Activation of the Alternative Complement Pathway by Fungal Melanins

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Melanins are complex biological pigments formed by the oxidative polymerization of phenolic and/or indolic compounds. These pigments have been implicated in the pathogenesis of some microbial infections, malignancies, degenerative disorders, and autoimmune diseases. Recent studies have demonstrated that melanins have antigenic and anti-inflammatory properties. These findings led us to further explore the interaction of melanins with the immune system. Melanin particles (“ghosts”) were isolated from in vitro-melanized Cryptococcus neoformans cells and Aspergillus niger conidia and then incubated in normal human serum containing 125I-labeled complement C3. The results demonstrated deposition of C3 fragments onto the melanin ghosts as early as 1 min after incubation, with maximum deposition occurring after 12 min for C. neoformans-derived melanin ghosts and after 25 min for A. niger-derived melanin ghosts. The blocking of classical pathway activation did not affect the kinetics or total deposition of C3 onto the melanin ghosts, indicating that melanins activate complement through the alternative pathway. Immunofluorescence analysis of lungs from BALB/c mice injected intratracheally with C. neoformans-derived melanin ghosts demonstrated deposition of C3 fragments onto the ghosts. Small granulomas were also observed surrounding the ghosts. However, melanization of the C. neoformans cell wall did not alter the kinetics or total deposition of C3 fragments onto the fungal cells. The finding that melanin surfaces can activate the complement system suggests a potential mechanism for the pathogenesis of some degenerative and/or autoimmune processes that involve melanized cells as well as another potential role for melanin in the virulence of melanin-producing microorganisms.

MATERIALS AND METHODS

Fungal cells. C. neoformans serotype D strains 3501 and 24067 and Aspergillus niger strain J9901 were purchased from the American Type Culture Collection (Manassas, Va.).

Melanization of fungal cells. C. neoformans 3501 was grown in a defined minimal medium (15 mM glucose, 10 mM MgSO4, 29.4 mM KH2PO4, 13 mM glycine, 3 μM vitamin B1; pH 5.5) with or without 1 mM L-3,4-dihydroxyphenylalanine (l-DOPA) (Sigma Chemical Co., St. Louis, Mo.) for 8 days at 30°C in a rotary shaker at 150 rpm. Only cells grown with l-DOPA became melanized. C. neoformans 24067 was grown for 15 days in minimal medium with l-DOPA.

A. niger was grown on Sabouraud dextrose agar (pH 5.6) (Difco Laboratories, Detroit, Mich.) for 5 months at 30°C to allow heavy melanization of the conidia.

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Isolation of fungal melanins. Melanin was extracted from melanized C. neoformans J9901 conidia and A. niger conidia by treatment with protolytic and glycolytic enzymes, with a denaturant, by chloroform extractions, and with boiling HCl, as described previously (34). Melanin particles, also referred to as melanin “ghosts” (40), were collected by centrifugation, washed extensively, and dialyzed against distilled water for 10 days. Melanin ghosts were then washed and suspended in phosphate-buffer saline (PBS; pH 7.4). Although the purity of the melanin ghosts cannot be established unequivocally because of the lack of knowledge about the structure of any melanin, the melanins used in this study consistently gave the same C/N/O ratios for the same melanin after quantitative analyses carried out as described in reference 35, which strongly suggests that the preparations were free of other cellular components.

**Serum and serum proteins.** Blood was collected from at least 10 donors. The sera were isolated, pooled, and stored at −85°C until use. C3 was isolated from frozen human plasma as described previously (20, 38) and labeled with 125I by using Iodogen (Pierce, Rockford, Ill.) according to the manufacturer’s instructions.

**Kinetic analyses of C3 binding in vitro.** The kinetics of activation and binding of C3 fragments to melanin ghosts or C. neoformans 3501 cells (melanized and nonmelanized) were assessed in 1-mL reaction mixtures consisting of (i) 40% normal human serum (NHS), (ii) GVB2* (5 mM sodium Veronal-buffered saline [142 mM] [pH 7.3] containing 0.1% gelatin, 0.15 mM CaCl2, and 1 mM MgCl2), or GVB-MgEGTA (5 mM sodium Veronal-buffered saline [142 mM] [pH 7.3] containing 0.1% gelatin, 10 mM EGTA, and 10 mM MgCl2), (iii) 125I-labeled C3 sufficient to provide a specific activity of 50,000 cpm/μg of C3 for the mixture of labeled and unlabeled C3 in serum, and (iv) 6.0 × 107 melanin ghosts or heat-killed cryptococcal cells. The tubes containing all of the reagents except melanin ghosts or cells were warmed for 5 min at 37°C, and the reaction was initiated by addition of the ghosts or cells. Duplicate 30-μL samples were then withdrawn at intervals of 1, 2, 4, 6, 8, 10, 12, 16, 20, and 25 min and placed into 150 μL of stop solution consisting of PBS with 0.1% sodium dodecyl sulfate and 20 mM EDTA. The melanin ghosts or cells were washed four times with PBS–0.1% sodium dodecyl sulfate by using Millipore (Bedford, Mass. USA) MABV-N12 filter plates fitted with BV 1.2-μm-pore-size filter membranes. The membranes were removed, and the amount of specifically bound C3 was determined by subtracting the radioactivity of samples that were heat-inactivated NHS from the total amount bound with NHS. Binding data are reported as the numbers of bound C3 molecules per melanin ghost or cryptococcal cell versus incubation time. For C. neoformans cells, C3 binding data were adjusted to the relative capsule size of cells grown in the presence or absence of L-DOPA to account for any differences in capsule size between the groups. Capsule sizes were determined by light microscopy by using an eyepiece with a grid with a 1-mm-mesh resolution. The data shown are representative of at least two replicate experiments for C. neoformans-derived melanin ghosts and from one experiment for A. niger-derived melanin ghosts.

**Immunofluorescence (IF) analysis of C3 binding to melanin.** C. neoformans-derived melanin ghosts (4 × 109) were incubated in 40% NHS for 2, 4, 8, and 16 min as described above, with the exception that 10% C3 was not added to the reaction mixture. After the reaction was stopped, the melanin ghosts were washed three times with PBS, resuspended in 100 μL of a 1/25 dilution of fluorescent isothiocyanate (FITC)-conjugated antiserum to human C3 (Kent Laboratories, Inc., Redmond, Wash.) in PBS containing 1% bovine serum albumin (Sigma Chemical Co.), and incubated for 1 h at 0°C. The melanin ghosts were washed twice with PBS, suspended in 1 drop of Vectashield (Vector Laboratories, Inc., Burlingame, Calif.), applied to poly-l-lysine-coated slides (Sigma Chemical Co.), and allowed to settle overnight at room temperature. The pattern of C3 deposition was determined by epifluorescence microscopy with a Nikon Eclipse E800 microscope with an oil immersion lens objective of 100×. Images were collected at 0.4-μm distances with a Photonic Science (Millham, United Kingdom) integrating charge-coupled-device camera driven by Image Pro Plus version 3.0 image analysis software (Media Cybernetics, Silver Spring, Md.).

**Immunohistochemical analysis of C3 binding to melanin.** Five female BALB/c mice (6 to 8 weeks old) (National Cancer Institute, Rockville, Md.) were injected intratracheally with 107 C. neoformans-derived melanin ghosts. Mice were killed 7 days after infection, and the lungs were collected for analysis. Tissue sections 4 μm thick were deparaffinized in xylenes and rehydrated by serial incubations in solutions with decreasing ethanol concentrations. The samples were blocked with SuperBlock blocking buffer (Pierce) for 4 h at room temperature to prevent nonspecific binding and then incubated with FITC-conjugated polyclonal goat anti-mouse C3 (ICN Pharmaceuticals, Inc., Aurora, Ohio) for 1.5 h at 37°C. As a control, melanin ghosts were incubated with FITC-conjugated polyclonal goat anti-mouse C3 as described above. The samples were washed with PBS, a mounting solution (50% glycerol and 0.1 M N-propyl gallate in PBS) and coverslips were applied to the slides, and the samples were examined with an Olympus America Inc. (Melville, N.Y.) AX70 microscope at a magnification of ×250.

**RESULTS**

**Complement activation by melanin in vitro.** Melanin ghosts were isolated from in vitro-melanized C. neoformans 24067 cells and A. niger J9901 conidia and then incubated in 40% NHS containing 125I-labeled C3. Deposition of C3 fragments onto the melanin ghosts occurred as early as after 1 min of incubation, reaching maximum depositions of approximately 1.9 × 106 C3 fragments per C. neoformans-derived melanin ghost after 12 min and 1.3 × 106 C3 fragments per A. niger-derived melanin ghost after 25 min (Fig. 1). The treatment of NHS with EGTA, which prevents activation of the classical pathway by chelating Ca2+ (13, 31), did not affect the kinetics or total deposition of C3 fragments onto the melanin ghosts (data not shown), indicating that complement activation by melanins occurs solely through the alternative pathway. Comparisons between the classical and alternative pathways are difficult to make, however, because of the 10-fold-higher concentration of MgCl2 used in the reaction mixture for alternative complement activation, which provided greater stability to the mixture. The concentration of MgCl2 was increased to account for chelation by EGTA. IF analyses of C. neoformans-derived melanin ghosts incubated in NHS revealed that the initial deposition of C3 fragments occurred at discrete focal sites. The number of discrete focal sites increased with increased incubation time, but the distribution of C3 never reached confluence (Fig. 2). We did not use synthetic melanin in these studies, because synthetic melanin is usually made by
incubating the precursor (e.g., L-DOPA) with chemical oxidants, such as H₂O₂, to increase the rate of polymerization into melanin. However, during the formation of synthetic melanin, these oxidants induce other modes of polymerization (33) as well as degradation of some of the key intermediates of the natural melanogenic pathway (37); therefore, the final product is likely to consist of a mixture of various polymerized structures. Thus, the commercially available synthetic melanin cannot be used as an adequate control for these studies. On the other hand, if chemical oxidants are not used, very large batches of reaction mixture (i.e., precursor in suspension) or a very long incubation time (over a year) would be required to yield enough melanin for use. In that event, there would be no guarantee that the synthetic product was the same as the material synthesized catalytically by the fungus.

**Complement activation by melanin in vivo.** Approximately 10⁷ C. neoformans-derived melanin ghosts were injected intratracheally into BALB/c mice to determine whether melanin ghosts could activate complement in murine tissue. IF analysis of lungs 7 days after injection demonstrated deposition of C3 fragments onto the melanin ghosts (Fig. 3A and B). Small granulomas were also observed surrounding the melanin ghosts (Fig. 3C).

**Kinetics of complement activation by melanized and nonm melanized C. neoformans in vitro.** Melanized and nonmelanized C. neoformans 3501 cells were incubated in 40% NHS containing ¹²⁵I-labeled C3 to determine whether melanin could affect complement activation by cryptococcal cells. For both melanized and nonmelanized C. neoformans, deposition of readily measurable amounts of C3 fragments onto the cells occurred only after a lag of approximately 4 min, reaching a maximum of approximately 1.5 × 10⁷ C3 fragments per cell after 16 min of incubation (Fig. 4). No appreciable differences in the numbers of bound C3 fragments between melanized and nonmelanized C. neoformans cells were observed.
nonmelanized cells were observed at any time period after the data were corrected to account for differences in capsule size between the melanized and nonmelanized cells.

**DISCUSSION**

Melanins are immunologically active molecules (1, 25, 27). Furthermore, melanization slightly increases the negative charge of *C. neoformans* cells (26) and affects their interaction with host effector cells (2, 37). These observations led us to explore the potential complement activation properties of melanins and whether melanization had an effect on complement activation by *C. neoformans*. Quantitative and IF analyses demonstrated that incubation of fungal melanins in NHS induced deposition of C3 fragments at numerous discrete focal sites that failed to become confluent over the surfaces of the melanin ghosts. This is an important qualitative difference from the pattern of C3 deposition observed with encapsulated and nonencapsulated *C. neoformans* cells, where deposition of C3 becomes confluent (21). The failure to deposit confluent C3 on the melanin ghosts may reflect a heterogenous surface and/or fundamental differences between the particles in the actions of the alternative pathway regulatory proteins factor H and factor I (30).

Approximately $1.9 \times 10^6$ C3 fragments bound to each *C. neoformans*-derived melanin ghost after 12 min of incubation in NHS, whereas approximately $1.3 \times 10^6$ C3 fragments bound to each *A. niger*-derived melanin after 25 min. The apparent difference in binding capacities may be due to the difference in size between the fungal melanins, as *C. neoformans*-derived melanin ghosts are approximately twice as large as *A. niger*-derived melanin ghosts (35) and therefore present a larger surface for the deposition of C3 fragments. IF analysis of murine lungs also demonstrated deposition of C3 fragments onto *C. neoformans*-derived melanin ghosts, indicating that melanin can activate the complement system in vivo.

Despite the ability of melanin to activate complement, the melanization of *C. neoformans* did not affect the kinetics or total deposition of C3 fragments onto the polysaccharide capsule of the cryptococcal cells. Melanin in *C. neoformans* is found in the cell wall (39), where it is shielded from the extracellular milieu by the polysaccharide capsule and therefore may not be able to interact with components of the complement system. The slight increase in negative charge in melanized cells may not be sufficient to significantly affect the interaction of the polysaccharide capsule with C3 molecules. Although we were unable to demonstrate a difference in levels of complement activation between melanized and nonmelanized cells, extracellular or cell wall-associated melanins produced by nonencapsulated pathogens may influence complement activation by the microbial surface. The interaction of exposed microbial melanins with the complement system may lead to complement depletion, which in turn may result in decreased susceptibility to complement-mediated phagocytosis by host effector cells. This would provide another mechanism for melanin in the virulence of microbial pathogens.

Recent studies have demonstrated that natural and synthetic melanins have anti-inflammatory properties (1, 25), which may be due to the ability of melanins to neutralize free radicals and oxidants (17, 23) and to suppress the production of proinflammatory cytokines (1, 25). The results from our study, however, indicate that melanins can activate the alternative complement pathway, suggesting a mechanism by which they can induce an inflammatory response. Complement activation by melanin particles would be expected to generate chemotactic and proinflammatory complement split products. In our studies, we noted that the injection of melanin into murine lungs elicited an inflammatory response, as indicated by the formation of small granulomas. Although these studies present contradictory properties for melanins, it is possible that the complex structures of these pigments allow for seemingly opposite roles. Our results suggest the possibility that other natural melanins, perhaps including human eumelanins, have the potential to activate complement in vivo. This ability would indicate a new biological activity for melanins which could contribute to the pathogenesis of several human autoimmune diseases and degenerative disorders.

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