Melanization of Cryptococcus neoformans reduces its susceptibility to the antimicrobial effects of silver nitrate*

J. GARCÍA-RIVERA* & A. CASADEVALL*,†
*Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA; †Division of Infectious Diseases of the Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

Cryptococcus neoformans is a human pathogenic fungus that is frequently found in avian feces and Eucalyptus trees. There is evidence that C. neoformans can make melanin-like pigment in pigeon excreta, a major natural environmental niche. Silver nitrate, AgNO₃, is a highly toxic compound for bacteria and fungi. In this study we investigated the effects of melanin production by C. neoformans on the susceptibility of this fungus to AgNO₃. C. neoformans was grown in media with and without the melanin precursor, l-dopa, for various times and susceptibility to AgNO₃ was determined by measuring percentage of survival after incubation in AgNO₃. There was an inverse association between time allowed for melanization and susceptibility to Ag⁺. Addition of melanin particles to a suspension of non-melanized C. neoformans cells reduced their susceptibility to AgNO₃, consistent with metal ion chelation by melanin. Binding of Ag⁺ to melanin particles was demonstrated by atomic absorption spectroscopy. The results indicate that melanization of C. neoformans reduces susceptibility to a toxic heavy metal. This suggests a role for melanin in environmental protection against heavy metal toxicity.

Keywords antimicrobial, Cryptococcus neoformans, melanin, silver nitrate

Introduction

Cryptococcus neoformans is a yeast that is frequently found in debris around pigeon roosts and in soil contaminated with decaying pigeon excreta [1]. It is a cause of life threatening infection primarily in immunocompromised hosts [2]. The incidence of cryptococcal infection in patients with acquired immune deficiency syndrome (AIDS) is estimated to be 6–10% in the USA and western Europe [2]. Among its virulence factors, C. neoformans has a laccase enzyme that catalyzes the synthesis of melanin-like pigments from various substrates, including catecholamines [3,4], aminophenols and diaminobenzene compounds [5]. In vitro studies of melanization in C. neoformans have demonstrated protection against environmental stressors such as extreme temperature [6], UV light [7], and nitrogen- and oxygen-related oxidants [8,9], as well as against host defenses such as antimicrobial peptides [10], and phagocytosis by macrophages [11]. C. neoformans can also produce melanin-like compounds in infected tissues [12–14]. These observations have led to the view that melanin plays a role in the protection of the microorganism [15].

The function of the C. neoformans laccase in the environment is unknown. In other microorganisms, laccase activity has been associated with lignolytic enzymes and degradation of wood [16,17]. C. neofo-
C. neoformans has recently been shown to be melanized in pigeon excreta, suggesting that melanin may have a role in protecting this fungus against various insults in the environment [18]. Melanins remain poorly understood polymers [19]. L-dopa-derived melanins are believed to be heteropolymers generated from various products of L-dopa oxidation [20]. Melanins are difficult to study because they are insoluble in acid, water, aqueous acids and common organic solvents [21]. They are known to have high affinity for a variety of metals that are potentially toxic to fungi including copper, calcium, magnesium and zinc [21]. Melanins have abundant carboxyl, phenolic, hydroxyl and amine groups that provide many potential binding sites for metal ions [21]. In this study we investigated the hypothesis that melanization of C. neoformans could protect this fungus against the antimicrobial effects of silver nitrate, AgNO$_3$. Silver was selected for study because it is the most toxic heavy metal for microorganisms [22, 23]. Binding of Ag$^+$ by melanin could prevent its internalization and subsequent reactions with internal molecules. The results indicate that melanization of C. neoformans is associated with reduced susceptibility to Ag$^+$, suggesting a potential role for melanin in protection against heavy metal toxicity in the environment.

Materials and methods

Cryptococcus neoformans

C. neoformans strain ATCC 24067 was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in a defined minimal medium (15 mM glucose [Sigma Chemical Co., St. Louis, MO, USA], 10 mM MgSO$_4$ [Sigma], 29.4 mM KH$_2$PO$_4$ [Fisher Scientific, Fairlawn, NJ, USA], 13 mM glycine [Sigma], 3 mM thiamine [Sigma]), pH 5.5, with and without 1-0 mM L-dopa (Sigma). All cultures were incubated at 30 °C in a rotary shaker at 130 rpm.

Silver toxicity experiments

C. neoformans (ATCC 24067) was grown in minimal medium overnight. An aliquot of 1 ml was used to inoculate 49 ml of minimal medium with and without 1-0 mM L-dopa. Cells grown in minimal medium with and without L-dopa were studied at 2, 5, 8 and 13 days of culture growth. Aliquots of 50 µl (10$^5$ cells) were added to a polystyrene 96-well enzyme-linked immunosorbent assay (ELISA) plate (Corning Glass Works, Corning, NY, USA) containing AgNO$_3$ (Sigma) solutions such that the final concentration of AgNO$_3$ was 1 × 10$^{-6}$ M or 5 × 10$^{-9}$ M. Cells were incubated for 1 h at room temperature. After incubation, cells were diluted in distilled water and plated on Sabouraud glucose agar (Becton Dickinson, Spark, MD, USA). Colonies were counted after incubation for 48 h at 30 °C to determine cfu (1 colony = 1 cfu). Results were expressed by averaging the cfu counts on five plates for each concentration. Data were expressed as percent of survival relative to non-exposed cells. Results are reported as means ± standard deviation of five repetitions from one experiment. Each experiment was performed twice.

Melanin protection against Ag$^+$

Melanin particles (ghosts) were isolated from melanized C. neoformans as described [24]. Briefly, C. neoformans cells were grown in defined minimal medium for 13 days with L-dopa to induce melanization. Cells were harvested and washed in 1-0 M sorbitol–0-1 M sodium citrate (pH 5.0). Glucanase (IntraSpex, San Mateo, CA, USA) was added at 30 mg ml$^{-1}$, and the suspension was incubated for 1 h at 30 °C. Cells were collected and suspended in 4-0 guanidinium isothiocyanate for 30 min at room temperature with frequent vortexing. The resulting suspension was then collected by centrifugation and suspended in 6-0 M HCl at 100 °C for 1 h. This procedure yields melanin particles, dissolving non-melanized cells completely. The resulting melanin particles were then dialyzed against dH$_2$O for 10 days to remove traces of HCl. Silver nitrate solutions (2 × 10$^{-8}$ or 1 × 10$^{-8}$ M) were incubated with ~3 × 10$^8$ melanin particles for 1 h at room temperature. An aliquot of 50 µl containing ~10$^8$ non-melanized cells was added to solutions of AgNO$_3$ (1 × 10$^{-5}$–5 × 10$^{-9}$ M) that had been incubated with and without melanin. The suspensions were then incubated at room temperature for 1 h. The cells were diluted and plated on Sabouraud agar plates to determine the cfu. This experiment was performed twice with five repetitions at each concentration. Results are reported as means ± standard deviations. Statistical analysis was performed using the Kruskal-Wallis test as provided by Primer of Biostatistics: The Program, Version 3.01 [25].

Atomic absorption analysis

Melanin ghosts (22–28 mg) were lyophilized and suspended in 1 × 10$^{-7}$, 1 × 10$^{-8}$ or 5 × 10$^{-9}$ M AgNO$_3$. After 1 h of incubation at room temperature, they were centrifuged and washed three times with distilled water. Bound Ag$^+$ was determined by atomic absorption at Quantitative Technologies Inc. (Whitehouse, NJ, USA).
Results are reported as microgram of Ag$^+$ per gram of melanin.

**Results**

In preliminary studies, melanized cells incubated with AgNO$_3$ in minimal medium for 24 h were less susceptible than non-melanized cells at Ag$^+$ concentrations ranging from $1 \times 10^{-5}$ M to $2.5 \times 10^{-7}$ M (data not shown). Furthermore, time killing experiments showed that 75% of the non-melanized cells were killed compared to only 49% of melanized cells after incubation in $1 \times 10^{-7}$ M AgNO$_3$ for 20 min (data not shown). In experiments in which *C. neoformans* cells were suspended in distilled water and exposed to AgNO$_3$ for 1 h, no survival was observed above $1 \times 10^{-6}$ M AgNO$_3$, whereas at concentrations below $1 \times 10^{-9}$ M more than 90% survival was observed (data not shown). Based on these results, we selected AgNO$_3$ concentrations of $1 \times 10^{-8}$ and $5 \times 10^{-9}$ M to study the susceptibility of non-melanized and melanized *C. neoformans*.

*C. neoformans* grown with and without L-dopa for various intervals was incubated in a series of concentrations of AgNO$_3$ and the percentage of survival was determined. Two- and 5-day-old cultures (in which cells were not yet heavily melanized) grown with and without L-dopa had comparable susceptibility to Ag$^+$. Early in the course of culture growth there was a consistent trend toward greater susceptibility to Ag$^+$ for the cells grown in L-dopa. Although the effect was small, it was reproducible (Fig. 1). The mechanism of this effect is unknown, but we speculate that Ag$^+$ toxicity could be potentiated by L-dopa polymerization intermediates. In our experimental conditions, melanization was visible after 5–6 days of growth in media with L-dopa. Cells from 8- and 13-day-old cultures grown with L-dopa were significantly less susceptible to Ag$^+$ than those grown without L-dopa (Fig. 1).

We hypothesized that the lower toxicity of Ag$^+$ for melanized cells was a result of silver chelation by melanin. To test this, we mixed AgNO$_3$ with $\sim 3 \times 10^5$ melanin particles for 1 h and then added this solution to non-melanized *C. neoformans*. Cells incubated with AgNO$_3$ pre-absorbed with melanin particles had higher survival than cells incubated with AgNO$_3$ alone. Addition of melanin particles to $1 \times 10^{-8}$ M AgNO$_3$ reduced toxicity for *C. neoformans* by more than 13-fold (Fig. 2). Moreover, Ag$^+$ binding to melanin ghosts was demonstrated by atomic absorption spectroscopy (Table 1). Incubation of 22.6 mg of melanin particles in a 0.001 M AgNO$_3$ solution reduced the amount of silver in solution by 98.8% with a concomitant increase in the percent silver content of melanin particles (data not shown).
Table 1  $\text{Ag}^+$ binding by melanin particles isolated from *Cryptococcus neoformans*

<table>
<thead>
<tr>
<th>$\text{[AgNO}_3\text{]}$ $\text{m}^+$</th>
<th>Melanin$\dagger$</th>
<th>$\text{Ag}^+$/melanin$\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>10.06</td>
<td>261.27</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>12.73</td>
<td>14.99</td>
</tr>
<tr>
<td>$1 \times 10^{-8}$</td>
<td>13.653</td>
<td>4.85</td>
</tr>
<tr>
<td>0</td>
<td>11.044</td>
<td>0</td>
</tr>
</tbody>
</table>

$\dagger$ Molar concentration of $\text{AgNO}_3$; $\ddagger$ amount of melanin used in experiment (mg); 1 mg of melanin ‘ghost’ material (see text for explanation) is approximately $3.3 \times 10^9$ particles; $\zeta$ ratio of $\text{Ag}^+$ in $\mu$g to melanin weight in g.

This suggests that melanin particles were saturated with $\text{Ag}^+$. Interestingly, we were unable to protect *C. neoformans* by mixing cells, melanin particles and $\text{Ag}^+$ at the same time presumably because toxicity is mediated faster than the metal is chelated by melanin. Direct evidence that melanin could bind $\text{Ag}^+$ was established by atomic absorption spectroscopy, which demonstrated absorption of $\text{Ag}$ by melanin particles. The amount of $\text{Ag}$ bound to melanin ghosts was proportional to its concentration in solution.

Melanin in *C. neoformans* is deposited in the cell wall where it forms an electron dense shield external to the cellular membrane [11]. It can thus bind potentially noxious substances in the extracellular space and protect the yeast cell. Other studies have shown that melanin has high binding capacity for metals, with a relative binding strength of $\text{Cu} > \text{Ca} > \text{Mg} > \text{Zn}$ [31,32]. In studies where albino and melanized strains of *Amorphotheca resinae* and *A. pullulans* were exposed to $\text{Cu}^{2+}$, melanized cells showed a higher uptake of $\text{Cu}^{2+}$ suggesting the role of melanin in biosorption [33]. Purified extracellular melanin has greater metal absorption capacity than melanized cells have, and also has a lower rate of metal ion release than do both albino and pigmented biomass [21].

Our observations in combination with evidence of melanization of *C. neoformans* in the environment suggest that melanin could protect this organism in its natural niche against a variety of insults, including heavy metals.

**Acknowledgements**

We would like to thank Angel L. Rosas for reviewing the manuscript. A. Casadevall is supported by NIH grants AI33774, AI13342 and HL59842, and is a recipient of a Burroughs Wellcome Fund Scholar Award in Experimental Therapeutics.

**References**


22 Golubovich VN. Toxic effect of silver ions on different groups of microorganisms. Mikrobiologia 1974; 43: 922–924.


