Pneumocystis Melanins Confer Enhanced Organism Viability

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Pneumocystis continues to represent an important opportunistic fungal pathogen of those with compromised immunity. Thus, it is crucial to identify factors that affect its viability and pathogenicity. We previously reported the first identification of melanins in Pneumocystis. In the present study, we sought to further characterize these components and define the function for these melanins. Melanins extracted from Pneumocystis and melanized Pneumocystis cells were analyzed by electron spin resonance spectroscopy, revealing spectra consistent with melanins from other fungi. Immunofluorescence assays using anti-melanin monoclonal antibodies showed that melanins are widely present across Pneumocystis host species, including mouse-, ferret-, and human-derived Pneumocystis organisms, as well as Pneumocystis carinii derived from rat. Using immunoelectron microscopy, melanins were found to localize to the cell wall and cytoplasm of P. carinii cysts, as well as to intracellular bodies within mature cysts. Next, the role of melanins on the maintenance of Pneumocystis viability was determined by using quantitative reverse transcription-PCR measurement of the heat shock protein mRNA under adverse environmental conditions. Using a new method to promote the melanization of Pneumocystis, we observed that strongly melanized Pneumocystis retained viability to a greater degree when exposed to UV irradiation or desiccation compared to less-pigmented organisms. These studies support our previous identification of Pneumocystis melanins across the genus, further characterize these Pneumocystis components, and demonstrate that melanins protect Pneumocystis from environmental stressors.

Pneumocystis organisms are opportunistic fungal pathogens that can cause life-threatening pneumonia in immunocompromised mammals, including humans. These fungi are absolutely host species specific (9), with Pneumocystis carinii infecting only rats and P. jirovecii infecting only humans. Pneumocystis organisms are ubiquitous in their geographic distribution, with reports of Pneumocystis-related disease on nearly every continent. Transmission of Pneumocystis has been determined to occur through an airborne route, but the transmissible form has not been identified. The lack of a consistent, long-term in vitro cultivation method necessitates the use of animal infection models.

The lack of a reliable culture system for Pneumocystis has caused significant difficulties in studying the basic biology of this fungus. Many of the most fundamental biological mechanisms have not been adequately described, since genetic manipulation of Pneumocystis is also not currently possible. The search for evidence of viability and virulence factors of Pneumocystis has been pursued in many investigations; however, relatively little has been learned. Common factors of other fungal pathogens include dimorphism, growth at 37°C, toxins, cell wall components, and melanins (13). Several cell wall components of Pneumocystis have been described as putative virulence factors, including major surface glycoprotein (11, 36), glucans (34), and melanins (19).

Melanins are negatively charged hydrophobic pigments of diverse molecular structure and high molecular weight. They are typically brown or black in color and exist in all animal kingdoms (8). Many fungal pathogens produce melanins, including Aspergillus, Cryptococcus, Histoplasma, Paracoccidioides, and Sporothrix species (2, 10, 12, 20, 26, 28). Melanins have been shown to influence viability and virulence in several of these fungal pathogens. Fungal melanins can be visualized with anti-melanin monoclonal antibodies (MAbs) in all of these fungal pathogens mentioned, including Pneumocystis carinii (10, 19, 25, 30, 31). Melanins have been shown to protect fungal pathogens from environmental stressors (3, 24) and host-induced damage (23, 29).

The presence of melanins in Pneumocystis and the association of melanins with virulence in other fungal pathogens prompted the present studies, in which we characterize melanins isolated from Pneumocystis carinii and begin to determine the importance of these pigments in the viability of this opportunistic fungal pathogen. We postulate that melanins promote the viability of Pneumocystis by providing protection from environmental stressors encountered during transmission.

MATERIALS AND METHODS

Isolation of Pneumocystis organisms. P. carinii organisms were harvested from the lungs of Long Evans rats that were immunosuppressed for ~12 weeks via 4 mg of dexamethasone (American Reagent, Shirley, NY)/ml in drinking water. To ensure consistent infection, rats were inoculated with P. carinii isolate via intratracheal injection after the first week of immunosuppression. P. carinii was harvested from the lungs of SCID mice that were further immunosuppressed for ~10 weeks via biweekly injection of each mouse with 2 mg of Solu-Cortef (Pharmacia, Michigan). Further, to ensure robust infection, SCID mice were inoculated at week 1 with P. carinii by intratracheal instillation. The minced rat or mouse lungs were homogenized in 10 ml of RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) plus 1% glutathione (Sigma, St. Louis, MO) for 10 min by using a Stomacher Lab Blender 80 (Tekmar, Cincinnati, OH). The homogenate from each rat or mouse lung was filtered through sterile gauze and then through a 10-µm pore-size TCTP Isopore membrane filter (Millipore, Bedford, MA). Human-derived P. jiroveci organisms were obtained from clinical bronchoalveolar lavage and/or induced sputum specimens provided by Laurence Huang (San Francisco General Hospital, San Francisco, CA). Human-derived P. jiroveci

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samples were obtained in the course of clinical evaluation of pneumonia, as previously described (14). The *Pneumocystis* organisms were washed in phosphate-buffered saline (PBS), flash-frozen, and stored at -70°C until analyzed. The human-derived *Pneumocystis* cell suspensions were homogenized in 1% glutathione and filtered as stated above. Ferret-derived *Pneumocystis* organisms were obtained by Francis Gigliotti (University of Rochester, Rochester, NY). The ferret *Pneumocystis* was generated as previously described, flash-frozen, and stored at -70°C until assayed (32). Aliquots of purified rat-, mouse-, or human-derived *Pneumocystis* were used for the isolation of melanin ghosts, immunofluorescence assays, immunohistochemistry assays, or quantitative reverse transcription-PCR (qRT-PCR) viability determination. All animals were handled and maintained following appropriate review and oversight of the Mayo Clinic Institutional Animal Care and Use Committee.

**Pneumocystis pigmentation.** Our earlier observations demonstrate that native *Pneumocystis* organisms exhibit surface melanins during the course of infection (15). However, preliminary studies further revealed that melanization of the organism could be significantly enhanced by incubating melanins in synthetic precursors including l-DOPA, thus providing a novel system to study the potential role of melanins in *Pneumocystis*. Accordingly, purified *P. carinii* and *P. murina* were incubated in the presence or absence of 100 mM l-DOPA (dissolved in 0.1 M citric acid and then diluted in PBS) at 30°C with rotation overnight to induce robust visible, black pigmentation. These highly melanized cells were used for electron spin resonance (ESR) analysis and qRT-PCR viability determinations. Pigmentation controls included normal rat lung tissue homogenate, supernatant from *Pneumocystis*-infected rat lung tissue, and l-DOPA alone.

**Role of phenoloxidase in *Pneumocystis* melanin pigment generation.** Phenoloxidase is an enzyme commonly required for the polymerization of melanin pigments in several other fungal species. We have previously modified a convenient colorimetric assay to measure phenoloxidase activity in *P. carinii* (16). Aliquots of 10^6 *P. carinii* organisms were incubated with 10 mM l-DOPA, the exogenous melanin precursor under study, in the presence of between 0 and 5,000 mg of glyphosate/ml, an agent that potently inhibits phenoloxidase activity (Sigma Chemical Co., St. Louis, MO). Incubations were conducted for 2 h at 30°C. Prior to reaction termination with 10 mM KCN (Sigma), subsequently the aliquots were centrifuged at 1,000 g for 5 min, and the absorbance of the supernatant at 480 nm was measured to quantify the extent of pigment formation.

**Isolation of *Pneumocystis* melanins.** Melanin ghosts were isolated by using previously described procedures from approximately 5 × 10^6 *P. carinii* organisms (36). *P. carinii* was incubated in 10 mg of Trichoderma sp. cell wall lysing enzymes (Sigma)/ml, dissolved in 1 M sorbitol-0.1 M sodium citrate (pH 5.3) overnight with rocking at 30°C. The fungi were centrifuged at 1,000 × g for 10 min, washed in PBS, and then incubated in 4 M guanidine thiocyanate (Sigma) overnight with rocking at room temperature. The cell debris was centrifuged, washed as described above, and then incubated in 1 mg of proteinase K (Invitrogen, Inc., Carlsbad, CA)/ml overnight at 37°C. The cell debris was centrifuged, washed as described above, and then incubated in 0.1 mg of proteinase K (Invitrogen)/ml at 37°C for 1 h. Slides were then submersed in 10 mM citric acid and heated in a microwave for 5 min. The samples were blocked in SuperBlock (Pierce Chemical Company, Rockford, IL) overnight with rocking at room temperature. The slides were subsequently incubated with 50 μg of primary antibody (Mab 6D2 an anti-melanin mouse immunoglobulin M [IgM] Mab obtained from Josh Nosanchuk, Albert Einstein College of Medicine, Bronx, NY) or mouse IgM isotype control (Sigma)/ml for 2 h at 37°C. The slides were then washed twice in 0.1% Tween for 5 min and twice in PBS for 5 min. A fluorescein isothiocyanate-goat anti-mouse IgM secondary antibody (1:250 dilution; Sigma) was added to the samples, followed by incubation for 2 h at 37°C, and then washed as described above. The slides were mounted with SlowFade Antifade reagent (Invitrogen/Molecular Probes, Carlsbad, CA), and the coverslips were sealed with clear nail polish. Samples were visualized under oil immersion using an Axioplan KS400 fluorescence microscope with fluorescein isothiocyanate and phase-contrast filters.

**Immunoelectron microscopy detection of *Pneumocystis* melanins.** *P. carinii* samples were fixed in 4% formaldehyde-0.2% glutaraldehyde-PBS for 16 to 24 h and then washed in fresh PBS. After fixation, samples were dehydrated in a series of increasing concentrations of ethanol and lowering temperatures (60% ethanol for 15 min at 4°C, 70% for 60 min at -20°C, 80% for 60 minutes at -20°C, 95% for 60 min at -20°C, and absolute ethanol for 60 min at -20°C). The samples were then infiltrated in 1:1 ethanol-LR White resin (Ted Pella, Inc., California) overnight at -20°C and fresh LR White for 60 min at -20°C. Embedded in LR White at room temperature, and then polymerized at 55°C for 2 days. Thin sections were cut and mounted on nickel grids and dried overnight. Nonspecific antigen sites were blocked in aqueous 1% glycine and again in 0.05% Tween 20-PBS (PBST) with 1% normal serum and 1% acetylated bovine serum albumin. The sections were incubated for 2 h at room temperature with the Mab 6D2 anti-melanin IgM antibody (diluted 1:100 in PBST plus normal goat serum) or IgM isotype control antibodies (Sigma). The sections were then rinsed in PBST and incubated for 60 min in goat anti-mouse IgM conjugated to 5-nm colloidal gold (Amersham Biosciences, New Jersey). After incubation, the sections were rinsed three times in PBST and water. Gold particles were silver enhanced with Aurion R-gent SE-em kit (Electron Microscopy Sciences, Pennsylvania) to a diameter of 15 to 20 nm and stained with uranyl and lead. Immunolabeled sections were viewed by transmission electron microscopy (model 6400: JEOL USA, Inc., Peabody, MA).

**UV irradiation, desiccation, and RNA isolation of *Pneumocystis***. We next sought to determine the role of *Pneumocystis* melanins in mediating organism viability. *Pneumocystis* organisms derived from three or four rats were pooled for each experimental run. Freshly isolated *P. carinii* containing the basal levels of melanin present during infection and highly melanized organisms, derived by preincubating organisms in l-DOPA, were maintained in 1 ml of a *Pneumocystis* culture medium (RPMI 1640 supplemented with 20% fetal bovine serum, 1% minimal essential medium vitamin mixture, 1% minimal essential medium non-essential amino acid mixture, glutamine, and 2% penicillin-streptomycin; modified from Chen and Cushion [4]) in 24-well tissue culture plates (10^5 organisms per well) in a humidified incubator at 35°C in 5% CO2. Tissue culture plates containing *Pneumocystis* (with or without melanin) or control (control) and the organisms with enhanced melanin content were handled in an identical fashion throughout the experiment, under the environmental stresses and during RNA isolation.

Cells were treated identically to avoid RNA degradation. Samples of each *Pneumocystis* sample were collected at 48 h after UV/desiccation treatment. Each sample was centrifuged for 5 min at 1,000 × g, and the supernatants were removed. The pelleted cells were resuspended in 100 μl of RNAlater (Ambion, Inc., Austin, TX) and stored at -20°C. Total RNA was extracted from each pooled sample using the Micro-to-Midi Total RNA Purification System (Invitrogen), followed by DNase I treatment to remove genomic DNA according to the manufacturer’s protocol, and stored at -80°C. All samples were treated similarly to assess any sample-to-sample variability.

**qRT-PCR viability measurement of *Pneumocystis***. Previous studies indicate that *Pneumocystis* RNA degradation can serve as a quantitative marker of loss of organism viability (17). Accordingly, measurement of *Pneumocystis* heat shock protein 70 message RNA was performed by qRT-PCR. The coding primer
sequence was PcHsp70A (5'-AGA CAA TTG GTA TT-3') (22) and the noncoding primer sequence was PcHsp70D (5'-GGA TTC ATA GCA ACT TG-3'). Each total RNA sample was analyzed by qRT-PCR using Script One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Inc., Hercules, CA). Each qRT-PCR was composed of the manufacturer's recommended reagents, with the exception that each reaction contained a final volume of 25 μl and the addition of 0.2 μl of RNaseOUT (Invitrogen). RNA standards were generated by isolating total RNA from \(\times 10^7\) Pneumocystis organisms and then making five 10-fold dilutions of this total RNA stock. Then, 1 μl of each total RNA standard and sample was added per reaction, and no-template controls were included. Reactions performed in the absence of reverse transcriptase did not reveal amplification.

The qRT-PCR was performed by using a Bio-Rad iCycler IQ using the following conditions: RT for 50°C for 10 min, hot start for 95°C for 5 min, denaturation for 95°C for 10 s, and annealing for 58°C for 30 s. All reactions were run for 50 cycles, SYBR Green fluorescence was collected during the anneal cycle, and a melting curve was generated for 72°C to 88°C. The data were analyzed by using iCycler Software v. 3.1.7050 and then exported to Excel XP for graphing.

Statistical analysis. All data are expressed as the mean ± the standard error of the mean. Differences between groups were determined by using a two-tailed Student's t test. Statistical testing was performed by using the SPSS/IM software program, with statistical differences considered significant if the P value was <0.05.

RESULTS

Melanization of Pneumocystis. Our prior studies have documented that P. carinii organisms are natively melanized during the course of infection (19). In our prior observations, the relative levels of melanin expression as assessed by immunofluorescence varied between individual Pneumocystis cyst and trophic forms. Specifically, some cysts and trophic forms within a given isolate were found to exhibit robust melanization, whereas other cysts and trophic forms in the same isolate exhibited low or undetectable levels of melanin (19). However, in every isolate studied, both cyst and trophic forms expressed some native level of melanin during infection. We recently discovered that substantially higher amounts of melanins than the native physiological melanin levels can be obtained in freshly isolated Pneumocystis organisms by incubating them with a potent melanin precursor substrate (Fig. 1A to C). For instance, Pneumocystis was incubated in the presence or absence of l-DOPA to strongly induce striking differences in melanin content. Figure 1A shows the difference between native melanized and highly melanized Pneumocystis organisms by visual detection, and Fig. 1B and C show these changes by electron microscopy. Controls of melanization included incubation of l-DOPA alone, incubation with uninfected rat lung tissue, and incubation with supernatant collected from Pneumocystis-infected rat lung tissue. All three controls showed no visible signs of melanin polymerization (data not shown), supporting our conclusion that Pneumocystis is capable of producing substantial amounts of melanins. This approach provides a novel means to drive melanin production in Pneumocystis, a necessary method for studying functional correlates of this pigment in this genetically intractable organism. This observation assumes further importance because we cannot genetically manipulate Pneumocystis and are therefore unable to knock out or overexpress melanin production by using traditional molecular strategies.

Our prior studies document that phenoloxidase activity is needed for the polymerization of melanin formation in Pneumocystis (16). Thus, we further sought to determine whether the exuberant pigment formation driven by l-DOPA similarly required phenoloxidase activity (Fig. 1D). P. carinii was capable of catalyzing the conversion of l-DOPA into a chromogenic pigment. Furthermore, in a dose-dependent fashion, glyphosate inhibited these reactions, strongly suggesting that phenoloxidase in P. carinii is required to polymerize l-DOPA precursors into melanin-like pigments.

ESR characterization of Pneumocystis melanins. ESR spectroscopy has been commonly used to verify the presence of melanins based on the common properties of unpaired elec-
Immunofluorescence detection of Pneumocystis melanins. Pneumocystis organisms consist of a variety of species, with each species specific for the mammalian host, which it infects (35). We next questioned whether melanin generation was unique to P. carinii derived from rats or whether it was a feature generalized across the genus. To address this, MAbs against fungal melanins were used to visualize melanins in P. carinii (rat), P. murina (mouse), ferret-derived Pneumocystis, and P. jirovecii (humans). The results from the immunofluorescence assays are depicted in Fig. 3, where the upper panels for each sample were incubated with MAAb 6D2 (recognizing fungal melanins) and the lower panels were incubated with mouse IgM isotype control antibody. The anti-melanin antibodies were found to bind to cysts and trophic forms of all four species of Pneumocystis tested, confirming that multiple Pneumocystis species contain melanins, including the species infecting humans (P. jirovecii). These data further demonstrate that cysts and trophic forms of P. carinii, P. murina, ferret-derived Pneumocystis, and P. jirovecii species bind the MAAb 6D2 antibody and thus contain melanins in both major life cycle forms of these organisms.

Immunoelectron microscopic localization of Pneumocystis melanins. We next sought to determine the subcellular localization of melanins in P. carinii. To accomplish this, immunoelectron microscopy was performed using the same anti-melanin MAb as in the immunofluorescence assays described above. The results of these analyses are shown in Fig. 4. Gold particles linked to the anti-melanin antibody were specifically binding to the cytoplasm. Interestingly, the Pneumocystis melanins also appear to strongly localize to intracystic bodies contained within mature cysts. Control experiments using isotypespecific nonimmune primary antibody showed no presence of gold particles binding to any structures of the Pneumocystis cyst or intracystic bodies (data not shown). These data confirm our previous immunofluorescence data and are consistent with melanin deposition in other fungi (25). Our electron microscopy data support the presence of Pneumocystis melanins in the cyst wall, cytoplasm, and intracystic bodies of the organism.

Pneumocystis melanins enhance organism viability in the presence of UV irradiation and desiccation. We next evaluated whether Pneumocystis melanins act to preserve organism viability in the presence of environmental stressors. To address this, we exposed native melanized Pneumocystis (basal amounts of melanins) and highly melanized organisms (excessive melanin production induced by L-DOPA) to either UV irradiation or desiccation. Pneumocystis viability was determined by qRT-PCR measurement of Pneumocystis heat shock protein 70 mRNA using a previously established method (22). Both conditions adequately stress Pneumocystis organisms, as evidenced by reduced organism viability. Under conditions of UV irradiation, natively melanized Pneumocystis exhibit a 17-fold net reduction in Hsp70 RNA copy number compared to unstressed natively melanized organisms. In the case of desiccation, organisms exhibit an eightfold reduction in Hsp70 RNA copy number compared to unstressed natively melanized organisms. For comparison, pentamidine (1 mg/ml), an agent known to very strongly suppress Pneumocystis viability, resulted in a 59-fold reduction of Hsp70 copy number over a similar time period. Strikingly, the highly melanized Pneumocystis melanins act to resonate in a magnetic field (7). This technique has been widely used to characterize melanins produced by a variety of fungal species, including C. neoformans and H. capsulatum (reviewed in reference 24). Our prior studies indicate that antibodies to fungal melanins recognize intact P. carinii organisms, as well as highly pigmented melanin ghosts derived from P. carinii, thus strongly suggesting the presence of melanins in Pneumocystis species (19). We therefore sought to use ESR spectroscopy as an independent means to confirm the presence of melanin pigments in Pneumocystis. Accordingly, ESR spectra were generated for highly melanized intact P. carinii organisms, highly melanized P. murina organisms, and native melanized P. carinii-derived melanin ghosts to determine whether these ESR spectra were consistent with ESR spectra from other fungal melanins. The ESR spectra results are depicted in Fig. 2, showing that all three Pneumocystis samples have ESR spectral patterns centered around 3.390 G. The observed ESR spectra were performed in an identical fashion to and yielded an ESR spectroscopic pattern identical to that recently published by Nosanchuk et al. for H. capsulatum (25). Thus, ESR analysis spectroscopically independently verified that the pigments produced by Pneumocystis are consistent with melanins.
cystis retained significantly greater viability compared to native melanized Pneumocystis under both the UV ($P < 0.004$) and desiccation ($P < 0.008$) conditions (Fig. 5). Over the time course of the desiccation experiments, the wells were reduced in volume, although they do not become completely dry. This was presumptively associated with increases in osmolarity, as well as some loss of membrane integrity and cell degradation. We selected desiccation because it closely simulates the situation of Pneumocystis organisms expelled as droplets from the host into the environment. These results are consistent with the function of melanins in other fungi and strongly support our hypothesis that Pneumocystis melanins enhance organism survival in the presence of environmental stressors that would typically be encountered during ex vivo transmission from one host to another.

**DISCUSSION**

*Pneumocystis* organisms inflict severe pneumonia upon immunocompromised hosts. While relatively little is known about the life cycle of these challenging organisms, accumulating evidence indicates that *Pneumocystis* are spread from susceptible host to host via the airborne route (33). Expectoration or exhalation of transmissible forms of the organism suspended as droplet nuclei (18) require that *Pneumocystis* has developed mechanisms to defend against environmental stressors that would otherwise reduce viability and inhibit transmission of the organism. The present study provides the first insight into one such mechanism that *Pneumocystis* may have evolved in order to survive the rigors of environmental transmission. Our investigations reveal that the cell walls of *Pneumocystis* contain melanin-like compounds, in both cystic and trophic forms of the fungus. These melanins are present widely across *Pneumocystis* organisms infecting a variety of mammalian host species and exhibit classical biochemical and spectroscopic activity consistent with other fungal melanins. Most importantly, we provide the first evidence that *Pneumocystis* cell wall melanins enhance the viability of organisms exposed to typical environmental stressors including desiccation and UV irradiation.

**FIG. 3.** Immunofluorescence visualization of *Pneumocystis* melanins. (A) *P. carinii* (rat); (B) *P. jirovecii* (human); (C) *P. murina* (mouse); (D) ferret-derived *Pneumocystis*. For each set of images, panel 1 represents staining with the anti-melanin MAb 6D2 and viewed under fluorescence microscopy, panel 2 is the phase-contrast image of the identical field shown in panel 1, panel 3 represents staining with nonimmune isotype control antibody and viewed under fluorescence microscopy, and panel 4 is the phase-contrast image of the identical field shown in panel 3.
Our previous studies demonstrate that *Pneumocystis* organisms contain melanins when freshly isolated from infected hosts (15). In an effort to manipulate the melanin content of *Pneumocystis*, we next incubated the organism in the presence of the melanin precursor L-DOPA and observed markedly enhanced pigmentation of *Pneumocystis*. The morphological differences between native *Pneumocystis* and organisms with enhanced melanin contents were demonstrated both by visual and by electron microscopic detection, showing a dramatic difference in the pigmentation of *Pneumocystis*. Interestingly, even at the level of electron microscopy, excessive electron dense deposits were noted in *Pneumocystis* with enhanced melanization. Multiple controls were performed to ensure that the *Pneumocystis* organisms were responsible for pigment production, including incubation of L-DOPA alone, incubation of L-DOPA with normal rat lung tissue, and incubation of L-

![FIG. 4. Immunoelectron microscopic localization of *Pneumocystis* melanins. Melanins were visualized by using MAb 6D2 antibody and immunogold particle detection. Panel A demonstrates a *Pneumocystis* cyst containing four visible intracystic bodies, and panel B is a magnified portion of panel A as indicated by the inset box in panel A.](image)

![FIG. 5. *Pneumocystis* melanins protect organisms viability. Shown are the *Pneumocystis* heat shock protein 70 content (in arbitrary units) as a means to assess the viability of native melanized and highly melanized (l-DOPA driven) *Pneumocystis* after exposure to environmental stressors. The left bars depict the relative amounts of *Pneumocystis* heat shock protein 70 in highly melanized and native melanized *Pneumocystis* after treatment with UV irradiation. Heat shock protein 70 measurements were collected 48 h after UV irradiation treatment. The right bars depict the relative amounts of *Pneumocystis* heat shock protein 70 in highly melanized and native melanized *Pneumocystis* after desiccation for 48 h. **, *P < 0.004; ***, *P < 0.008 (comparing native melanized to highly melanized *Pneumocystis*).](image)
DOPA with supernatants collected from *Pneumocystis*-infected rat lung tissue homogenates. In all of these control conditions no pigments were produced, confirming that *Pneumocystis* generated these pigments. Since *Pneumocystis* cannot be grown in vitro or manipulated genetically, the ability to further induce melanization after the organisms are isolated from their host is also a significant finding, since it provides the first available means to study the functional correlates of higher and lower melanin contents in differential *Pneumocystis* populations.

We further utilized ESR spectra to verify that *Pneumocystis* organisms contain melanins. ESR spectroscopy has been widely used to identify fungal melanins based on the properties of unpaired electrons of the melanin polymer (reviewed in reference 24). Both *Pneumocystis* organisms derived from rat and mouse and isolated *P. carinii* melanin ghosts provided typical ESR spectra, further defining the presence of the pigments. This approach provided an important independent strategy, beyond immune detection, to document the presence of melanin pigments in this organism.

Recent investigations indicate that separate species of *Pneumocystis* infect specific mammalian host species. In that light, it is noteworthy that we were able to detect melanins in *Pneumocystis* species derived from rats, mice, ferrets, and humans, indicating that the fungal pigments are widely expressed across the genus. We further performed immunoelectron microscopy to identify the precise location(s) of melanins within the *Pneumocystis* cyst. Melanins were found predominantly in the *Pneumocystis* cyst cell wall and cytoplasm, as well as in the intracystic body cell wall and cytoplasm. This pattern of melanin distribution in *Pneumocystis* is consistent with that found in other fungi, mainly exhibiting cell wall deposition (30). Cell wall localization of this pigment would place it in a position most likely to protect the organisms from adverse environmental challenges, including ambient UV light and drying.

Interestingly, anti-melanin antibodies do not appear to bind to all *Pneumocystis* cysts in freshly isolated populations of *Pneumocystis*, using either the immunofluorescence or immunoelectron microscopy assays. This finding may be due to variation in melanin expression during different stages of the *Pneumocystis* life cycle. Other explanations could include that the anti-melanin antibody may not bind to all types of melanins and that there may be synthesis of more than one type of melanin by *Pneumocystis*. In addition, one might postulate differential exposure of melanin domains under various circumstances that are not recognized by the MAb. Alternatively, the apparent absence of melanins in some of the *Pneumocystis* cysts may reflect the presence of more than one species or strain of *Pneumocystis* in our rat colony, suggesting that not all species of rat *Pneumocystis* may synthesize melanins. If the latter case is true, differences in melanin production may factor into competition efficiencies of *Pneumocystis* species within the rat lung. It is common for an individual rat to harbor more than one species of *Pneumocystis*, and these species have been shown to compete for resources within the lung (15–17).

Beyond the initial identification of melanins in *Pneumocystis*, it was crucial to determine the function of *Pneumocystis* melanins related to the survival of the organism. Native melanized *Pneumocystis* and *Pneumocystis* with excess melanin production were treated with UV irradiation and desiccation to determine whether melanins offer protection from these common environmental stressors. The viability of the excessively melanized *Pneumocystis* was significantly greater than native *Pneumocystis* for both treatments. These data are consistent with the putative roles of melanins in other fungal species (1, 27) and further support our hypothesis that melanins are a potential virulence factor for *Pneumocystis*. In this case, melanins would preserve *Pneumocystis* viability as the organisms spread from one host to another (via airborne transmission) by protecting the fungus from sunlight and/or the less humid conditions outside the host lung. Melanins have also been shown to provide cell wall strength and rigidity and to serve as free radical scavengers. These activities provide potential mechanisms for protection against UV irradiation and desiccation.

It is possible that environmental stresses such as UV irradiation or desiccation may in fact upregulate the expression of Hsp70, since this protein is known to respond in other cell system stressors. However, if anything, upregulation of Hsp70 in response to stress would serve to reduce the observed difference in Hsp70 expression between treated and untreated *Pneumocystis*. Despite this, we still observed significant differences in viability *Pneumocystis* with basal melanin and in organisms with enhanced production of melanin. Thus, we believe that Hsp70 mRNA determination is a useful method to determine organism viability in *Pneumocystis* during environmental stress.

Melanins have been reported to exhibit a wide range of activities in other fungal species. The molecular precursors and the long-chain polymeric structure of fungal melanins strongly suggest that they act as potent cross-linking agents to stabilize the fungal cell wall (20). Indeed, melanized fungal cell walls are resistant to hydrolysis, as well as enzymatic digestion (5, 37). Other investigations strongly implicate melanin production in fungal pathogenesis. Melanized species and strains of *Cryptococcus*, namely, *C. neoformans*, are particularly associated with pulmonary and central nervous system infection in both humans and animals (21). Furthermore, melanin-deficient mutants of *Wangiella dermatitidis* are also associated with reduced virulence (6). Additional mechanisms through which melanins may promote enhanced pathogenesis have been proposed. These include melanin acting as a redox buffer, protecting the fungus against oxidative and free radical-based killing within the host (20). Similar to our present studies, cell wall melanins has also been linked with resistance to UV light in *C. neoformans* (37). Studies of the pathogenic role of melanins in *Pneumocystis* are currently challenging, since they would require isolation of *Pneumocystis* strains permanently lacking or having constitutively enhanced melanin production. However, the current study provides the first solid evidence that *Pneumocystis* widely expresses this important cell wall component and that it enhances survival of the organism under common environmental conditions.

In summary, these investigations demonstrate that *Pneumocystis* can synthesize melanins, that these melanins are associated with the cyst and troph cell wall and cytoplasm, and that melanins protect *Pneumocystis* from environmental insults as the organism is transmitted from one host to the next. Evidence of melanins in *P. carinii* is of great interest due to the roles melanins play in pathogenic fungi. Our investigation further demonstrates the first convenient laboratory method through which *Pneumocystis* can be induced to synthesize ex-
cess melanins, providing a useful system to study the various potential roles of these pigments in *Pneumocystis*. Additional studies to fully understand the activities of melanins in *Pneumocystis* pathogenesis, defense against additional environmental stressors, and alteration of host-induced damage, as well as the biochemical pathways used by *Pneumocystis* to synthesize melanins, are currently under way.

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