*Medical Mycology* 2001, **39**, 353–357 Accepted 16 October 2000

# 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 a melanin-like pigment in pigeon excreta, a major natural environmental niche. Silver nitrate, $AgNO_3$, 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 $AgNO_3$. *C. neoformans* was grown in media with and without the melanin precursor, L-dopa, for various times and susceptibility to $AgNO_3$ was determined by measuring percentage of survival after incubation in $AgNO_3$. 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 $AgNO_3$, 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 cryptococal 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. neofor-*

Correspondence: A. Casedevall, Albert Einstein College of Medicine, Golding 701, 1300 Morris Park Ave., Bronx, NY 10461, USA. Tel.: +1 718 4302215; fax: +1 718 4308968; e-mail: casadeva@aecom.yu.edu

*The data in this paper are from a thesis to be submitted by Javier García-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

*mans* 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 media (15 mM glucose [Sigma Chemical Co., St. Louis, MO, USA], 10 mM $MgSO_4$ [Sigma], 29·4 mM $KH_2PO_4$ [Fisher Scientific, Fairlawn, NJ, USA], 13 mM glycine [Sigma], 3 μM 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 ($\sim 10^5$ 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 $1 \times 10^{-8}$ M or $5 \times 10^{-9}$ 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 M 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 $dH_2O$ for 10 days to remove traces of HCl. Silver nitrate solutions ($2 \times 10^{-8}$ or $1 \times 10^{-8}$ M) were incubated with $\sim 3 \times 10^5$ melanin particles for 1 h at room temperature. An aliquot of 50 μl containing $\sim 10^5$ non-melanized cells was added to solutions of $AgNO_3$ ($1 \times 10^{-8}$–$5 \times 10^{-9}$ 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 $1 \times 10^{-7}$, $1 \times 10^{-8}$ or $5 \times 10^{-9}$ 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 $2{\cdot}5 \times 10^{-7}$ 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).

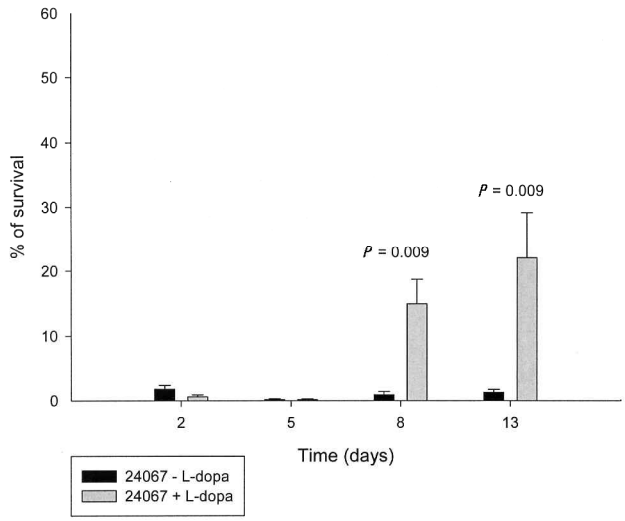


**Fig. 1** Survival of *C. neoformans* cells grown with and without L-dopa after exposure to $1 \times 10^{-8}$ M $AgNO_3$ for 1 h. Results are expressed as percentage of survival relative to cells not exposed to $AgNO_3$, and are the average of five measurements. Error bars represent standard deviation. *P*-values for the comparison of cells grown with and without L-dopa at various times were calculated using the Kruskal-Wallis test. The experiment was performed twice with similar results.

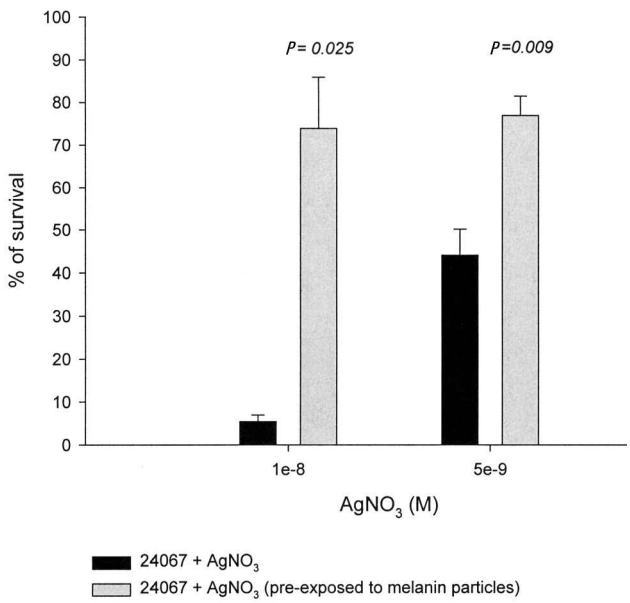


**Fig. 2** Effect of pre-incubation with melanin particles reduces $AgNO_3$ toxicity for non-melanized *C. neoformans* cells. Results are expressed as percentage of survival relative to non-exposed cells, and are the average of five measurements. Error bars represent standard deviation. *P*-values were calculated using the Kruskal-Wallis test. The experiment was performed twice with similar results.

**Table 1** $Ag^+$ binding by melanin particles isolated from *Cryptococcus neoformans*

| $[AgNO_3]$ M* | Melanin† | $Ag^+$/melanin‡ |
|---|---|---|
| $1 \times 10^{-3}$ | 10·06 | 261·27 |
| $1 \times 10^{-6}$ | 12·73 | 14·99 |
| $1 \times 10^{-8}$ | 13·653 | 4·85 |
| 0 | 11·044 | 0 |

*, Molar concentration of $AgNO_3$;
†, amount of melanin used in experiment (mg); 1 mg of melanin 'ghost' material (see text for explanation) is approximately $3·3 \times 10^8$ particles;
‡, ratio of $Ag^+$ in μg to melanin weight in g.

These findings are consistent with the notion that melanin protects *C. neoformans* against $Ag^+$ by chelating the metal ion.

## Discussion

Silver ions displace hydrogen bonds between adjacent nitrogen complementary base pairs in DNA molecules [26]. This is believed to prevent replication of the DNA and subsequent cell division [26]. Also, silver is believed to bind to negatively charged amino acids and may become toxic by interfering with protein function [26]. Growth of microorganisms in low doses of $Ag^+$ ions can result in the emergence of resistant variants. For example, a resistant strain of *Escherichia coli* was isolated from a patient with burn injuries treated with silver sulphadiazine [27]. Resistance was associated with a decrease in $Ag^+$ accumulation, an increase in production of hydrosulfuric acid and intracellular acid labile hydrosulfuric acid ($HS^-$), and increased cell surface hydrophobicity [28]. Experiments on *Debaryomyces hansenii*, *Candida albicans*, *Saccharomyces cerevisiae*, *Rhodotorula rubra* and *Aureobasidium pullulans* have demonstrated that exposure to $Ag^+$ results in the appearance of electron-dense granules in and around cell walls and in the external medium which presumably contain $Ag^+$ [29].

Incubation of *C. neoformans* cells with silver nitrate resulted in a killing of both non-melanized and melanized cells at concentrations greater than $1 \times 10^{-6}$ M $AgNO_3$. This concentration is comparable to that required to fully inhibit *E. coli* growth in LB media ($1·8 \times 10^{-7}$ M) [30]. Similarly, a concentration of $1·77 \times 10^{-5}$ M prevented the replication of *Pseudomonas aeroginosa* in nutrient broth and $8·83 \times 10^{-6}$ M was sufficient to inactivate bacteriophage T2 in broth [26]. Our experiments were performed in water to avoid interference from halide ions that can bind $Ag^+$ ions.

In protection experiments, a survival of approximately 70% was observed at $1 \times 10^{-8}$ and $5 \times 10^{-9}$ M $AgNO_3$. This suggests that melanin particles were saturated with $Ag^+$. Interestingly, we were unable to protect *C. neoformans* by mixing cells, melanin particles and $Ag^+$ at the same time presumably because toxicity is mediated faster than the metal is chelated by melanin. Direct evidence that melanin could bind $Ag^+$ was established by atomic absorption spectroscopy, which demonstrated absorption of Ag by melanin particles. The amount of 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 Cu > Ca > Mg > Zn [31,32]. In studies where albino and melanized strains of *Amorphotheca resinae* and *A. pullulans* were exposed to $Cu^{2+}$, melanized cells showed a higher uptake of $Cu^{2+}$, 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

1 Buchanan KL, Murphy JW. What makes *Cryptococcus neoformans* a pathogen? *Emerg Infect Dis* 1998; **4:** 71–83.
2 Mitchell TG, Perfect JR. Cryptococcosis in the era of AIDS – 100 years after the discovery of *Cryptococcus neoformans*. *Clin Microbiol Rev* 1995; **8:** 515–548.
3 Polacheck I, Hearing VJ, Kwon-Chung KJ. Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. *J Bacteriol* 1982; **150:** 1212–1220.
4 Polacheck I, Platt Y, Aronovitch J. Catecholamines and virulence of *Cryptococcus neoformans*. *Infect Immun* 1990; **58:** 2919–2922.
5 Chaskes S, Tyndall RL. Pigment production by *Cryptococcus neoformans* and other *Cryptococcus* species from aminophenols and diaminobenzenes. *J Clin Microbiol* 1978; **7:** 146–152.

6 Rosas AL, Casadevall A. Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold [published erratum appears in *FEMS Microbiol Lett* 1997; **156:** 171]. *FEMS Microbiol Lett* 1997; **153:** 265–272.

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

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

9 Jacobson ES, Emery HS. Catecholamine uptake, melanization, and oxygen toxicity in *Cryptococcus neoformans*. *J Bacteriol* 1991; **173:** 401–403.

10 Doering TL, Nosanchuk JD, Roberts WK, Casadevall A. Melanin as a potential cryptococcal defence against microbicidal proteins. *Med Mycol* 1999; **37:** 175–181.

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

12 Nosanchuk JD, Valadon P, Feldmesser M, Casadevall A. Melanization of *Cryptococcus neoformans* in murine infection. *Mol Cell Biol* 1999; **19:** 745–750.

13 Nosanchuk JD, Rosas AL, Lee S-HC, Casadevall A. Melanization of *Cryptococcus neoformans* in infected human brain tissue. *Lancet* 2000; **355:** 2049–2050.

14 Rosas AL, Nosanchuck JD, Feldmesser M, Cox GM, McDade HC, Casadevall A. Synthesis of polymerized melanin by *Cryptococcus neoformans* in infected rodents. *Infect Immun* 2000; **68:** 2845–2853.

15 Hamilton AJ, Holdom MD. Antioxidant systems in the pathogenic fungi of man and their role in virulence. *Med Mycol* 1999; **37:** 375–389.

16 Heinzkill M, Bech L, Halkier T, Schneider P, Anke T. Characterization of laccases and peroxidases from wood-rotting fungi (family Coprinaceae). *Appl Environ Microbiol* 1998; **64:** 1601–1606.

17 Mansur M, Suarez T, Fernandez-Larrea JB, Brizuela MA, Gonzalez AE. Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197. *Appl Environ Microbiol* 1997; **63:** 2637–2646.

18 Nosanchuk JD, Rudolph J, Rosas AL, Casadevall A. Evidence that *Cryptococcus neoformans* is melanized in pigeon excreta: implications for pathogenesis. *Infect Immun* 1999; **67**: 5477–5479.

19 Hill HZ. The function of melanin or six blind people examine an elephant. *Bioessays* 1992; **14:** 49–56.

20 Enochs WS, Nilges MJ, Swartz HM. A standardized test for the identification and characterization of melanins using electron paramagnetic resonance (EPR) spectroscopy. *Pigment Cell Res* 1993; **6:** 91–99.

21 Fogarty RV, Tobin JM. Fungal melanins and their interactions with metals. *Enzyme Microb Technol* 1996; **19:** 311–317.

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

23 Berger TJ, Spadaro JA, Bierman R, Chapin SE, Becker RO. Antifungal properties of electrically generated metallic ions. *Antimicrob Agents Chemother* 1976; **10:** 856–860.

24 Wang Y, Aisen P, Casadevall A. Melanin, melanin ''ghosts'', and melanin composition in *Cryptococcus neoformans*. *Infect Immun* 1996; **64:** 2420–2424.

25 Glantz SA, Slinker BK. *Primer of Applied Regression and Analysis of Variance*. McGraw-Hill, 1990.

26 Richards RM. Antimicrobial action of silver nitrate. *Microbios* 1981; **31:** 83–91.

27 Starodub ME, Trevors JT. Silver resistance in *Escherichia coli* R1. *J Med Microbiol* 1989; **29:** 101–110.

28 Starodub ME, Trevors JT. Silver accumulation and resistance in *Escherichia coli* R1. *J Inorg Biochem* 1990; **39:** 317–325.

29 Kierans M, Staines AM, Bennett H, Gadd GM. Silver tolerance and accumulation in yeasts. *Biol Metals* 1991; **4:** 100–106.

30 Zhao G, Stevens SE Jr. Multiple parameters for the comprehensive evaluation of the susceptibility of *Escherichia coli* to the silver ion. *Biometals* 1998; **11:** 27–32.

31 Sainz-Jimenez C, Shafizadeh F. Iron and copper binding by fungal phenolic polymers: an electron spin resonance study. *Current Microbiol* 1984; **10:** 281–286.

32 Zunino H, Martin JP. Metal-binding organic molecules in soil. 1. Hypothesis interpreting the role of soil organic matter in the translocation of metals ions from rocks to biological system. *Soil Science* 1977; **123:** 65–76.

33 Gadd GM, de Rome L. Biosorption of copper by fungal melanin. *Appl Microbiol Biotechnol* 1988; **29:** 610–617.