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Phagocytosis of *Cryptococcus neoformans* by, and Nonlytic Exocytosis from, *Acanthamoeba castellanii*▽†

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***Cryptococcus neoformans*, an encapsulated, pathogenic yeast, is endowed with a variety of virulence factors, including a polysaccharide capsule. During mammalian infection, the outcome of the interaction between *C. neoformans* and macrophages is central to determining the fate of the host. Previous studies have shown similarities between the interaction of *C. neoformans* with macrophages and with amoebae, resulting in the proposal that fungal virulence for mammals originated from selection by amoeboid predators. In this study, we investigated the interaction of *C. neoformans* with the soil amoeba *Acanthamoeba castellanii*. Comparison of phagocytic efficiency of the wild type, nonencapsulated mutants, and complemented strains showed that the capsule was antiphagocytic for amoebae. Capsular enlargement was associated with a significant reduction in phagocytosis, suggesting that this phenomenon protects against ingestion by phagocytic predators. *C. neoformans* var. *neoformans* cells were observed to exit amoebae several hours after ingestion, in a process similar to the recently described nonlytic exocytosis from macrophages. Cryptococcal exocytosis from amoebae was dependent on the strain and on actin and required fungal viability. Additionally, the presence of a capsule was inversely correlated with the likelihood of extrusion in certain strains. In summary, nonlytic exocytosis from amoebae provide another parallel to observations in fungus-macrophage interactions. These results provide additional support for the notion that some mechanisms of virulence observed during mammalian infection originated, and were selected for, by environmental interactions.**

The encapsulated yeast *Cryptococcus neoformans* is an environmental organism that is capable of causing human disease. This fungus is a facultative intracellular pathogen with a unique pathogenic strategy, despite no obvious need for replication in an animal host as part of its life cycle (10). *C. neoformans* is known to interact with protozoa, some of which have been shown to be effective predators for this fungus (6, 26), and amoebae appear to be important for the control of *C. neoformans* in the environment (28). Previously, we reported that the interaction of *C. neoformans* with *Acanthamoeba castellanii* directly paralleled the interaction with human macrophages (33). Similarities between *C. neoformans* interactions with amoebae and macrophages included intracellular replication in a phagosome and the release of polysaccharide-containing vesicles into the cytoplasm (33). Furthermore, passage of avirulent *C. neoformans* and *Histoplasma capsulatum* through slime mold and amoebae was shown to increase virulence in mice (31, 32). On the basis of these observations, it was proposed that the capacity for mammalian virulence emerged from interactions with phagocytic predators, such as amoebae and slime mold, in the environment (7, 17, 30). Consequently, single-cell protists have emerged as important systems for the study of *C. neoformans* virulence, and subsequent studies have investigated the interaction of this fungus with slime mold and paramecia (9, 31). Additional evidence for this concept comes from studies of insect fungal pathogens, which suggest that the capacity for insect pathogenicity may follow preadaptation from interactions with amoebae in the environment (4). Understanding the mechanisms by which virulence emerges in environmental microbes is important considering that global warming has been hypothesized to bring about new fungal diseases in the coming century (13).

Recent work in our laboratory and in that of Robin May simultaneously uncovered a novel strategy of avoiding macrophage killing whereby yeast cells were expulsed without lysis of the host cell (2, 19). The process is remarkable in that extrusion of the *C. neoformans*-filled phagosome is accompanied by the survival of both the host cells and the yeast cells. Phagosome extrusion or fungal exocytosis appears to be a *C. neoformans*-dictated event that is dependent on both the presence of the polysaccharide capsule and on the depolymerization of actin. A corollary of the hypothesis that *C. neoformans* virulence emerged from interactions with environmental predators is that phenomena observed with mammalian cells are likely to have a counterpart in free-living phagocytic cells. Consequently, the observation of an apparently unique event such as phagosomal extrusion from mammalian macrophages suggested a need to search for similar events in *C. neoformans* interactions with environmental phagocytic predators.

In this study, we investigated parallels between the intracellular pathogenic strategy of *C. neoformans* in both macrophages and *A. castellanii*, focusing on characterizing the impact of the capsule on protozoan phagocytosis and on ascertaining whether fungal cells could also exit amoebae, including the role of the capsule in that possible mechanism. Using time-lapse microscopy, we observed the exocytosis of *C. neoformans* from *A. castellanii*. While there are significant differences in

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the nonlytic exocytosis process when comparing amoebae and macrophages, the observation of this phenomenon in amoebae provides additional support for the idea that the virulence of *C. neoformans* was selected for, and is maintained, by interactions in the environment with other soil organisms.

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MATERIALS AND METHODS

Yeast strains and culture conditions. *Cryptococcus neoformans* strains 24067 (obtained from the American Type Culture Collection [ATCC], Rockville, MD), 11, 13, 14, 16, J4, J8, J10, J11, J20, and J40 (21) were grown from frozen stocks and maintained in Sabouraud dextrose broth. All strains were agitated at 150 to 180 rpm in 30°C incubators. The *C. neoformans* var. *grubii* strain, H99, was obtained from John Perfect (Durham, NC). The acapsular strain, CAP67, was obtained from the ATCC. The CAP59 mutant, parent, and complemented strains were obtained from K. J. Kwon-Chung (Bethesda, MD) and grown in yeast nitrogen base (YNB) for 3 days at 30°C. *C. neoformans* strain 24067 cells were killed by heating at 65°C for 1 h.

Amoebae. *Acanthamoeba castellanii* strain 30324 was acquired from the ATCC. This strain was cultured at 28°C in peptone-yeast extract-glucose broth (PYG; ATCC medium 354) as described previously (22). *A. castellanii* cells were passaged every 7 to 10 days and used when they were confluent on the bottom of the flasks.

Induction of capsule. *C. neoformans* capsules were induced through overnight incubation in the presence of 10% heat-inactivated fetal calf serum as described previously (35). India ink staining and light microscopy verified the increase in capsule size as previously described (35). Briefly, cells were stained with India ink and imaged using an Olympus AX70 microscope, and photos were taken with a QImaging Retiga 1300 digital camera using the QCapture suite V2.46 software (QImaging, Burnaby, British Columbia, Canada). Capsule diameters were measured with Adobe Photoshop CS for Windows (San Jose, CA) where the diameter was defined as the difference between the total cell and the cell body.

Phagocytosis assays. Confluent *A. castellanii* cells were removed from culture flasks and counted using a hemocytometer. After *A. castellanii* cells were plated in 96-well plates at 1×10^6 cells/ml, they were allowed to settle for 1 h at 28°C. During this time, *C. neoformans* cells were also isolated from fungal cultures by centrifugation, washed in phosphate-buffered saline (PBS), counted with a hemocytometer, and resuspended at a density of 1×10^6 cells/ml. *C. neoformans* cells were then incubated with *A. castellanii* at 28°C for variable times. Following incubation, the microtiter plates were centrifuged so that *A. castellanii* would settle to the bottom of the plate, and the supernatant was removed by careful aspiration. A 100-μl volume of ice-cold methanol was added to each well, and the plates were incubated at 4°C for 30 min. The methanol was then removed, and PBS was added 2 times to wash the wells. To visualize *A. castellanii* cells, a 1:20 dilution of Giemsa stain was added for 30 min. After the plates were rinsed twice with PBS, they were viewed at a magnification of ×40 and counted to determine the phagocytic index. Internalization of *C. neoformans* was confirmed through experiments with fluorescent *C. neoformans*. *C. neoformans* cells were labeled with fluorescent 18B7 and used in phagocytosis assays. With an Olympus AX70 microscope, the internalization observed using bright-field microscopy could be confirmed with observation of internalized *C. neoformans* visualized with fluorescence. The phagocytic index is defined as the number of *A. castellanii* with internalized yeast per total number of *A. castellanii* cells and shown as a percentage.

Imaging of amoebae. Amoebae were plated at a density of 1×10^5 or 1×10^6 cells/ml on polylysine coverslips at the bottom of wells on MatTek plates (Ashland, MA) in PBS and allowed to settle for 1 h at 28°C. *C. neoformans* cells of the various strains tested were then added. The cell suspension was then allowed to incubate for an additional hour before centrifuging the plates so that the *A. castellanii* cells would settle on the coverslip. The plates were then washed with PBS, and the amoebae were resuspended in feeding medium for subsequent imaging.

Reducing amoeba motility. Protoslo quieting solution (Carolina Biological Supply, Burlington, NC) was used to suspend the organisms for imaging. Additionally, the organisms were suspended in increasing concentrations of soft agar diluted in PBS.

Analysis of exocytosis events. An Axiovert 200 M inverted microscope was used in conjunction with an AxiocamMR camera controlled by the Axio Vision 4.4 software (Carl Zeiss Micro Imaging, New York). The microscope was encased in a Plexiglas box with conditions as previously described (2). Time-lapse movies were generated by collecting images of amoeba-cryptococcus interactions at magnifications of ×10 and ×20 every 30, 60, 120, or 240 s for 18 to 24 h and then assembling these images into animated movies using ImageJ software (1). For each condition, at least 150 amoebae were observed by eye and their fate was recorded, and each condition was done in triplicate on different days with independent cultures.

Actin depolymerization and polystyrene beads. The effect of actin polymerization was tested using cytochalasin D. Cytochalasin D (2 μM) was added to the medium containing *C. neoformans* strain 24067 and *A. castellanii* after the cells were washed with PBS; this resulted in the same amoeba viability but a decrease in motility. To determine whether inert particles were expulsed from *A. castellanii*, 5.2-mm polystyrene beads (Spherotech, Lake Forest, IL) were added to a suspension of *A. castellanii*, and the interaction was recorded as described above.

Statistical analysis. Statistics, including averages, standard deviations, and correlations, were performed using Microsoft Excel and Graphpad Prism 5 (La Jolla, CA).

RESULTS

The efficacy of phagocytosis of *C. neoformans* by *A. castellanii* is inversely proportional to the diameter of the capsule. The capsule of *C. neoformans* has long been known to inhibit the phagocytosis of fungal cells by mammalian macrophages (15). To investigate the role of the capsule in the interaction of *C. neoformans* with *A. castellanii*, we measured the phagocytic index for 8 *C. neoformans* strains (Fig. 1A). We then correlated capsule size to phagocytic efficacy and found that there was significant strain-to-strain variation in phagocytic efficacy, and no correlation was observed between capsule size and phagocytic efficacy. Given that our prior study suggested a role for the capsule in resisting phagocytosis (33), we inferred that the lack of correlation between strains reflected variation between strains in the contribution of noncapsule variables to the fungus-protist interaction. Consequently, we changed our experimental approach to explore the role of the capsule within strains, and the phagocytic efficacy was determined for each strain before and after capsule growth induction. For all strains, capsule enlargement was associated with a reduction in phagocytic efficacy relative to that measured for the smaller capsule forms (Fig. 1B), and a pair-wise comparison of phagocytic efficacy before and after capsular enlargement showed a statistically significant reduction in phagocytic index in response to capsule growth (Fig. 1B). To further investigate the role of the capsule in amoeba phagocytosis, we compared *A. castellanii* phagocytic efficacy for an isogenic strain trio, the wild-type strain (B3501), an acapsular mutant resulting from a defined deletion of the CAP59 gene (C536), and the complemented strain (C538). For this strain set, encapsulation was associated with a major reduction in phagocytic efficacy, consistent with an antiphagocytic role for the capsule (Fig. 1C).

Yeast phagocytosis and exocytosis. Incubation of *A. castellanii* with *C. neoformans* strains H99 and 24067 resulted in the ingestion of both cryptococcal strains (Fig. 2B and C). However, after a certain time, yeast cells of *C. neoformans* strain 24067 were occasionally expulsed from amoebae (Fig. 3; see Video S1 in the supplemental material), whereas no expulsion events were observed with strain H99 within 24 h in the course of analyzing the results of three separate experiments that included at least 500 *A. castellanii* cells. The earliest expulsion with strain 24067 cells was observed 4 h after the experiment commenced. The exocytosis phenomenon always involved sin-

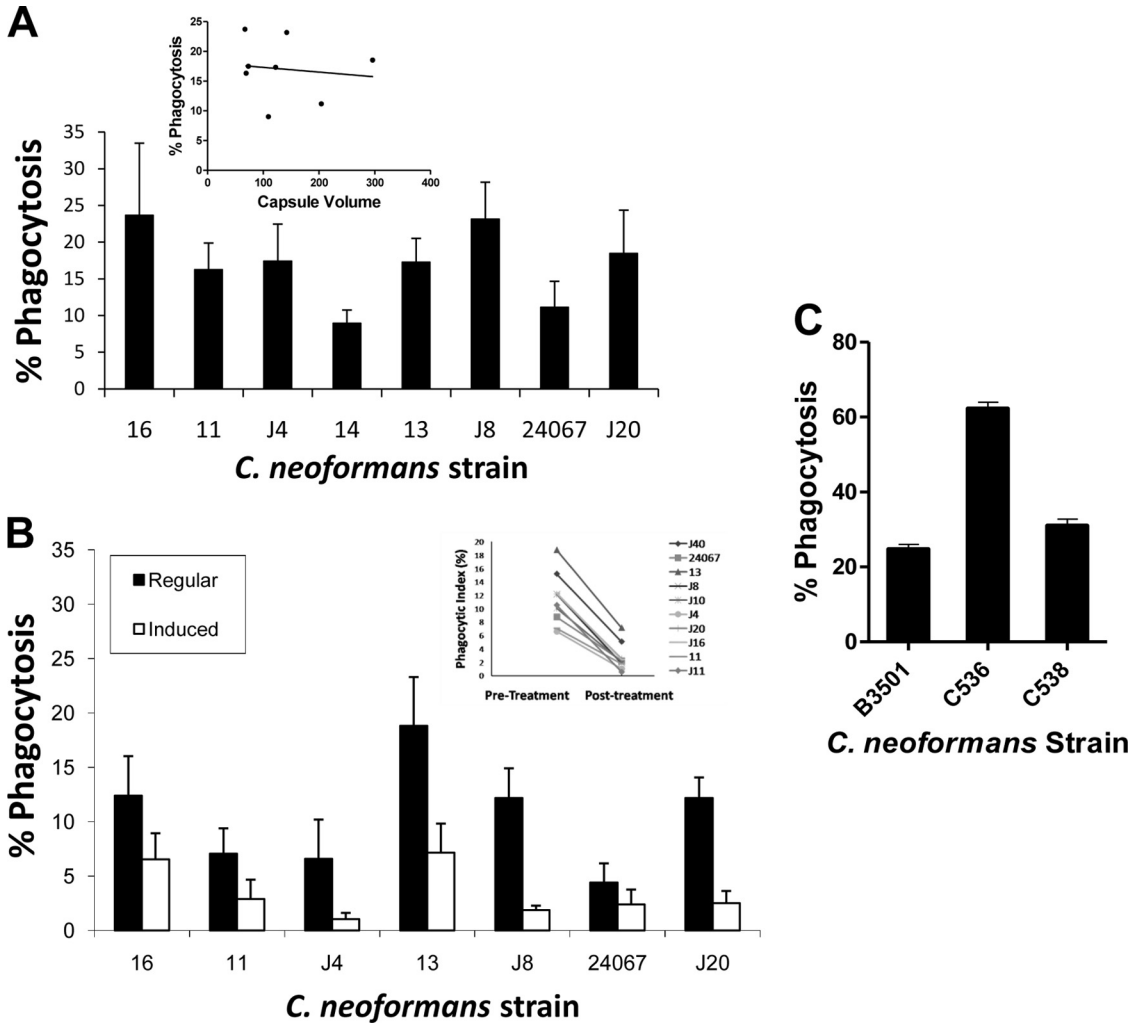

FIG. 1. (A) Percent phagocytosis for 8 different *C. neoformans* strains from *A. castellanii*. After incubation at a 1:1 ratio, 96-well plates were washed, cells were stained, and a minimum of 200 cells/well were counted to determine the percent phagocytosis. This value is defined as the number of *A. castellanii* with internalized yeast per total number of *A. castellanii* cells counted shown as a percentage. (Inset) Linear regression of capsule volume (μm^3) versus percent phagocytosis ($R^2 = 0.01518$). (B) Phagocytosis assays after capsule induction. *A. castellanii* cells were allowed to phagocytose various strains of *C. neoformans* before and after the strains had been placed in media to increase the size of the polysaccharide capsule. All strains showed a decrease in the phagocytic index after the capsules had enlarged. (Inset) Comparison of percent phagocytosis before and after capsular enlargement ($P < 0.05$). The experiments shown in panels A and B were done at different times, and the differences in the values of phagocytosis measured for individual strains are within the experimental error of this assay. (C) Phagocytosis assay with acapsular mutants and capsule complemented strain. *A. castellanii* phagocytosis assays were performed with the wild type, CAP59 mutant (an acapsular mutant, C536), and complemented strains of *C. neoformans* (C538).

gle yeast cells and occurred in up to 17% of amoebae with internalized *C. neoformans* when observed over a period of 18 to 24 h. However, this number may be an underestimate given the high mobility of amoebae. Amoebae moved rapidly in the field and often in the direction away from the light beam. This limited our ability to track all individual host cells during the course of time-lapse microscopy, as many migrated outside the field (approximately 45% of amoebae left the field in the course of each experiment). Attempts to slow the amoeba, including the use of soft agar and Protoslo, were unsuccessful. If the medium was too solid, the amoebae encysted. On the other hand, if the medium was diluted to allow mobility, the protists retained their high motility and exited the microscope viewing field. Extruded yeast cells consistently remained attached to the amoeba surface as the cell moved about the field (Fig. 4; see Video S2 in the supplemental material). Attachment was broken by contact with other amoebae. After exocytosis events, amoebae and their attached fungal cells continued to move about the field, and host cells with their attached fungal cells moved sufficiently to leave the field of vision (see Videos S1 and S2 in the supplemental material).

Actin polymerization. To investigate the effect of actin polymerization, the organisms were observed after the addition of cytochalasin D. Cytochalasin D did not affect the viability of the amoebae; however, the motility was decreased. In the presence of cytochalasin D, no exocytosis events were observed compared to extrusion rates without the addition of cytochalasin D (0% versus 17.4%; $P = 0.0082$, Fisher exact test).

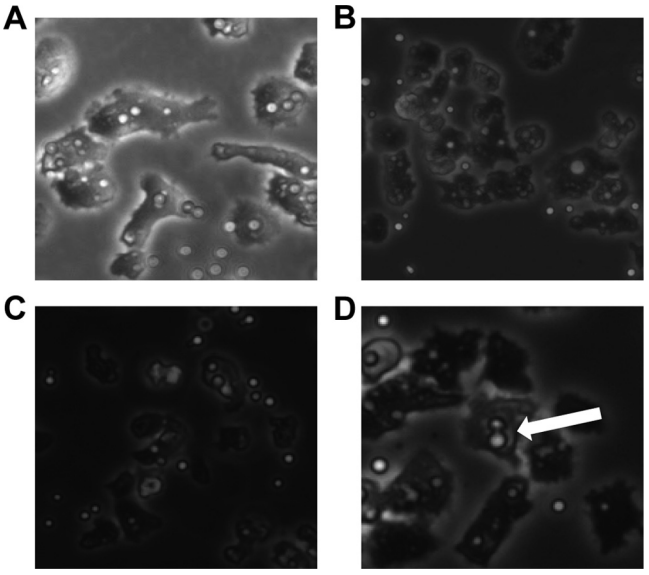

FIG. 2. *A. castellanii* phagocytoses *C. neoformans* strains 24067, H99, and C536 and polystyrene beads. For imaging of extrusion, movies were recorded following incubation of amoeba and *C. neoformans* for 1 h and subsequent washing of extracellular *C. neoformans*. Recording then commenced, and the images represent still photos taken from movies at a magnification of ×10. (A) *A. castellanii* with ingested 5.2-mm polystyrene beads. (B) *A. castellanii* with ingested *C. neoformans* strain 24067. (C) *A. castellanii* with ingested *C. neoformans* strain H99. (D) *A. castellanii* with ingested *C. neoformans* strain C536. The large white arrow shows the internal replication.

Interaction with polystyrene beads, acapsular mutants, and heat-killed *C. neoformans*. Incubation of *A. castellanii* with polystyrene beads resulted in highly efficient phagocytosis of the beads (Fig. 2A), but no exocytosis events were observed in the course of analysis (data not shown). Additionally, no exocytosis events were observed after *A. castellanii* organisms were coincubated with heat-killed *C. neoformans* strain 24067 (0% versus 17.4%; $P = 0.0064$, Fisher exact test), implying that fungal viability was required to exit the host cell. When *A. castellanii* was incubated with the acapsular *C. neoformans* strains, CAP67 and CAP59, high levels of exocytosis resulted, averaging 17% of the cells with internalized *C. neoformans*. For strain CAP67, the extrusion rate was significantly higher than for the parental wild-type strain (B3501), which exhibited no extrusion events in the course of analysis. As in the case of the wild-type cells, the extruded acapsular cells were observed to replicate following exocytosis. The exocytosis of the acapsular strains may represent an early burst of exocytosis, as they were observed to extrude within 30 min of the start of the movies, as opposed to 4 h for other wild-type strains.

DISCUSSION

Nonvertebrate hosts have emerged as powerful tools for the study of microbial virulence (25). In recent years, amoebae, slime mold, worms, and insects have become useful models for evaluating the virulence characteristics of fungi (3, 23, 24, 31, 32). This study represented a further investigation of the interaction between two soil-dwelling organisms, *C. neoformans* and *A. castellanii*, that were previously shown to interact in the laboratory (6, 26, 33) and may do so in the environment. Amoebae are associated with biological control of *C. neoformans* in the environment (28). Prior studies had established that many aspects of the intracellular pathogenic strategy of *C. neoformans* for macrophages could be reproduced in amoebae (33). Here, we considered two phenomena associated with *C. neoformans* and macrophage interactions, namely, that the capsule inhibited phagocytosis and that *C. neoformans* was capable of nonlytic exocytosis, and investigated whether these also applied to *C. neoformans-A. castellanii* interactions.

The capsule is known to have an antiphagocytic function in *C. neoformans*-macrophage interactions such that phagocytosis is extremely inefficient unless antibody and/or complement opsonins are present (14). In contrast, amoebae are able to ingest encapsulated cryptococci without opsonins, and the role of the capsule is uncertain in this fungus-protozoan interaction (33). A prior study involving a limited number of strains had suggested a role for the capsule in protecting cryptococci against phagocytosis (33). Two lines of evidence in this study indicate that the capsule has an antiphagocytic role in *C. neoformans* interactions with *A. castellanii*. First, capsular enlargement was associated with major reductions in phagocytic efficacy for all 7 strains tested. Hence, capsular enlargement, a phenomenon associated with evasion of phagocytosis by macrophages and virulence in mice, also has a corresponding protective function in interactions with amoebae (34, 36). Second, comparison of phagocytic efficacy of three isogenic strains differing in capsular phenotype revealed that, in the absence of capsule, *C. neoformans* was much more easily ingested by *A. castellanii*. However, when we compared the relationship between uninduced capsule size and phagocytic efficacy across diverse strains, we observed considerable interstrain variability, and no correlation was apparent between capsule size and phagocytic efficacy. These experimental results suggest the existence of variables other than capsular diameter that contribute to amoeba phagocytic efficacy. In this regard, we note that *C. neoformans* strains manifest differences in polysaccharide capsule structure which could affect the interaction of the capsule with *A. castellanii* receptors as was observed with *C. neoformans* var. *gattii* (20), and that *C. neoformans* makes antiphagocytic proteins (18). Overall, these results confirm that the *C. neoformans* capsule is antiphagocytic for environmental amoeboid predators, implying a major defensive role for this distinctive fungal structure and establishing another correlate for cryptococcal interactions between amoebae and macrophages.

Given the recent observation that *C. neoformans* is capable of nonlytic exocytosis from mammalian phagocytic cells (2, 19), we investigated whether similar phenomena occurred following ingestion of yeast cells by *A. castellanii*. Cells of *C. neoformans* strain 24067 (serotype D) were observed to exit *A. castellanii* after phagocytosis. Hence, we now report the phenomenon of cryptococcal nonlytic exocytosis from protozoan phagocytic cells. This observation echoes similar findings with mammalian macrophages with the caveat that there were significant differences between exit from mammalian macrophages and from protozoan phagocytic cells. First, the exocytosis events in amoebae involved single yeast cells whereas macrophage exocytosis events often involve numerous yeast cells exiting a giant phagosome. In this regard, the exit of *C. neoformans* from amoebae is similar to that described by Ma

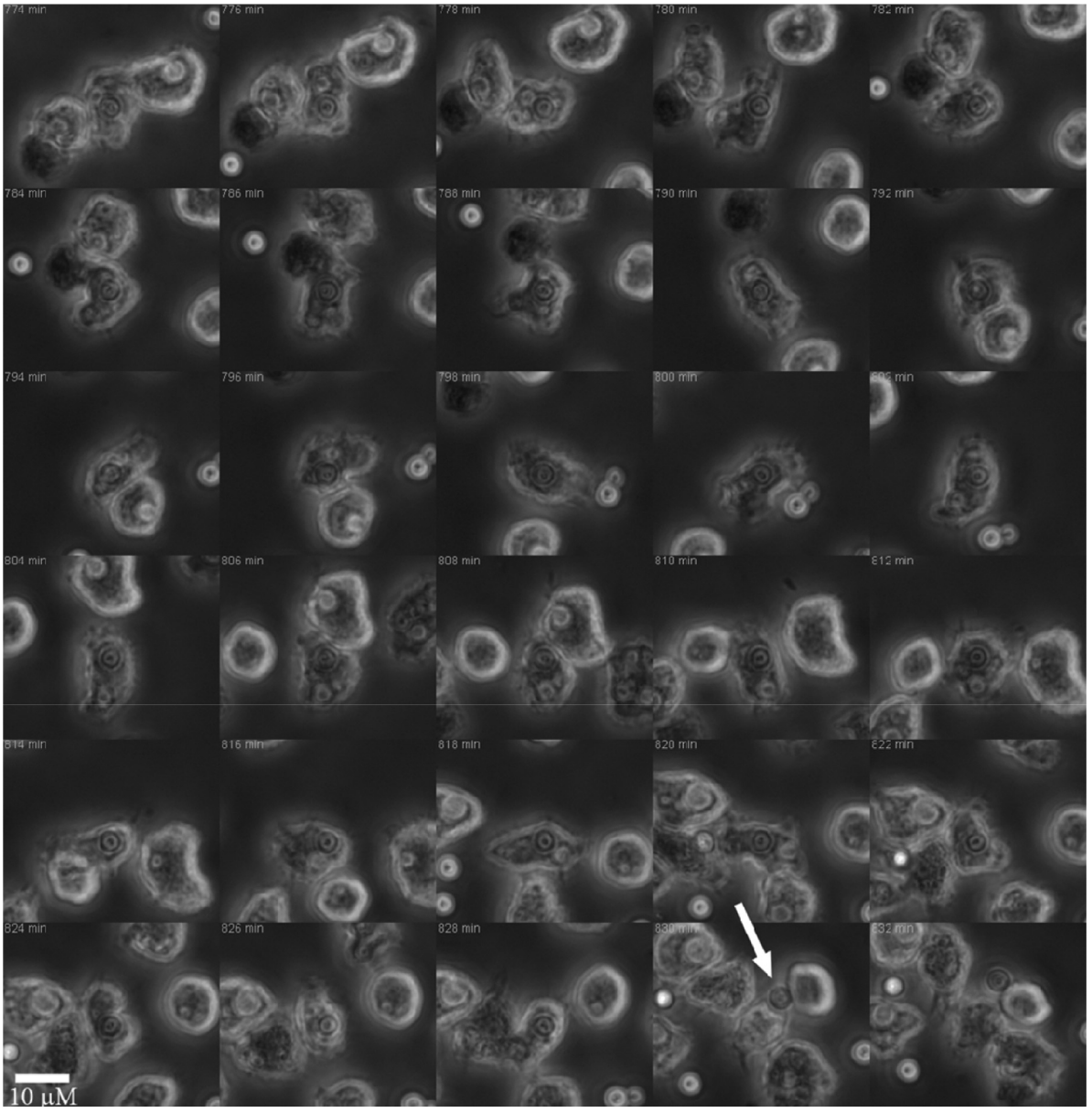

FIG. 3. Exocytosis of *C. neoformans* strain 24067 from *A. castellanii*. Montage taken from a video of interactions between the two organisms. Video was started 1 h after the commencement of phagocytosis, and the frames are labeled accordingly. The large white arrow indicates the expulsion event, which was followed by both organisms continuing to move about the field. Magnification, ×10.

et al. who reported exocytosis of single yeast cells from mammalian macrophages (19). Second, we observed strain-related differences in the ability of *C. neoformans* to exit amoebae, as no exit events were observed with yeast cells of *C. neoformans* var. *grubii* strain H99 (serotype A). In contrast, all cryptococcal strains studied in macrophages were found capable of nonlytic exocytosis from those cells. Furthermore, in macrophages, the efficiency of exocytosis was higher for strain H99 than for strain 24067 (2), whereas the former was never observed to exit *A. castellanii*. Given the limitations encountered in observing amoeba-cryptococcus interactions over a prolonged time, relating to their extreme mobility and microscope light aversion, we cannot rule out a false-negative result in the absence of observable exocytosis events with strain H99. Nevertheless, if these exocytosis events occur, exocytosis appears to be a much more frequent event with strain 24067 than with strain H99. Third, exocytosis from amoebae occurred significantly later than macrophage exocytosis, which can occur as early as 2 h after ingestion. Fourth, the addition of the actin inhibitor cytochalasin D abrogated yeast expulsion in amoebae, while the same drug hastens it in macrophages. Comparison of CAP59 and CAP67 mutants to encapsulated strains suggested a role for the capsule in extrusion. The lack of capsule greatly increased the rate of expulsion, something not observed with the macrophage experiments.

C. neoformans exocytosis from amoebae was a microbe-me-

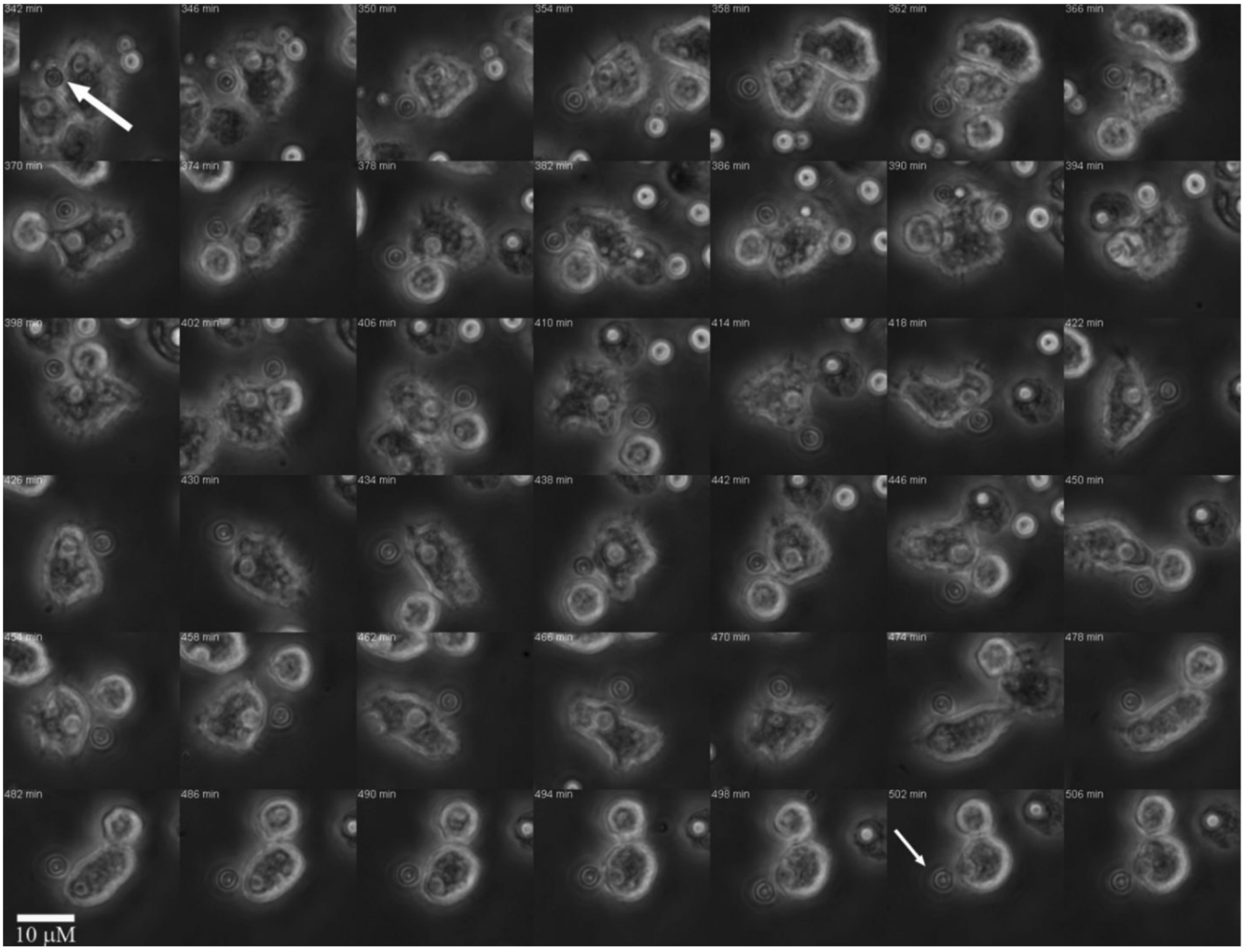

FIG. 4. Persistence of *C. neoformans-A. castellanii* attachment following exocytosis of yeast cells from the host phagocytic cell. The large white arrow indicates the expulsion of a *C. neoformans* cell from inside *A. castellanii*. Following the expulsion, the amoeba actively moves about the field while the yeast cell remains attached, indicated by the small white arrow. Magnification, ×10.

diated process, as no exit events were observed with heat-killed yeast or polystyrene beads. Yeast exocytosis from amoebae appeared to be a different phenomenon than the cytoplasmic expulsion reported when amoebae are injected with foreign cytoplasm, since yeast exocytosis followed active phagocytosis and occurred much later than cytoplasmic expulsion (11). The capacity of yeast cells to exit amoebae would be an effective survival strategy for escaping predatory protozoa. We note that bacteria have recently been shown to exit species of the protozoan *Tetrahymena* in a process that can alter bacterial virulence properties (5). Alternatively, it is possible that the amoeba cell expels ingested yeast with a pathogenic potential for that host. In this regard, it is known that *Dictyostelium discoideum* expulses zymosan particles after ingestion (27). Since fungal passage in amoebae can alter virulence properties, one can imagine how cycles of ingestion and exocytosis could affect the virulence of *C. neoformans* (31, 32).

In summary, we report that *C. neoformans* has the capacity for nonlytic exocytosis from amoebae, thus echoing similar observations with macrophages, but there appears to be significant differences in the mechanisms involved (2, 19). The observation that amoebae ingest and occasionally extrude *C. neoformans* is consistent with the proposal that fungus-protozoan interactions are both common and ancient and represents a mechanism for the selection of fungal traits that confer virulence in the setting of appropriate fungus-animal interactions (7, 30, 33). In considering the implications of these findings, we caution that there are innumerable species of protozoa capable of yeast predation in the environment and that the findings observed here were limited to one amoeba species that has been adapted to laboratory conditions. In fact, the strain of *A. castellanii* used in this study is unusual among amoeba strains in its capacity to grow in axenic media as a result of laboratory adaptations. Consistent with the notion that each fungus-protozoan interaction may be unique, we recently described the interaction of *C. neoformans* with *Paramecium* spp. and noted that it was very different from that described for amoebae (12). Nevertheless, the occurrence of *C. neoformans* nonlytic exocytosis from amoebae establishes another important global correlate between fungus-protozoan interactions and fungus-mac-

rophage interactions, despite significant differences in the details of this process. An enhanced understanding of the interactions of fungi with pathogenic potential with environmental protists is important in light of the hypothesis that continued climate warming will be associated with new and more frequent fungal diseases (13). Considering the enormous evolutionary time that separates protozoa and mammals, the global similarities between *C. neoformans* interactions with amoebae and macrophages is consistent and supportive of the proposal that many virulence-associated characteristics emerged from environmental selection pressures (8).

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