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Cryptococcus neoformans Virulence Is Enhanced after Growth in the Genetically Malleable Host *Dictyostelium discoideum*

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***Cryptococcus neoformans* is an encapsulated, environmental fungus that can cause life-threatening meningitis. Pathogenicity of *C. neoformans* for macrophages and vertebrate hosts may be a mechanism selected in evolution for protection against environmental predators. In this study, we investigated whether *Dictyostelium discoideum* could serve as an alternate host for *C. neoformans*. *D. discoideum* has a defined genetic system which provides significant advantages for the study of fungus-amoeba interactions. Our results show that *D. discoideum* is susceptible to infection with *C. neoformans* and that the interactions are similar to those described previously for this fungus with macrophages and *Acanthamoeba castellanii*. Acapsular *C. neoformans* cells did not replicate when coincubated with *D. discoideum*. However, incubation of acapsular *C. neoformans* with *D. discoideum* mutants defective in myosin VII synthesis resulted in infection, validating the concept that avirulent organisms can be virulent in impaired hosts even at the unicellular level. Phagocytosis of *C. neoformans* by *D. discoideum* could be inhibited with capsule-specific antibodies and various sugars. Passage of an encapsulated *C. neoformans* strain through *D. discoideum* cultures increased virulence and was accompanied by larger capsules and faster time to melanization. These results add to the evidence implicating soil ameboid predators as important factors for the maintenance of *C. neoformans* virulence in the environment and suggest that *D. discoideum* promises to be an extremely useful system for studying the interaction of *C. neoformans* with phagocytic cells.**

Cryptococcus neoformans is an encapsulated fungus found predominately in soils contaminated with pigeon excreta, and human infection is widely assumed to result from inhalation of desiccated yeast particles or basidiospores (23). *C. neoformans* is macrophage tropic in mammalian infection, but the fungus also infects environmental microorganisms, such as the amoeba *Acanthamoeba castellanii* and the nematode *Caenorhabditis elegans* (46, 52). This pathogenic fungus has developed elaborate mechanisms to evade macrophage, nematode, and amoeba phagocytosis and killing, including a unique intracellular pathogenic strategy that causes the release of polysaccharide capsule into cytoplasmic vesicles (24, 46, 52). *C. neoformans* infections in immunocompetent hosts often result in latency and persistence, with the yeast residing inside macrophages (27, 30). The reactivation of these sequestered fungal cells can result in active disease in immunocompromised individuals, which usually presents clinically as a life-threatening meningitis (20, 27, 42). *C. neoformans*' infection of *A. castellanii* and macrophages show striking similarities; therefore, it was postulated that the pathogenicity of *C. neoformans* for macrophages was the result of evolutionary selection as a mechanism for protection against phagocytic environmental predators (52). This hypothesis was further supported by a study using *C. elegans*, which determined that fungal virulence factors played an important role in nematode killing (46).

Virulence is a microbial characteristic that is expressed and maintained in a susceptible host (12). *C. neoformans* can occasionally lose virulence during in vitro culture conditions (25). However, virulence can also be restored by passage of an avirulent *C. neoformans* strain through mice (47). Since *C. neoformans* is an environmental fungus and does not depend on an animal host for replication, it is remarkable that soil isolates are virulent for many animal species, and virulence remains relatively stable in the laboratory. *C. neoformans* laboratory isolates consistently retain traits associated with virulence, including the formation of a polysaccharide capsule, the ability to make melanin pigments, and the capacity to express certain enzymes, such as phospholipase, urease, and superoxide dismutase (9, 15, 18, 19, 41). Previous studies with *Legionella pneumophila* demonstrated an increase in virulence of the bacterium after infection of amoebae (7). This precedent together with the observations made with *A. castellanii* and *C. elegans* suggest that *C. neoformans* virulence for mammals is maintained in the environment by selection resulting from interactions with other soil organisms (46, 52).

Dictyostelium discoideum, a free-living soil amoeba, has been exploited to study professional phagocytic processes and is a model system organism (22, 49, 51). *D. discoideum* is a haploid, genetically malleable amoeba and therefore is ideal for studying host-pathogen interactions. In addition, the endolysosomal and phagosomal pathways of *D. discoideum* have been studied extensively, and many mutants are available (10, 38). Here we explore the interaction of *C. neoformans* with *D. discoideum* and show that the latter is a suitable host for the fungus. Furthermore, infection of *D. discoideum* by the fungus can affect subsequent fungal virulence for mice.

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MATERIALS AND METHODS

Organisms and culture conditions. Stock cultures of *D. discoideum* strains were maintained at −80°C. For experimental work, *D. discoideum* cultures were grown axenically in HL5 medium supplemented with 100 μg of penicillin and streptomycin (GibcoBRL, Carlsbad, Calif.)/ml at 22°C with shaking at 150 rpm for no more than 10 passages (53). Alternatively, *D. discoideum* was grown as plaques on lawns of *Klebsiella aerogenes* plated on SM/5 agar (53). *D. discoideum* AX-4 was a gift from J. E. Segall (Albert Einstein College of Medicine, Bronx, N.Y.) and was used for most experiments unless otherwise noted. *D. discoideum* HTD17-1, a myosin VII null mutant, and G1-21, a nonhomologous recombinant control for HTD17-1, were gifts from M. Titus (University of Minnesota, Minneapolis, Minn.) (58). These strains were maintained in HL5 medium supplemented with 10 μg of Blasticidin S (ICN)/ml. *rtoA D. discoideum*, a gene disruption mutant with defects in endosomal fusion and pH regulation as well as exocytosis, and DH1 wild-type cells were a gift from R. Gomer (Rice University, Houston, Tex.) and were maintained in HL5 medium as described previously (31, 63). The DH1 cell cultures were supplemented with uracil (31, 63).

C. neoformans serotype D strains 24067 and 3501 were obtained from the American Type Culture Collection (Rockville, Md.). Strain F7 is a stable pseudohyphal mutant of 24067 (26); strain Cap67, an acapsular mutant of 3501, was obtained from E. Jacobson (Richmond, Va.) (11). The *C. neoformans* serotype A strain H99 was obtained from J. Perfect (Duke University Medical College, Durham, N.C.). Yeast cultures were started from frozen stock maintained at −80°C and grown in Sabouraud dextrose (SAB) broth for 48 h at 30°C with shaking at 150 rpm, collected by centrifugation, and washed three times with phosphate-buffered saline (PBS) (0.137 M NaCl, 0.003 M sodium phosphate [pH 7.4]).

Cytotoxicity assays. *C. neoformans* replication in and cytotoxicity for *D. discoideum* was measured by CFU and trypan blue exclusion assays, respectively. *D. discoideum* viability determinations were confirmed with PFU on bacterial agar plates. The fungal and amoeba cells were washed with PBS, counted with a hemocytometer, and suspended at 10^5 cells/ml. Cell counts were confirmed by CFU on SAB agar plates incubated at 30°C. *C. neoformans* cells were added at a 1:1 effector-to-target ratio to *D. discoideum* in 96-well tissue culture plates and incubated in PBS at 22°C. The numbers of viable yeast and amoeboid cells were determined at 0, 24, and 48 h by measuring CFU and the number of *D. discoideum* that excluded the trypan blue dye, respectively. For CFU analysis, *D. discoideum* and *C. neoformans* cells were removed from the wells by placing the tissue culture plate on ice for 10 min followed by gentle agitation. *D. discoideum* cells were lysed by pulling the contents of the wells through a 27-gauge needle five times (43). At each time interval, five tissue culture wells per amoeba or fungal strain were used to ascertain viable yeast cells, and serial dilutions were plated in duplicate onto SAB agar plates, which were incubated at 30°C for 48 h. Using a trypan blue exclusion assay, the viability of *D. discoideum* was assessed at 0, 24, and 48 h. A 1:10 dilution of trypan blue in PBS was added to the undisrupted tissue culture wells at each time interval. The 96-well tissue culture plates were centrifuged at 220 × *g* for 10 min and viewed at magnification ×200. *D. discoideum* cells were considered dead if they were unable to exclude the blue dye. The percentage of dead *D. discoideum* cells was calculated from the trypan blue cytotoxicity assay by counting the number of blue cells per 100 *D. discoideum* cells. Five wells per experimental condition were counted. To confirm trypan blue results, PFU were assayed. The contents from wells containing a 1:1 ratio of *D. discoideum* to *C. neoformans* were removed gently after incubation on ice for 10 min. Serial dilutions were plated onto SM/5 agar with *K. aerogenes*, and the plates were incubated at 22°C. Plaques were visible and counted within 4 days of plating.

Phagocytosis index. *D. discoideum* cells were collected by centrifugation at 600 × *g*, washed with PBS, and suspended in HL5 media. Washed *C. neoformans* cells from strains H99, 24067, 3501, F7, and Cap67 were added to the *D. discoideum* cells at an effector-to-target ratio of 1:1, as determined by hemocytometer counts, and incubated at 22°C for 2 h. The medium was aspirated, and the cells were fixed with ice-cold methanol for 30 min at 4°C. Cells were not washed prior to fixing to avoid removal of loosely adhered *D. discoideum* cells. Wells were washed with 2× PBS and stained with a 1:10 dilution of Giemsa for 45 min. The plates were viewed at magnification ×200, and five wells per experimental condition were used to determine the phagocytic index, defined as the number of amoebae with at least one internalized fungal cell (8).

Transmission electron microscopy. Fungal and AX-4 amoeba cells were washed as described above and incubated at a 1:1 ratio in HL5 media for 2.5 h with gentle rocking. The cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate at room temperature overnight, and the samples were prepared according to published methods and viewed with a model 102 electron microscope (Siemens, Berlin, Germany) (59).

Phagocytosis inhibition assays. Carbohydrate phagocytosis inhibition assays were used to determine what receptors might be involved in the phagocytosis of *C. neoformans* by *D. discoideum* AX-4. Concentrations ranging from 1 to 1,000 mM of D-mannose, D-galactose, D-xylose, and mannitol (Sigma, St. Louis, Mo.) were added to *D. discoideum* in 96-well plates and incubated for 1 h prior to adding *C. neoformans* H99 as described above. Five wells per condition were counted.

To assess the effect of antibody specific for the *C. neoformans* capsule, the immunoglobulin G1 (IgG1) monoclonal antibody (MAb) 18B7 and *C. neoformans* 24067 were added to *D. discoideum* in 96-well plates prepared as mentioned above for phagocytosis assays. Concentrations of MAb 18B7 were varied between 1, 10, and 100 μg/ml. The IgG1 MAb 3665 was used as an irrelevant isotype-matched control (48). Phagocytic index was determined by averaging results from five wells.

Murine studies. *C. neoformans* strain 24067 cells were passaged through *D. discoideum* AX-4 by coincubating *C. neoformans* and *D. discoideum* cells, and virulence was determined by murine survival. Four preparations were evaluated for virulence in mice: (i) *D. discoideum* cells with *C. neoformans* cells, (ii) killed *D. discoideum* cells with *C. neoformans* cells, (iii) *D. discoideum* alone, and (iv) *C. neoformans* alone. All cells were washed, counted, and suspended in HL5 medium in 175-ml tissue culture flasks (Corning, Corning, N.Y.). Live fungal cells were added at a 1:10 ratio to *D. discoideum*. *D. discoideum* cells were killed by sonication, which was confirmed by trypan blue exclusion. All flasks were incubated at 22°C for 72 h. As determined by light microscopy, there were no whole *D. discoideum* cells present in the culture that contained both *C. neoformans* and *D. discoideum* cells at 72 h. Cells were removed from the tissue culture flasks and washed four times with PBS and suspended in PBS. A total of 10^7 *C. neoformans* cells or *D. discoideum* cells, prepared as noted above, were injected into the peritoneum of A/Jcr female mice, 7 to 9 weeks of age, from the National Cancer Institute (Bethesda, Md.). The survival time was recorded.

Phenotypic analysis of passaged *C. neoformans* cells. To ascertain if there were phenotypic differences between the passaged and nonpassaged *C. neoformans* cells, we determined the growth rate, time to melanization, and capsule size for both groups. Immediately after coincubation with *D. discoideum*, the growth rate of *C. neoformans* was determined in SAB broth at either 30 or 37°C for 5 days by counting cells and CFU as a function of time. Cells were also streaked onto agar plates containing minimal media with 1 mM L-Dopa, incubated in the dark, and checked daily for pigmentation change, indicative of melanization (59, 62). The experiment was simultaneously performed with nonpassaged *C. neoformans* cells. To measure capsule size differences in *C. neoformans*, fungal cells were grown at 37 and 30°C for 24 h, and capsule size was measured using India ink exclusion. Micrographs were taken at ×400, and the capsule area was measured using Scion image software (National Institutes of Health, Bethesda, Md.). The capsule and cell body size of 20 cells per condition were counted, and capsule size was determined as the total organism area minus the area of the cell body in pixels.

Statistical analysis. All experiments were performed at least two independent times. Student *t* test analysis and cytotoxicity graphs were compiled in Excel 2000 (Microsoft; Redmont, Wash.). Survival analysis was performed using log rank analysis (SPSS, Chicago, Ill.). (This work is from a thesis to be submitted by J.N.S. in partial fulfillment of the requirements for a Ph.D. in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Bronx, N.Y.)

RESULTS

Growth of *C. neoformans* with *D. discoideum*. *D. discoideum* AX-4 was infected with *C. neoformans*, and fungal growth was determined by counting CFU. Wild-type *C. neoformans* strains were able to grow in the presence of *D. discoideum*, as evidenced by an increase in the number of CFU with time (Fig. 1A, B, and C). Increases in CFU were evident for both serotype A (H99) and serotype D (24067 and 3501) strains. For both strain H99 and strain 3501, there was more growth between 24 and 48 h; however, strain 24067 had the greatest growth increase in the first 24 h. The number of CFU increased between 12-fold for H99 and almost 50-fold for 24067. The CFU increases at 48 h were significant compared to CFU of *C. neoformans* alone for all three wild-type strains ($P \leq 0.001$). In

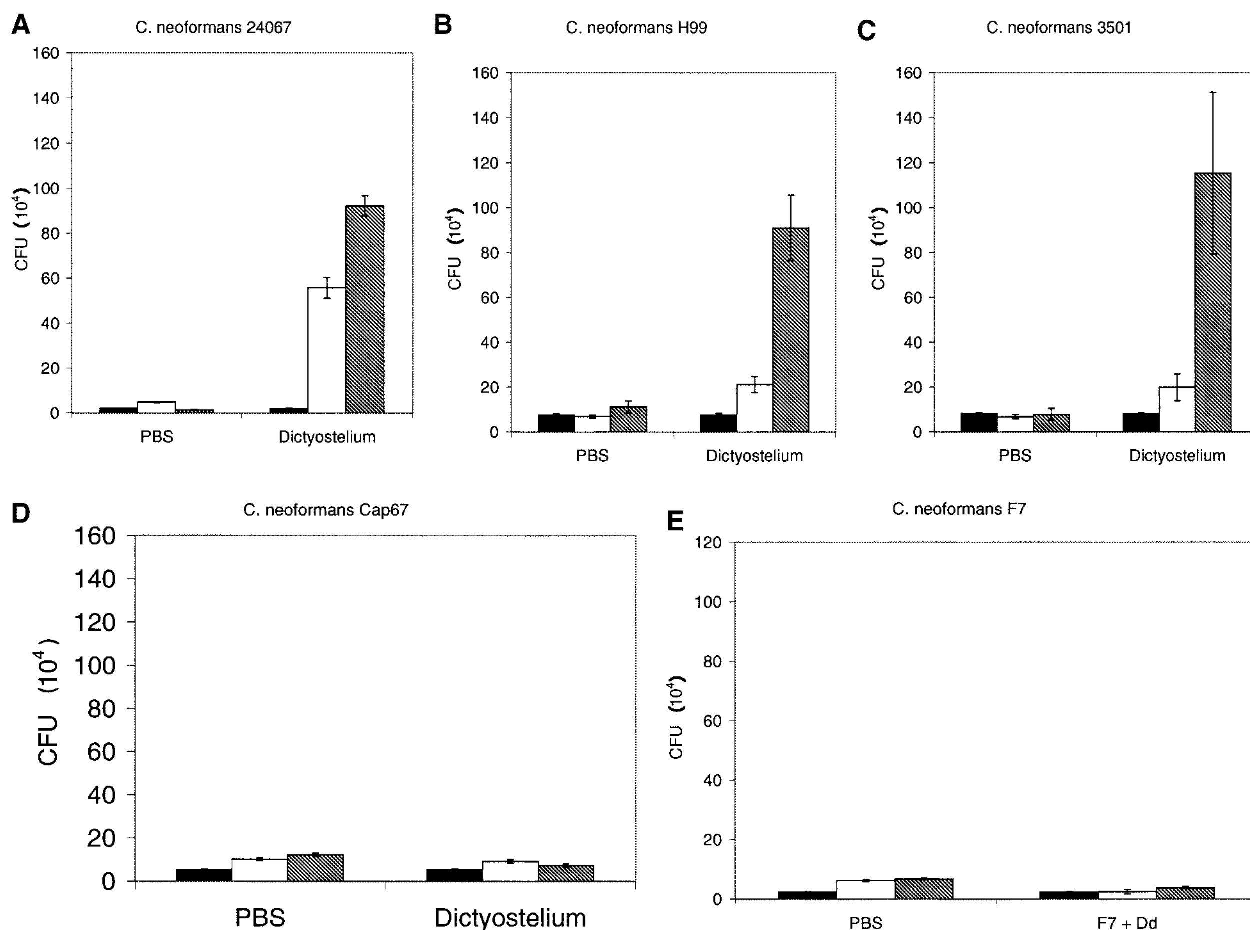

FIG. 1. Fungal CFU after incubation of *C. neoformans* with or without *D. discoideum*. Solid bars denote CFU at 0 h, open bars denote CFU at 24 h, and hatched bars denote CFU at 48 h. The error brackets represent one standard deviation. The differences between the CFU of *C. neoformans* strains 24067, H99, and 3501 incubated with *D. discoideum* and the corresponding fungal cells in PBS at 48 h were significant ($P \leq 0.001$). Numbers of CFU for *C. neoformans* Cap67 were similar in PBS and *D. discoideum* for each time interval. Numbers of CFU for *C. neoformans* strains 24067 (serotype A) (A), H99 (serotype D) (B), 3501 (serotype A) (C), Cap67 (acapsular variant of 3501) (D), and F7 (pseudohyphal variant of 24067) (E) are shown. Each experiment was repeated with similar results.

addition, results with Cap67, an avirulent acapsular mutant of 3501, indicate that the capsule is required for *C. neoformans* growth in *D. discoideum* (Fig. 1D). There was no change in the number of CFU of Cap67 when exposed to *D. discoideum*. At 48 h there was no difference in CFU between *C. neoformans* Cap67 grown in PBS or *D. discoideum* conditions ($P = 0.445$). Similar results were demonstrated for F7, a hypovirulent, pseudohyphal variant of 24067 (Fig. 1E).

Changes in *D. discoideum* viability were assessed using both a trypan blue exclusion assay and PFU enumeration. The two methods produced similar results. Trypan blue exclusion results showed that *C. neoformans* killed *D. discoideum* (Fig. 2A). The greatest killing of *D. discoideum* occurred with *C. neoformans* strains H99 and 3501, with 53 and 52%, respectively, at 24 h. Incubation of *D. discoideum* with *C. neoformans* 24067, H99, and 3501 resulted in a decrease in *D. discoideum* viability at 48 h as indicated by the number of PFU (Fig. 2B). This decrease was significant when compared to *D. discoideum* in PBS only ($P \leq 0.001$). In contrast, the acapsular mutant, *C. neoformans* Cap67, caused no significant decrease in PFU numbers from that for *D. discoideum* in PBS only ($P = 0.397$). The results from the trypan blue exclusion and PFU assay parallel each other, indicating that wild-type *C. neoformans* strains were lethal to *D. discoideum.*

Phagocytosis of *C. neoformans* by *D. discoideum*. Phagocytosis assays were used to confirm that *D. discoideum* cells ingested *C. neoformans* cells. The phagocytic index showed that up to 80% of wild-type AX-4 *D. discoideum* cells phagocytosed at least one *C. neoformans* fungal cell (Fig. 3). The majority of *D. discoideum* cells internalized one fungal cell, though two and three *C. neoformans* cells were found internalized in occasional amoeboid cells. The hypovirulent *C. neoformans* strains F7 and Cap67 were phagocytosed in significantly higher numbers than virulent parental strains.

Intracellular interaction of *C. neoformans* with *D. discoideum*. Transmission electron microscopy was used to investigate the interactions of *C. neoformans* and *D. discoideum*. Figure 4A depicts *D. discoideum* phagocytosing a *C. neoformans* strain 3501 cell. *D. discoideum* pseudopods can be seen surrounding the encapsulated yeast as the fungal cell is en-

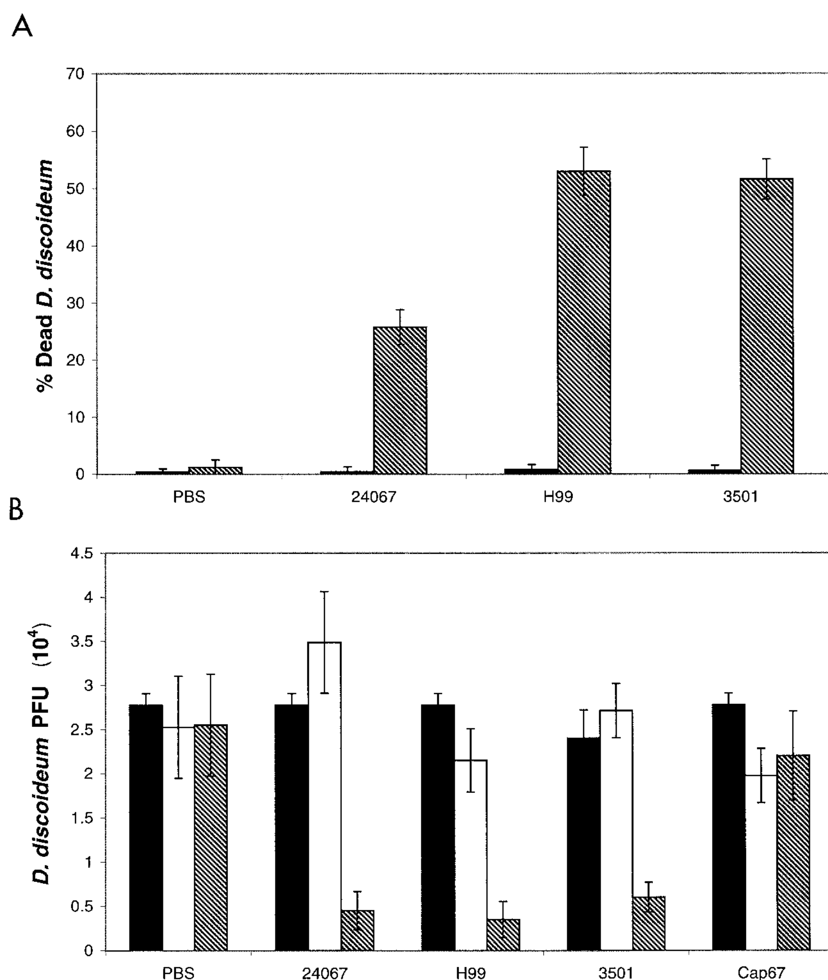

FIG. 2. Viability of *D. discoideum* after incubation with *C. neoformans*. Solid bars represent viability at 0 h, open bars represent viability at 24 h, and hatched bars represent viability at 48 h. Error brackets denote one standard deviation. (A) Percentages of amoebae that are trypan blue positive. At 48 h, *P* values were $\leq$0.001 for comparisons of amoebae incubated with any of the *C. neoformans* strains with *D. discoideum* to amoebae alone. (B) Numbers of PFU representing the total numbers of viable *D. discoideum* cells. At 48 h a significant decrease in PFU of *D. discoideum* cells was measured after incubation *C. neoformans* strains 24067, H99, and 3501 ($P \leq 0.001$). The number of PFU of *D. discoideum* incubated with *C. neoformans* Cap67 was unchanged throughout the assay. The experiment was done twice with similar results.

gulfed. Intracellular replication by *C. neoformans* cells was suggested by the observation of budding fungal cells inside *D. discoideum* (Fig. 4B). Ingested fungal cells were contained in a membrane-enclosed vacuole inside *D. discoideum*. Some *D. discoideum* cells appeared to have participated in at least two phagocytic events, as suggested by the presence of separate phagocytic vacuoles containing *C. neoformans* cells. The micrographs shown are representative for all wild-type *C. neoformans* strains tested and confirm that the *C. neoformans* cells are ingested by *D. discoideum*.

***D. discoideum* mutants affect growth of *C. neoformans*.** The genetics of *D. discoideum* are well defined, and an assortment of known null mutants is available. Previously characterized amoeba mutants defective in phagocytoses and exocytosis were analyzed to explore the usefulness of *D. discoideum* mutants to study the interaction of amoeboid cells with *C. neoformans*.

The *myoi D. discoideum* mutant is a null mutant for myosin VII (58). *D. discoideum* myosin VII is important in cell adhesion and particle adhesion during phagocytosis (58). Phagocytosis of the hypovirulent *C. neoformans* strain Cap67 by the *myoi* null *D. discoideum* mutant was only 49% of the G1-21 nonhomologous control *D. discoideum* phagocytic index ($P = 0.042$) (Fig. 5A). Also, the *D. discoideum myoi* null mutant was more permissive to growth of *C. neoformans* strain Cap67 (Fig. 5B). The number of CFU of Cap67 increased dramatically compared to the absence of growth for Cap67 cells grown with *D. discoideum* G1-21 control cells ($P = 0.002$). When evaluating the effect of the *myoi* mutant on the wild-type *C. neoformans* strain 3501, a less dramatic result was seen; however, the *myoi* null *D. discoideum* cells were still more permissive to growth of 3501 ($P = 0.041$) (Fig. 5C).

A second mutant was used to evaluate the contribution of the amoeboid cell exocytosis phenotype to the interaction of *C. neoformans* and *D. discoideum*. Through homologous recombination, *rtoA* mutants were created which had normal endocytosis but reduced and poor exocytosis (5, 63). *rtoA* mutants are defective in endosomal vesicle fusion and the regulation of both endosomal and cytosolic pH (5, 63). Surprisingly, phago-

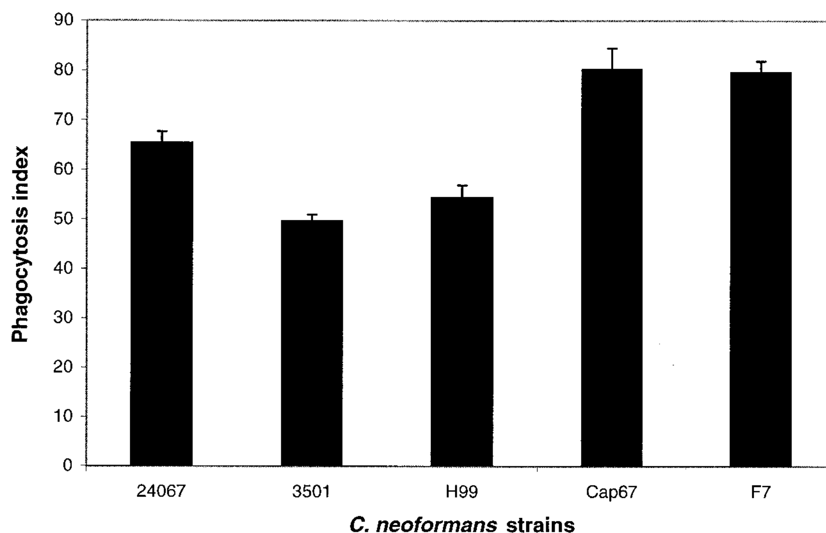

FIG. 3. Phagocytosis of *C. neoformans* strains 24067, 3501, H99, F7, and Cap67 by *D. discoideum*. Bars represent the numbers of phagocytic events by *D. discoideum*, and error brackets denote 1 standard deviation. The phagocytosis index was determined by counting the total number of internalized fungal cells per 100 amoebae. For each experimental condition the number of repetitions was five. This experiment was repeated on different days and yielded similar results.

cytosis assays revealed that the *rtoA D. discoideum* cells phagocytosed fewer fungal cells than parental *D. discoideum* DH1 controls for *C. neoformans* (Fig. 5E) ($P \leq 0.001$). This reduced phagocytosis, however, did not translate to reduced growth as determined by numbers of CFU (Fig. 5). The *rtoA* mutant supported growth as well as DH-1 control cells ($P = 0.665$).

Phagocytosis inhibition studies. To understand the interaction between *D. discoideum* and *C. neoformans*, we evaluated the efficiency of phagocytosis using several potential inhibitors. Four sugars were investigated for their ability to inhibit phagocytosis: D-mannose, D-xylose, D-galactose, and mannitol. At sugar concentrations of 0.5 M or higher, there was a marked

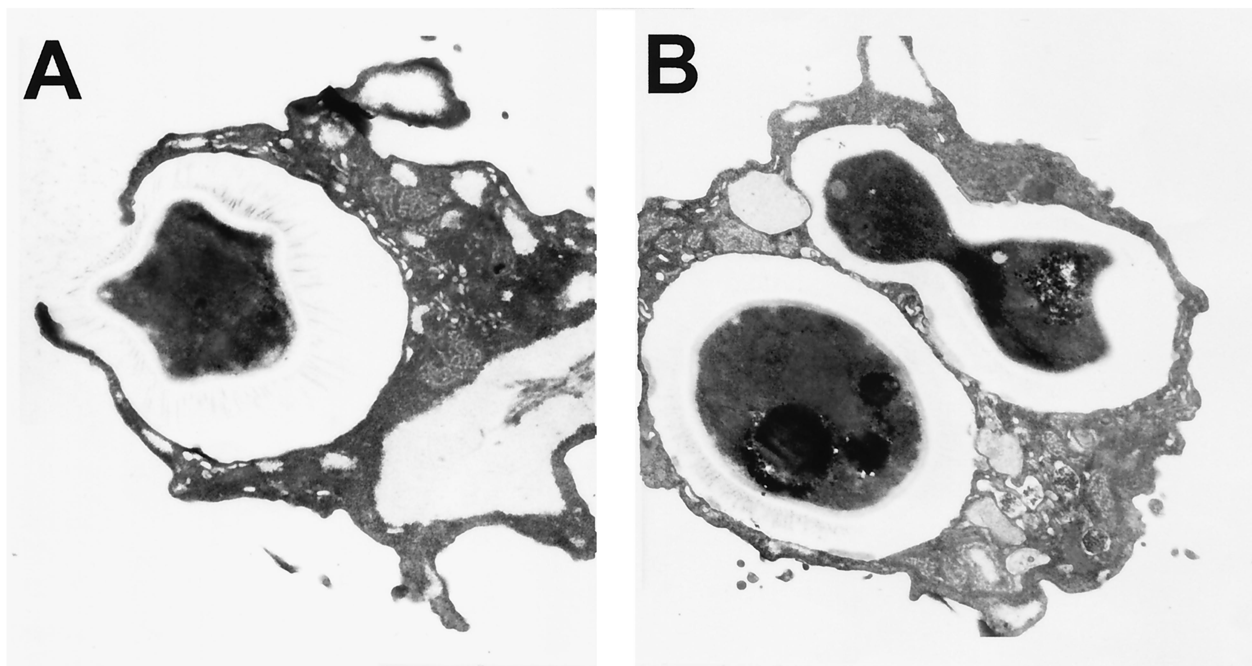

FIG. 4. Transmission electron micrographs of *C. neoformans* strain 3501 cells interacting with *D. discoideum*. (A) *C. neoformans* is being engulfed by the pseudopods of a *D. discoideum* cell 2 h postinfection. (B) Two individual phagocytic events by one *D. discoideum* cell 2 h postinfection. In one event the *C. neoformans* cells are in membrane-bound vacuoles. The second event shows a budding *C. neoformans* cell in a vacuole. (Magnifications: ×24,000 [A] and ×18,000 [B]). The micrographs shown are representative of what was observed under the microscope.

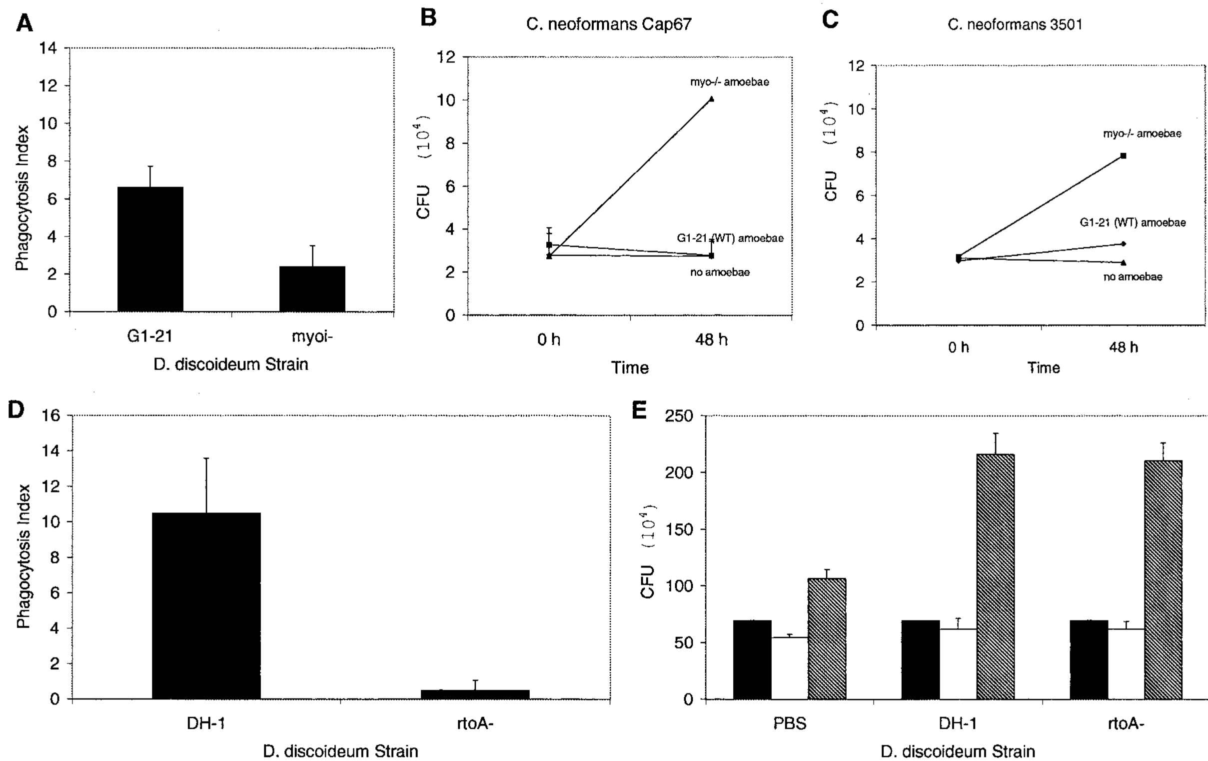

FIG. 5. Interaction of *C. neoformans* strains with *D. discoideum myoi* and *rtoA* mutants. (A) Phagocytosis of *C. neoformans* Cap67 cells by *D. discoideum myoi* or wild-type cells. Wild-type *D. discoideum* cells phagocytosed significantly more fungal cells than *myoi* null cells ($P = 0.042$). Solid bars represent the numbers of phagocytic events by *D. discoideum*, and error brackets denote one standard deviation. The phagocytosis index was determined by counting the number of *D. discoideum* cells with at least one internalized *C. neoformans* cell. For each experimental condition the number of repetitions was five. (B) Number of CFU of Cap67 after incubation with *myoi* null *D. discoideum* cells (■), wild-type *D. discoideum* cells (◆), and alone (▲). Error bars denote one standard deviation. (C) Number of CFU of 3501 after incubation with *myoi* null *D. discoideum* cells (■), wild-type *D. discoideum* cells (◆), and alone (▲). Error bars denote one standard deviation. (D) Phagocytosis of *C. neoformans* H99 cells by *D. discoideum rtoA* or wild-type cells. Wild-type *D. discoideum* cells phagocytosed significantly more fungal cells than *rtoA* null cells ($P \leq 0.001$). Solid bars represent the numbers of phagocytic events by *D. discoideum*, and error brackets denote one standard deviation. For each experimental condition the number of repetitions was five. (E) *C. neoformans* H99 cell counts after incubation with either wild-type or *rtoA D. discoideum* cells. Bars represent numbers of CFU at different times; solid bars denote CFU at 0 h, open bars denote CFU at 24 h, and hatched bars denote CFU at 48 h. The error brackets represent one standard deviation. There are no significant differences between *C. neoformans* growth with wild-type and *rtoA D. discoideum* cells. These experiments were repeated on different days and yielded similar results.

decrease in phagocytosis of *C. neoformans* by *D. discoideum* strain AX-4 with each compound (data not shown). Concentration of 1 M D-mannose inhibited *C. neoformans* phagocytosis by 90% (Fig. 6). Trypan blue exclusion assays ascertained that 1 M mannose was not cytotoxic to the *D. discoideum* cells (data not shown). In fact, *D. discoideum* cells with added sugars had a slightly higher percentage of live cells than did those incubated in PBS alone.

The *C. neoformans* capsule is primarily composed of glucuronoxylomannan, and antibodies against this polysaccharide are opsonic. The IgG1 MAb 18B7 binds glucuronoxylomannan and enhances phagocytosis of *C. neoformans* cells by macrophages (45). Since it has been shown that the *C. neoformans* capsule is essential for fungal virulence and capsule-specific antibodies enhance phagocytosis by macrophages, we investigated the effect of 18B7 binding to yeast cells on *D. discoideum* phagocytosis (13), (36). Binding of 18B7 to the capsule of *C. neoformans* inhibited phagocytosis by *D. discoideum* (Fig. 5). In fact, 18B7 inhibited phagocytosis in a dose-dependent manner, since 1 μg of antibody/ml had no inhibitory affect while both 10

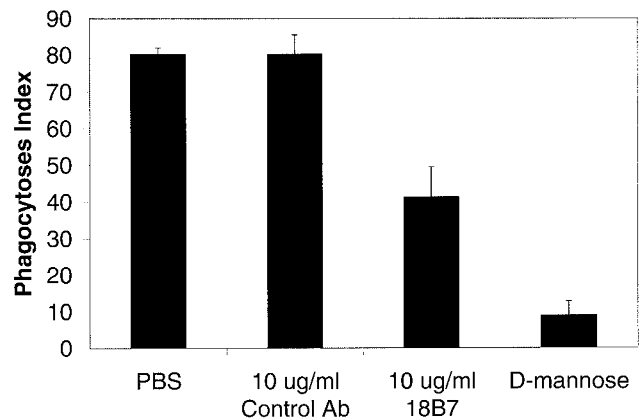

FIG. 6. Inhibition of *D. discoideum* phagocytosis of *C. neoformans* 24067 cells by 1 M mannose and MAb 18B7. Solid bars represent the number of phagocytic events by *D. discoideum*, and error brackets denote one standard deviation. The phagocytosis index was determined by counting the number of *D. discoideum* cells with at least one internalized *C. neoformans* cell. For each experimental condition the number of repetitions was five.

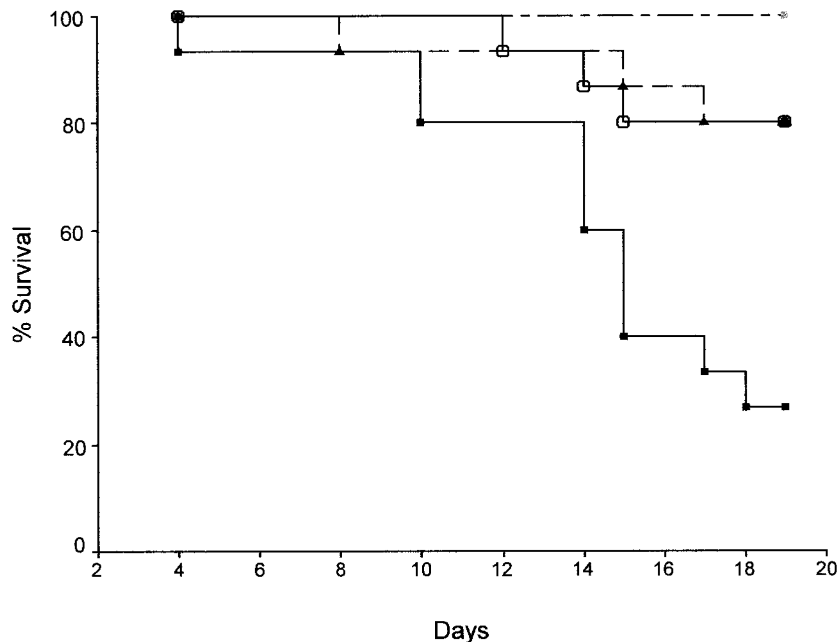

FIG. 7. Survival of A/J mice infected with either 10^7 *C. neoformans* cells grown with live *D. discoideum* (−■−) (n = 15), 10^7 *C. neoformans* cells grown with killed *D. discoideum* (−○−) (n = 15), 10^7 *C. neoformans* cells (-▲-) (n = 15), or 10^7 live *D. discoideum* cells (-×-) (n = 5). The graph shows that *C. neoformans* 24067 primed by growth with live *D. discoideum* is more lethal than *C. neoformans* grown either alone or with killed *D. discoideum* ($P \leq 0.005$ for both). Also, *D. discoideum* alone was not pathogenic. There were no significant differences in the survival of mice infected with *C. neoformans* alone and *C. neoformans* grown with killed *D. discoideum* ($P = 0.974$). This experiment was performed twice with similar results.

and 100 μg/ml of 18B7 significantly inhibited phagocytosis of *C. neoformans* by *D. discoideum* ($P = 0.005$ and $P \leq 0.001$, respectively) (data not shown). In contrast, an isotype-matched control antibody that does not bind to the capsule had no effect on phagocytosis ($P = 0.749$).

Incubation of *C. neoformans* with *D. discoideum* increases virulence in mice. Mice infected with *C. neoformans* grown with live *D. discoideum* AX-4 had significantly shorter survival times than mice infected with *C. neoformans* grown alone or with killed *D. discoideum* ($P \leq 0.001$) (Fig. 7). Mice infected with *C. neoformans* grown with killed *D. discoideum* lived as long as mice infected with *C. neoformans* grown in medium only. No deaths occurred for mice infected with *D. discoideum* alone.

We analyzed *D. discoideum*-passaged and nonpassaged *C. neoformans* cells for growth rate, time to melanization, and capsule size to assess phenotypic differences that may be associated with alterations in virulence. The capsule size of fungal cells passaged with *D. discoideum* was larger than that of cells grown in medium at 37°C ($P \leq 0.001$) (Fig. 8A). The average capsule size of the *D. discoideum*-passaged *C. neoformans* cells grown at 37°C was more than twice the size of the capsule from *C. neoformans* grown alone. However, cells grown at 30°C had no noticeable capsule size difference. There were no differences in the growth rates between *C. neoformans* cells passaged with or without *D.* discoideum (Fig. 8B). *C. neoformans* passaged through *D. discoideum* melanized more rapidly than the nonpassaged control at both 30 and 37°C. The difference in melanization became noticeable at day 4, with pronounced differences by day 5.

DISCUSSION

A. castellanii was the first phagocytic organism to be identified as an alternative host for *C. neoformans* infection (52). Recent studies have extended the alternative host range for *C. neoformans* to the nematode *C. elegans* (46). Here we broaden the consortium of alternative host models for the study of *C. neoformans* virulence to include *D. discoideum*. The availability of *D. discoideum* as an alternative host is of particular significance because this organism can be easily maintained, and the genetics are well defined. Furthermore, *D. discoideum* is recognized as a model system for study of phagocytic processes and microbial pathogenesis (49). Many *D. discoideum* mutants are available with phenotypes ranging from defects in phagocytic processes to impaired motility. In this study we show that *D. discoideum* is suitable as a model host for *C. neoformans* infection and can affect cryptococcal virulence.

C. neoformans was readily ingested by *D. discoideum*, and the interaction between the fungal and amoeboid cells resulted in the death of the host cell and proliferation of the yeast. *D. discoideum* mutants in myosin and exocytosis were more susceptible to *C. neoformans* as illustrated by the fact that an acapsular mutant which is avirulent for wild-type *D. discoideum* replicated and killed *D. discoideum* mutants. Most interestingly, *C. neoformans* virulence was enhanced by passage through live *D. discoideum* culture. These results significantly extend prior findings made with *A. castellanii* to demonstrate that interaction of amoeboid cells with *C. neoformans* cells can alter fungal virulence.

Myosin VII is a member of a large family of proteins, but its

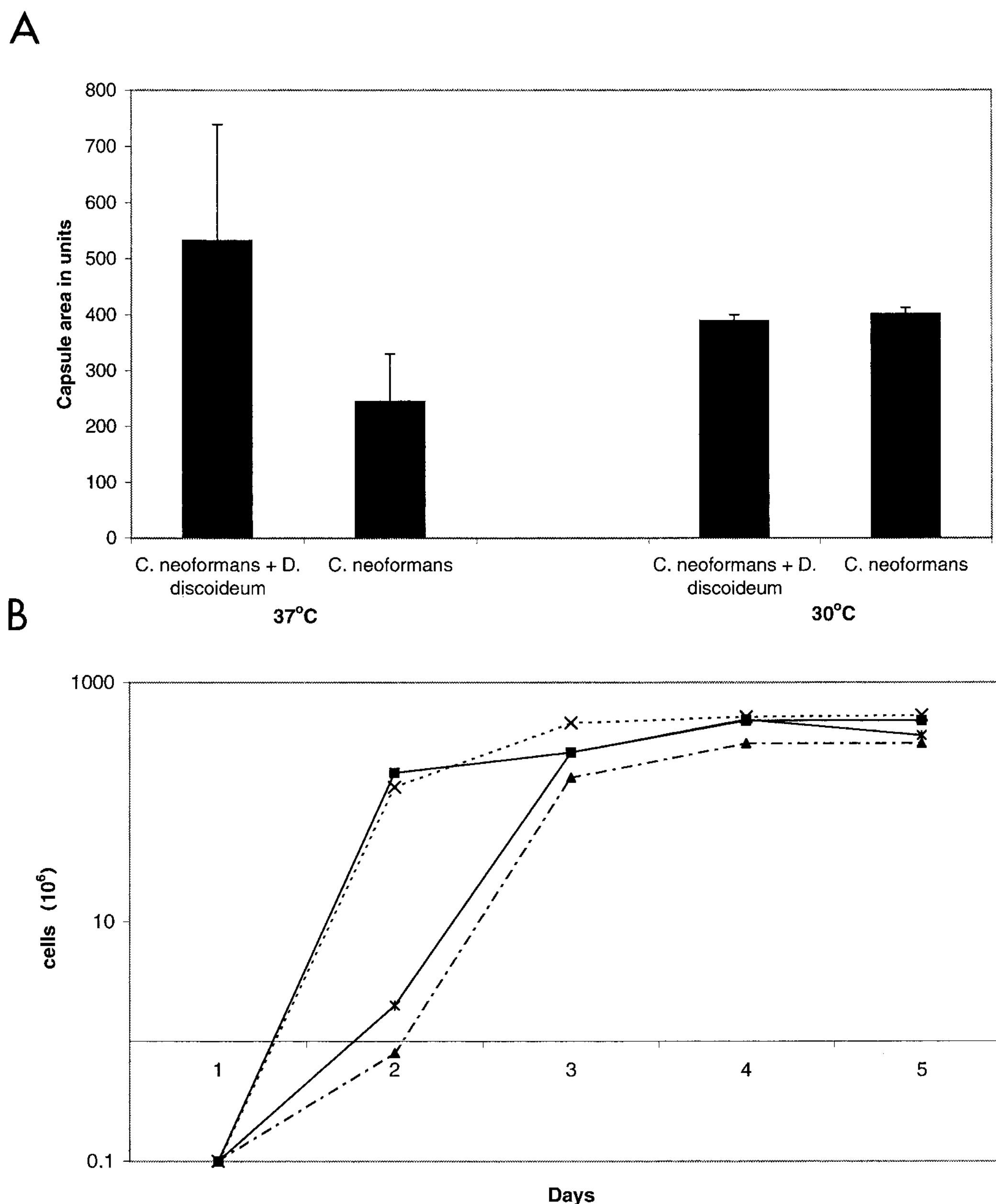

FIG. 8. Phenotypic differences between *D. discoideum* passaged or unpassaged *C. neoformans* cells. (A) Capsule area of *C. neoformans* cells grown at 37 or 30°C after passage. Bars represent capsule area, and error brackets denote one standard deviation. At 37°C there is a difference between *D. discoideum* passaged *C. neoformans* cells and unpassaged cells ($P \leq 0.001$). (B) Growth curves at 37 and 30°C of passaged or unpassaged *C. neoformans* cells. Growth curves are similar for passaged *C. neoformans* grown at 37°C (×) compared to unpassaged *C. neoformans* at 37°C (■) and for passaged *C. neoformans* at 30°C (*) compared to unpassaged *C. neoformans* at 30°C (▲). This experiment was repeated with similar results.

specific function is not well understood. Earlier studies have associated myosin VII mutations with deafness in humans (37). Recent studies with *D. discoideum* suggest that it has important roles in adhesion and phagocytosis, specifically with particle adhesion and phagocytosis (56, 58). Myosin VII null cells, *myoi* cells, were reported to phagocytose particles at only 20% of the wild-type rate due to a decrease in particle binding (56, 58). In our experiments, the *myoi* mutant was less efficient in ingesting *C. neoformans* cells than the G1-21 wild-type *D. discoideum* control. However, the *myoi* mutant was also significantly more permissive to *C. neoformans* growth than wild-type *D. discoideum*, suggesting a role for myosin VII in amoeboid defense against live yeast. These results may seem paradoxical given that *C. neoformans* is a facultative intracellular parasite (24). The specific defect in the *myoi* null *D. discoideum* cells was determined to involve particle binding, cell-to-cell adhesion, and cell-to-substratum adhesion; however, these characterization assays employed either latex beads or heat-killed *Saccha-*

romyces cerevisiae in a nonadherent cell assay (58). Our live fungal cells could have exerted additional stresses on the *D. discoideum* cells that were not present in assays using killed yeast cells. Similar phagocytic differences were observed in experiments using *L. pneumophila* in which a myosin I double mutant was more permissive to bacterial growth only in adherent culture conditions (51).

rtoA is an important gene for exocytosis and phagosomal pH regulation for *D. discoideum* (5, 6, 63). Prior studies using both beads and nonpathogenic yeast cells in a nonadherent cell assay suggested that phagocytosis was not impaired in this mutant (5, 6). However, for *C. neoformans*, *D. discoideum* cells deficient in RtoA manifested significantly reduced phagocytosis. *C. neoformans* cells are encapsulated, and the interaction of amoeboid cells with this yeast may be significantly different than that with nonpathogenic yeasts. It was proposed that RtoA is required for lipid bilayer fusion through transient cell membrane association (5). Therefore, RtoA− *D. discoideum* mutants may be defective in phagocytosis in our adherent cell assay compared to the case in previous assays, which identified the RtoA mutational affect in a nonadherent amoeba cell assay. Hence, the interaction of *C. neoformans* with RtoA-deficient *D. discoideum* revealed an unexpected phenotype in the host cells. It is interesting that both parental control strains G1-21 and HTD-17 were less efficient at phagocytosing *C. neoformans* cells than the AX-4 wild-type *D. discoideum* strain used for all other amoeba experiments.

C. neoformans infection follows the inhalation of yeast cells into the lungs. This initial infection is generally contained by lung granulomas involving macrophages (1, 14, 28, 29). However, if the host becomes immunocompromised, asymptomatic infections can reactivate and cause cryptococcosis (20). Patients with underlying phagocyte deficiencies are well known to be more susceptible to disease resulting from *C. neoformans* infection (4, 40, 55). In parallel with the human experience, where immune deficiencies can enhance susceptibility, the *myoi* and *rtoA D. discoideum* mutants were also more susceptible to *C. neoformans* infection. Therefore, these experiments validate the concept that host defects increase susceptibility to pathogenic fungi for unicellular host-pathogen interactions and suggest that the interaction of *C. neoformans* with mutant *D. discoideum* is analogous to that observed for some immunocompromised states in mammals. Furthermore, identification of susceptibility genes in *D. discoideum* suggests that homologous genes in mammals have a similar function with regard to fungal pathogenesis.

Phagocytosis is mediated through cell surface receptor binding particles, which initiate a signal transduction cascade resulting in actin polymerization and particle internalization into a phagosome (32). This process of receptor-mediated phagocytosis is similar in *Dictyostelium* and macrophages (2, 3). To identify the mechanism by which *D. discoideum* interacted with *C. neoformans* for phagocytosis, we investigated the effect of specific sugars and antibodies on the efficiency of phagocytosis. The monoclonal antibody 18B7, specific for the *C. neoformans* polysaccharide capsule, inhibits *C. neoformans* phagocytosis by *D. discoideum*. Fc-like receptors are unlikely to be involved in particle internalization processes of *D. discoideum*, since these are vertebrate receptors that mediated their activity through tyrosine kinase activity, a process not required for *D. discoideum* phagocytosis (50). Furthermore, there is no reason to suppose that *D. discoideum* would have a receptor for ingesting antibody-coated *C. neoformans*, since immunoglobulins are products of multicellular animals. However, surface-expressed glycoproteins have been implicated as *D. discoideum* phagocytic receptors, and antibodies designed against oligosaccharides of these *D. discoideum* glycoproteins inhibited phagocytosis and cross-reacted with a range of membrane proteins (16, 17). The mechanism by which antibody binding to the capsule reduces *D. discoideum* phagocytosis is most likely due to blocking sites in the polysaccharide that are recognized by amoeba phagocytic receptors. Alternatively, antibody binding could cause a conformational change of the *C. neoformans* capsule, masking the C*. neoformans* residues that are recognized by *D. discoideum* phagocytic receptors (54).

Phagocytosis was also inhibited by the four carbohydrates tested, with D-mannose having the greatest effect, suggesting that carbohydrate receptors on the surface of *D. discoideum* may be involved in recognition and ingestion of yeast cells. These results would be more suggestive of a specific effect if one of the sugars did not affect phagocytosis of *C. neoformans* by *D. discoideum*. However, the interaction of *D. discoideum* with *C. neoformans* through a mannose-like receptor would imply that it is similar to the interaction of *C. neoformans* with macrophages and dendritic cells, where the mannose receptor is used to bind cryptococcal cells through the cryptococcal mannoprotein (39).

Passaging *C. neoformans* through *D. discoideum* cultures significantly increased virulence for mice, as evidenced by shorter survival times for mouse groups infected with these passaged cells. To our knowledge this is the first demonstration that the interaction of the fungus with a nonmammalian host can modify *C. neoformans* virulence. To investigate the mechanism for increased virulence, we analyzed three phenotypes that are associated with virulence: capsule size, melanin formation, and growth rate. The capsule is essential in virulence and has antiphagocytic as well as cytotoxic properties, while melanization protects against oxygen and nitrogen free radicals and microbicidal peptides (24, 33–36, 60, 61). Passaged *C. neoformans* cells expressed larger capsules and melanized faster than nonpassaged cells. Given recent studies linking capsule size to virulence (21) and the high likelihood that more-rapid melanization could have enhanced survival of yeast cells in mice, these observations suggest an explanation for the enhanced *C. neoformans* virulence through microevolution. Although the molecular and genetic mechanisms responsible for this phenomenon are not understood, it is clear that *C. neoformans* is capable of rapid phenotypic change. The ability of an organism to undergo microevolution is believed to be a vital characteristic for survival, allowing a pathogen to change its virulence and specificity (44). The passage experiments provide a powerful example of the capacity of this organism to undergo rapid change when confronted with certain selective pressures. In this regard, our findings suggest that the *D. discoideum* system could provide an excellent model for studying the effects of microevolution of *C. neoformans* virulence.

The capsule size increase was noted only in passaged cells grown at 37°C, which may be linked to the cAMP-PKA signal transduction pathway in *C. neoformans*, which has been identified as a regulatory pathway for capsule production, melanin

formation, mating, and virulence (57). Similar changes in regulation could be responsible for the decrease in time to melanization observed for the passaged *C. neoformans* cells. Interestingly, passaging *C. neoformans* through *D. discoideum* did not alter the results of the amoeba killing assay. Unpassaged and passaged cells had nearly identical abilities to kill the amoebae and to grow in the presence of amoebae. This may be due to host specificity and possibly to capsule and melanin expression being already optimal for *D. discoideum* pathogenicity. Nevertheless, murine survival studies with the passaged *C. neoformans* cells illustrate a clear change in *C. neoformans* virulence after *D. discoideum* passage.

In summary, our results indicate that (i) *D. discoideum* can be used to study *C. neoformans* phagocytosis, (ii) *C. neoformans* virulence is enhanced after passage through *D. discoideum*, and (iii) enhancement of virulence is correlated with capsule size and melanization. The results with the *D. discoideum* mutants suggest new roles for the myosin VII and RtoA genes, illustrating the power of this system for study of host genetic factors that are important in antifungal defense. The findings in this study are consistent with and support the hypothesis that *C. neoformans* virulence for mammalian hosts is maintained in the environment by predatory microorganisms in soils.

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