Reduced virulence of melanized Cryptococcus neoformans in Galleria mellonella

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Fungal melanins are important in the virulence of many pathogenic fungi. In this study, we examined the role of melanin in the interaction between Cryptococcus neoformans and the invertebrate host, Galleria mellonella. C. neoformans was able to melanize in the presence of G. mellonella homogenate, indicating the presence of melanin substrates. Melanization was confirmed by the recovery of acid-resistant particles that were recognized by anti-melanin antibodies. In addition, we tested the effect of fungal melanization on virulence. Surprisingly, G. mellonella larvae infected with melanized fungal cells lived longer than those infected with non-melanized fungi. When the cellular immune response of G. mellonella to melanized and non-melanized cells was compared, inflammatory nodules were observed in both groups. However, the response was stronger in larvae infected with melanized cells. These results suggest that fungal melanin activates the immune response of G. mellonella, thereby resulting in the decreased virulence observed with melanized cells.

Introduction

Infectious disease research relies on experimental animal models for investigation of virulence, treatment, and immunity. Vertebrates, such as mice, are traditional models of choice for these studies. However, concerns about animal welfare and costs have prompted researchers to seek out alternatives. Larvae of the waxmoth, Galleria mellonella, are an increasingly popular alternative host for the study of numerous fungal and bacterial pathogens.1,2 Some of the advantages to using the larvae are their low cost, commercial availability, and ease of use.3

Cryptococcus neoformans is a human fungal pathogen that causes one million cases of cryptococcosis annually worldwide.4 Disease is prevalent among populations that are immune compromised, such as AIDS patients. C. neoformans infections result from the inhalation of spores or yeast into the lungs. In the absence of an effective immune response, infection of the brain and central nervous system can lead to life-threatening illness.5 In the laboratory, C. neoformans can infect a variety of organisms, including mice, insects, amoeba and plants.6-8 G. mellonella larvae are susceptible to systemic infection with C. neoformans. Furthermore, several C. neoformans virulence factors that are important in mammals are also important in infection of G. mellonella, including laccase, an enzyme that is required for the production of melanin.9

Melanins are pigments that are found in the cell walls of many fungal pathogens and have roles in fungal virulence as well as survival in the environment. Melanins are polymers of phenolic or indolic subunits. They share certain physical and chemical characteristics, such as dark color, low solubility and resistance to acid hydrolysis. Research suggests that fungal melanin is composed of aggregates of small particles or granules. Exogenous substrates, such as the catecholamine 1,3,4-dihydroxyphenylalanine (1-DOPA) are required for melanin production by C. neoformans. Synthesis of melanin occurs when laccase catalyzes the initial oxidation of 1-DOPA to form dopaquinone, a highly reactive molecule. Further spontaneous steps produce melanin, an amorphous polymer.10-12

Melanin also plays a central role in the host defense of Galleria mellonella and other invertebrates. These organisms have an innate immune system that consists of both humoral and cellular defenses. The humoral responses include a variety of secreted molecules with antimicrobial properties, including phenoloxidase, which defends against pathogens by catalyzing the formation of melanin and toxic melanin intermediates. The cellular immune responses include phagocytosis and nodulation. Nodulation occurs when microbes are recognized by the immune system and surrounded by layers of hemocytes, where they may be trapped and killed. The humoral and cellular components can also work together as melanization occurs inside of nodules.13,14

The goal of this study was to analyze the role of melanin in the interaction of C. neoformans and G. mellonella. Specifically, we tested for the presence of melanin substrates in G. mellonella larvae. In addition, we examined the effect of fungal melanization on virulence and host defense.

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Results

Detection of melanin substrates in *G. mellonella* larvae

The ability of *C. neoformans* to produce melanin depends on the availability of substrates. Therefore, we tested for the presence of melanin substrates in *Galleria mellonella* larvae. This information is key to understanding the role of laccase and melanization in the virulence of *C. neoformans* in *G. mellonella*. Medium was made with homogenized larvae and inoculated with *C. neoformans*. After five days of incubation, growth of the wild-type strain appeared light tan. The color intensity increased over time until a brown color was achieved at two weeks of incubation (Fig. 1A). Longer incubation times did not produce darker pigmentation, nor did doubling the concentration of larvae homogenate in the medium (data not shown). Furthermore, pigmentation was dependent on the laccase activity of *C. neoformans*, because a laccase deficient mutant did not become pigmented on this medium (Fig. 1A). In general, the pigmentation observed with *G. mellonella* homogenate was not as dark as with l-DOPA. Additional wild-type cryptococcal strains of other serotypes were tested for melanization in the presence of *G. mellonella* homogenate. Of the strains tested, H99 (serotype A) appeared the most pigmented in the presence of l-DOPA or *Galleria* homogenate (Fig. 1B and C).

The pigment produced in the presence of *G. mellonella* homogenate was analyzed to determine whether it was melanin. Acid-resistant particles resembling cryptococcal cells (melanin “ghosts”) were recovered from cultures grown in the presence of homogenate (Fig. 2A). Furthermore, the particles were detected by immunofluorescence with an anti-melanin antibody (Fig. 2A). No fluorescence was observed when the particles were incubated with the secondary antibody alone (data not shown). On the basis of dark color, acid insolubility, and reactivity with a melanin-binding mAb we tentatively concluded that the pigment was a melanin. Next, histology of larvae infected with *C. neoformans* cells that had been cultured with or without l-DOPA. At 32 °C
pre-melanization of \textit{C. neoformans} decreased virulence in \textit{G. mellonella} or had no effect (Fig. 3; Table 1). In three out of four separate trials the mean survival times for larvae infected with melanized cells was longer than for those infected with non-melanized cells. The experiment was then repeated at human physiological temperature. A slight decrease in virulence of melanized cells was observed at 37 °C (Table 1). The effect of laccase on virulence was evaluated under similar conditions. There was no difference in the survival of \textit{C. neoformans} strain H99 after 5 d, larvae were sacrificed and fixed. Sections were stained with hematoxylin and eosin. Asterisks indicate pigmented \textit{C. neoformans} cells. Scale bars, 5 μm.

Figure 2. Analysis of melanin produced by \textit{C. neoformans}. (A) Melanin “ghosts” were isolated from fungal cells grown in the presence of \textit{G. mellonella} homogenate or L-DOPA. The melanin ghosts were visualized by light microscopy (top row) and immunofluorescence with a melanin-binding antibody (bottom row). (B) \textit{G. mellonella} was infected with \textit{C. neoformans} strain H99. After 5 d, larvae were sacrificed and fixed. Sections were stained with hematoxylin and eosin. Asterisks indicate pigmented \textit{C. neoformans} cells. Scale bars, 5 μm.

Table 1. Summary of survival experiments

| Incubation temperature | Melanized | Non-melanized | P value | N |
|------------------------|-----------|---------------|---------|---|
| Survival results with larvae from New York Worms* |
| 32°C                   | 5.3       | 3.1           | 0.037   | 11 |
| 39°C                   | 9         | 5.3           | 0.019   | 10 |
| 6.1                    | 4.7       | 6.1           | 0.076   | 15 |
| 5.6                    | 5.7       | 6.84          | 0.684   | 15 |
| Survival results with larvae from Vanderhorst Wholesale, Inc.* |
| 32°C                   | 8.2       | 5.7           | 0.096   | 10 |
| 37°C                   | 4.5       | 4.4           | 0.238   | 14 |

*Mean survival of larvae (days)

When using larvae supplied from New York Worms rather than Vanderhorst, there was no difference in the survival of \textit{G. mellonella} infected with either a wild-type strain (H99) or a double laccase mutant (QGC8) at both 32 °C and 37 °C (P > 0.05).

Since there was some variability in the survival results, the experiment was repeated with larvae from another supplier (Vanderhorst). Although there was a trend toward longer survival of larvae infected with melanized cells, the results were not significant (Table 1). Thus, we were more likely to observe a significant difference between melanized and non-melanized cells when using larvae supplied from New York Worms rather than Vanderhorst.

Fungal burden was assessed in addition to virulence. For both melanized and non-melanized cells, there was little change in the amount of CFUs present in the larvae at day two. By day five, both melanized and non-melanized cells had increased CFUs by approximately 100-fold. To determine how well the yeast reproduced in the \textit{G. mellonella} larvae, a growth curve was generated by plotting the starting inoculum and day five fungal burden.
**Table 2. Nodule formation in G. mellonella larvae**

|          | Day 2       | Day 5       |       |       |       |
|----------|-------------|-------------|-------|-------|-------|
|          | Number of   | Size of     | Number | Size of |       |
|          | nodulesa    | nodulesb    | nodulesa| nodulesb|     |
| Melanized| 23          | 4829        | 110    | 10349  |      |
|          | 22          | 3744        | 87     | 12767  |      |
| Non-melanized| 9          | 2119        | 42     | 4230   |      |
|          | 13          | 1054        | 175    | 5304   |      |
| PBS      | 4           | 2075        | 0      | NA     |      |
|          | 0           | NA          | 0      | NA     |      |

*aThe number of nodules observed per longitudinal section of infected larvae. Each row represents an individual larva; bThe mean area of nodules observed in the larvae.

The slope of the resulting line was used as a measure of growth. The growth of the non-melanized C. neoformans was higher than the melanized (6.6 × 10^6 and 1.29 × 10^6 CFU/day, respectively). However, this trend was not significant (P > 0.05).

**Nodule formation in response to melanized fungi**

To determine whether there were differences in the fates of melanized and non-melanized fungal cells, infected larvae were analyzed by histology. Larvae were fixed at 2, 5, and 8 d post-infection, corresponding to early, middle, and late time points, respectively. Fixed larvae were embedded in paraffin and stained with hematoxylin and eosin. The size and number of nodules in each larva was measured (Table 2). At day 2 post-infection, a robust cellular immune response was observed, with fungal cells visible inside inflammatory nodules (Fig. 4A). This response was stronger in larvae infected with melanized cells. In these larvae the nodules were significantly larger. The average nodule size was 4298 μm^2 for the melanized group and 1490 μm^2 for the non-melanized group (P < 0.02). At day 2, there was a trend toward more nodules in the melanized group.

By day 5, very large nodules were observed with numerous yeast cells inside (Fig. 4A). Similar to day 2, the melanized group had significantly larger nodules (11417 μm^2 for melanized and 5096 μm^2 for non-melanized (P < 0.02). There was no consistent trend in the number of nodules at day 5 for the melanized vs. non-melanized groups. Some tissue damage was apparent, with empty spaces seen in the larvae sections for both the melanized and non-melanized groups, which possibly confounded the nodule counts (Fig. 4B). By 7 d post-infection, the interiors of the larvae showed extensive tissue damage and large areas were filled with fungal cells for both groups (Fig. 4B).

Since the histology results suggested an enhanced immune response in the larvae infected with melanized cells, a qualitative evaluation of phenoloxidase activation was made by observing the color of the larvae. No obvious differences in melanization were observed between the melanized and non-melanized groups. However, the larvae from New York worms were more heavily melanized than the Vanderhorst larvae (Fig. 5).

**Discussion**

Melanin is produced by many fungal pathogens and has an important role in the ability of fungi to infect hosts. G. mellonella are a useful tool in infectious disease research. Studies can be done easily and with low cost. In this study, the role of melanization in C. neoformans infection of G. mellonella larvae was analyzed. Specifically, our goal was to address the potential use of the G. mellonella model for investigating melanin as a virulence factor.

*C. neoformans* cannot produce melanin without being supplied a substrate. This trait has allowed researchers to easily control melanin production and determine that it is important for *C. neoformans* to survive UV irradiation, predation by amoeba, and killing by host phagocytes. A fundamental question in understanding the role of melanin in the interaction of *C. neoformans* and *G. mellonella* is whether melanin substrates are present in the larvae. We observed laccase-dependent pigment production when *C. neoformans* was cultured on medium containing *G. mellonella* homogenate. Acid-resistant particles that were recognized by an anti-melanin antibody were recovered from the homogenate cultures. Together, these results suggest that melanin substrates are present in the larvae and that *C. neoformans* may be able to produce melanin inside of the larvae. Pigmented cells were visible in histology sections of infected larvae. However, the possibility that this was melanin produced by *G. mellonella* phenoloxidase cannot be ruled out.

Melanin is an established virulence factor in numerous fungal species. To further explore the question of melanin and virulence, we assessed melanized and non-melanized cells in *G. mellonella* infection by growing wild-type cells with l-DOPA. In a mouse model, pre-melanization of *C. neoformans* resulted in higher fungal burdens in the lungs and brain at early times post-infection. This suggested that melanin is important for fungal virulence in mice. In contrast, our results suggest that melanin is not a critical virulence factor in *G. mellonella*. Pre-melanization of *C. neoformans* with l-DOPA resulted in decreased virulence in *G. mellonella*. In addition, the presence of the genes for laccase (*LAC1* and *LAC2*) had no effect on virulence under the experimental conditions of this study. This was somewhat surprising since previous research showed that a *LAC1* deletion strain had reduced virulence in *G. mellonella*. However, this may be explained by the fact that the studies were done using *C. neoformans* strains with different genetic backgrounds (serotype A and serotype D). The two serotypes diverged over 18 million years ago in evolution and differences in their overall virulence have been reported in mice as well as in *G. mellonella*. Furthermore, the
Figure 4. For figure legend, see page 614.
effects of certain virulence factors varied between serotypes A and D, including protein kinase A, 20 mating type, 21 and macrophage killing. 22

We found variability in the survival of larvae in our experiments, including larvae from different suppliers as well as different batches of larvae from the same supplier. The variability was not surprising since G. mellonella larvae are not maintained for scientific research purposes and there may be differences in overall health, genetics, and rearing conditions that affect the outcome of survival experiments. Previous research showed that cold or hot thermal shock of the larvae resulted in differences in survival outcomes and activation of the immune response. 23 This implies that results may be affected by temperatures experienced by the larvae during storage and transport. Larvae in our experiments were stored at cold temperature prior to infection, which may have enhanced the immune effects and differences in survival.

Although the virulence results were surprising in terms of what is known about melanin as a virulence factor, they agree with other studies on fungal virulence in G. mellonella. The decreased virulence of the melanized C. neoformans cells in G. mellonella is consistent with what has been observed with Aspergillus fumigatus. This fungus produces melanin via a multi-enzyme polyketide synthase pathway. When color mutants in A. fumigatus were obtained from Stuart Chaskes, G. mellonella larvae were obtained from New York Worms and Vanderhorst Wholesale, Inc. Larvae were stored at 4 °C prior to experiments.

Medium and culture conditions

Fungal cells were prepared for infection by culturing the C. neoformans H99 strain in chemically defined minimal medium (15 mM dextrose, 10 mM magnesium sulfate, 29.4 mM potassium phosphate, 13 mM glycine, 3 μM thiamine, pH 5.5) at 30 °C and 150 RPM for one week. To obtain melanized cells, 1-DOPA (1 mM) was added to the cultures.

G. mellonella larvae medium

G. mellonella larvae were homogenized in 2x chemically defined minimal medium. Homogenization was performed on ice. The homogenate was centrifuged to pellet debris (5 min at 2500 rpm, 4 °C) and filter sterilized. The resulting solution was mixed with molten agar (3%) and immediately poured into petri dishes. The medium was inoculated the following day. Twenty larvae (approximately 5 g) were homogenized to make 200 mL of medium. The liquid medium was made in a similar manner, but without the addition of agar. The medium was briefly heated to inactivate G. mellonella phenoloxidase.

Infection of G. mellonella

Washed fungal cells were suspended in PBS at a concentration of 5 × 10^6 CFU/mL. G. mellonella larvae weighing approximately 250 mg (± 25) were selected and randomly sorted into equal sized groups per treatment. G. mellonella larvae were infected by injection into the last proleg. A repeating dispenser fitted with a disposable syringe delivered precisely 20 μL of inoculum to each larva. Larvae were infected with 1 × 10^6 CFU. To prevent contamination with bacteria, streptomycin (20 mg/kg bodyweight) was administered. After infection, larvae were placed in a sterile
petri dish and maintained in a 32 or 37 ± 2 °C incubator. G. mellonella survival was monitored daily. Between 10 and 15 larvae were infected per treatment group. This sample size represents a power of 0.53–0.69 to correctly reject the null hypothesis that melanized and non-melanized C. neoformans have equal virulence in G. mellonella (with an α of 0.05 and an effect size of 0.8). Fungal burden was determined by sacrificing 4–5 larvae on days 2 and 5 post-infection and homogenizing the larvae in PBS with a hand-held homogenizer equipped with a 7 mm probe (Omni International). Separate infections were performed for the survival, histology, and fungal burden analyses.

**Immunofluorescence of melanin ghosts**

C. neoformans strain H99 was cultured in medium with G. mellonella larvae homogenate for approximately 1 mo. Melanin was then isolated from the cultures in a multi-step procedure that included enzymatic digestion, chemical denaturation, chloroform extraction, and boiling in concentrated acid, as described previously. The resulting particles were analyzed by immunofluorescence with a melanin-binding monoclonal antibody (6D2). mAb 6D2 was purified from cell culture supernatants. The secondary antibody was a goat anti-mouse IgM conjugated to TRITC. Each antibody was diluted to a concentration of 10 μg/mL in 1% BSA. Antibody incubations were done for 1 h at 37 °C. As a control, the particles were incubated with secondary antibody alone, showing that 6D2 binding to the melanin ghosts was required to observe fluorescence.

**Analysis of virulence**

Three groups of larvae were infected for each experiment: (1) melanized C. neoformans (2) non-melanized C. neoformans, and (3) PBS control. The number of surviving larvae was recorded daily. Larvae were considered dead if they did not respond to sustained touch stimulus. Kaplan–Meier survival curves were created with the statistics program SPSS 18 (SPSS, Inc.). Differences between treatment groups were compared by the Log-rank method.

**Fixation and histology**

At indicated time points larvae were collected and incubated on ice for 15 min. Larvae were then fixed in 4% paraformaldehyde in PBS for 10 d. A needle was used to poke holes in the larvae to facilitate entry of fixative. After 10 d, the larvae were transferred to 30% sucrose overnight, and then transferred to 70% ethanol for storage. Fixation was performed at 4 °C. Larvae were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Analysis of slides**

Slides were scanned visually with a microscope to identify nodules. Digital microscope images of the nodules were analyzed with ImageJ software (NIH) to determine their size by the following method. First, a scale of pixels to microns was established with a stage micrometer. The size was then determined by drawing an outline around each nodule and measuring its area with the software. Nodule area was analyzed using the non-parametric Wilcoxon Rank Sums test. A P value < 0.05 was considered significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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