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Report

Expulsion of Live Pathogenic Yeast by Macrophages

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Summary

Phagocytic cells, such as neutrophils and macrophages, perform a critical role in protecting organisms from infection by engulfing and destroying invading microbes [1]. Although some bacteria and fungi have evolved strategies to survive within a phagocyte after uptake, most of these pathogens must eventually kill the host cell if they are to escape and infect other tissues [2, 3]. However, we now demonstrate that the human fungal pathogen *Cryptococcus neoformans* is able to escape from within macrophages without killing the host cell by a novel expulsive mechanism. This process occurs in both murine J774 cells and primary human macrophages. It is extremely rapid and yet can occur many hours after phagocytosis of the pathogen. Expulsion occurs independently of the initial route of phagocytic uptake and does not require phagosome maturation [4, 5]. After the expulsive event, both the host macrophage and the expelled *C. neoformans* appear morphologically normal and continue to proliferate, suggesting that this process may represent an important mechanism by which pathogens are able to escape from phagocytic cells without triggering host cell death and thus inflammation [6].

Results and Discussion

The facultative pathogenic yeast *Cryptococcus neoformans* is the causative agent of cryptococcosis, a fatal infection of immunocompromised patients. After inhalation, *C. neoformans* first establishes an infection in the lung but then rapidly spreads to the central nervous system and causes a meningoencephalitis that is fatal unless rapidly treated [7]. The mechanism by which *C. neoformans* is able to spread to the brain is currently unknown, but there has been much speculation that this may occur by a so-called "Trojan horse" mechanism [8]. This model proposes that cryptococci are engulfed by phagocytic cells at an early stage of infection and then trafficked by these host cells into distal tissues without being exposed to the full onslaught of the immune system. This hypothesis has received strong support with the discovery that *C. neoformans* can be isolated from circulating monocytes in infected mice [9] and from the observation that *C. neoformans* can proliferate inside the phagosome of macrophages in vitro [10–12]; this proliferation leads to lysis of the phagosomal membrane and eventually death of the host macrophage [12].

However, we now show that live *C. neoformans* cells can also escape from within macrophages via a mechanism that does not result in host cell death. This expulsive process may play an important role in the dissemination of *C. neoformans*, and potentially other pathogens, in infected individuals.

We observed cryptococcal expulsion in both cultured J774 cells (a murine macrophage line derived from a reticulosarcoma; Table 1 and see also Movie S1 in the Supplemental Data available with this article online) and human primary macrophage cells (Table 2 and Movie S2). It appears to occur when a mature phagosome containing one or more *C. neoformans* cells fuses with the plasma membrane and thus releases the yeast cell(s) into the extracellular medium (Figure 1 and Movies S1 and S2). Neither the host macrophage nor the *C. neoformans* cell is killed by this event, and both appear morphologically normal for the duration of the experiment thereafter (several hours). Indeed, in several cases, we were clearly able to observe *C. neoformans* cells that had been expelled subsequently undergo replication (See Movie S1 for an example); such findings demonstrate that the yeast cells were alive when ejected by the macrophage.

Cryptococcal expulsion appears to occur independently of the initial route of phagocytic uptake because both antibody or serum opsonized and nonopsonized yeast cells were capable of being expelled (Tables 1 and 2). However, we never observed expulsion of dead (heat-killed) *C. neoformans* cells nor of inert latex beads, suggesting that this process requires the presence of a live organism within the phagosome (Table 1).

Based on our observations, cryptococcal expulsion is a relatively rare event in cultured J774 cells. The majority of phagocytosed *C. neoformans* either proliferate or remain latent within the host macrophage rather than become ejected (Table 1). This probably explains why previous studies (e.g., [12] and [13]) have not reported it. Interestingly, however, the rate of expulsion in human primary macrophages is significantly ($p < 0.001$) higher than that in J774 cells, although the time distribution (Figure 2) does not differ between the two cell types ($p > 0.2$). This rate is particularly remarkable in light of the fact that our results are likely to underestimate the true frequency of this process because events that occur outside of the filming time (10 hr) will remain undetected in our experimental setup.

Previous studies have demonstrated that *C. neoformans* is well adapted to survive within the acidic

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Table 1. Intracellular Behavior of Different Target Particles in J774 Macrophages

Target Particles	Number of Cells Observed	Macrophages with Internalized CN	Intracellular CN Undergoing Proliferation	Occurrences of Expulsion
mAb 18B7 opsonized CN	603	134 (22.2%)	63 (47.0%)	13 (9.7%)
Nonopsonized CN	903	45 (5.0%)	28 (62.2%)	3 (6.7%)
Latex beads (3 μm)	336	157 (46.7%)	N/A	0
Latex beads (12 μm)	299	97 (32.4%)	N/A	0
Heat-killed CN with mAb 18B7	277	80 (28.8%)	N/A	0

The rate of intracellular proliferation and cryptococcal expulsion recorded for five different particle types with J774 cells. Note that the phagocytosis of nonopsonized *C. neoformans* is extremely inefficient because of the antiphagocytic effects of the cryptococcal capsule [21], resulting in a lower number of macrophages with intracellular yeast cells. CN, *C. neoformans*; N/A, not applicable; and mAb, monoclonal antibody.

environment of the phagosome and, unlike other many other pathogens, it does not inhibit phagolysosomal fusion [14]. We therefore wondered whether cryptococcal expulsion might occur only after maturation of the phagosome.

We blocked phagosome maturation by using both 100 nM concanamycin (a V-ATPase inhibitor that inhibits acidification of phagosome [15], maintaining it at a pH of approximately 7.5 [4]) and 10 μM chloroquine (a weak base that accumulates within the phagosome by ion trapping and raises the pH to approximately 6.5 when used at this concentration [16]). We found that cryptococcal expulsion was not blocked in the presence of either drug, suggesting that this process is not dependent on phagosome maturation. Interestingly, the rate of expulsion appears to be enhanced by the presence of 10 μM chloroquine (Table 2). It is possible that this effect may explain Levitz and his colleagues' earlier finding that this concentration of chloroquine suppresses intracellular growth of *C. neoformans* to a level that cannot be explained simply by its ability to inhibit phagosome acidification [4, 5].

The molecular mechanism that drives cryptococcal expulsion remains to be elucidated. Given the speed with which expulsion occurs, we considered a possible role for the actin cytoskeleton in providing the required force. To test this, we exposed J774 macrophages to cytochalasin D, a drug that blocks barbed-end growth of actin filaments. Interestingly, we continued to observe cryptococcal expulsion even at concentrations of up to 10 μM of cytochalasin D (Movies S3 and S4), suggesting that actin-filament polymerization is not strictly required for this process. However, because expulsion is an infrequent event, we cannot rule out the possibility that a low level of actin polymerization is still occurring in the few cells that show this effect in the presence of cytochalasin D.

Table 2. Cryptococcal Expulsion in Drug-Treated J774 and Primary Macrophages

Cell Type/Treatment	Number of Cells Observed	Macrophages with Internalized CN	Occurrences of Expulsion
J774 untreated (data from Table 1)	603	134 (22.2%)	13 (9.7%)
J774 treated with Concanamycin A (100 nM)	449	90 (20.0%)	3 (3.3%)
J774 treated with Chloroquine (10 μM)	408	68 (16.7%)	14 (20.6%)
Primary human macrophages	661	177 (26.8%)	47 (26.6%)

The rate of particle expulsion recorded for J774 cells after treatment to block phagosome maturation and for primary human macrophages. CN, *C. neoformans*; N/A, not applicable; and mAb, monoclonal antibody. Each value represents the total number of recorded events from three or more independent time-lapse experiments.

Currently, we do not know whether this process is unique to *C. neoformans* or whether it is a more widespread cellular phenomenon. Some bacteria and fungi have evolved strategies to survive within a phagocyte after uptake, but most of them must eventually kill the host cell if they are to escape and infect other tissues [2, 3]. Cryptococcal expulsion is thus unique in representing a nondestructive mechanism by which pathogens can reemerge from infected host cells. We suggest that the high incidence of cryptococcal expulsion in primary macrophage cells may have significant clinical implications because it represents a mechanism by which *C. neoformans* may be trafficked between tissues without triggering the localized inflammation that would occur if the host phagocyte was lysed [6]. In addition, it is possible that expulsion may also occur in cells other than macrophages, making this a plausible mechanism by which *C. neoformans* may be released into the central nervous system after being phagocytosed by endothelial cells of the blood-brain barrier [17].

It is interesting to note that the phagocytic amoeba *Dictyostelium discoideum* has been observed to expel the remains of digested yeast cells in a process that is morphologically similar to cryptococcal expulsion [18]. The fact that we do not observe the expulsion of heat-killed *C. neoformans* or of inert latex beads (Table 1) would argue against this process being a general mechanism for the expulsion of indigestible particles, although it is possible that such particles are expelled with a lower efficiency (and thus not detectable by our experimental setup). In addition, some retroviruses appear to be expelled from their host cell in vesicles termed "viral exosomes" [19]. Although these vesicles are one to two orders of magnitude smaller than the "expulsive phagosomes" observed in our system, it is possible that expulsion of *C. neoformans* occurs by a related mechanism and thus represents a previously unrecognized form of exocytosis. Nonetheless, to the best of our knowledge, this is the first report of such an expulsive process in a vertebrate phagocyte and the first demonstration that live pathogens can be expelled from phagocytic cells.

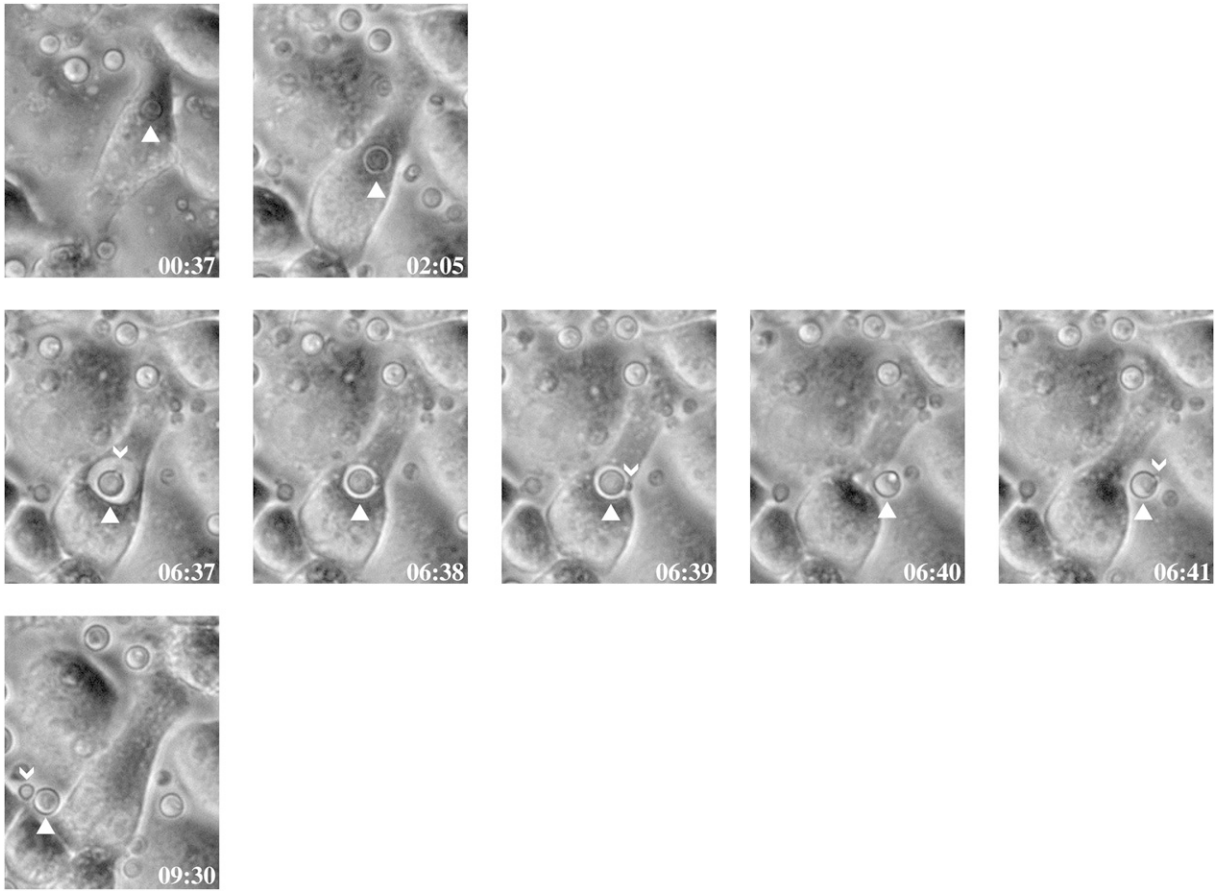

Figure 1. Representative Images from Movie S1

The internalized *Cryptococcus neoformans* (marked by a large arrow) is initially contained within a "tight" (phase-dark) phagosome. Two hours later, the phagosome begins to swell—the yeast cell is surrounded by a phase-bright region between the cell wall and the phagosome membrane; this region is believed to contain secreted capsular polysaccharide [12]. By 6 hr into the time series, the phagosome has grown dramatically; note that the *C. neoformans* cell is proliferating and a small daughter bud (marked by a small arrow) is clearly detectable. The *C. neoformans* containing phagosome suddenly fuses with the plasma membrane 400 min after the onset of filming. The phase-bright material is released and, 2 min later, the yeast cell is clearly outside of the host macrophage. Importantly, both the macrophage and the yeast cell are still alive 3 hr later, by which time the *C. neoformans* daughter cell (marked by a small arrow) has clearly grown in size.

Experimental Procedures

Yeast Strain and Growth Conditions

Cryptococcus neoformans strain JEC21 was grown overnight in YPG medium (1% yeast extract, 1% peptone, and 2% glucose) with 50 μg/ml ampicillin at 25°C with shaking. To avoid yeast clumps, we vigorously shook the fungal suspensions and allowed them to sediment briefly before use. The top suspension, containing mostly individual yeast cells, was then used. For experiments with heat-killed *C. neoformans*, overnight cultures were killed by incubating at 55°C for 30 min.

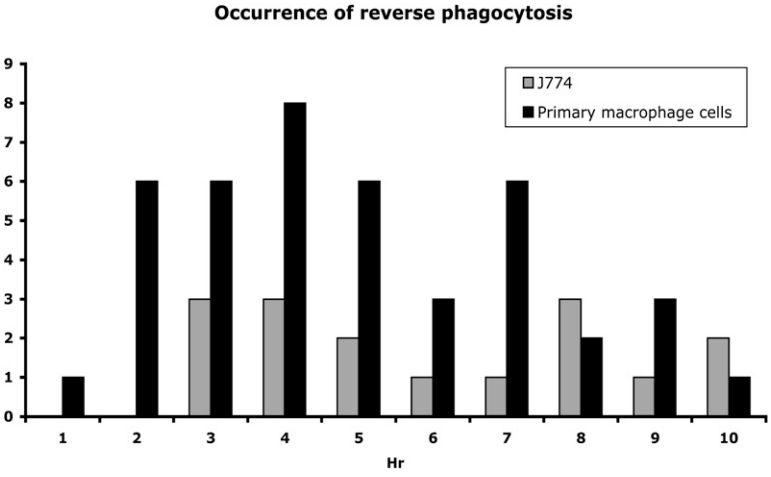

Figure 2. The Distribution of Expulsive Events over the Course of the Time-Lapse Recordings

Data are pooled from all time-lapse recordings (15 independent experiments) of J774 macrophages (11 experiments, 1506 cells, and 179 with internalized *C. neoformans*) and human primary macrophages (4 experiments, 661 cells, and 177 with internalized *C. neoformans*). Although the rate of cryptococcal expulsion is higher in primary macrophages than in J774 cells, the distribution of expulsive events over the 10 hr of time-lapse recording does not differ between these cell types ($p > 0.2$, two-tailed Student's t test).

Cell Line and Culture Media

J774 cells were grown at 37°C in 5% CO_2 in DMEM with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cell line was used between five and 20 passages after thawing.

Human primary blood macrophage cells (PBMC) were prepared as described previously [20] and resuspended at 6×10^6 cells/ml in RPMI 1640 medium containing 2% pooled AB^+ male human serum and 2 mM L-glutamine. The PBMC were then seeded into 175 cm tissue-culture flasks. Nonadherent cells were removed by extensive washes with PBS, and adherent monocytes were incubated overnight in RPMI medium + 5% human serum supplemented with GM-CSF (100 IU/ml). After overnight incubation, adherent cells were removed from flasks by incubation on ice in prechilled PBS and cultured for 4-5 days in RPMI + 5% human serum and GM-CSF (100 IU/ml). The cells were adjusted to 1×10^6 cells/ml and then aliquoted into 3 cm plastic Petri dishes in 3 ml cultures. The cell media was replenished on days 3 and 5 and yielded adherent, confluent macrophage cultures at day 7. When required, the macrophages were activated with LPS (1 μg/ml) and IFNγ (1000 IU/ml), which were added to the culture dishes 24 hr prior to infection with *C. neoformans*.

Phagocytosis Assay

J774 cells (6×10^5) were plated into a 35 mm tissue-culture-treated plate 16–24 hr before the assay. Shortly before use, cells were incubated for 1 hr in serum-free DMEM medium (Complete DMEM medium without heat-inactivated FBS) containing 150 ng/ml PMA. Similarly, the primary macrophage cells (which have been activated with LPS and IFNγ) were incubated for 1 hr in serum-free DMEM medium.

At the same time, *C. neoformans* cells were washed three times with phosphate-buffered saline (PBS [pH 7.2]) and counted in a haemocytometer. For experiments with opsonized *C. neoformans*, both live and heat-killed fungal cells were incubated with 10 μg/ml of the monoclonal antibody 18B7 (a kind gift of Arturo Casadevall) or fresh human serum at 37°C for 1 hr. For nonopsonized *C. neoformans* and latex beads, mAb18B7 was replaced with water.

For commencement of the assay, the medium containing PMA was removed and replaced by normal serum-free medium containing *C. neoformans* at a ratio of ten yeast cells per macrophage. We allowed phagocytosis to proceed for 2 hr at 37°C in a 5% CO_2 atmosphere. We then removed noninternalized yeast cells by three successive washes with serum-free medium and maintained cells in serum-free medium with 25 mM HEPES for time-lapse imaging.

For experiments with drugs for disrupting phagosome maturation, 100 nM concanamycin A was added during the onset of phagocytosis (because this drug inhibits phagocytosis if it is added before phagocytosis commences), whereas 10 μM chloroquine was added 1 hr prior the onset of phagocytosis. Both drugs remained in the culture medium throughout the time-lapse imaging. For experiments with cytochalasin D for blocking actin polymerization, this drug was added to the culture medium immediately before commencing imaging, as described in the legends for the Supplemental Movies.

Image Capture and Analysis

Cells were maintained at 37°C with a temperature-controlled chamber (Solent Scientific) and imaged on a Zeiss Axiovert 100 inverted microscope with a 32× dry objective lens. Time-lapse movies were made with OpenLab (Improvision), capturing one frame every 60–90 s for 10 hr on a QICAM camera. The cell number, phagocytosis rate, intracellular proliferation rate, and the occurrence and timing of expulsive events were scored manually by eye. All experiments were repeated three or more times on different days with independent cultures of macrophages and *C. neoformans* JEC21.

For producing Figure 2 and the Supplemental Movies, the original time-lapse movie was decompiled into individual TIFF images with ImageJ (http://rsb.info.nih.gov/ij/). These were then cropped to the region of interest and sharpened. A semiopaque mask and arrow were added to the first frame (to indicate the cell of interest) with Adobe Photoshop 7.0 before the TIFF image stack was recompiled into a Quicktime movie with ImageJ. For producing Figure 1, selected TIFF images from the movie were compiled and annotated with arrows with Adobe Illustrator 10.

Supplemental Data

Supplemental Data include four movies and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/21/2156/DC1/.

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