as variations in cell populations and/or the use of a different instrument and were performed

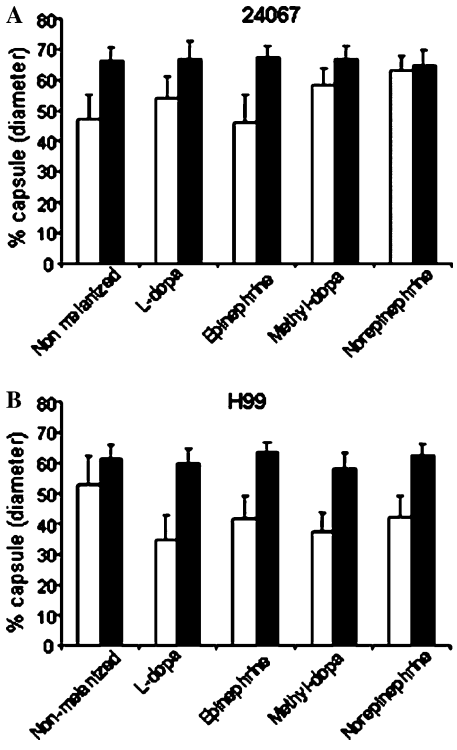

Fig. 7. Cells from (A) 24067 and (B) H99 strains were grown in minimal medium in the absence or presence of L-dopa, epinephrine, methyl-dopa, and norepinephrine for 11 days at 30 °C. Then, the cells were placed in capsule growth inducing medium as described in Materials and methods, and capsule size was measured before (open bars) and after (close bars) capsule size induction. At least 50 cells were measured in each case, and the mean value and standard deviation are shown. Statistical differences were calculated by *t* test.

with a lower concentration of yeast cells and in 10 mM NaCl rather than 1 mM KCl. For example, the capsule thickness previously reported for nonmelanized and melanized cells was smaller than what we observed (Nosanchuk and Casadevall, 1997). Despite this variation in the magnitude of the particle zeta potential, the results are consistent in the sense that both measured a significantly higher negative charge for encapsulated cells and the finding that melanization affected zeta potential.

Reactivity of the mAb 6D2 was observed with each of the ghosts derived from the various substrates. Nonetheless, significantly lower reactivity was observed with epinephrine derived particles, which suggest that the epitope recognized by mAb 6D2 is reduced. Also, ghosts isolated from epinephrine had a different appearance compared to the other substrates when viewed by light or electron microscopy. The differences in properties of epinephrine ghosts are more pronounced when the ESR results are considered. Out of the four substrates, only epinephrine lacked a spectrum characteristic of melanin. Furthermore, the absence of both eumelanin and pheomelanin oxidation products in the chromatogram of epinephrine ghosts combined with lack of ESR signal points to the conclusion that epinephrine is not sufficient for the production of melanin. This result is relevant to assays for laccase activity based on epinephrine oxidation (Rhodes, 1986). Furthermore, it suggests that epinephrine is not an ideal substrate for studies of *C. neoformans* melanization and implies the need for caution, when concluding that dark pigments in *C. neoformans* are melanins.

Measurements of cells grown under different substrate conditions revealed that cell and capsule size could be affected by the substrates used for melanization. Although the mechanisms responsible for substrate effects on cell and capsule size were not elucidated, we hypothesize that chemical differences in the melanin deposited in the cell wall could impose certain physical constrains that translate into cell body and capsule size differences. Given that capsule components are synthesized in the cytoplasm and must be exported through the cell wall, one might expect that melanization and the type of melanin could affect both cell size and capsule size.

This study provides the basis for further characterization of melanin particles. It is conceivable to generate melanin

ghosts with properties of different substrates by growing the strain in culture with two or more substrates. Such approaches may contribute to understanding the structure of melanin produced by *C. neoformans* in vivo, where the substrate(s) for melanization are not known. Presumably, multiple catecholamines are present in tissues that can be used as substrates for melanization. These studies will be relevant to understanding the pathogenesis of *C. neoformans*.

Acknowledgments

J. Garcia-Rivera is supported by the Ruth L. Kirschstein National Research Service Award GM64100. A. Casadevall and J. Nosanchuk are supported by the National Institutes of Health Awards AI52733. E. Dadachova is supported by NIH Grant AI060507. H. Eisenman is supported by NIH training Grant T32CA09173. The data presented in this paper are from a thesis to be submitted by Javier Garcia-Rivera in partial fulfillment of the requirements for the degree of doctor of philosophy in the Sue Golding Graduate Division of Medical Science, Albert Einstein College of Medicine, Yeshiva University, Bronx, NY, USA.

References

Brush, L., Money, N.P., 1999. Invasive hyphal growth in *Wangiella dermatitidis* is induced by stab inoculation and shows dependence upon melanin biosynthesis. Fungal Genet. Biol. 28, 190–200.

Buchanan, K.L., Murphy, J.W., 1998. What makes *Cryptococcus neoformans* a pathogen? Emerg. Infect. Dis. 4, 71–83.

Casadevall, A., Rosas, A.L., Nosanchuk, J.D., 2000. Melanin and virulence in *Cryptococcus neoformans*. Curr. Opin. Microbiol. 3, 354–358.

Chang, Y.C., Kwon-Chung, K.J., 1994. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. Mol. Cell. Biol. 14, 4912–4919.

Chaskes, S., Tyndall, R.L., 1975. Pigment production by *Cryptococcus neoformans* from *para*-and *ortho*-Diphenols: effect of the nitrogen source. J. Clin. Microbiol. 1, 509–514.

Eisenman, H.C., Nosanchuk, J.D., Webber, J.B., Emerson, R.J., Camesano, T.A., Casadevall, A., 2005. Microstructure of cell wall-associated melanin in the human pathogenic fungus *Cryptococcus neoformans*. Biochemistry 44, 3683–3693.

Enochs, W.S., Nilges, M.J., Swartz, H.M., 1993. A standardized test for the identification and characterization of melanins using electron paramagnetic resonance (EPR) spectroscopy. Pigment Cell Res. 6, 91–99.

Feldmesser, M., Kress, Y., Casadevall, A., 2001. Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. Microbiology 147, 2355–2365.

Garcia-Rivera, J., Casadevall, A., 2001. Melanization of *Cryptococcus neoformans* reduces its susceptibility to the antimicrobial effects of silver nitrate. Med. Mycol. 39, 353–357.

Garcia-Rivera, J., Chang, Y.C., Kwon-Chung, K.J., Casadevall, A., 2004. *Cryptococcus neoformans* CAP59 (or Cap59p) is involved in the extracellular trafficking of capsular glucuronoxylomannan. Eukaryot. Cell 3, 385–392.

Glatman-Freedman, A., Martin, J.M., Riska, P.F., Bloom, B.R., Casadevall, A., 1996. Monoclonal antibodies to surface antigens of *Mycobacterium tuberculosis* and their use in a modified enzyme-linked immunosorbent spot assay for detection of mycobacteria. J. Clin. Microbiol. 34, 2795–2802.

Hill, H.Z., 1992. The function of melanin or six blind people examine an elephant. Bioessays 14, 49–56.

Ito, S., Fujita, K., 1985. Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. Anal. Biochem. 144, 527–536.

Jacobson, E.S., 2000. Pathogenic roles for fungal melanins. Clin. Microbiol. Rev. 13, 708–717.

Knorle, R., Schniz, E., Feuerstein, T.J., 1998. Drug accumulation in melanin: an affinity chromatographic study. J. Chromatogr. B Biomed. Sci. Appl. 714, 171–179.

Leonowicz, A., Cho, N.S., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D., Rogalski, J., 2001. Fungal laccase: properties and activity on lignin. J. Basic Microbiol. 41, 185–227.

Liu, L., Wakamatsu, K., Ito, S., Williamson, P.R., 1999. Catecholamine oxidative products, but not melanin, are produced by *Cryptococcus neoformans* during neuropathogenesis in mice. Infect. Immun. 67, 108–112.

McFadden, D.C., Casadevall, A., 2001. Capsule and melanin synthesis in *Cryptococcus neoformans*. Med. Mycol. 39, 19–30.

Money, N.P., Caesar-TonThat, T.C., Frederick, B., Henson, J.M., 1998. Melanin synthesis is associated with changes in hyphopodial turgor, permeability, and wall rigidity in *Gaeumannomyces graminis var. graminis*. Fungal Genet. Biol. 24, 240–251.

Nosanchuk, J.D., Casadevall, A., 1997. Cellular charge of *Cryptococcus neoformans*: contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. Infect. Immun. 65, 1836–1841.

Nosanchuk, J.D., Rosas, A.L., Lee, S.C., Casadevall, A., 2000. Melanisation of *Cryptococcus neoformans* in human brain tissue. Lancet 355, 2049–2050.

Nosanchuk, J.D., Casadevall, A., 2003a. Budding of melanized *Cryptococcus neoformans* in the presence or absence of L-dopa. Microbiology 149, 1945–1951.

Nosanchuk, J.D., Casadevall, A., 2003b. The contribution of melanin to microbial pathogenesis. Cell Microbiol. 5, 203–223.

Perfect, J.R., Ketabchi, N., Cox, G.M., Ingram, C.W., Beiser, C.L., 1993. Karyotyping of *Cryptococcus neoformans* as an epidemiological tool. J. Clin. Microbiol. 31, 3305–3309.

Polacheck, I., Hearing, V.J., Kwon-Chung, K.J., 1982. Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. J. Bacteriol. 150, 1212–1220.

Prota, G., 1988. Progress in the chemistry of melanins and related metabolites. Med. Res. Rev. 8, 525–556.

Rhodes, J.C., 1986. A simplified assay for cryptococcal phenoloxidase. Mycologia 78, 867–868.

Rosas, A.L., Nosanchuk, J.D., Feldmesser, M., Cox, G.M., McDade, H.C., Casadevall, A., 2000a. Synthesis of polymerized melanin by *Cryptococcus neoformans* in infected rodents. Infect. Immun. 68, 2845–2853.

Rosas, A.L., Nosanchuk, J.D., Gomez, B.L., Edens, W.A., Henson, J.M., Casadevall, A., 2000b. Isolation and serological analyses of fungal melanins. J. Immunol. Methods 244, 69–80.

Tian, S., Garcia-Rivera, J., Yan, B., Casadevall, A., Stark, R.E., 2003. Unlocking the molecular structure of fungal melanin using ^{13}C biosynthetic labeling and solid-state NMR. Biochemistry 42, 8105–8109.

Vecchiarelli, A., Pietrella, D., Dottorini, M., Monari, C., Retini, C., Todisco, T., Bistoni, F., 1994. Encapsulation of *Cryptococcus neoformans* regulates fungicidal activity and the antigen presentation process in human alveolar macrophages. Clin. Exp. Immunol. 98, 217–223.

Wakamatsu, K., Ito, S., 2002. Advanced chemical methods in melanin determination. Pigment Cell Res. 15, 174–183.

Wang, Y., Casadevall, A., 1994a. Decreased susceptibility of melanized *Cryptococcus neoformans* to UV light. Appl. Environ. Microbiol. 60, 3864–3866.

Wang, Y., Casadevall, A., 1994b. Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. Infect. Immun. 62, 3004–3007.

Wang, Y., Aisen, P., Casadevall, A., 1995. *Cryptococcus neoformans* melanin and virulence: mechanism of action. Infect. Immun. 63, 3131–3136.

Zaragoza, O., Fries, B.C., Casadevall, A., 2003. Induction of capsule growth in *Cryptococcus neoformans* by mammalian serum and CO_2. Infect. Immun. 71, 6155–6164.

Zaragoza, O., Casadevall, A., 2004. Experimental modulation of capsule size in *Cryptococcus neoformans*. Biol. Proced. Online 6, 10–15.