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Galleria mellonella as a Model System To Study *Cryptococcus neoformans* Pathogenesis

Eleftherios Mylonakis,[1]* Roberto Moreno,[1] Joseph B. El Khoury,[1,3,4] Alexander Idnurm,[5] Joseph Heitman,[5,6,7,8] Stephen B. Calderwood,[1,9] Frederick M. Ausubel,[2,10] and Andrew Diener[2]

Division of Infectious Diseases,[1] Department of Molecular Biology,[2] Center for Immunology and Inflammatory Diseases,[3] and Division of Rheumatology, Allergy, and Immunology,[4] Massachusetts General Hospital, Boston, Massachusetts 02114; Department of Molecular Genetics and Microbiology,[5] Division of Infectious Diseases,[6] Department of Medicine,[7] and Howard Hughes Medical Institute,[8] Duke University Medical Center, Durham, North Carolina 27710; and Department of Microbiology and Molecular Genetics[9] and Department of Genetics,[10] Harvard Medical School, Boston, Massachusetts 02115

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Evaluation of *Cryptococcus neoformans* virulence in a number of nonmammalian hosts suggests that *C. neoformans* is a nonspecific pathogen. We used the killing of *Galleria mellonella* (the greater wax moth) caterpillar by *C. neoformans* to develop an invertebrate host model system that can be used to study cryptococcal virulence, host immune responses to infection, and the effects of antifungal compounds. All varieties of *C. neoformans* killed *G. mellonella*. After injection into the insect hemocoel, *C. neoformans* proliferated and, despite successful phagocytosis by host hemocytes, killed caterpillars both at 37°C and 30°C. The rate and extent of killing depended on the cryptococcal strain and the number of fungal cells injected. The sequenced *C. neoformans* clinical strain H99 was the most virulent of the strains tested and killed caterpillars with inocula as low as 20 CFU/caterpillar. Several *C. neoformans* genes previously shown to be involved in mammalian virulence (*CAP59*, *GPA1*, *RAS1*, and *PKA1*) also played a role in *G. mellonella* killing. Combination antifungal therapy (amphotericin B plus flucytosine) administered before or after inoculation was more effective than monotherapy in prolonging survival and in decreasing the tissue burden of cryptococci in the hemocoel. The *G. mellonella*-*C. neoformans* pathogenicity model may be a substitute for mammalian models of infection with *C. neoformans* and may facilitate the in vivo study of fungal virulence and efficacy of antifungal therapies.

The yeast *Cryptococcus neoformans* is amenable to a variety of genetic manipulations, making it an excellent model fungal pathogen in which to identify and study fungal virulence factors. *C. neoformans* is an important human pathogen that infects individuals with compromised immune function and less commonly infects immunocompetent hosts as well (30, 36). Recent studies have taken advantage of the broad host range of *C. neoformans* to develop facile invertebrate model systems utilizing the amoeba *Acanthamoeba castellanii* (44), the nematode *Caenorhabditis elegans* (31), the slime mold *Dictyostelium discoideum* (45, 46), and the insect *Drosophila melanogaster* (3) as hosts for the study of established virulence attributes or to identify novel genes involved in virulence (32). Some of these previously developed alternative models are limited by the inability of the host system to survive at 37°C, the difficulty of administering exact fungal inocula, or the difficulty of administering antifungal chemotherapy. Moreover, wild-type *D. melanogaster* is extremely resistant to systemic infection by a number of fungi, including *C. neoformans* (3).

To address these limitations, we developed a new system using caterpillars of the greater wax moth (*Galleria mellonella*) as an alternative model for the study of *C. neoformans*. We report here that various serotypes of *C. neoformans* proliferate inside the hemocoel and kill the caterpillar, even though the fungi undergo phagocytosis by *G. mellonella* hemocytes. Caterpillar killing correlates with the number of CFU of *C. neoformans* inoculated, and virulence factors involved in mammalian cryptococcal infection play a significant role in *G. mellonella* killing. The efficacy of antifungal agents in this system is similar to results obtained in studies with humans, suggesting that the model may be developed for study of the in vivo efficacy of antifungal therapy.

* Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, Gray-Jackson 504, 55 Fruit St., Boston, MA 02114. Phone: (617) 726-3812. Fax: (617) 726-7416. E-mail: emylonakis@partners.org.

MATERIALS AND METHODS

Strains and media. The *C. neoformans* strains used in these experiments are summarized in Table 1 or described in the text. Fungal cultures were maintained on yeast peptone dextrose (YPD) agar (Difco) and as frozen stocks. *C. neoformans* strains were grown at 30°C and strains of *Cryptococcus laurentii* were grown at 24°C. All strains were grown in YPD with aeration.

***G. mellonella* killing assay.** *G. mellonella* caterpillars in the final instar larval stage (Vanderhorst, Inc., St. Marys, Ohio) were stored in the dark and used within 7 days from the day of shipment. Caterpillars (330 ± 25 mg in body weight) were employed in all assays. Twelve to 16 randomly chosen caterpillars of the required weight were used per group.

A 10-μl Hamilton syringe was used to inject 10-μl aliquots of the inoculum into the hemocoel of each caterpillar via the last left proleg (9). Before injection, the area was cleaned using an alcohol swab. A total of 20 mg of ampicillin/kg of body weight was coadministered to prevent infection by bacteria naturally present on the surface of the caterpillar. After injection, caterpillars were incu-

TABLE 1. Fungal strains used and their interaction with *G. mellonella*[a]

C. neoformans strain (reference[5])	Relevant characteristics or phenotype (reference [5])	Median time to mortality for *G. mellonella* killing at 37°C	*P* value
H99, ATCC 208821 (19, 41)	Serotype A; clinical isolate; genome sequence available	5 days after injection of 1.5×10^4 CFU/larva (killing by other inocula detailed in Fig. 1)	
H99 *gpa1* (2)	*GPA1* encodes a G-protein alpha-subunit homolog; mutant is attenuated in mammalian models (2) and *C. elegans* (28)	8.5 days after injection of 1.5×10^4 CFU/larva (Fig. 3A)	0.01
H99 *gpa1* + *GPA1* (2)	Complementation of the *gpa1* mutant with wild-type *GPA1* restored virulence in mammals (2) and *C. elegans* (31)	5 days after injection of 1.5×10^4 CFU/larva[b]	
H99 *pka1* (12)	*PKA1* encodes the major cAMP-dependent protein kinase catalytic subunit; mutant attenuated in mammalian models (12) and *C. elegans* (31)	9 days after injection of 1.5×10^4 CFU/larva (Fig. 3A)	0.01
H99 *pka1* + *PKA1* (12)	Complementation of the *pka1* mutant with wild-type *PKA1* restored virulence in mammals (12) and *C. elegans* (31)	5 days after injection of 1.5×10^4 CFU/larva[b]	
H99 *ras1* (1)	*ras1* mutant is avirulent in an animal model of cryptococcal meningitis (1) and hypovirulent in *C. elegans* (31)	>16 days after injection of 1.2×10^4 CFU/larva (Fig. 3A)	<0.001
H99 *ras1* + *RAS1* (1)	Complementation of the *ras1* mutant with wild-type *RAS1* restored virulence in rabbits (1) and *C. elegans* (31)	5 days after injection of 1.5×10^4 CFU/larva[b]	
H99 *cap59* (33, 34)	*CAP59* is essential for capsule formation (33, 34); mutant is avirulent in mammals and *A. castellanii* (44) and hypovirulent in *C. elegans* (31) and *D. melanogaster* (3)	>16 days after injection of 1.5×10^4 CFU/larva (Fig. 3A)	<0.001
H99 MFα1::GFP (11)	As virulent as H99 in rabbits (11)	5 days after injection of 1.5×10^4 CFU/larva[b]	
KN99**a** (35)	Congenic H99 mating parent, *MAT***a**; in mice, virulence similar to H99 and KN99α (35)	5 days after injection of 1.5×10^4 CFU/larva[c]	
KN99α (35)	Congenic H99 mating parent, *MAT*α; in mice, virulence similar to H99 and KN99**a** (35)	5 days after injection of 1.5×10^4 CFU/larva[d]	
KN99-5 (35)	Fifth backcross in creation of KN99**a** and KN99α	5 days after injection of 1.5×10^4 CFU/larva[e]	
KN99α *kin1* + *KIN1* (32)	*MAT*α product of *kin1* + *KIN1-NEO*	5 days after injection of 1.5×10^4 CFU/larva[c]	
ATCC 62068 (26)	Serotype A; isolated from bovine lymph node	5 days after injection of 1.5×10^4 CFU/larva	
NIH 444; ATCC 32609 (22)	Serotype B; MATα; sputum isolate, Washington	11 days after injection of 1.5×10^4 CFU/larva	
NIH 76, ATCC 34877	Serotype B; isolated from cerebrospinal fluid	10 days after injection of 1.5×10^4 CFU/larva	
C. neoformans var. *gattii* R265 (15)	Serotype B; clinical isolate	At inoculum of 2×10^3 CFU/larva, strain was avirulent, while inoculum of 10^4 CFU/larva was required to reach 100% killing with a mean time to mortality of 12 days	
NIH 312, ATCC 34880, CDC B3182	Serotype C; MATα mating type	Avirulent at 5×10^3 CFU/larva or less, killing reached 60% by day 14 at inocula 1.5×10^4 CFU/larva and reached 75% at 1.75×10^4CFU/larva (with mean time to mortality 11 days at this inoculum)	
JEC20, ATCC 96910; JEC21, ATCC 96909 (23)	Serotype D strains; a pair of congenic MAT**a** (JEC20) and MATα (JEC21) strains; genome sequence available	Day 8.5 for JEC21 versus day 16 for JEC20 after injection of 2.2×10^4 CFU/larva	0.001
ATCC 208820, 2e-tuc4 (40)	Serotype D; MATα *CNLAC1*; laccase positive	Day 8 after injection of 2.5×10^4 cfu/larva (Fig. 4)	
ATCC 208819, 2e-tu4 (40)	Serotype D; MATα *cnlac1*; laccase negative	Day 12 after injection of 2.5×10^4 CFU/larva (Fig. 4)	0.001[f]
C. neoformans ATCC 32308	Environmental isolate, India	13 days after injection of 1.5×10^4 CFU/larva	
C. laurentii ATCC 76483	Nonpathogenic in *C. elegans* (31)	>17 days after injection of 2.5×10^4 CFU/larva	

[a] All experiments included in Table 1 were performed at 37°C. *P* values report comparisons to parent strain, when relevant.
[b] *P* value indicates no difference from parent strain.
[c] *P* value indicates no difference compared to strains H99 and KN99α.
[d] *P* value indicates no difference compared to strains H99 and KN99**a.**
[e] *P* value indicates no difference compared to strains H99, KN99α, and KN99**a.**
[f] *P* value compared to 2e-tuc4.

bated in plastic containers, and the number of dead caterpillars was scored daily. Caterpillars were considered dead when they displayed no movement in response to touch.

Antifungal drugs were injected using the same technique. For experiments that required multiple injections, a different proleg was used for each injection, starting from the left last proleg and rotating left to right and moving proximally (i.e., injecting through the left last proleg, right last proleg, penultimate left proleg, and penultimate right proleg, as needed). Antifungal agents used were amphotericin B (Sigma), fluconazole (Toronto Research Chemicals), and flucytosine (InvivoGen). All antifungal drugs were diluted in water, and each antifungal agent was administered once by a separate injection.

To heat kill *C. neoformans*, yeast cells were exposed to 60°C for 45 min. Heat killed *C. neoformans* cells were washed three times, resuspended in sterile phosphate-buffered saline (PBS), diluted to the appropriate density, and inoculated into the caterpillars. For preparation of the inocula, cultures were diluted as needed, using a hemocytometer to determine the exact dose administered. The concentration of cryptococcal cells in the inoculum was confirmed by plating serial dilutions on plates with YPD medium containing ampicillin (100 μg/ml) and streptomycin (100 μg/ml). After inoculation, caterpillars were incubated at 37°C, unless otherwise specified. A mock inoculation with PBS was performed in each experiment to monitor killing due to physical injury or infection by pathogenic contaminants. In most experiments, no caterpillars in this control group died. Rare experiments with more than two dead caterpillars in the control group were discarded and repeated. For simplicity, the control group is not included in some figures.

Killing curves were plotted and estimation of differences in survival (log rank and Wilcoxon tests) analyzed by the Kaplan-Meier method using STATA 6 statistical software (Stata). The same software program was used for the statistical analysis of the CFU of *G. mellonella* in the hemocoel (Mann-Whitney and Kruskal-Wallis tests). A P value of <0.05 was considered significant. Each experiment was repeated at least three times, and each independent experiment gave similar results. Data presented here are from a representative experiment.

Tissue burden culture studies. For the evaluation of the tissue burden of *C. neoformans* in caterpillars over time, five caterpillars per group were weighed and homogenized in 1 ml sterile PBS with a Tissue Tearor (model 398; Biospec Products), and serial dilutions of the homogenates were plated on YPD agar plates containing ampicillin (100 μg/ml), streptomycin (100 μg/ml), and nourseothricin (100 μg/ml). Plates were incubated at 30°C for 72 h before colonies were counted.

Collection of hemocytes. At selected time points after inoculation, 6 to 10 caterpillars per group were bled by insertion of a lancet into the hemocoel. Hemolymph was collected into ice-cold anticoagulant saline (54) with or without EDTA (Fluka). Hemolymph was centrifuged, the supernatant was discarded, and cells were diluted in Grace's insect medium (Gibco). Specimens were fixed in 4% paraformaldehyde (Electron Microscopy Sciences). For clarity in experiments, we used cryptococcal cells that were stained before inoculation with fluorescein isothiocyanate (FITC; Molecular Probes) or, after specimen collection, with calcofluor white (Polysciences).

Some experiments involving cryptococcal strains containing the pYGFP3 plasmid (expressing the synthetic green fluorescent protein [GFP]) (11) used undiluted hemolymph. For these latter experiments, caterpillars were bled into an ice-cold test tube containing 1.5 mg phenylthiourea (Sigma) to prevent melanization and then used for microscopy without any further processing.

To determine if the cryptococcal cells interacting with the hemocytes were intracellular or extracellular, we incubated the mixture of cells containing the hemocytes and FITC-labeled *C. neoformans* with the dye trypan blue (Sigma). Trypan blue quenches FITC fluorescence but does not get inside the hemocytes, so only intracellular FITC-labeled *C. neoformans* were visualized in this manner, as previously described (48).

Light and fluorescence microscopy were performed using an Olympus BX51 microscope, and images were captured and processed using the digital camera Qimaging Retiga (Burnaby) and Olympus Microsuite Software. Vectashield mounting medium was used for fluorescence microscopy (Vector Laboratories).

RESULTS

Killing of *G. mellonella* by *Cryptococcus neoformans* var. *grubii* strain H99 (ATCC 208821). As shown in Fig. 1, inoculation of *G. mellonella* with the *C. neoformans* var. *grubii* sequenced strain H99 (ATCC 208821) resulted in rapid killing of the caterpillars. Killing depended on the number of cryptococcal cells injected (Fig. 1). In addition, caterpillar killing depended on the incubation temperature following inoculation; overall, the rate of killing was slower at 30°C. For example, after injection of 1.5×10^4 CFU/larva, median time to mortality was 9 days at 30°C (compared to 5 days at 37°C). Although a number of temperature-sensitive virulence traits of *C. neoformans* have been described, this is the first system in which *C. neoformans* demonstrated a higher degree of virulence at 37°C than at 30°C in the same host.

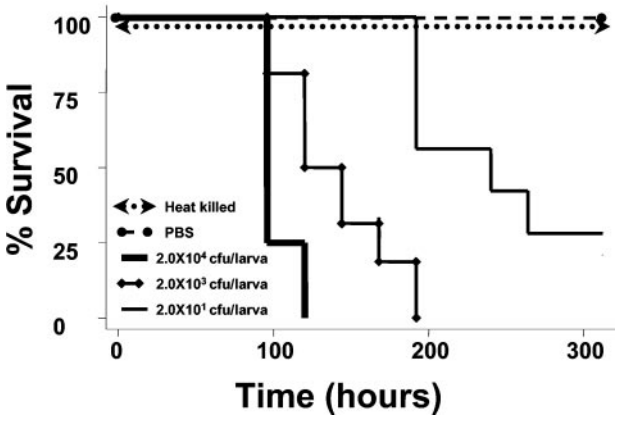

FIG. 1. Killing of *G. mellonella* caterpillars by *C. neoformans* depends on the number of cryptococcal cells inoculated. Kaplan-Meier plots of *G. mellonella* survival after injection of different inocula of *C. neoformans* strain H99 (ATCC 208821). There was no killing of caterpillars that received PBS or heat-killed cryptococcal cells of the same *C. neoformans* strain (10^5 cells/larva).

To evaluate whether *G. mellonella* killing was caused by a noninfectious reaction (for example, in the cryptococcal capsule), we determined the survival of caterpillars after injection of heat-killed *C. neoformans* or of yeast cells of the nonpathogenic *C. laurentii* (strain ATCC 76483). The *C. laurentii* capsule was considered to have extensive similarities to the capsule of *C. neoformans*. There was no killing in the group that received heat-killed *C. neoformans* (up to 10^5 cells/larva) or the group that received live *C. laurentii* (up to 10^5 cells/larva) (Table 1 and data not shown).

Killing of *G. mellonella* is not a unique feature of *C. neoformans* strain H99. We determined whether different varieties of *C. neoformans* could kill *G. mellonella* in addition to H99. As shown in Table 1, all *C. neoformans* strains tested were able to kill *G. mellonella* caterpillars, although strain H99, the most widely used *C. neoformans* strain for the study of pathogenesis, was the most virulent among the strains tested. Strain H99 killed caterpillars faster than other *C. neoformans* strains tested and at inocula as low as 20 CFU/larva. Substantially higher numbers of inocula were needed for killing of the caterpillars by other strains. For example, strain NIH 312 (ATCC 34880; CDC B3182) was avirulent at 5×10^3 CFU/larva or less. Killing reached 60% by day 14 at inocula of 1.5×10^4 CFU/larva and reached 75% at 1.75×10^4 CFU/larva (with mean time to mortality of 11 days at this inoculum). Similar results were obtained with several other *C. neoformans* strains (Table 1), including the clinical isolates of *C. neoformans* var. *grubii* strain ATCC 62068 (serotype A), *Cryptococcus neoformans* var. *gattii* strains ATCC 34877 and ATCC 32609 (NIH 444) (sero-

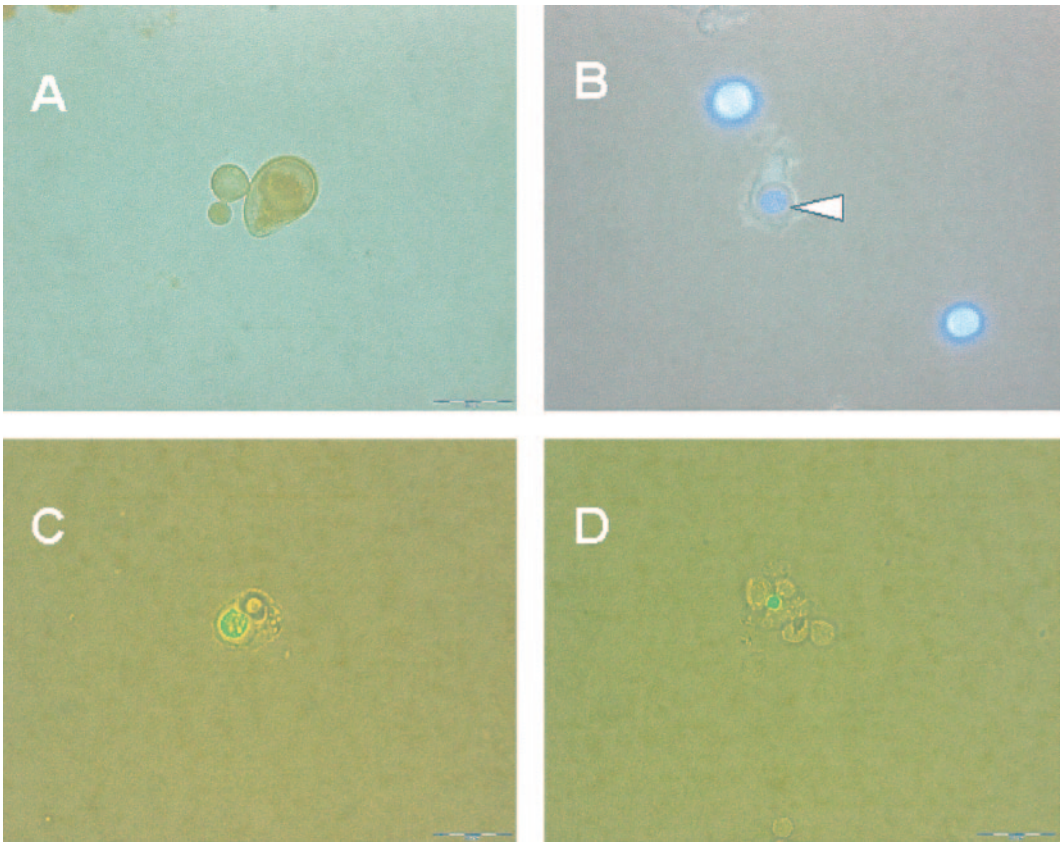

FIG. 2. Binding, phagocytosis, and nodulation of *C. neoformans* cells by *G. mellonella* hemocytes. *C. neoformans* fungal cells binding to (A) or phagocytosed by *G. mellonella* hemocytes (B and C) are shown. (D) A *C. neoformans* fungal cell is surrounded by layers of hemocytes in a process known as nodulation that has been described previously for *G. mellonella* in association with pathogens. Fungal cells (B) were stained with calcofluor white, and a phagocytosed *C. neoformans* is identified by a white arrowhead. (C and D) *C. neoformans* cells were stained with FITC before inoculation as described in Materials and Methods. Original magnification in all panels, ×100; bar, 50 μM.

type B) (22), and *Cryptococcus neoformans* var. *neoformans* strain ATCC 96909 (serotype D).

Tissue burden of *C. neoformans* in *G. mellonella* and the role of hemocytes. Because *G. mellonella* larvae might be colonized by other fungi, we used a strain resistant to the antimicrobial nourseothricin (KN99α [*kin1* + *KIN1*], designated KN99α *kin1* + *KIN1*) to evaluate the fungal burden in *G. mellonella*. This enabled us to plate homogenates of caterpillars on medium containing nourseothricin, along with broad-spectrum antibacterials, to inhibit growth of other yeasts and bacteria. Strain KN99α *kin1* + *KIN1* is congenic with H99 and has a level of virulence similar to that of H99 with mice and *C. elegans* (32), as well as with *G. mellonella* (Table 1). After injection of 2.2×10^3 CFU/larva, the proliferation in *G. mellonella* was slow at the beginning, but by day 3, the organism burden reached levels (mean ± standard deviation) of $4.2 \times 10^4 \pm 4.1 \times 10^3$ CFU/mg. The cryptococcal burden increased only slightly by the following day, when the caterpillars began to die. A control group of caterpillars was injected with PBS, and homogenates from these caterpillars were plated using a similar protocol. No organisms grew from this control group.

We also monitored the interaction between *C. neoformans* strain H99 and hemocytes. The host response of *G. mellonella* to infection consists of structural and passive barriers, as well as cellular and humoral responses that are performed by hemocytes within the hemolymph. Six types of hemocytes in *G. mellonella* have been identified, and the insect response includes phagocytosis by plasmatocytes and granulocytes and nodulation by layers of hemocytes, to encapsulate large invading pathogens (reviewed in reference 18). Over the first hours after injection, cryptococcal cells were bound to hemocytes (Fig. 2A), followed by a progressively increasing number of *C. neoformans* cells within hemocytes, indicating successful phagocytosis (Fig. 2B and C). By 24 h, we found a significant number of cryptococcal cells surrounded by layers of hemocytes (Fig. 2D) in a process known as nodulation, which has been described previously with *G. mellonella* in association with pathogens (21) and that may have some similarities with the granulomas associated with cryptococcal infection in mammals (16–18, 32).

Genes of *C. neoformans* associated with pathogenesis in mammals cause enhanced killing of *G. mellonella*. The most extensively studied *C. neoformans* virulence factors are the polysaccharide capsule (8, 37) and production of melanin (40, 52, 53). Capsule formation and melanin production are regulated by at least two signal transduction cascades. The first cascade is the cyclic AMP (cAMP)-dependent protein kinase A (PKA) signaling pathway. PKA is composed of a catalytic

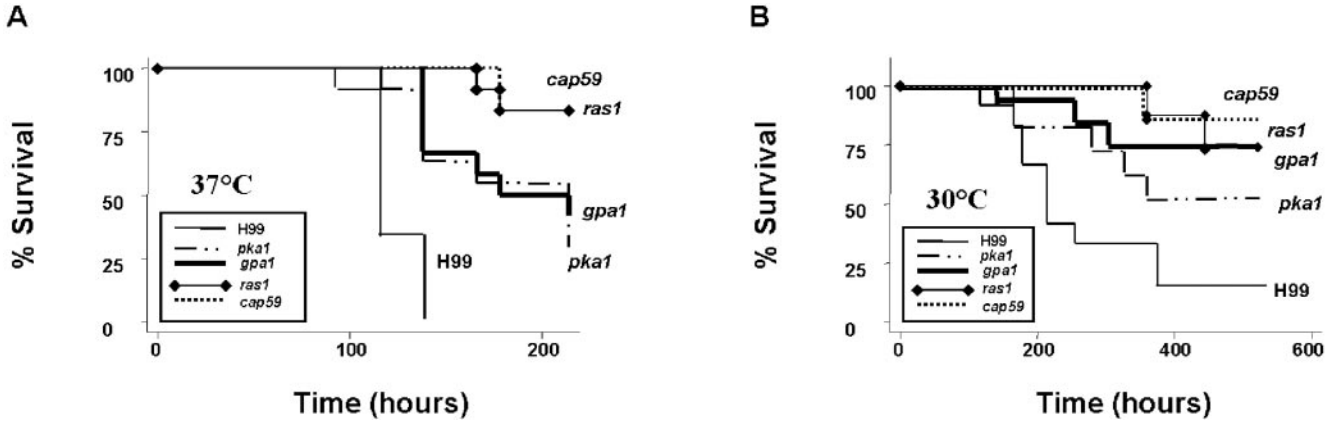

FIG. 3. *C. neoformans* virulence factors important for mammalian infection also enhance killing of *C. elegans.* Survival of *G. mellonella* after injection of 1.5×10^4 CFU/larva of wild-type C. *neoformans* strain H99 or mutants with disruptions in the genes encoding CAP59 (essential for capsule formation) or the G protein-cAMP-PKA or the RAS1-controlled signal transduction cascades demonstrated hypovirulence (*cap59*, *gpa1*, *ras1*, and *pka1*). There was a significant decrease in virulence between the wild type and the mutants at both 37°C (A) and 30°C (B). *P* values were <0.01 for each of the mutants compared to the parental strain H99 (Table 1).

subunit encoded by the *PKA1* gene (2, 12). Strains in which the *PKA1* gene is disrupted display attenuated virulence in murine models of cryptococcal infection (12) and *C. elegans* (31). In addition to the PKA signaling pathway, another regulatory pathway is a *RAS1*-specific signaling cascade (1). As shown in Fig. 3 and Table 1, strains with mutations involving the genes in these signaling pathways and the acapsular strain *cap59* were less virulent in *G. mellonella* than in the parent strain H99 at both 37°C and 30°C. Reconstitution of each gene reversed the attenuated virulence in *G. mellonella*, similar to findings with mammals (Table 1).

Melanin is a potent free radical scavenger and, as noted above, has been associated with cryptococcal virulence in murine studies (40, 52, 53) and with *C. elegans* (31). A strain of *C. neoformans* mutated in the gene encoding laccase (an enzyme essential for melanin production) was less virulent than the wild type in the *G. mellonella* system ($P = 0.001$) (Fig. 4).

Interesting similarities also existed in the role of the mating

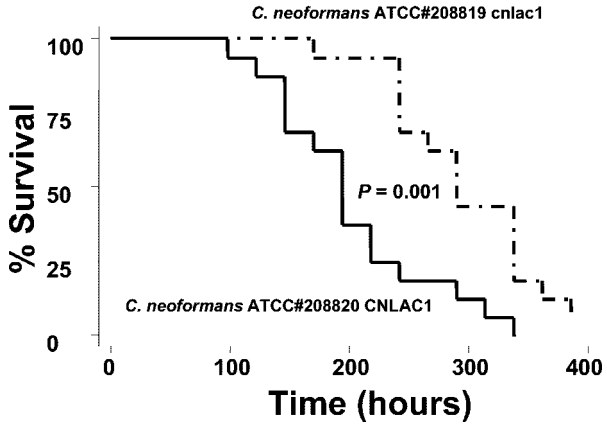

FIG. 4. Melanin biosynthesis is involved in *G. mellonella* killing by *C. neoformans*. A mutation involving the gene that encodes laccase (an enzyme essential for melanin biosynthesis) renders *C. neoformans* less virulent in the *G. mellonella* system at 37°C than the wild type ($P = 0.001$) (in this experiment, caterpillars received 2.5×10^4 CFU/larva).

locus in *C. neoformans* pathogenesis in *G. mellonella* to that studied previously in mammalian models of cryptococcal pathogenesis. *C. neoformans* can reproduce asexually by budding or can form spores when cells of opposite mating types (MATα and MAT**a**) undergo conjugation. Studies with mammalian models (23) and *C. elegans* (31) demonstrate that strain JEC21, the model α mating strain of *C. neoformans* var. *neoformans*, is more virulent than the otherwise isogenic **a** mating parent JEC20 (23). However, in *C. neoformans* var. *grubii*, congenic **a** and α mating type strains demonstrate equivalent virulence in both mammalian models (35) and *C. elegans* (E. Mylonakis, K. Nielsen, and J. Heitman, unpublished data); it is not known why this difference between *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* strains exists. Even in the case of *C. neoformans* var. *grubii*, cells containing the pMFα1::GFP reporter gene demonstrate specific GFP expression in the central nervous system (CNS) of infected rabbits (pMFα1 regulates production of the α mating type pheromone), suggesting that the MFα1 gene is induced during the proliferative stage of a CNS infection (11).

Our findings in *G. mellonella* were similar to those in mammalian models in all these aspects. Specifically, the rate of killing of *G. mellonella* caterpillars that received MATα strain JEC21 was significantly faster than in the group that received the otherwise-isogenic MAT**a** strain JEC20 (Table 1). Similarly, we found that there was no pMFα1::GFP expression of *C. neoformans* cells in *G. mellonella* hemolymph during the first 48 h after injection but that there was significant GFP expression by day 3 after injection, suggesting that in *G. mellonella*, the *MFα1* gene is induced during the proliferative stage of the infection, similar to the findings with rabbit CNS (11) and *C. elegans* (31) models (Fig. 5). Finally, killing of *G. mellonella* by *C. neoformans* var. *grubii* strains H99, KN99**a**, KN99α, and KN99-5 was identical, similar to findings with mammals (Table 1) (35).

Study of antifungal agents in the *G. mellonella-C. neoformans* system. Although *G. mellonella* has been used extensively for the study of microbial pathogenesis, there is no previous experience studying antimicrobial (especially antifungal) agents

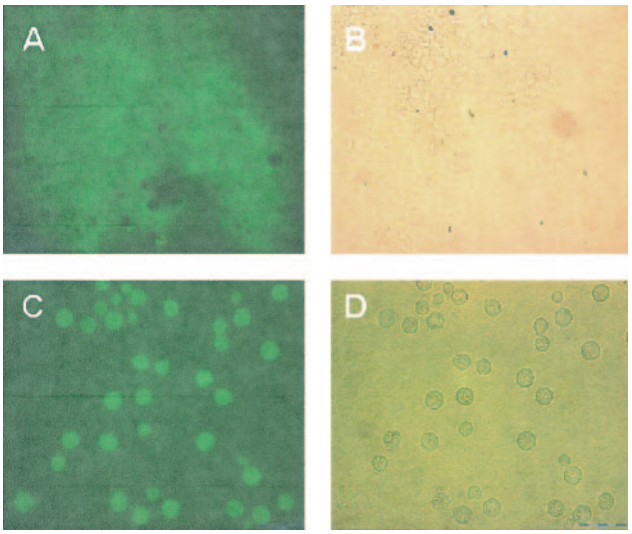

FIG. 5. The *C. neoformans* MFα1 promoter is expressed in the *G. mellonella* hemocoel during the proliferative stage. Fluorescent microscopy of *G. mellonella* hemolymph on day 3 (proliferative stage) after injection with 1.25×10^4 CFU/larva of *C. neoformans* strain H99 expressing GFP fused to the MFα1 promoter. (A and B) Hemolymph was undiluted to provide a better appreciation of the fungal burden within the insect hemolymph. (C and D) Cells were fixed as detailed in Materials and Methods. Images in panels A and C are fluorescence images, and images in panels B and D are the corresponding confocal images. There was no fluorescence observed on day 1 or 2 of this experiment.

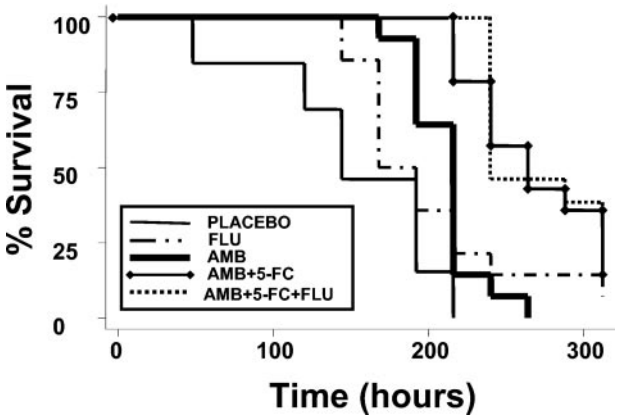

FIG. 6. Antifungal drugs prolong the survival of *G. mellonella* caterpillars after challenge with *C. neoformans*. We examined the role of the most commonly used agents for *C. neoformans* infection by administering a single dose of amphotericin B (AMB; 1.5 mg/kg), fluconazole (FLU; 14 mg/kg), or flucytosine (5-FC; 20 mg/kg) alone or in combination 48 h after the inoculation of caterpillars with 1.2×10^3 CFU of *C. neoformans* strain H99 per larva. A control group received the *C. neoformans* inoculum and PBS instead of antifungal drugs. Monotherapy with amphotericin B prolonged the survival of *G. mellonella* caterpillars ($P = 0.001$ compared to control). Also, there was a trend suggesting that fluconazole was effective ($P = 0.072$). The combination of amphotericin B plus flucytosine was significantly more effective than amphotericin B alone ($P = 0.0002$).

in this system. To evaluate if the *G. mellonella-C. neoformans* system could be used to study antifungal agents, we investigated the role of the most commonly used agents for *C. neoformans* infection by administering a single dose of amphotericin B (1.5 mg/kg), fluconazole (14 mg/kg), or flucytosine (20 mg/kg), alone or in combination 48 h after the inoculation of caterpillars with 1.2×10^3 CFU *C. neoformans* strain H99 (Fig. 6). Monotherapy with amphotericin B or flucytosine prolonged the survival of *G. mellonella* caterpillars (mean time to mortality was 6 days in the control group, compared to 9 days in the amphotericin B and the flucytosine groups; in both cases, *P* values were 0.001 compared to those of the control). Also, there was a trend that did not reach statistical significance that suggested that fluconazole monotherapy was effective ($P = 0.0721$; mean time to mortality, 7.5 days). The combination of amphotericin B plus flucytosine (the most commonly used combination for management of severe human cryptococcosis (39) was significantly more effective than amphotericin B alone ($P = 0.0002$) and was as effective as triple therapy (amphotericin B, flucytosine, and fluconazole); in both cases, mean time to mortality was 16 days.

We also compared the efficacy of dosing with amphotericin B (1.0 mg/kg), flucytosine (20 mg/kg), fluconazole (14 mg/kg), or a combination of amphotericin B (1.0 mg/kg) with flucytosine (20 mg/kg) either 4 h before (prophylaxis group) or 4 h after inoculation (treatment group) with *C. neoformans* (1.25×10^3 CFU/caterpillar). Amphotericin B and flucytosine were again the two most effective agents when used as monotherapy (in both cases, $P < 0.0001$ in the prophylaxis and $P = 0.03$ in the treatment group, compared to control). In this experiment, administration of fluconazole significantly prolonged survival ($P = 0.01$ in the prophylaxis and $P = 0.04$ in the treatment groups, compared to placebo), although the level of efficacy of fluconazole monotherapy was lower than that of amphotericin B or the amphotericin B-plus-flucytosine combination ($P = 0.01$ compared to amphotericin B and $P < 0.001$ compared to amphotericin B plus flucytosine). The combination of amphotericin B plus flucytosine was again more effective than amphotericin B monotherapy ($P = 0.002$ in the treatment and $P = 0.04$ in the prophylaxis group). As a control, to rule out any killing of larvae by antifungal agents, we administered the antifungal agents alone to control groups of caterpillars. There were no deaths in these groups.

In addition to monitoring the survival of *G. mellonella* caterpillars, we also evaluated the impact of antifungal agents on the tissue burden of *C. neoformans* within the hemocoel. As noted above, because *G. mellonella* larvae are often colonized by other fungi, we used a nourseothricin-resistant strain (KN99α *kin1* + *KIN1*) to evaluate the fungal burden in *G. mellonella*. For this, we injected caterpillars with *C. neoformans* (2.4×10^3 CFU of *C. neoformans* strain KN99α *kin1* + *KIN1* per caterpillar). After 48 h, we injected caterpillars (five caterpillars per group) with PBS (control group) or antifungal drugs (amphotericin B, fluconazole, and flucytosine either as monotherapy or in combination). All antifungal agents significantly decreased the number of fungi in the caterpillars, but fluconazole was significantly less effective than the other antifungal therapies we studied. The fungal load (mean ± standard deviation) in the control group that received no antifungal drugs was $5.5 \times 10^4 \pm 1.4 \times 10^3$ CFU/mg but was only somewhat lower in the fluconazole-treated group ($2.1 \times 10^4 \pm 9.9 \times 10^3$ CFU/mg; $P = 0.016$ compared to control). In contrast, both

flucytosine (356 ± 225 CFU/mg; $P = 0.009$ compared to control) and amphotericin B (384 ± 234 CFU/mg; $P = 0.009$ compared to control) dramatically lowered the fungal load. The combination of amphotericin B with flucytosine was more effective at decreasing the tissue burden of *C. neoformans* than either agent alone (the tissue burden in the amphotericin B-plus-flucytosine group was 54 ± 33 CFU/mg; $P = 0.008$ compared to the groups that received amphotericin B or flucytosine monotherapy). Interestingly, triple therapy was the most effective therapy in decreasing the tissue burden of *C. neoformans* in the caterpillars (9 ± 6 CFU/mg; $P = 0.035$ compared to the amphotericin B with flucytosine group), even though as noted above, this therapy did not prolong the survival of *G. mellonella* compared to dual therapy with amphotericin B plus flucytosine. None of the therapeutic regimens studied sterilized the caterpillars following infection; instead, the prolongation of survival meant that infected caterpillars treated with antifungals had delayed mortality.

DISCUSSION

We report the novel observation that after injection, *C. neoformans* propagates in the *G. mellonella* hemocoel and leads to killing of the larva despite the ability of *G. mellonella* hemocytes to phagocytose cryptococcal cells. Caterpillar killing is associated with virulence traits known to be involved in mammalian pathogenesis. Finally, we show that the *G. mellonella* model can be utilized for study of the in vivo efficacy of antifungal agents. These findings suggest that the *C. neoformans-G. mellonella* pathosystem is a facile model that complements more cumbersome and expensive mammalian models of fungal pathogenesis.

Studies of microbial pathogenesis in nonvertebrate hosts during the past decade have resulted in important insights into the molecular mechanisms of microbial pathogenesis and host defense. It is now apparent that many of the same microbial virulence factors are involved in pathogenesis in evolutionarily disparate hosts (3, 31, 32, 44, 46). *G. mellonella* caterpillars have previously been used to study infection by various pathogens, including *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli*, *Bacillus cereus*, and the insect pathogenic fungus *Metarhizium anisopliae* (13, 20, 27–29, 50, 51). Among human fungal pathogens, *Candida* spp. were also shown to kill *G. mellonella* when injected into the hemocoel of the insect caterpillars (6, 10, 14, 42, 43). Brennan et al. reported that in the case of *Candida albicans*, there is a correlation between virulence in *G. mellonella* and virulence measured by systemic infection of mice (6). Of note is that for most strains of *Candida* spp., at least 2×10^5 cells/larva are usually needed for killing (10), whereas *C. neoformans* is considerably more virulent.

G. mellonella has also been used to study *Aspergillus flavus* pathogenicity. Conidia of *A. flavus* were not virulent when applied to the surface of healthy caterpillars but killed the caterpillars (100% mortality within 48 h) when injected (47). Recently, Reeves et al. reported that *G. mellonella* is susceptible to *Aspergillus fumigatus* strain ATCC 26933 and suggested a role in virulence for gliotoxin in promoting tissue penetration (38).

Our findings suggest that, similar to the findings reported by St. Leger and coworkers with *A. flavus* and *A. fumigatus* (47), *G. mellonella* hemocytes are able to phagocytose *C. neoformans*. However, the fact that inocula as low as 20 fungal cells of *C. neoformans* strain H99 were able to kill caterpillars suggests that successful phagocytosis does not necessary translate into fungal cell clearance. Possibly, phagocytosis is more effective with other *C. neoformans* strains than H99; this may explain why significantly higher numbers of inocula were needed for killing of caterpillars by a variety of *C. neoformans* strains.

The work reported here provides several examples of correlations between *C. neoformans* factors involved in mammalian pathogenesis and those necessary for *G. mellonella* killing. The *C. neoformans* polysaccharide capsule, as well as several *C. neoformans* genes previously shown to be involved in mammalian virulence (such as *GPA1*, *PKA1*, and *RAS1*), were shown to play a role in *G. mellonella* killing. Of note is that not only was strain *cap59* attenuated compared to parent strain H99 ($P < 0.001$ at 37°C and $P = 0.003$ at 30°C), but killing of caterpillars by this mutant in capsule production was similar to that in the control groups that received PBS or heat-killed H99. This finding resembles findings with mammalian models (5, 8) and the amoeba system (25, 44), where acapsular strains of *C. neoformans* are completely avirulent. In the *C. elegans* (31) and *D. melanogaster* (3) systems, in contrast, although *cap59* is attenuated, it results in killing of more than half of the animals. Also, heat-killed *C. neoformans* cells kill *C. elegans* but not wax moth caterpillars. Similarly, a *ras1* mutant was avirulent in *G. mellonella* (Fig. 3 and Table 1), while *pka1* and *gpa1* mutants that were avirulent in mammalian models were still able to kill more than half of the caterpillars. This suggests that the RAS1 pathway may be more important in the caterpillar model than the Gα protein-cAMP-PKA signaling pathway (2, 12). Previous work with *D. melanogaster* demonstrated that these virulence traits are involved in the killing of nonmammalian hosts after feeding (3). Coupled with the current studies, this suggests that *C. neoformans* factors involved in mammalian pathogenesis are involved in the survival of *C. neoformans* within nonmammalian hosts.

The choice of treatment for human *C. neoformans* infection depends on both the anatomic site of involvement and the immune status of the host. For management of severe cryptococcal infection involving the CNS, guidelines (39) based on a number of clinical trials suggest the initial use of amphotericin B plus flucytosine, followed by fluconazole consolidation therapy (4, 7, 24, 49). In the *G. mellonella* system reported here, amphotericin B, flucytosine, and fluconazole all prolonged survival of caterpillars, but combination therapy with amphotericin B plus flucytosine was the most effective therapy. Further studies evaluating the tropism of *C. neoformans* within *G. mellonella* and the concentration of antifungal drugs within tissues are needed to obtain a more thorough understanding of the efficacy of antifungal agents in this model.

Larvae of *G. mellonella* are inexpensive and relatively easy to manipulate, and their use may reduce the need to employ mammals for some in vivo studies for the efficacy of antifungal agents. This model may be a particularly useful addition to laboratories that study fungal pathogenesis but do not want to allocate the resources and commitment necessary to study *C. elegans* or *D. melanogaster*. However, important limitations of the *G. mellonella* model need to be considered. First, com-

pared to other invertebrate models (such as *C. elegans* and *D. melanogaster*), the *G. mellonella* system has limited genetic tractability; the genome of this organism has not been sequenced. *G. mellonella* (similar to flies and nematodes) does not have an adaptive immune system. It is unlikely that all virulence traits that are important for mammalian infection will be significant in *G. mellonella* killing. For example, a clinical isolate of *C. neoformans* var. *gattii* that was associated with an outbreak of severe cryptococcal infection in Vancouver Island (15; http://ftp.cdc.gov/pub/infectious_diseases/iceid/2002/pdf/starr.pdf) was not considerably more virulent in the caterpillar model than other strains we tested (Table 1). Moreover, the impact of increased temperature on the host response of *G. mellonella* has not been studied in detail, and this may contribute to the increased killing of larvae at mammalian temperature that we noted in our experiments. Finally, a practical consideration for the use of *G. mellonella* is that caterpillars are unable to undergo an indefinite number of multiple injections, as this increases the mortality from trauma or infection from pathogenic contaminants on the insect cuticle.

In conclusion, a positive correlation exists between the pathogenicity of *C. neoformans* evaluated in the insect *G. mellonella* and in other model systems, including mammals. The *G. mellonella* model allows the administration of precise fungal inocula and the study of cryptococcal virulence at mammalian temperatures. The correlation between virulence in *G. mellonella* and mammalian models suggests that the *G. mellonella-C. neoformans* system can be used for the identification of new genes in *C. neoformans* involved in virulence, as well as for the in vivo evaluation of new antifungal agents.

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