Temporal Kinetics and Quantitative Analysis of *Cryptococcus neoformans* Nonlytic Exocytosis

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Cryptococcus neoformans is a facultative intracellular pathogen and the causative agent of cryptococcosis, a disease that is often fatal to those with compromised immune systems. *C. neoformans* has the capacity to escape phagocytic cells through a process known as nonlytic exocytosis whereby the cryptococcal cell is released from the macrophage into the extracellular environment, leaving both the host and pathogen alive. Little is known about the mechanism behind nonlytic exocytosis, but there is evidence that both the fungal and host cells contribute to the process. In this study, we used time-lapse movies of *C. neoformans*-infected macrophages to delineate the kinetics and quantitative aspects of nonlytic exocytosis. We analyzed approximately 800 macrophages containing intracellular *C. neoformans* and identified 163 nonlytic exocytosis events that were further characterized into three subcategories: type I (complete emptying of macrophage), type II (partial emptying of macrophage), and type III (cell-to-cell transfer). The majority of type I and II events occurred after several hours of intracellular residence, whereas type III events occurred significantly ($P < 0.001$) earlier in the course of macrophage infection. Our results show that nonlytic exocytosis is a morphologically and temporally diverse process that occurs relatively rapidly in the course of macrophage infection.

Cryptococcus neoformans is a facultative intracellular pathogen that causes life-threatening disease in immunocompromised individuals (1, 2). Individuals can become infected after inhalation of desiccated yeast cells or spores (3) from the environment. Most human infections are asymptomatic, but immunocompromised individuals are particularly vulnerable to cryptococcal disease, which often presents itself as meningoencephalitis. After inhalation of fungal cells or spores, resident macrophages in the alveolar space presumably provide the first line of host defense. The initial interactions between host macrophages and cryptococcal cells have been extensively studied and clearly defined (4, 5). *C. neoformans* has an antiphagocytic polysaccharide capsule (6) which requires the presence of opsonins in the form of complement or capsule-binding antibodies (Ab) for phagocytosis to occur (3). Fc receptor polymorphisms were associated with susceptibility to *C. neoformans* (7, 8), suggesting a link between phagocytosis and the outcome of infection. Once inside the macrophage, the fungus resides in a mature phagosome and can survive acidification and phagocytic bursts (4, 9). The polysaccharide capsule is believed to aid in intracellular survival by providing a free radical sink that interferes with fungicidal oxidative products (4, 6). In human hosts, there is evidence that both host and fungal factors can contribute to the outcome of infection (10).

There are three outcomes to the macrophage-*C. neoformans* interaction: death of the fungal cell, death of the macrophage, or nonlytic exocytosis of fungal cells with the survival of both the macrophage and cryptococcal cells. During nonlytic exocytosis, which is also known by the more colorful term “vomocytosis” (11), a macrophage releases all or some of its phagosomal contents into the surrounding extracellular environment (12). This action is believed to affect the outcome of infection. For example, *C. neoformans* cells exiting the lung in infected macrophages may be disgorged into the body tissues, promoting the spread of infection (4, 5, 13). This process has also been described for other fungal pathogens such as *Candida albicans* and could represent a general strategy for fungal cells to exit phagocytic cells (14).

There is evidence that both fungal and macrophage factors

contribute to nonlytic exocytosis (15). Host cells may use brief bursts of “actin flashes” to inhibit escape of cryptococcal cells from the phagosome (16), and the activity of the WASP and SCAR homologue (WASH) was shown to be critical in the process, as a knock down of this protein causes a decrease in nonlytic exocytosis (17). The process requires *C. neoformans* to be alive as well as to have an intact polysaccharide capsule, since neither heat-killed *C. neoformans* nor live acapsular yeast cells nor latex beads are readily expelled from macrophages (12). Cryptococcal phospholipase was shown to play a role in nonlytic exocytosis, as a knockout of the enzyme caused a reduction in the process (18). Nonlytic exocytosis has been observed in murine (12), human (19), and insect (20) cells, which would suggest that this process is a conserved mechanism for exiting phagocytic cells. The fact that nonlytic exocytosis of *C. neoformans* also occurs in amoeba is consistent with the idea that many aspects of cryptococcal intracellular pathogenesis were selected by interactions with protozoan predators encountered in the environment (21).

Despite considerable progress since nonlytic exocytosis was independently described by two groups in 2006 (11, 12), many details of this process remain unknown. In this study, we used time-lapse microscopy to establish the temporal kinetics and the morphological events associated with nonlytic exocytosis from infected macrophages. Our data show that nonlytic exocytosis can be categorized into three different types of events that differ in

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temporal dynamics and that the process occurs in minutes and begins in the early stages of infection.

MATERIALS AND METHODS

***Cryptococcus neoformans* growth conditions.** Two different strains of *C. neoformans* were used for imaging experiments: H99 (serotype A) and 24067 (serotype D). Strain H99 was used for all experiments unless otherwise noted. For both strains, one colony was incubated in 10 ml of Sabouraud dextrose broth (Difco) for 24 h at 30°C with constant agitation (150 to 180 rpm). Before infection, yeast cells were washed three times with sterile phosphate-buffered saline (PBS) and used at a multiplicity of infection (MOI) of 1:5 for all experiments.

Preparation of primary murine macrophages. C57BL/6 mice were obtained from the National Cancer Institute and used as the source of primary macrophages. All femurs and tibias were collected from mice, and muscle tissue was removed. Intact bones were disinfected using 70% ethanol, and bone marrow was subsequently flushed out of bones. All cell clumps were disrupted, and the cell suspension was centrifuged for 10 min at $650 \times g$. The pellet was resuspended in 10 ml of growth media, consisting of Dulbecco's modified Eagle's medium (Cellgro, Manassas, VA) supplemented with 20% L929 media, 10% fetal calf serum (FCS) (Atlanta Biologicals, Lawrenceville, GA), 10% NCTC-109 medium (Gibco), 1% HEPES (Gibco), Glutamax-1 (Gibco), minimal essential medium (MEM) nonessential amino acids (Cellgro, Manassas, VA), penicillin-streptomycin (Cellgro, Manassas, VA), and 0.1% 2-mercaptoethanol (Gibco), and plated on sterile tissue culture dishes. After 3 days of growth, fresh medium was added, and after 6 days of growth, the media were replaced and cells were ready for use. All mice were between the ages of 6 and 8 weeks when macrophages were collected.

Preparation of human-derived macrophages from whole blood. Macrophages were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Blood was aliquoted into 50-ml Falcon tubes and diluted at a ratio of 4:1 in sterile PBS to bring the total volume to 35 ml. Very slowly, 15 ml of Ficoll-Paque Plus was overlaid onto the blood-PBS mixture, and this solution was centrifuged for 30 min at $420 \times g$. The mononuclear layer was removed and diluted with PBS to a final volume of 50 ml. Cells were centrifuged at 4°C for 20 min at $360 \times g$. The resulting pellet was resuspended in 1 ml of a red blood cell (RBC) lysis mixture (0.1 M NH_4Cl , 10 mM NaHCO_3 , 10 mM EDTA) and incubated for 10 min at room temperature. After lysis incubation, the volume of the pellet was brought up to 50 ml with PBS and the reaction mixture was centrifuged for 10 min at $230 \times g$ and 4°C. The cell suspension was centrifuged at $230 \times g$ for 10 min at 4°C. The resulting pellet was resuspended in feeding media: RPMI medium (Cellgro, Manassas, VA) supplemented with 10% FCS (Atlanta Biologicals, Lawrenceville, GA), 1% HEPES (Gibco), 1% penicillin-streptomycin (Cellgro, Manassas, VA), 1% Glutamax-1 (Gibco), 5% human serum AB+ (Cellgro, Manassas, VA), and 10 ng/ml of macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ). Cells were plated on Mat-tek glass petri dishes (MatTek Brand Corporation) and allowed to adhere for 6 days, with media being changed every 3 days.

SEM and TEM. Murine macrophages were cultured in 6-well tissue culture dishes to 80% confluence and allowed to adhere overnight in media as described above. The next day, cells were infected with *C. neoformans* and phagocytosis was allowed to occur for 5 min, 10 min, 2 h, 6 h, and 24 h for scanning electron microscopy (SEM) and 5 min, 10 min, 2 h, 6 h, and 24 h for transmission EM (TEM). Samples were washed $3 \times$ with sterile PBS to remove extracellular fungal cells. Prior to imaging by SEM, the macrophage samples were fixed in 2.5% glutaraldehyde–0.1 M sodium cacodylate–0.2 M sucrose–5 mM MgCl_2 (pH 7.4), and fixing was followed by dehydration through immersion in a graded ethanol series. The fixed and dehydrated sample was then dried in a Tousimis Samdri 795 Critical Point Drier (Rockville, MD) at the critical point using liquid carbon dioxide. Samples were then sputter coated with chromium in a Quorum EMS 150T ES system (Quorum Technologies Ltd., United King-

dom). SEM imaging was done using a Zeiss Supra field emission scanning electron microscope (Carl Zeiss Microscopy, LLC) with an accelerating voltage of 5 kV. For TEM, the samples were first fixed with 2% paraformaldehyde–2.5% glutaraldehyde–0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide followed by 1% uranyl acetate, and then dehydrated by immersion in a graded series of ethanol concentrations followed by embedding in LX112 resin (LADD Research Industries, Burlington, VT). A Reichert Ultracut UCT instrument was used to cut ultrathin (80-nm-thick) sections that were then stained with uranyl acetate followed by lead citrate. TEM was done using a JEOL 1200EX transmission electron microscope at 80 kV.

Nonlytic exocytosis assay and time-lapse imaging. Experiments were carried out as described in reference 19. Briefly, primary bone marrow macrophages were plated (10^5 cells/well) on Mat-tek glass petri dishes (MatTek Brand Corporation) and incubated at 37°C with 10% CO_2 overnight in media containing lipopolysaccharide (LPS; Sigma) at 1 $\mu\text{g}/\text{ml}$ and gamma interferon ($\text{IFN-}\gamma$; Roche) at 50 $\mu\text{g}/\text{ml}$ as previously described. On the following day, macrophages were infected with monoclonal antibody (MAb) 18B7-opsonized *C. neoformans*. Phagocytosis was allowed to occur for 2 h, and then the monolayer was thoroughly washed with sterile PBS to remove extracellular yeast cells. Fresh medium was added to the dish. Using a Zeiss Axiovert 200 M inverted microscope with a $10\times$ objective with the dish housed in an enclosed chamber under conditions of 5% CO_2 and 37°C, images were taken every 4 min for the duration of the experiment.

Cinematographic analysis. For each time-lapse movie, frames were collected every 4 min and analyzed using Image J 1.46 software. All frames were compiled into a movie, and about 100 to 200 infected macrophages were analyzed by eye for the occurrence of nonlytic exocytosis. Once a cell was determined to have undergone nonlytic exocytosis, the timing of the event in the infection period was calculated using the equation $x = (4 \times \text{the frame number})/60$. Five separate movies were analyzed.

Transfection of J744.16 macrophage like-murine cells. J744.16 cells were seeded at 80% confluence on 6-well plates. Various concentrations of Lipofectamine 2000 reagent (Invitrogen) and diluted plasmid DNA containing the mCherry-CAAX construct cloned in a pcDNA 3.1 transfection vector in Opti-MEM medium (Invitrogen) were divided into aliquots, combined, and then incubated for 5 min at room temperature. This DNA-complex mixture was then added to macrophages. Cells were then incubated for 1 day, and media containing G418 at a concentration of 1 mg/ml were then added to select for transfectants. Fresh medium was added every 3 days to generate a stable cell line of fluorescently labeled macrophages.

Statistical analysis. Proportions with 95% confidence intervals (CI) were calculated for the three types of nonlytic exocytosis events. Proportions of event types with a time interval ≤ 8 h were compared with odds ratio determinations (95% confidence interval) and a chi-square test. A nonparametric Spearman's rho correlation was performed to examine the monotonic association of time interval with event type, both expressed as ordinal variables. Statistical analyses were performed with SPSS for Windows (Version 20). A two-tailed alpha of 0.05 was used to denote statistical significance.

RESULTS

Ultrastructure of *C. neoformans*-macrophage interactions. Transmission electron microscopy was used to analyze *C. neoformans*-infected macrophages throughout 24 h of *C. neoformans* infection. Yeast cells can clearly be seen undergoing ingestion as soon as 5 min after infection as noted by the extension of the cellular membrane engulfing individual yeast cells (Fig. 1A). In macrophages infected for 6 h, most *C. neoformans* cells were fully internalized, as the fungal cells were within the confines of the macrophage (Fig. 1B). Images taken at 24 h showing *C. neoformans* at the edge of discontinuous cellular membrane can be interpreted to indicate that the yeast cell was in the beginning stages

FIG 1 Transmission electron microscope images of *C. neoformans*-macrophage interactions. (A) Cellular membrane protrusions can be seen surrounding individual cryptococcal cells 5 min after exposure to the pathogen. The image was taken with an 8,000 $\times$ objective. (B) Hours after infection, there was complete phagocytosis and internalization of the yeast cells. The image was taken with an 4,000 $\times$ objective. (C) *C. neoformans* cells can be seen near breaks at the edge of the cellular membrane (indicated by the arrowhead) after being infected for 24 h. The image was taken with a 3,000 $\times$ objective.

of exiting the macrophage (Fig. 1C). To study the surface morphology of macrophages during cryptococcal infection, scanning transmission microscopy was utilized using early and late infection time intervals. Early in the interaction with *C. neoformans*, macrophages displayed numerous surface ruffles characteristic of such cells initiating phagocytosis (Fig. 2A). After 24 h, the surface of macrophages was much smoother, with the disappearance of characteristic macrophage cell membrane ruffles. Yeast cells can be seen breaking through the cellular membrane of the macrophage in a manner that would be consistent with exocytosis or possible lysis (Fig. 2B and C).

Types of nonlytic exocytosis events. Our first goal was to characterize the process of nonlytic exocytosis by visually studying many infected macrophages. This was made possible by using microscopic cinematography at a magnification that allowed visual-

ization of a large field with sufficient resolution to ascertain the cellular events involved. We analyzed about 800 macrophages containing intracellular *C. neoformans* in five separate experiments (Table 1) and identified 163 exocytosis events that were then further characterized. For each exocytosis event, we were able to follow the process of individual infected cells. Three types of nonlytic exocytosis events were identified, and we have classified them as types I, II, and III. The three types share the characteristic that *C. neoformans* exits a cell without lysing it. Type I involved the full emptying of the intracellular fungal burden into the extracellular space. In type I events, there is a defined outline of the cryptococcus-filled phagosome inside the macrophage that migrates to the edge of the cell membrane, where all of the cryptococcal cells are subsequently released in one burst (Fig. 3). After the release of its fungal burden, the cell continues to move about, indi-

FIG 2 Scanning electron microscope images of early and late stages of *C. neoformans* infection. (A) Primary macrophages were infected with *C. neoformans* for 5 min. The macrophage membrane (shown in purple) reaches over to phagocytose the yeast cell (shown in yellow) seen on its surface. (B) *C. neoformans* infection was allowed to proceed for 24 h. An infected cell's membrane can be seen separating to release cryptococcal cells. Images A to C were artificially colored using Photoshop CS4. Images D to F represent raw data images. The scale bar represents 1 μ m.

TABLE 1 Frequency of nonlytic exocytosis events in primary murine macrophages

Experiment	Time to completion (h)	Total no. of infected cells	Total no. (%) of nonlytic exocytosis events
1	20	121	29 (24)
2	26	220	49 (22)
3	24	154	18 (12)
4	24	204	48 (24)
5	24	167	19 (11)

cating that it is still alive and has not lysed (see Movie S1 in the supplemental material). In some instances, the process of nonlytic exocytosis did not release the entire phagosomal content; instead, the result was partial phagosomal emptying. We have categorized this partial emptying event as a type II nonlytic exocytosis event. Type II events included the exit of one cryptococcal cell from the macrophage (Fig. 4A) or exocytosis of the majority of the cryptococcal cells while leaving only one yeast cell inside the phagosome (Fig. 4B). In this example (see Movie S2 in the supplemental material), an infected macrophage is seen moving throughout the field containing multiple *C. neoformans* cells. As the movie progresses, the intracellular fungal burden appears to increase and a portion of it is released, leaving a single yeast cell inside the macrophage. Type I and II nonlytic exocytosis events appear to be qualitatively similar except that they differ in the degree of phagosome emptying.

Lastly, we observed a type of nonlytic exocytosis event that was qualitatively different from types I and II in that the cryptococcal cells were not released to the extracellular space but were instead transferred to a nearby macrophage. *C. neoformans* cell-to-cell transfer between macrophages was previously described (22), and we have categorized this event as type III nonlytic exocytosis, since the fungal cell leaves the original cell without host cell lysis (Fig. 5). Infected macrophages interacted to form a cell-to-cell bridge that allowed multiple yeast cells to be passed back and forth between different cells without being exposed to the extracellular space (see Movie S3 in the supplemental material). When we observed multiple cryptococcal cells being passed between two macrophages,

we scored this transfer as a single event. It is also very clear that this is not macrophage-macrophage fusion, as there is no engulfment of either cell and, after transfer, the macrophages are seen moving in opposite directions. In our observations, for cell transfer to occur, macrophages needed to be in close proximity to one another. After cell transfer events, the macrophages were active and motile, consistent with the notion that they were relatively healthy despite having been infected with *C. neoformans*.

Visualizing membrane dynamics during type III nonlytic exocytosis (cell-to-cell transfer). Using macrophages transfected with a plasmid containing a mCherry-CAAX construct, we visualized the changes in the cellular membrane as it underwent cell-to-cell transfer or type III nonlytic exocytosis. The formation of cellular membrane around the fungal cell begins as the *C. neoformans* nears the edge of the macrophage. From there, the transfer of the *C. neoformans* into the uninfected macrophage is accompanied by an increase of cellular membrane at the site of *C. neoformans* transfer as inferred due to an increase in fluorescence in the recipient cell (Fig. 6). The process is rapid, and once the *C. neoformans* cell is in the recipient cell, the fluorescence decays in a manner consistent with membrane breakdown (see Movie S4 in the supplemental material).

Temporal kinetics of nonlytic exocytosis. After categorizing the various types of nonlytic exocytosis, we then set out to determine the duration and timing of nonlytic exocytosis events. This was achieved by analyzing five separate movies over a period of 20 to 26 h, following roughly 100 to 200 infected macrophages in each movie frame by frame. Since the pictures were taken every 4 min, the error of the measurement is at least equal to this interval. For the majority of events, nonlytic exocytosis required 4 to 12 min (Fig. 7A). The start of an event was determined when a cryptococcal cell was seen nearing the edge of the plasma membrane, and it reached completion when it was clear that the fungal cell was outside the confines of the macrophage and in the extracellular space. We then ascertained the timing of nonlytic exocytosis events within a 24-h infection period and found that >50% of the events occurred within the first 8 h after the initial infection period (Fig. 7B). During the prolonged imaging required for these experiments, we observed minimal cell death, as evident by the fact that the overwhelming majority of macrophages continued to be motile. However, the possibility that the decrease of events with time

FIG 3 Macrophage undergoing complete nonlytic exocytosis (type I nonlytic exocytosis). (A and B) An infected primary macrophage, indicated by the white arrowhead, has a well-defined fungal burden. (C to F) As the frames progress, the phagosomal membrane shifts to accommodate the moving yeast cells. (G and H) Yeast cells are then emptied out near the top of the cell. (I and J) By the end, the macrophage is left empty with free-floating yeast cells surrounding it. All images were collected with a 10 $\times$ objective, and scale bars represent 25 μ m.

FIG 4 Partial expulsion of cryptococcal cells (type II nonlytic exocytosis). (a) The phagosome in an infected macrophage (A), indicated by the white arrowhead, pinches off part of itself to create two separate phagosomes (B to E). The phagosome containing just one cryptococcal cell is released (F and G), and several yeast cells remain in the macrophage (H). (b) Cryptococcal cells can be seen contained in a phagosome that takes up a majority of the macrophage, indicated by a white arrowhead (A to D). A portion of the phagosome expunges some of the cryptococcus while leaving one cell behind (E to H). All images were collected with a 10 $\times$ objective, and scale bars represent 25 μ m.

reflects some cellular damage cannot be ruled out. Analysis of 163 nonlytic exocytosis events revealed that type I and II events occurred at frequencies of 22% and 26%, respectively, while type III events had a significantly ($P < 0.05$) greater frequency of 52% with a 95% confidence interval of 44% to 59% (Table 2). When the frequencies of the three event types were analyzed as a function of the time interval (Table 3), it became apparent that type I and II events tended to occur in the later stages of macrophage infection whereas type III events occurred earlier (Spearman's rho of -0.63 ; $P < 0.001$). Comparing type III events to type I and II events combined showed that type III had an odds ratio of 16.9 (95% CI, 7.2 to 40.2) of having a time interval ≤ 8 h ($P < 0.001$). Further experiments were carried out using human-derived macrophages as well as with primary murine macrophages infected with *C. neoformans* 24067 (serotype D). We observed that the frequencies of type I, II, and III events in human-derived macrophages were 29%, 65%, and 6%, respectively. With strain 24067, we observed that frequencies of type I, II, and III events were 24%, 31%, and 45%, respectively. Hence, the overall phenomenon of different types of exocytosis events is reproducible with other strains and

host cells of different species but the frequencies with which the different events occur can differ.

DISCUSSION

It is well documented that *C. neoformans* can survive and replicate inside a macrophage, but significantly less information is available regarding nonlytic exocytosis (1, 3, 4). We have reported the occurrence of nonlytic exocytosis *in vivo* (23), but the role that this process plays in *C. neoformans* pathogenesis remains uncertain. In this study, we utilized TEM and SEM, along with time-lapse microscopy, to characterize the morphology, temporal kinetics, types, and frequency of nonlytic exocytosis following ingestion of *C. neoformans* by primary murine macrophages. Phagocytosis of opsonized *C. neoformans* is a regulated process that is required for both control of infection and nonlytic exocytosis. Using both TEM and SEM, we show that the engulfment of the yeast cells by the macrophage occurs by extension of the pseudopods surrounding *C. neoformans*. This morphology looks similar to the established conventional model of phagocytosis (24). While the observation of cryptococci protruding from macrophages at a later time

FIG 5 Macrophages exhibiting cryptococcal cell-to-cell transfer (type III nonlytic exocytosis). (A) Time-lapsed images show multiple infected macrophages are within close proximity to each other. (B to H) One macrophage reaches over and creates a cell-to-cell bridge (C and D) multiple times with its neighboring macrophage to receive cryptococcal cells. After the transfer is complete, the macrophages separate (E and F). A second event occurs as seen in panel G, and cells start moving away in panel H. All arrowheads indicate cell-to-cell bridges. All images were collected with a 10 $\times$ objective, and scale bars represent 25 μ m.

interval in the cellular infection does not allow us to unambiguously state that these represent exocytosis or even nonlytic exocytosis events, we have confidence that we have captured this process, since the timing of the analysis would strongly argue against late ingestion. The experimental protocol used involved the removal of extracellular cryptococci and the opsonin, both of which would be required for phagocytosis. Furthermore, in studying these images, there is a clear morphological difference in the smoothness of the macrophage cell surface compared to the cell surface appearance during phagocytosis. The reduction in membrane ruffles late in the course of infection could reflect increased demands for internal membranes in the very large phagosomes that form in macrophages with ingested cryptococcal cells.

Outcomes of nonlytic exocytosis events had been previously classified into two categories, outcome 2 being when a single yeast cell escapes and outcome 3 being when the entire fungal burden is completely expunged (25), but we now characterize these events into three subcategories: type I (complete emptying of macrophage), type II (partial emptying of macrophage), and type III (cell-to-cell transfer). While cell-to-cell transfer was previously described (22, 26), we suggest treating this phenomenon as another form of exocytosis, since the yeast cell exits the macrophage. Each type is dependent on how the phagosomal contents are released and on the amount of cryptococcal cells expunged. These findings provide insight into the complexity of the nonlytic exocytosis process and indicate new parameters to consider when carrying out studies to establish in detail the cellular mechanisms that underlie the process.

This study used primary macrophages, which are known to undergo nonlytic exocytosis *in vivo* (23). Each movie was assembled from frames generated every 4 min over a roughly 24-h time period. This allowed us to estimate the duration of the various nonlytic exocytosis events. We found that most events took between 4 to 12 min to complete. In some instances, a single macrophage supported multiple nonlytic exocytosis events, whereas some events took longer to complete, which could be an indication of the health of the cell. By analyzing the distribution of nonlytic exocytosis events as a function of time, we found that the

majority of events occurred during the initial stages of infection, a finding consistent with the results reported in reference 11, even though their experiment was limited to 10 h. While temporal differences *in vitro* may not be reflective of *in vivo* conditions, if one were to put this into the context of an *in vivo* infection, this could suggest that many nonlytic exocytosis events occur in the lung early in the course of infection. The data would also predict that most of these events would be type III, which could allow yeast cells to relocate from macrophage to macrophage without exposure to the extracellular space. Later in infection, most nonlytic exocytosis events tended to be of the type I or type II variety. *C. neoformans* manifests quorum-sensing effects in culture (27), and it is possible that similar effects occur in the cramped spaces of cryptococcal phagosomes over time that affect the type of exocytosis. In studying each event frame by frame, we have gained a new insight into the cellular events that accompany this process which is critical for future studies of macrophage-fungus interactions and the changes that accompany them. We did note significant interexperimental variations in the frequency of exocytosis, suggesting that when experiments are set up under the same conditions, there must be unidentified variables that affect this complex process.

For many events, the morphology of the phagosome appeared to rapidly change preceding individual nonlytic exocytosis events. For example, prior to nonlytic exocytosis, macrophages would become very active and motile. The elongated shape that is characteristic of primary macrophages changed to a more rounded appearance (see Movie S1 in the supplemental material). Within the macrophage itself, the phagosomal compartment would become quite large possibly due to the increase in the numbers of *C. neoformans* cells, and in certain instances, the phagosome volume would increase to nearly the size of the macrophage. Our prior study noted fusion of multiple phagosomes within a macrophage to form a giant phagosome containing many cells prior to nonlytic exocytosis (22). However, our observations in this study suggest the existence of another mechanism for phagosomal enlargement, since some phagosomes contained only one yeast cell and still grew to large sizes. We postulate that this could have been due to

FIG 6 Cell-to-cell transfer visualized using fluorescently labeled macrophages. J774.1 macrophage-like murine cells were transfected with a mCherry-CAAX construct which allowed us to visualize membrane dynamics during the transfer of cryptococcal cells between macrophages (A). An uninfected macrophage reaches (B to D) over to the infected macrophage to form a cell-to-cell bridge, indicated by an arrowhead (E). As a result, there is an accumulation of membrane surrounding the transferred yeast cell as indicated by the increase in mCherry (F to I). At the end of the event, the yeast cell can be seen inside the receiving macrophage (I). All images were taken with a 63 $\times$ objective, and scale bars represent 50 μ m.

FIG 7 Temporal kinetics of nonlytic exocytosis throughout fungal infection. (A) Primary murine macrophages were incubated with *C. neoformans* opsonized with 18b7 MAb for 2 h. Infection was allowed to occur for 24 h, with an image being captured every 4 min. Each individual event was determined to start when yeast cells neared the cellular plasma membrane and to conclude when they were seen in the extracellular space. (B) The actual start time (in relation to the 24-h infection) of each nonlytic exocytosis event was determined by the following calculation: $(4 \times \text{the frame number})/60$. We show that nonlytic exocytosis occurs more frequently in the first 8 h of infection.

an enlargement in the cryptococcal capsule and/or release of capsular polysaccharide in the phagosome.

Type I and II exocytosis events appeared to be qualitatively similar and differed only in how the yeast cells exited the macrophage: either all at once or in stages. These events may give clues as to whether all the yeast cell-containing phagosomes fuse prior to nonlytic exocytosis or whether large phagosomes break off into smaller ones to facilitate type II nonlytic exocytosis. Unfortunately, the resolution of our movies does not permit an analysis of phagosomal dynamics. The mechanism by which the *C. neoformans* phagosome can fuse with the membrane to discharge its contents into the extracellular space without lysis of the host cell has not been fully solved. We previously described the macrophage-to-macrophage transfer of *C. neoformans* between cells (22), but now we extend that observation to the transfer of live yeast cells that appear to be in phagosomes. Previous studies of cell-to-cell transfer suggested that it was, in fact, a relatively rare event (26), but here we report that cell-to-cell transfer of phagosomes containing yeast cells was a relatively common event, with type III

TABLE 2 Frequency of nonlytic exocytosis types during a fungal infection

Type ^a	Total no. of events	Frequency of events (%)
I	36	22
II	42	26
III	84	52

^a Type I, complete nonlytic exocytosis; type II, partial nonlytic exocytosis; type III, cell-to-cell transfer.

TABLE 3 Distribution of nonlytic exocytosis types throughout infection

Interval (h) ^a	No. of nonlytic exocytosis events ^b		
	Type I	Type II	Type III
0–4	3	5	54
4–8	8	12	22
8–12	5	10	5
12–16	9	11	1
16–24	6	9	2

^a Interval, time interval after 2-h phagocytosis incubation period.

^b Type I, complete nonlytic exocytosis; type II, partial nonlytic exocytosis; type III, cell-to-cell transfer. Spearman's rho for correlation of interval categories with type = -0.63, $P < 0.001$.

nonlytic exocytosis representing approximately 52% of all events studied. By transfecting J774.1 murine-like macrophage cells with a fluorescently labeled membrane protein, we visualized cells participating in cell-to-cell transfer events. To our knowledge, this is the first time that this event has been observed in this manner. We observed an accumulation of cellular membrane both around the yeast cell that was being transferred and at the site of entry in the recipient macrophage. While we initially thought that entire phagosomes must be transferred, a supposition that may still be the case in some type III events, in the particular event shown in the movie, it would appear that, when individual yeast cells are transferred, a membrane coat is formed to shuttle them into another cell. This provides direct evidence that the yeast cells are in fact protected from the extracellular environment when they undergo this process. Studies using *Bacillus subtilis* as a model show that the bacteria form intercellular nanotubes to transport fluorescently labeled molecules and even antibiotic resistance to neighboring bacterial cells (28). Although these are clearly very different structures, the topology of a cell-to-cell bridge created between two macrophages during type III nonlytic exocytosis is reminiscent of this structure but on a much larger scale. This event, which could involve the transport of a phagosome or phagosomal load across two cell membranes without the spilling of its contents or lysis of either the donor or recipient, is a newly revealed biological phenomenon that raises many new questions as to how the process is achieved and what other cellular components are involved.

Nonlytic exocytosis is a unique phenomenon that could provide clues as to how cryptococcal cells disseminate throughout the host. More studies are needed to determine the various host cell and pathogen components that contribute to this process. Furthermore, it is likely that different types of molecular machinery were used in the different types of events. The future discovery of macrophage cell lines and/or cryptococcal cells that possibly do not undergo nonlytic exocytosis, or the utilization of possible drugs that block the event from occurring, could hasten our understanding of molecular events associated with this phenomenon. One could imagine that during these events, the cytoskeleton must be rearranged to allow for cryptococcal release or transfer between macrophages. Studies researching actin molecules, microfilaments, and microtubules during this process could be insightful for deciphering the mechanism. To summarize, our analysis reveals kinetic and morphological heterogeneity in exocytosis events and provides new parameters for studying this enigmatic process.

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