EUKARYOTIC CELL, Apr. 2004, p. 413–419 Vol. 3, No. 2
1535-9778/04/$08.00+0 DOI: 10.1128/EC.03.2.413–419.2004
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Challenge of *Drosophila melanogaster* with *Cryptococcus neoformans* and Role of the Innate Immune Response

Yiorgos Apidianakis,[1,2] Laurence G. Rahme,[1,2] Joseph Heitman,[3,4,5] Frederick M. Ausubel,[6,7] Stephen B. Calderwood,[8,9] and Eleftherios Mylonakis[8]*

Department of Surgery,[1] Department of Molecular Biology,[7] and Division of Infectious Diseases,[8] Massachusetts General Hospital, and Shriner's Burns Institute,[2] Boston, Massachusetts 02114; Department of Genetics[6] and Department of Microbiology and Molecular Genetics,[9] Harvard Medical School, Boston, Massachusetts 02115; and Department of Medicine,[3] Department of Molecular Genetics and Microbiology,[4] and Howard Hughes Medical Institute,[5] Duke University Medical Center, Durham, North Carolina 27710

Received 16 February 2004/Accepted 20 February 2004

We found that the ingestion of *Cryptococcus neoformans* by *Drosophila melanogaster* resulted in the death of the fly but that the ingestion of *Saccharomyces cerevisiae* or the nonpathogenic *Cryptococcus kuetzingii* or *Cryptococcus laurentii* did not. The *C. neoformans* protein kinase A and RAS signal transduction pathways, previously shown to be involved in virulence in mammals, also played a role in killing *Drosophila*. Mutation of the Toll immune response pathway, the predominant antifungal pathway of the fly, did not play a role in *Drosophila* defense following ingestion of the yeast. However, the Toll pathway was necessary for the clearance of *C. neoformans* introduced directly into the hemolymph of *D. melanogaster* and for the survival of systemically infected flies.

Fungal infections are a common cause of morbidity and mortality among immunocompromised patients, including human immunodeficiency virus-infected individuals (42). Cryptococcal infections are a particularly common and often fatal complication of immune suppression (21, 24, 42, 52). In immunocompromised patients, cryptococcal infection is caused by *Cryptococcus neoformans* var. *neoformans*, while the other variety, *C. neoformans* var. *gattii*, is usually associated with infections in immunocompetent individuals in tropical and subtropical areas. However, a significant epidemic caused by *C. neoformans* var. *gattii* on Vancouver Island has challenged this understanding, and some believe that this outbreak may represent the emergence of a new disease (50; http://ftp.cdc.gov/pub/infectious diseases/iceid/2002/pdf/starr.pdf).

Studies of the pathogenic mechanisms of *C. neoformans* have been enhanced by the development of transformation protocols, homologous recombination for genetic manipulations, and the establishment of several host models (7, 17, 28, 38, 53). The most important *C. neoformans* virulence factors identified to date include the polysaccharide capsule (7, 46) and melanin (5, 14, 22, 44, 45). Signal transduction cascades leading to the production of these *C. neoformans* virulence factors have also been elucidated (1, 15, 40). These and other studies conducted during the past decade have established *Cryptococcus* as an important human pathogen and a model yeast for the study of fungal pathogenesis (41).

Recently, the development of nonmammalian host models for *Cryptococcus* infection has emerged as a promising tool to facilitate the study of *C. neoformans* pathogenesis. Steenbergen et al. reported the use of the free-living amoeba *Acanthamoeba castellanii* as a model for the study of *C. neoformans* survival strategies following ingestion by macrophages (48, 49). These investigators found that *C. neoformans* was phagocytosed by *A. castellanii* and that, once intracellular, *C. neoformans* replicated, eventually killing the amoeba. The process was remarkably similar to the events following the phagocytosis of *C. neoformans* by mammalian macrophages (37). Recently, it was reported that *C. neoformans* is ingested by and kills the nematode *Caenorhabditis elegans*. It was shown that the *C. neoformans* polysaccharide capsule, as well as several *C. neoformans* genes previously shown to be involved in mammalian virulence, also play a role in the killing of *C. elegans* (40).

During the past decade, the well-studied fruit fly *Drosophila melanogaster* has been extensively used to study the host innate response to microbial pathogens, leading to the discovery of a high degree of conservation in the innate immune signaling pathways between mammals and insects (27). In *D. melanogaster*, activation of the immune response is regulated by at least two pathways: the Toll pathway and the Imd pathway, two parallel signaling cascades which both contribute to the *Drosophila* response against microbes (30, 33, 54). Following fungal infection, the Toll receptor on the surface of fat body cells is activated by a cleaved form of a cytokine-like protein, Spatzle (Spz), which is present in the *Drosophila* hemolymph. The physical interaction between Spz and Toll initiates an intracellular cascade that triggers signal transduction through the threonine-serine kinase Pelle (26, 57). This signal leads to the phosphorylation and degradation of Cactus, the release and subsequent nuclear translocation of the Rel family transcription factors Dorsal and Dif, and the synthesis of antifungal and antibacterial peptides (57). Similarly, the Imd pathway leads to the activation of the Rel family transcription factor Relish (29) and the synthesis of antibacterial peptides. The *Drosophila* Toll and Imd signaling pathways exhibit striking

* Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit St., Boston, MA 02114-2696. Phone: (617) 726-3812. Fax: (617) 726-7416. E-mail: emylonakis@partners.org.

TABLE 1. Yeast strains used in this study and their characteristics[a]

Strain name (reference)	Relevant characteristic(s) or phenotype(s)	LT_{50} (h)	*P* value	LB_{50} (CFU)
C. neoformans				
H99 ATCC 208821 (25)	Serotype A; clinical isolate; genome sequence being determined	40		5×10^4
H99 *pka1* (15)	*PKA1* encodes the major cyclic AMP-dependent protein kinase catalytic subunit; mutant attenuated in mammalians	90	<0.001	5×10^4
H99 *pka1* + *PKA1* (15)	Complementation of the *pka1* mutant with wild-type *PKA1* restored virulence in mammals	38		3×10^4
H99 *ras1* (1, 56)	*ras1* mutant is avirulent in animal models of cryptococcal meningitis	60	<0.001	4×10^4
H99 *ras1* + *RAS1* (1)	Complementation of the *ras1* mutant with wild-type *RAS1* restored virulence in rabbits	35		2×10^4
H99 *cap59* (43)	A capsular mutant; *CAP59* is essential for capsule formation	48	0.01	5×10^3
H99 *pkr1* (15)	*PKR1* encodes the PKA regulatory subunit; in mice, a *pkr1* mutant overproduces capsule and is hypervirulent	32	0.01	5×10^3
ATCC 62068 (39)	Serotype A; clinical isolate	50		ND
ATCC 34877 (47)	Serotype B/C	40		ND
ATCC 36556 (32)	Serotype D; clinical isolate	44		ND
ACT::GFP (11)	Serotype A; strain H99 containing the inducible GFP gene fused to the actin promoter	40		ND
C. laurentii				
ATCC 18803 (19)	Environmental isolate	>168	<0.001	ND
C. kuetzingii				
ATCC 42276	Clinical isolate	>168	<0.001	ND
S. cerevisiae				
YJM145 (23)	Clinically derived strain	>168	<0.001	ND
YJM237 (23)	Strain isogenic to sequenced strain S288c	>168	<0.001	ND

[a] Time (LT_{50}) and yeast burden per fly (LB_{50}) when 50% of the flies died are shown for each individual strain. Note that although the LT_{50} values differ between strains, the LB_{50} values are very similar for most strains. The survival kinetics *P* value for each strain (LT_{50}) compared to the H99 strain is also displayed where significant.

similarities to the Toll-like receptor and tumor necrosis factor alpha pathways, respectively, which regulate NF-κB activity in vertebrates, suggesting common evolutionary roots (27).

Because recent reports suggest that the virulence factors of *C. neoformans* involved in mammalian pathogenesis may have evolved as a consequence of the interaction of yeast with environmental predators such as amoebae and nematodes (6) and because *D. melanogaster* has been a valuable model for the study of host-pathogen interactions (3, 9, 12, 31, 33), we studied the interaction between *C. neoformans* and *D. melanogaster*. Here, we show that *C. neoformans* is a potent pathogen of *Drosophila* when it is ingested but not when it is injected. By analyzing the role of the Toll and Imd innate immune signaling pathways, we show that the Toll pathway does not appear to play any role in conferring resistance to ingested *C. neoformans* on *D. melanogaster* but is necessary for protection against systemic *C. neoformans* infection of the fly following injection.

MATERIALS AND METHODS

Strains and media. The *C. neoformans* strains used in these experiments are listed in Table 1. *Cryptococcus laurentii* strain ATCC 76483 and *Cryptococcus kuetzingii* strain ATCC 42276 were obtained from the American Type Culture Collection (ATCC). The sources of the other strains are indicated in Table 1. Yeast cultures were maintained on yeast peptone dextrose (YPD; Difco) agar.

The Oregon-R (OR) strain of *D. melanogaster* was used as the wild type. Stocks and crosses were maintained on a standard cornmeal medium. The imd^1 and $imd^1; spz^{rm7}$ lines used in this study have been described (36). The spz^{rm7} (34) and spz^{197} (Bloomington stock center) alleles were used to obtain *spz* mutant adults. All experiments were performed at 25°C.

Infection protocols. We initially assessed different media for the growth of *C. neoformans* and other yeasts prior to ingestion by the flies. We found that diluted YPD (1/3 YDP) agar medium (17 g of YPD/liter instead of the generally used concentration of 50 g/liter) enhanced the virulence of *C. neoformans* compared to regular YPD agar (50 g/liter) or brain heart infusion (BHI) medium (Difco); therefore, for all assays involving infection by ingestion, yeasts were grown on 1/3 YPD agar medium and grown overnight to form a lawn covering the medium surface. For the ingestion assay, 50 μl of an overnight liquid YPD medium culture of *C. neoformans, C. kuetzingii, C. laurentii*, or *Saccharomyces cerevisiae* was spread in a fly vial containing 2 ml of 1/3 YPD agar medium. For each experimental condition tested, approximately 80 male flies were divided in three vials and transferred to new vials every 24 h. Results for female flies were qualitatively similar to those for males, although some quantitative differences between genders were evident. Each experiment was performed in duplicate or triplicate; similar results were always obtained, and results of representative experiments are shown in the figures.

Injection assay. The dorsal part of the fly thorax was pricked with a sharpened needle (100-μm diameter) dipped into pelleted yeast cultures that had been grown overnight at 30°C. Approximately 400 yeast cells were introduced into each fly, as judged by counting CFU immediately after injection (see below). For each experimental condition, 50 to 70 male adults aged 2 to 4 days were inoculated. Similar results were obtained with female flies, although minor quantitative differences between genders were evident. Flies that died within 3 h after injection (less than 5% of the total) were not considered in the analysis.

The STATA 6 statistical software package (Stata, College Station, Tex.) was used to plot killing curves by the Kaplan-Meier method and to estimate statistical differences in fly survival. The time required for 50% of the flies to die (LT_{50}) was calculated with Prism, version 2.00, software (GraphPad) by using the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/[1 + 10^{(\log ET_{50} - X) - \text{Hill slope}}]$, where X is the logarithm of the number of hours, Y is the average number of dead flies, ET_{50} is the 50% effective time, Top and Bottom are the highest and lowest plateaus, respectively, and Hill slope is the degree of curve inclination.

Yeast CFU were enumerated as previously described (8), by plating serial dilutions of homogenates of five adults on YPD plates containing ampicillin,

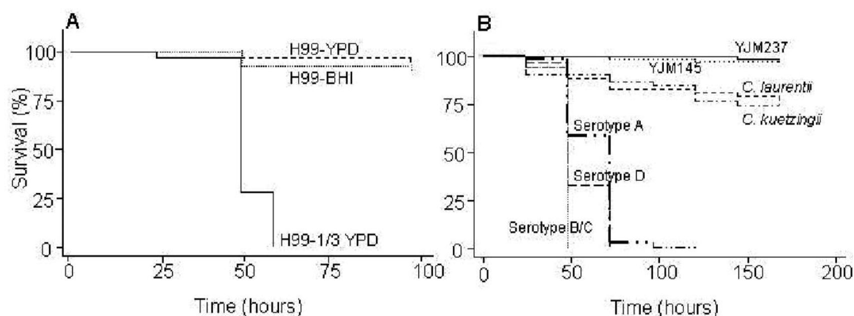

FIG. 1. Killing of *D. melanogaster* following exposure to *C. neoformans*. (A) Survival of wild-type *D. melanogaster* (OR) exposed to lawns of *C. neoformans* (H99) grown on different agar media (1/3 YPD, YPD, and BHI). (B) Survival of wild-type *D. melanogaster* (OR) exposed to lawns of *C. neoformans* serotypes A (ATCC 62068), B/C (ATCC 34877), and D (ATCC 36556), *C. laurentii* (ATCC 18803), *C. kuetzingii* (ATCC 42276), and *S. cerevisiae* (YJM 145 or YJM 237) grown on 1/3 YPD agar medium. *P* values are less than 0.001 for all *C. neoformans* strains compared to *C. laurentii*, *C. kuetzingii*, or either *S. cerevisiae* strain.

streptomycin, and kanamycin to prevent bacterial contamination. Prior to homogenization, flies ingesting cryptococci were washed by pipetting in water to remove surface yeast. For the enumeration of yeast in injected flies, the inoculum site was dissected away prior to homogenization and culturing to avoid counting organisms embedded in the wound clot; this process involved the removal of ca. one-fourth of the dorsal thorax with a pair of microsurgical scissors. Plates were incubated at 30°C. Each experiment was performed in duplicate, and the standard deviation was calculated.

Extraction of hemolymph for culture. To assess the presence or absence of yeast in the hemolymph, a 1-μl sample was extracted as follows. First, a hole was made in the dorsal thoracic cuticle with a sharpened metal needle (diameter, 100 mm). Then, the tip of a Pasteur pipette, manipulated in a flame to decrease its diameter, was inserted through the cuticle hole in a left-to-right direction and dorsal enough to avoid the fly midgut. Negative pressure was applied, and the extracted hemolymph was added to 100 μl of phosphate-buffered saline and spread onto plates.

RESULTS

Killing of *D. melanogaster* through feeding on *C. neoformans*. To investigate the interplay between *C. neoformans* and *D. melanogaster*, we fed flies with wild-type *C. neoformans* (H99) grown on various media differing in richness (1/3 YPD, YPD, and BHI media, described in Materials and Methods). Interestingly, we observed *Drosophila* killing only when *C. neoformans* was grown on 1/3 YPD medium prior to feeding (Fig. 1A). This result is in agreement with observations that *Cryptococcus* virulence is enhanced by growth under nutrient-limiting conditions (1, 2).

To assess whether different human pathogenic strains of *C. neoformans* are virulent following ingestion by flies, we used different serotypes of *C. neoformans*. All serotypes tested killed 100% of the flies within 2 to 3 days (Fig. 1B). In contrast, two nonpathogenic cryptococci, *C. laurentii* and *C. kuetzingii*, killed only approximately 25% of the flies over 7 days (Fig. 1B). Two *S. cerevisiae* strains (YJM145 and YJM237) had no effect on the life spans of the flies (Fig. 1B), confirming that this yeast, which is often used for *D. melanogaster* maintenance in the laboratory, is nonpathogenic. Importantly, when fed heat-killed cells of *C. neoformans* (H99), more than 90% of the flies survived over an 8-day period (data not shown), indicating that only live *C. neoformans* organisms are virulent for *D. melanogaster*.

Genes associated with pathogenesis in mammals cause enhanced killing of *Drosophila* by *C. neoformans*. Capsule and melanin production in *C. neoformans* are regulated by a cyclic AMP-dependent protein kinase A (PKA) signaling pathway. PKA is composed of catalytic and regulatory subunits encoded by the *PKA1* and *PKR1* genes, respectively (15). Strains in which the *PKA1* gene is disrupted display attenuated virulence in murine and *Caenorhabditis elegans* cryptococcosis infection models, whereas strains with disruptions in the *PKR1* gene overproduce capsule and are hypervirulent (15, 16). As shown in Fig. 2, similar to the results obtained for mice and nematodes, the *pka1* mutant was significantly less virulent than the isogenic wild type ($P < 0.001$) or the reconstituted strain, which contains the *PKA1* gene integrated into the *pka1* mutant genome (2, 15, 16), whereas the *pkr1* mutant was hypervirulent (flies fed on *pkr1* died 8 h faster than flies fed on the parental strain, H99) (Table 1). In addition to the PKA signaling pathway, another regulatory pathway associated with *C. neoformans* infection in mammalian hosts involves a *RAS1*-specific signaling cascade (1). In the *D. melanogaster* feeding assay, the virulence of a *C. neoformans ras1* mutant was also attenuated compared to those of the wild type and a *ras1+RAS1* reconstituted strain (Fig. 2 and Table 1). These results suggest that, at least in the case of the PKA and RAS signaling pathways,

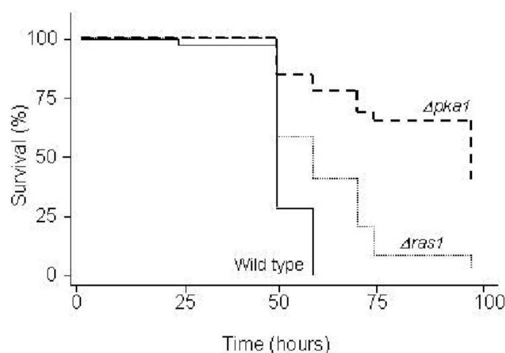

FIG. 2. *C. neoformans* virulence factors for mammalian infection also enhance the killing of *D. melanogaster*. Shown is the survival of *D. melanogaster* (OR) flies exposed to wild-type (H99) or mutant *C. neoformans*. Mutations refer to disruptions in the genes encoding the PKA- or the Ras1-controlled signal transduction cascades. *P* values are less than 0.001 for each of the mutants compared to the parental strain, H99.

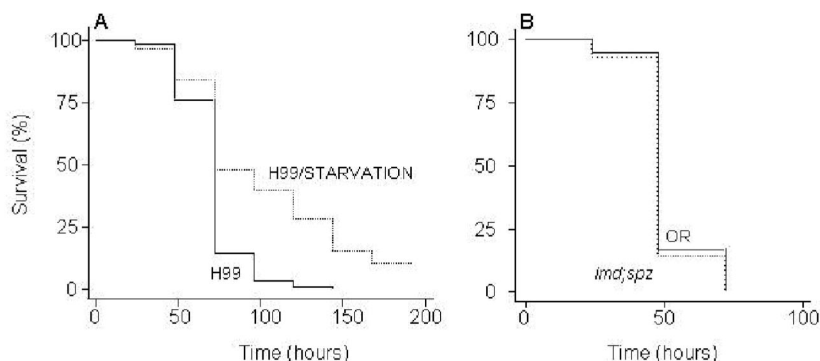

FIG. 3. Role of starvation and *D. melanogaster* immune system in survival following intestinal exposure to *C. neoformans*. (A) Killing of wild-type (OR) flies feeding continuously on *C. neoformans* (H99) or feeding for 18 h per day and then starved for the remaining 6 h. (B) Killing rates for wild-type (OR) and mutant (*imd; spz*) flies following feeding on *C. neoformans* (H99) are identical. *P* values are less than 0.001 for the comparison of the curves in panel A.

there is a close correlation between cryptococcal factors required for virulence in three disparate hosts: *Caenorhabditis elegans*, *Drosophila*, and mammals (mice and rabbits). The induction of the PKA/PKR and RAS pathways and the formation of a capsule in *C. neoformans* have previously been associated with growth in minimal media (1, 2). Similarly, in our experiments, *C. neoformans* strain H99 did not kill flies when grown on a rich medium such as undiluted YPD or BHI agar (Fig. 1A) but did when grown on more minimal media (1/3 YPD).

In mammalian hosts, the *C. neoformans* polysaccharide capsule, which distinguishes *C. neoformans* from many other pathogenic fungi, protects against phagocytosis and killing by immune effector cells and also blocks the presentation of antigen to T cells and the production of cytokines (4, 13, 18). To determine the role of the capsule in *Drosophila* killing, we tested *cap59*, an acapsular derivative of H99 (43). Interestingly, although differences between *cap59* and H99 are evident (flies fed on *cap59* died 8 h later than flies fed on the parental strain H99 [Table 1]), the *cap59* mutation did not cause a major diminution in virulence, indicating that the capsule is not a major virulence factor in the *Drosophila*-*C. neoformans* interaction.

Cryptococcal accumulation in the fly following ingestion. We determined the number of CFU of *C. neoformans* that had accumulated in the fly following ingestion as described in Materials and Methods. Cryptococcal cells accumulated in wild-type flies, reaching titers of 1.4×10^3, 3.3×10^3, 1.1×10^4, and 5×10^4 after 6, 12, 24, and 48 h of exposure, respectively. We selected time points only up to 48 h because more than 50% of wild-type flies were already dead by that time (Fig. 2). Independent experiments showed that 50% mortality correlated with a fungal burden of 2×10^4 to 5×10^4 per fly. Similar results were obtained with the *pka1* and *ras1* mutants (Table 1). In contrast, 50% mortality for the *cap59* and *pkr1* mutants corresponded to a fungal burden of approximately 5×10^3 cells per fly (Table 1).

Role of starvation in killing of *Drosophila* by *C. neoformans*. To rule out the possibility that flies feeding on *C. neoformans* were dying simply due to starvation, because this yeast is a nonnutritional source of food, we compared the survival of flies continuously exposed to *C. neoformans* with the survival of flies exposed for 18 h per day and starved for the remaining 6 h. Reduction of the exposure of flies to *C. neoformans* from 24 to 18 h per day yielded significantly longer survival times (Fig. 3A), demonstrating that starvation per se is not the sole mechanism by which *Cryptococcus* kills the fly.

Role of *Drosophila* innate immune signaling pathways in defense against ingested *C. neoformans*. To evaluate whether previously described innate immune signaling pathways of *D. melanogaster* are involved in an effective response to the ingestion of *C. neoformans*, we tested various fly strains mutated in the Imd and/or Toll pathways, the two most prominent pathways dictating immune responses, for survival following the ingestion of *C. neoformans*. Surprisingly, strains with a mutation in either the Imd or the Toll pathway were not more susceptible to killing than wild-type flies (data not shown). Among the strains tested, *imd; spz* double mutants were defective in both antifungal and antibacterial responses. Still, these flies survived as well as the wild type (Fig. 3B). This finding shows that either the fly immune system does not provide resistance to *C. neoformans* following ingestion or *C. neoformans* effectively blocks the immune response.

Yeast cells are undetectable in fly hemolymph following ingestion of *C. neoformans*. The fact that the *Drosophila* immune system did not affect killing in the *C. neoformans* ingestion assay suggests that killing may not be a consequence of the systemic growth of *C. neoformans* in the *Drosophila* hemolymph. To test this hypothesis, we extracted samples of hemolymph (~1 μl each) at 24 and 48 h following the initiation of feeding. Analyzing six flies at each time point, we detected virtually no yeast (an average of 0.8 CFU per fly) in the hemolymph. As a control, in parallel experiments (see below) in which ca. 400 CFU of H99 was injected directly into the hemolymph, ca. 20 yeast cells per fly could be recovered 3 h later.

As an alternative approach to detecting the systemic spread of *C. neoformans* following its ingestion by *D. melanogaster* flies, we fed flies a strain of *C. neoformans* that constitutively expresses green fluorescent protein (GFP) (Table 1) (11). Following the ingestion of this strain, flies were examined by fluorescence microscopy at 24 and 48 h. While we detected fluorescence in the fly gastrointestinal tract (this fluorescence

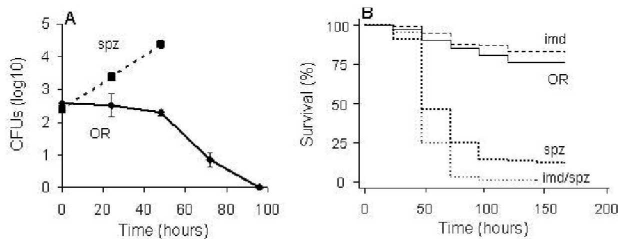

FIG. 4. The *D. melanogaster* immune response is necessary for clearing systemic *C. neoformans* infection. (A) Accumulation of *C. neoformans* within mutant (*spz*) or wild-type (OR) flies following injection with ~400 CFU. CFU in surviving flies were enumerated. (B) Survival of wild-type (OR) and mutant (*spz*, *imd*, or *imd; spz*) flies following injection with ~400 CFU of *C. neoformans*.

could be due to yeast cells and/or the autofluorescence of consumed yeast media), no light emission was detected in any other part of the fly (data not shown). These experiments, in addition to the enumeration of CFU (detailed above) and the finding that wild-type flies are able to clear significant numbers of systemically introduced cryptococci (detailed below), suggest that there is either no systemic spread or very limited systemic spread of yeast cells following the ingestion of *Cryptococcus* by *D. melanogaster* flies.

Clearance of systemic infection by *C. neoformans* depends on the innate immune response of the fly. The immune system of *Drosophila* has proved to be extremely efficient in clearing systemic infections of various fungi (35). To investigate the effectiveness of the innate immune signaling pathways against *C. neoformans*, we tested whether *Drosophila* can clear *C. neoformans* (H99) cells directly injected into the fly hemolymph and survive systemic infection. Following the injection of ca. 400 CFU of *C. neoformans* into the hemolymph, wild-type flies eliminated yeast cells in 3 to 4 days (Fig. 4A) and most of these flies survived the infection (Fig. 4B); this result indicates that the wild-type fly is capable of clearing a systemic infection. As was the case for wild-type flies, no killing was observed when *imd* mutant flies were injected with *C. neoformans* (Fig. 4B) and the *imd* mutant flies rapidly cleared the yeast (data not shown). In contrast to wild-type and *imd* mutant flies, however, *spz* mutant flies and *spz; imd* double mutants were highly susceptible to *C. neoformans* killing following injection (Fig. 4B) and *C. neoformans* multiplied in these mutants following injection (Fig. 4A and data not shown). Taken together, these findings provide evidence that the well-established antifungal pathway of the fly plays a pivotal role following systemic infection with *C. neoformans*.

DISCUSSION

D. melanogaster typically feeds on yeast as a natural source of nutrients (51). In this paper, we report the novel observation that strains of the pathogenic yeast *C. neoformans* kill *D. melanogaster*, in contrast to the nonpathogenic yeasts *C. laurentii, C. kuetzingii*, and *S. cerevisiae*. We excluded the possibility that the killing of flies is simply a consequence of starvation, because flies that were exposed to *C. neoformans* for a portion of the day and starved for the rest of the day lived significantly longer than flies exposed to *C. neoformans* continuously. Also, when fed heat-killed cells of *C. neoformans* (H99), more than 90% of the flies survived over an 8-day period, indicating that *C. neoformans* can provide adequate nutrients. Hence, it is likely that live *C. neoformans* organisms cause an infection-like process leading to mortality.

Following its ingestion by *D. melanogaster*, *C. neoformans* was restricted to the digestive tract and did not gain access to the hemolymph. Different observations support this notion: (i) we detected few if any yeast cells in extracted hemolymph samples, and (ii) we failed to detect fluorescent cryptococci outside the fly gut. Nevertheless, wild-type flies are able to clear significant numbers of systemically introduced cryptococci; thus, we cannot exclude the possibility that a number of yeast cells adhere to or penetrate into the fly during feeding.

Interestingly, the virulence of *C. neoformans* for flies was reduced when the yeast was grown on rich media, suggesting that factors inducible in minimal media affect virulence. Indeed, genes associated with the PKA and RAS signal transduction pathways that are known to be induced in minimal media and that were previously shown to be involved in the mammalian virulence of *C. neoformans* were also shown to play a significant role in the killing of *Drosophila* by *C. neoformans*. Despite the fact that the PKA and RAS signaling pathways are involved in capsule production, however, the virulence of the acapsular *cap59* mutant was not dramatically reduced in *Drosophila*, a result that was similar to our findings for *Caenorhabditis elegans* (40). In addition to capsule formation, the PKA pathway is known to affect melanin production and the alpha mating factor, and *RAS1* affects at least the last process as well (1, 16). Because mutations in these pathways have a more severe effect on virulence in *Drosophila* than the *cap59* mutation does, we conclude that PKA- and Ras1-regulated factors unrelated to *C. neoformans* capsule formation could be primarily responsible for fly killing.

In contrast to the observation that the virulence of the *C. neoformans* acapsular mutant is not profoundly affected, the observation that the *C. neoformans pkr1* mutant, which overproduces capsule, is hypervirulent and lethal in lower doses (Table 1) suggests that the capsule may play a role in the killing of *Drosophila*. On the other hand, *C. laurentii* also forms a capsule, and this organism is rarely identified in clinical specimens and is not pathogenic to nematodes or flies. Thus, it is possible that factors unrelated to the capsule, which may also be altered in the *pkr1* mutant, are responsible for the hypervirulence of this strain.

Our data suggest that *Cryptococcus*-mediated killing of *Drosophila* following ingestion involves a variety of factors relevant to mammalian pathogenesis and is therefore a promising model with which to study fungal pathogenesis. This notion is strengthened by the recent appreciation that *C. neoformans* pathogenesis may be a consequence of adaptations that have evolved during the interaction of *C. neoformans* with environmental predators such as free-living nematodes and amoebae and that these adaptations may have selected for virulence factors that also play key roles in human disease (6). Thus, either *Drosophila* could be a natural predator for *C. neoformans*, since it is known to feed on decayed fruits in the wild (54), or it may resemble a natural predator, as has been proposed for *Caenorhabditis elegans* (40).

In *Drosophila*, fungi and bacteria activate the Toll receptor

pathway, which in turn leads to the production of antimicrobial peptides. Gram-negative bacteria primarily activate a parallel pathway (the Imd pathway), which also leads to the induction of antibacterial peptides. Additional evidence indicates that cross talk occurs between the two pathways (10) and that there may be activation of distinct sets of effector molecules in response to different categories of pathogens. In our experiments, the antifungal immune response mediated by the Toll pathway in flies was important in clearing systemic infection by *C. neoformans*, but neither the Toll nor the Imd pathway protected against killing after the ingestion of *C. neoformans*. Our results are in agreement with previous work reporting that the expression of the antifungal effector Drosomycin in the fly digestive system is not dependent on Toll signaling (20) and that the Toll-mediated host responses, which are highly expressed in the fat body following systemic infection (34), are apparently absent from the fly intestine (20, 55).

In conclusion, *C. neoformans* pathogenesis may be a consequence of adaptations that have evolved during the interaction of *C. neoformans* with environmental predators; our data suggest that the ingestion of *C. neoformans* by *D. melanogaster* may help to model certain aspects of mammalian pathogenesis of this yeast. Our data also suggest that there are significant differences in the systemic and intestinal responses of *D. melanogaster* to pathogens. Comparative studies of *Drosophila* responses to systemic versus intestinal exposure to *C. neoformans* may lead to a deeper understanding of host-fungus interactions and innate immunity.

ACKNOWLEDGMENTS

We thank Andy Alspaugh, Gary Cox, Jenny Lodge, and John McCusker for generous gifts of strains.

Financial support was provided by the Howard Hughes Medical Institute; by the Pfizer fellowship in medical mycology of the Infectious Disease Society of America; by the Massachusetts General Hospital Center for the Study of Inflammatory Bowel Disease; by the Harvard Medical School Center for AIDS Research; by the New Scholar Award in the Global Infectious Diseases of the Ellison Medical Foundation; by the Charles E. Culpeper Biomedical Pilot Initiative to E.M; by a grant from Aventis SA to F.M.A., L.G.R., and S.B.C.; and by NIH grant GM48707, awarded to F.M.A. The authors have no commercial or other association that might pose a conflict of interest.

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