Aspergillus fumigatus conidia survive and germinate in acidic organelles of A549 epithelial cells

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Summary

***Aspergillus fumigatus* is an environmental mould that can cause invasive disease in the immunocompromised host. Previous work has shown that conidia can be internalized by lung epithelial cells (A549) and murine macrophages (J774) in vitro. Therefore, the purpose of this study was to determine the fate of *A. fumigatus* conidia within the endosomal network of these cells. Co-localization of conidia expressing green fluorescent protein with proteins present in the early endosomal (CD71) and lysosomal (CD63, LAMP-1) membrane was assessed using confocal microscopy. In J774 cells, 75% of internalized conidia were found in phagosomes containing LAMP-1 120 minutes post-infection. In A549 cells, 55% and 58% of internalized conidia were found to co-localize with LAMP-1 and CD63 by 24 hours. Cathepsin D also co-localized with internalized conidia in A549 cells. Phagosomes containing conidia were shown to be acidified in both cell types. Less than 1% of the initial inoculum survived in J774 cells by 12 hours post-infection. After 24 hours, 3% of internalized conidia survived in A549 cells and 34% of these had germinated. By 36 hours, the germlings were able to escape the phagosome and form extracellular hyphae without lysis of the host cell.**

Key words: *Aspergillus fumigatus*, Phagosome, Germination, A549, J774

Introduction

Infections due to opportunistic fungal pathogens are a growing concern in industrialized nations. As the success of organ transplants and cancer chemotherapies increases, so does the number of neutropenic patients who are susceptible to these previously uncommon fungal diseases (van Burik and Magee, 2001). *Aspergillus fumigatus* is a saprophytic soil fungus responsible for the majority of invasive mold infections worldwide. Inhalation of infectious conidia by immunoscompromised individuals may lead to germination and growth of the fungus in the lung, followed by progression to a serious disease called invasive pulmonary aspergillosis (IPA) (Bodey and Vartivarian, 1989). Treatment of IPA is difficult due to problems with early and accurate diagnosis (Groll et al., 1996) and the toxicity of drugs used to treat the illness (Graybill, 2000). Despite medical advances, mortality rates exceed 65% (Denning, 1996).

Intracellular pathogens have evolved many mechanisms to survive in the hostile environment of the host. Invasion of host cells by the pathogen is one of several possible outcomes once a microbe has successfully colonized a host. Once internalized, intracellular pathogens are protected from the host immune system and have access to preformed nutrients. These intracellular microbes employ several different strategies to grow and reproduce within host cells. For example, *Shigella flexneri* and *Listeria monocytogenes* degrade the phagosome and gain access to the nutrient rich cytoplasm (Meresse et al., 1999), whereas *Chlamydia pneumoniae* does not enter a phagolysosome but is intimately associated with early endosomes (Al-Younes et al., 1999). *Brucella abortus* escapes the endocytic pathway and is found in a vacuole resembling an autophagosome (Pizarro-Cerda et al., 1998). Finally, *Mycobacterium tuberculosis* prevents phagosome acidification by exclusion of the vesicular proton (Sturgill-Koszycki et al., 1994). All of these diverse mechanisms have the common theme of allowing the pathogen to avoid degradation and create a favorable niche within which to grow and replicate.

Some pathogenic fungi also evade phagocytic degradation by mechanisms similar to those used by bacteria. Phagosomes containing *Histoplasma capsulatum* fail to acidify while still retaining fusion competence (Eissenberg et al., 1993). In macrophages, *Candida albicans* develops germ tubes within phagolysosomes and eventually escapes from and destroys the macrophage (Káposzta et al., 1999). In contrast, in brain endothelial cells, virulent *C. albicans* strains invade and transcytose without affecting the integrity of the endothelial cell monolayer (Jong et al., 2001).

Previous work by our laboratory and others has determined that A. *fumigatus* conidia are internalized by lung cells (DeHart et al., 1997; Paris et al., 1997; Wasylnka and Moore, 2002) endothelial cells (Paris et al., 1997; Wasylnka and Moore, 2002) and macrophages (Nessa et al., 1997; Wasylnka and Moore, 2002) in vitro. We have previously demonstrated that 100% of conidia internalized by A549 lung epithelial cells survive for at least 3 1/2 hours following uptake, whereas 70% of conidia internalized by J774 murine macrophages are killed within 6 hours. This finding prompted us to determine whether there were differences in the fate of *A. fumigatus* conidia within the endosomal networks of A549 and J774 cells. Therefore, the objectives of this study were: (1) to determine co-localization

of conidia-containing phagosomes with endosomal/lysosomal proteins in A549 and J774 cells; (2) to determine the pH of the conidia-containing phagosomes within these cells, and (3) to determine the viability of conidia within A549 and J774 cells after extended incubation times.

Materials and Methods

A. fumigatus growth conditions

A previously described *A. fumigatus* 13073 strain that constitutively expresses green fluorescent protein (GFP) (Wasylnka and Moore, 2002) was used for all experiments. Fungi were grown on YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 0.5% dextrose) containing 200 μg/ml of hygromycin for 3-4 days at 37°C until conidia were fully mature. Conidia were harvested as described previously (Wasylnka and Moore, 2000).

Tissue culture

The type II human pneumocyte cell line A549, and murine macrophage line J774, were obtained from the ATCC and maintained in RPMI 1640 media containing 10% fetal bovine serum (Canadian Life Technologies, Burlington, Ontario, Canada), 100 mg/l streptomycin and 16 mg/l penicillin (both from Sigma-Aldrich Canada, Oakville, Ontario, Canada). Cells were maintained at 37°C in humidified 5% CO_2 incubators.

Measurement of conidia survival in A549 and J774 cells

A549 cells and J774 cells were seeded at 5.0×10^5 cells/well in 24 well plates and grown for 16 hours. Following cell growth, wells were blocked for 30 minutes in minimum essential media (MEM) (Canadian Life Technologies) containing 0.1% (w/v) bovine serum albumin (BSA) (ICN Pharmaceuticals, Montreal, Canada) at 37°C. Cells were infected with a 2:1 multiplicity of infection (M.O.I.) of *A. fumigatus* gGFP conidia and incubated in MEM/10% (v/v) FBS for 3 hours at 37°C. After incubation, unbound spores were removed by washing wells three times with PBS/0.05% Tween 20 (PBS-T). Extracellular conidia were killed with nystatin as described previously (Wasylnka and Moore, 2002). Cells were lysed with 0.5% Triton X-100 and serial dilutions of released conidia were plated onto YM agar (three replicate plates/well). Some samples were incubated in MEM for an additional 6 hours (both cell lines) or 18 hours (A549 only) at 37°C prior to lysis and plating. For A549 cell incubations, 5 μg/ml nystatin was added to the medium. To maintain high viability of the J774 cells, 1% FBS was added to the incubation media during the extended incubation times. Nystatin was not added because after 6 hours all spores were intracellular.

Confocal microscopy

Endosomal trafficking assays

A549 and J774 cells were seeded at 5.0×10^5 cells/well onto 12 mm Number 1 coverslips in 24 well plates (Falcon, Becton-Dickinson Canada, Mississauga, Ontario, Canada) and grown for 16 hours. Cells were infected with 1 ml of $1\text{-}2\times10^6$ spores/ml in MEM/10% (v/v) FBS (M.O.I. of 2:1 for A549 and 4:1 for J774 cells) for the indicated times at 37°C and then washed with PBS-T. A549 cells were treated with nystatin as described above. Extracellular spores were labelled using an anti-*Aspergillus* cell wall polyclonal antibody (Wasylnka and Moore, 2002). Cells were fixed for 1 hour with PBS/4% (w/v) paraformaldehyde, pH 7.4 and then permeabilized for 1 hour with PBS/10% goat serum/0.05% saponin. The A549 antibodies used were anti-human cathepsin D (Oncogene Research Products, Boston, MA), anti- human CD71 (Sigma), anti-human LAMP-1 (clone H4A3, Developmental Studies Hybridoma Bank (DHSB), University of Iowa) and anti-human CD63 (clone H5C6, DHSB). J774 antibodies were anti-murine LAMP-1 (clone 1D4B, DHSB) and anti-murine CD71 (Sigma). Antibodies were diluted in PBS/10% goat serum and used at 1:5 (1D4B), 1:100 (cathepsin D, anti-human CD71 and H4A3), 1:200 (H5C6), or 1:500 (anti-murine CD71). Cells were incubated with primary antibodies for 60 minutes and washed three times with PBS. The secondary antibody goat anti-mouse/rat Alexa 647 (Molecular Probes, Eugene, OR), was diluted 1:100-1:400 in PBS/10% goat serum and incubated with cells for 45 minutes. Wells were washed with PBS and coverslips were mounted onto slides with ProLong antifade from Molecular Probes. Cells were viewed with a Zeiss LSM-410 confocal microscope equipped with a krypton/argon laser (Omnichrome), using a 63× 1.4 numerical aperture lens. Green fluorescence was captured with a 515-540 nm band pass filter, red fluorescence with a 590-610 band pass filter and blue fluorescence with a 670-810 nm bandpass filter. Images were processed in Adobe PhotoShop 6.0 (Adobe Systems Incorporated, San Jose, CA). At least 100 conidia-containing phagosomes in two separate fields were analyzed for each treatment and infection time.

LysoTracker co-localization

Cells were seeded at 3×10^5 cells/well in 8-well chambered coverslips (Nalge Nunc International Corporation, Naperville, IL) and grown for 16 hours. Cells were infected for the indicated times with a 2:1 M.O.I. of *A. fumigatus* conidia. (A549 cells infected for 24 hours were treated as in the survival assay). The wells were washed with PBS-T and then incubated for 30 minutes at 37°C with pre-warmed LysoTracker Red DND-99 (Molecular Probes) diluted to 50 nM in MEM. The loading solution was replaced with fresh warm media, and red and green fluorescence was captured on the Zeiss LSM-410 confocal microscope as described above. At least 100 conidia in two separate fields were analyzed for each treatment and infection time.

Penetration of the A549 epithelial barrier

A549 cells were seeded onto 12 mm diameter No. 1 coverslips at 5.0×10^5 cells/well in 24-well plates and grown for 16 hours. Cells were infected with a 2:1 M.O.I. of *A. fumigatus* gGFP conidia for 3 hours at 37°C, washed with PBS-T and then incubated with 50 μg/ml nystatin in MEM for 3 hours. Cells were washed once with PBS and then incubated for 18 hours with MEM containing 5 μg/ml nystatin. After 24 hours, the cells were washed with PBS and extracellular conidia/hyphae were either immediately labelled with the anti-*Aspergillus* cell wall antibody as described (Wasylnka and Moore, 2002), or incubated a further 12 hours in MEM/2% FBS prior to labelling. Cells were viewed on the Zeiss LSM-410 confocal microscope as described above.

Nystatin control assay

Conidia were harvested and 10^6 spores were diluted in MEM media and incubated at 37°C for 3 hours to initiate germination. Nystatin (dissolved in DMSO) was then added to the media at 50 μg/ml and incubated with the spores for another 3 hours at 37°C. Aliquots were diluted and plated (along with untreated spores) onto YM agar. The media was then diluted 10-fold to decrease the nystatin concentration to 5 μg/ml and the samples were incubated for an additional 18 hours. The next day the solution was diluted and plated to determine the number of surviving spores.

Cytotoxicity of cultured cells during *A. fumigatus* infection

To test the viability of the infected and uninfected cells during the survival assay, 50 μl of supernatant was removed at various time points post-infection and the level of lactate dehydrogenase (LDH) was determined using the Cytotox 96 Non-radioactive assay kit from

Promega (Madison, WI). The assay quantitatively measures LDH, a stable cytosolic enzyme that is released upon cell lysis. Uninfected wells were treated with either 100 μl of 10% Triton X-100 (1% v/v final concentration) or PBS to serve as positive and negative controls for LDH release. Samples were incubated with LDH substrate for 5 minutes and then the absorbance was read on a microplate reader at 490 nm.

The Student *t*-test was used for statistical analysis of data.

Results

A. fumigatus conidia fuse quickly with lysosomes after phagocytosis by A549 or J774 cells

Previous work by us (Wasylnka and Moore, 2002) and others (DeHart et al., 1997; Nessa et al., 1997; Paris et al., 1997) have shown that *A. fumigatus* conidia are phagocytosed by lung epithelial and macrophages cells in vitro. Internalization of conidia by type II pneumocytes such as A549 cells may be important in the development of aspergillosis in vivo, as sequestration by these cells may allow conidia to evade the immune response of the host. However, the long-term viability of internalized conidia is unknown. We have also demonstrated that germination of internalized conidia inside A549 cells was delayed compared to extracellular conidia (Wasylnka and Moore, 2002). Therefore, we investigated the location of conidia within the endosomal network of A549 cells following uptake to determine whether internalized conidia were targeted for degradation or could survive within the cell. As a positive control, we first examined the trafficking of *A. fumigatus* conidia by J774 cells, as previous experiments had shown that up to 90% of the initial inoculum was internalized by these professional phagocytes (Wasylnka and Moore, 2002). J774 cells were infected with *A. fumigatus* conidia expressing green fluorescent protein (AfGFP) for 25, 45 or 120 minutes. Cells were then incubated with an antibody for the anti-transferrin receptor (CD71) (found on early endosome membranes) and with antibodies to the lysosomal-associated membrane protein 1 (LAMP-1). After 25 minutes, we occasionally observed co-localization of conidia in CD71 positive phagosomes, which was visualized by a ring of CD71 fluorescence around the internalized spore (Fig. 1A,a). These structures were not found in uninfected cells (data not shown). At 120 minutes post-infection we observed many conidia in LAMP-1 positive phagosomes (Fig. 1A,b). To quantify the number of conidia within early endsomes and lysosomes, the number of internalized conidia surrounded by a ring of marker label was scored in three independent experiments. After 25 minutes, only 3% of internalized conidia were found in phagosomes containing CD71, whereas 30% of the conidia were already co-localized with LAMP-1 (Fig. 2B). At 45 minutes and 120 minutes post-infection, less than 1% of the internalized conidia were still in early endosomes, most were in LAMP-1 positive phagosomes. The percentage of conidia that co-localized with LAMP-1 increased from 50% at 45 minutes to 75% by 120 minutes.

To determine whether the kinetics of conidia transport through the endosomal network was similar in A549 cells, we infected cells with *A. fumigatus* conidia under identical conditions and then labelled them with CD71 and LAMP-1 antibodies as well as antibodies to CD63 (found on late endosome/lysosome membranes) and cathepsin D. Cathepsin D is a soluble lysosomal hydrolase found in late endosomes/lysosomes (Bohley and Seglen, 1992). Our previous experiments have shown that internalization of

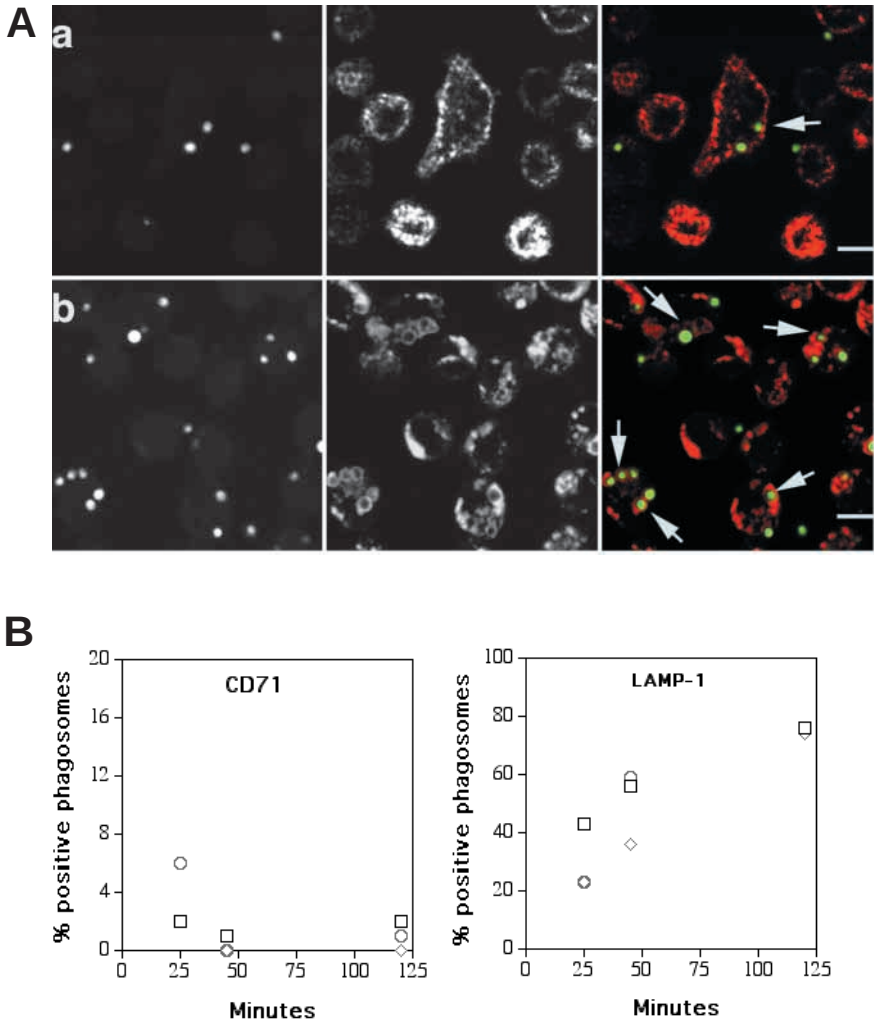
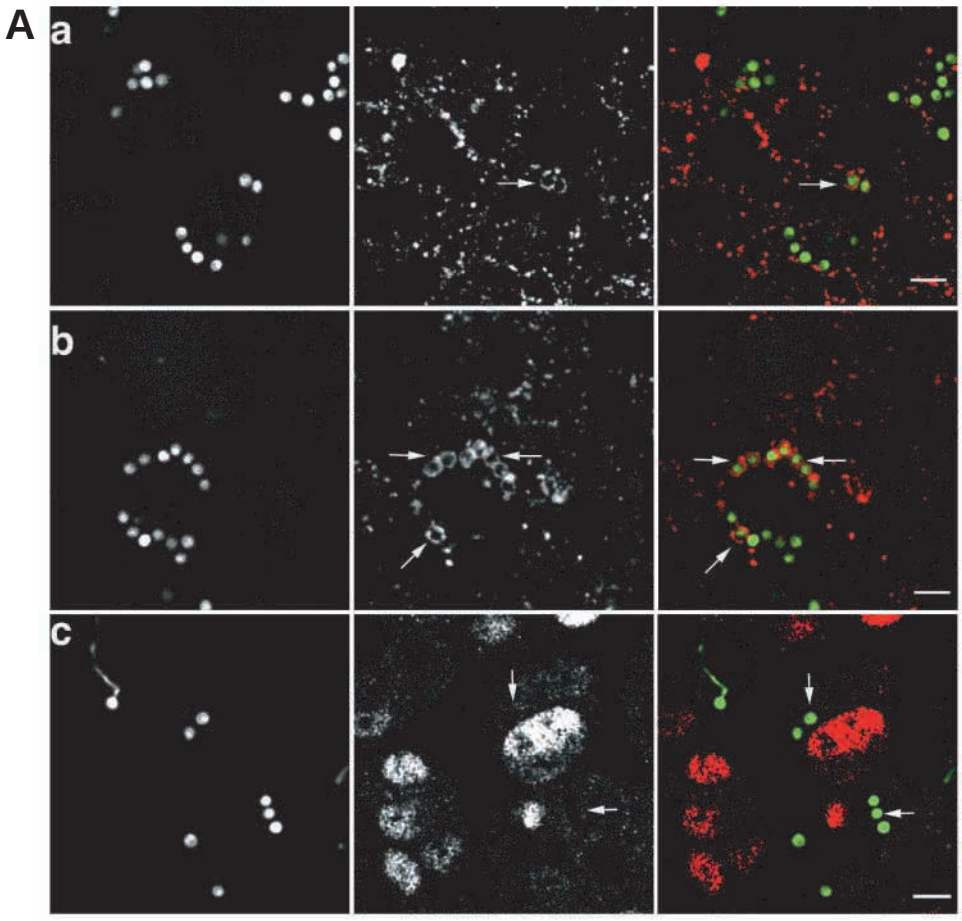


Fig. 1. Co-localization of *A. fumigatus* conidia with endosomal markers in J774 cells. (A) J774 cells were infected with *A. fumigatus* conidia for 25 minutes (a) or 120 minutes (b) and then stained with an antibody to CD71 (a) or LAMP-1 (b). From left to right: fluorescence image showing the green channel (conidia); fluorescence image of the blue channel (red pseudocolor) (endosomes/lysosomes); and a merged overlay of both images. Bars, 10 μm. (B) The percentage of *A. fumigatus* phagosomes that contained either CD71 or LAMP-1 at 25, 45 or 120 minutes was scored by analysis of at least 100 internalized conidia in three separate experiments, each represented by a different symbol (circles, squares and diamonds).



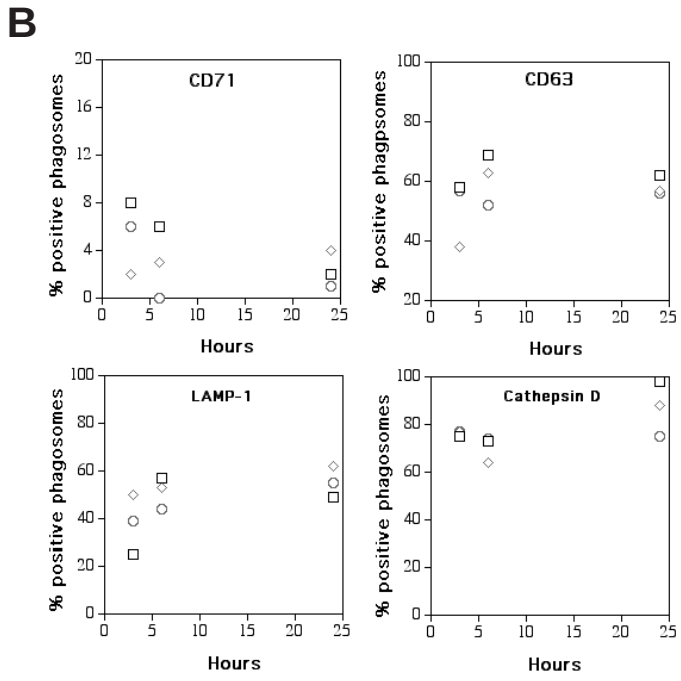


Fig. 2. Co-localization of *A. fumigatus* conidia with endosomal markers in A549 cells. (A) A549 cells were infected with *A. fumigatus* conidia for 3 (a,b) or 24 hours (c) and then stained with an antibody to CD71 (a), CD63(b) or cathepsin D (c). From left to right: fluorescence image showing the green channel (conidia); fluorescence image of the blue channel (red pseudocolor) (endosomes/lysosomes); and a merged overlay of both images. Bars, 10 μm. (B) The percentage of *A. fumigatus* phagosomes that contained either CD71, CD63, LAMP-1 or cathepsin D at 3, 6 or 24 hours was scored by analysis of at least 100 internalized conidia in three separate experiments, each represented by a different symbol (circles, squares and diamonds).

conidia by A549 cells occurs between 2.5-3 hours post-infection (Wasylnka and Moore, 2002). Since A549 cells are non-professional phagocytes, we hypothesized that trafficking through the endosomal system would be slower than in J774 cells. Therefore, we looked at co-localization of conidia phagosomes with endosomal markers at 3, 6 and 24 hours post-infection. As we had observed with J774 cells, very little co-localization with CD71 occurred (Fig. 2A,a), but there were many CD63 (Fig. 2A,b) and LAMP-1 positive phagosomes at 3 hours (data not shown). Conidia-containing phagosomes also acquired cathepsin D (Fig. 2A,c). The co-localization of conidia within endosomal/lysosomal markers was also quantified for A549 cells. Only 5% of conidia were in early endosomes after 3 hours, whereas 38% were co-localized with LAMP-1 and 51% with CD63 (Fig. 2B). After 6 hours, only 3% of conidia co-localized with CD71, but 51% and 61% were found with LAMP-1 and CD63 respectively. These values remained similar up to 24 hours post-infection (2% CD71, 55% LAMP-1, 58% CD63). Cathepsin D was also found to co-localize early with internalized conidia (76% after 3 hours) and increased to 87% after 24 hours. The percentage of conidia that associated with cathepsin D was higher than that seen with LAMP-1 and CD63, as phagosomes were scored positive for the presence of cathepsin D even when the intensity of the signal was very weak (Fig. 2A,c). Together, this data suggests that conidia fuse rapidly with late endosomes/lysosomes following uptake by J774 or A549 cells.

A. fumigatus conidia reside in acidic organelles of A549 and J774 cells

A large majority of the conidia were co-localized with late endosome/lysosome markers, and since these organelles typically have a pH of less than 6, we hypothesized that the phagosomes would also be acidic. Therefore, we investigated whether the pH of the phagosomes containing conidia was neutral or acidic. Cells were infected with AfGFP conidia for 1, 2 or 6 hours (J774 cells) or 3, 6 and 24 hours (A549 cells) and then labelled with a fluorescent probe, LysoTracker, which

selectively accumulates in the membranes of acidic organelles. The LysoTracker probe was observed to co-localize with GFP conidia as seen by rings of red fluorescence surrounding the green spores (Fig. 3A,a-c). Germinating conidia were seen in A549 cells after 6 or 24 hours and after 6 hours in J774 cells, however germination was observed much less frequently in these cells. We observed that the fluorescence extended around the germlings (Fig. 3A), suggesting that these germlings were still contained within membrane-bound acidic organelles. Occasionally, we also noticed some red phagosomes that had no conidia associated with them (Fig. 3A,b, blue arrows). These phagosomes may have contained conidia that had already been degraded.

We quantified the proportion of phagosomes that contained conidia that also showed staining with LysoTracker. After 1 hour in J774 cells, 29% of the internalized conidia were in acidic organelles (Fig. 3B) and this number rose to 70% after 6 hours. This data is consistent with the antibody co-localization experiments in which 75% of the conidia co-localized with LAMP-1 after 2 hours.

In contrast, conidia-containing phagosomes within A549 cells were slower to acidify; only 17% of internalized conidia were in acidic organelles after 3 hours, 24% after 6 hours and this increased to 50% after 24 hours. However, the data at 3 and 6 hours may underestimate the proportion of conidia in acidic phagosomes. The number of co-localized conidia was determined by dividing by the number of ringed conidia by the total conidia/field but at 3 and 6 hours post-infection not all of the conidia had been internalized. Moreover, this experiment must be done using live cells, so the actual number of intracellular conidia cannot be determined as the cells cannot be immunostained. Based on our previous findings, approximately 30% of added conidia are internalized after 3 hours (Wasylnka and Moore, 2002). Thus, the adjusted value of conidia co-localization with LysoTracker after 3 hours is approximately 50%. Therefore, it is probable that up to 50% of conidia fuse with acidic organelles at 3 hours and this number remains constant up to 24 hours post-infection.

Thus, *A. fumigatus* conidia reside in acidic organelles of J774 and A549 cells and these phagosomes are positive for LAMP proteins and lysosomal acid hydrolases.

Survival of *A. fumigatus* in A549 and J774 cells

The results from the antibody co-localization and LysoTracker experiments suggested that conidia were trafficking to acidic late endosomes or lysosomes. Furthermore, conidia were able to germinate within lysosomes in both cell lines (Fig. 3A). Therefore, it was of interest to determine the viability of the conidia in A549 and J774 cells over a 6-24 hour time period. Either J774 or A549 cells were incubated with conidia for 6, 12 or 24 hours and a nystatin protection assay was used to measure the viability of the internalized spores. After 6 hours, there was a fourfold difference in the number of conidia recovered from the two cell lines; 8% of the initial inoculum was still viable in A549 cells, whereas only 2% survived in J774 cells (Table 1). By 12 hours, the difference was tenfold; 6.5% remained in A549 cells versus 0.6% in J774 cells (Table 1).

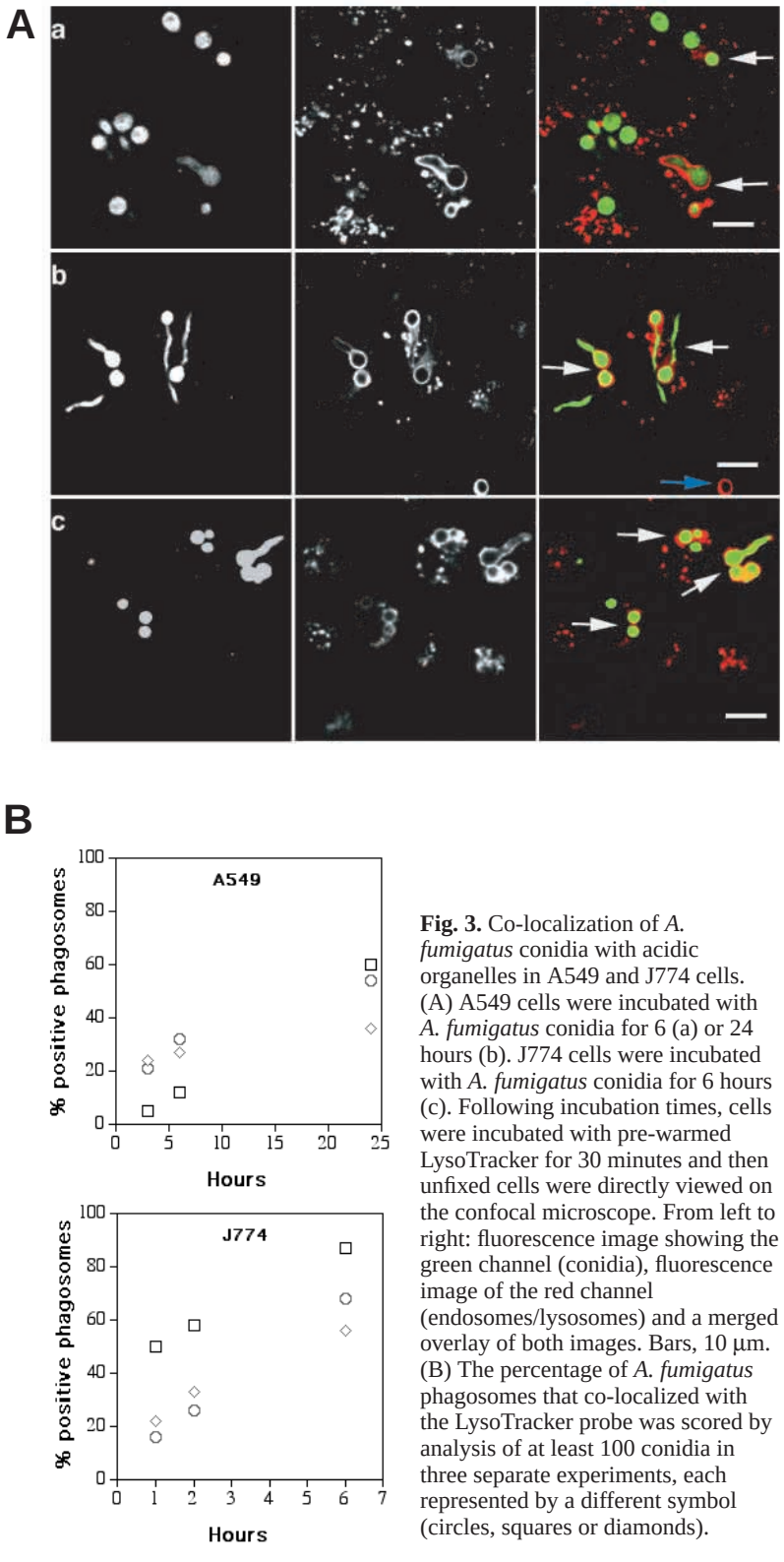


Fig. 3. Co-localization of *A. fumigatus* conidia with acidic organelles in A549 and J774 cells. (A) A549 cells were incubated with *A. fumigatus* conidia for 6 (a) or 24 hours (b). J774 cells were incubated with *A. fumigatus* conidia for 6 hours (c). Following incubation times, cells were incubated with pre-warmed LysoTracker for 30 minutes and then unfixed cells were directly viewed on the confocal microscope. From left to right: fluorescence image showing the green channel (conidia), fluorescence image of the red channel (endosomes/lysosomes) and a merged overlay of both images. Bars, 10 μm. (B) The percentage of *A. fumigatus* phagosomes that co-localized with the LysoTracker probe was scored by analysis of at least 100 conidia in three separate experiments, each represented by a different symbol (circles, squares or diamonds).

Table 1. Survival of *A. fumigatus* conidia in A549 lung epithelial cells versus J774 macrophages*

	Cell line	
Time (h)†	A549	J774
6	7.9±1.1	2.0±0.8‡
12	6.5±2.0	0.6±0.1‡
24	3.0±0.4	ND

*Cells were infected with *A. fumigatus* conidia at a M.O.I. of 2:1 for the indicated times. At the end of the incubation period, cells were lysed and released conidia were diluted and plated. Data is expressed as the percentage of the initial inoculum that remained viable.

†Time elapsed after addition of conidia to the cells.

‡Significantly different (P<0.05) from A549 as determined by the Student's t-test

ND, not determined.

To determine whether conidia could survive for longer periods in A549 cells, the incubation step was increased to 24 hours. (J774 cells were not investigated as essentially all of the conidia were killed after 12 hours.) After 24 hours, 3% of the initial inoculum was still viable (Table 1) and 34±3% of the intracellular conidia had germinated.

To determine whether intracellular conidia had any cytotoxic effect on the cells, we measured the release of the cytosolic enzyme lactate dehydrogenase (LDH) into the supernatant after 6, 12 or 24 hours. In both A549 and J774 cells, there was no difference in LDH release between infected and uninfected cells after 6 or 12 hours (Fig. 4A,B). In contrast, after 24 hours in A549 cells, infected cells displayed a small but significant increase in cytotoxicity over uninfected cells (P<0.05).

Intracellular conidia can germinate and penetrate the epithelial barrier

The finding that conidia could germinate and survive inside acidic organelles within A549 cells, prompted us to determine whether internalized germlings could escape the phagosome and penetrate the A549 cell membrane. To observe this process by confocal microscopy, cells were infected for 24 or 36 hours and stained with an *Aspergillus* cell wall antibody under conditions that only label extracellular conidia or hyphae. After 24 hours, we observed many intracellular conidia and germlings, as well as some extracellular germlings. The extracellular germlings usually had longer germ tubes than the intracellular ones (data not shown). We also observed many germlings that were only partially labelled by the antibody at the ends of the germ tubes (Fig. 5a, arrows). Thus, the ends of the germ tube were extracellular and accessible to the antibody, but the remainder of the germling was still inside the cell. This data suggests that after 24 hours, germlings begin to penetrate the A549 cell from within, but the plasma membrane is not so disrupted that antibodies can access the cytoplasm and stain intracellular germlings.

To determine whether these germlings could develop into hyphae, the incubation period was extended to 36 hours and nystatin was removed from the media for the final 12 hours. Infected cells were treated for 3 hours with 50 μg/ml nystatin, for 18 hours with 5 μg/ml nystatin and then the nystatin was removed. In control experiments with no cells, this treatment kills all added conidia by the end of the incubation. Therefore, any extracellular germlings/hyphae present after 24-36 hours should represent conidia that originated from within the A549 cells. As shown in Fig. 5 (b), germlings had grown into extracellular hyphae by 36 hours post-infection. Interestingly, LDH release was not significantly greater than after 24 hours, which suggests that the hyphal extension had not yet caused significant damage to the host cells (data not shown).

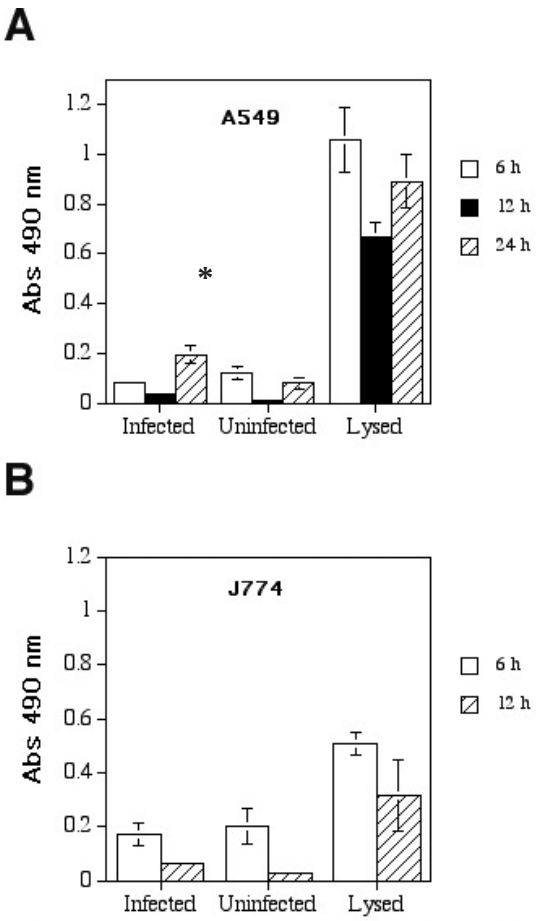


Fig. 4. Infection of cultured cells with *A. fumigatus* conidia does not induce cellular cytotoxicity. A549 (A) or J774 (B) cells were incubated with *A. fumigatus* conidia for 6, 12 or 24 hours and then the supernatant was assayed for LDH activity. Infected samples contained cells plus conidia and uninfected samples contained cells only. Lysed represents uninfected cells that were treated with 1% Triton X-100 prior to sampling. Samples were incubated with LDH substrate for 5 minutes and then the absorbance was read on a microplate reader at 490 nm. *P<0.05.

Discussion

We have presented data demonstrating that *A. fumigatus* conidia are internalized by J774 and A549 cells, and that phagosomes containing conidia fuse quickly with late endosomes/lysosomes. Passage through the endosomal network of J774 cells was rapid and more than 99% of the added conidia were killed by 12 hours post-infection. Similarly, conidia internalized by A549 cells fused rapidly with late endosomal markers. However, in contrast to J774 cells, a

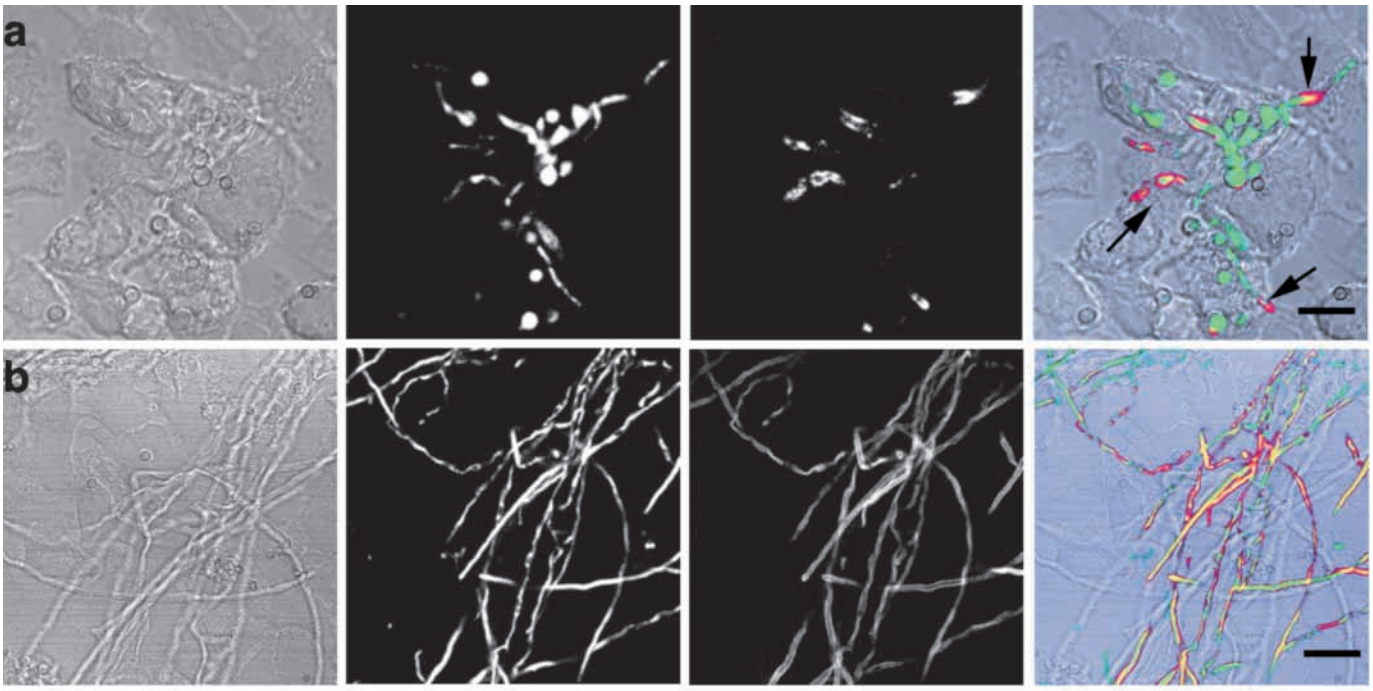


Fig. 5. Intracellular germlings escape from the A549 phagosome and grow into hyphae in the extracellular medium. A549 cells were incubated with *A. fumigatus* conidia for 24 (a) or 36 (b) hours. Following infection, samples were processed for immunofluorescence. From left to right: differential interference contrast (DIC) image, fluorescence image showing the green channel (total conidia/hyphae), fluorescence image of the red channel (extracellular conidia), and a merged overlay of all images. Bars, 10 μm. The results are representative of two independent experiments. Note that some conidia/hyphae are seen in the DIC image but not the green channel since the fluorescent image represents a single 0.2 μm section that only detects signals from spores in the same plane.

significant number of intracellular conidia survived and germinated in acidic phagosomes of A549 cells. After 24 hours, 3% of the initial inoculum persisted in these cells and some *A. fumigatus* germlings had penetrated the plasma membrane. By 36 hours post-infection, there was significant hyphal outgrowth from the cell into the extracellular medium.

Uptake of particulate material by cultured cells results in the formation of a phagosome that can have several fates within the endosomal network, depending on the nature of the internalized particle. Phagosomes containing inert materials such as latex beads initially possess early endosome markers such as rab 5 and gradually accumulate late endosome markers such as rab 7 and lamp 2 (Desjardins et al., 1994). Based on elegant video microscopy studies, Desjardins et al. (Desjardins et al., 1994) have suggested that phagolysosome formation is a dynamic process that relies on the gradual acquisition of proteins from endocytic organelles. Many microbial pathogens reside in phagosomes that can alter this normal trafficking behaviour. For example, *Salmonell*a-containing phagosomes (SPs) possessed significant amounts of rab 5 and N-ethylmaleimide-sensitive factor and were very fusogenic with early endosomes (Mukherjee et al., 2000). Furthermore, SPs contained no mannose 6-phosphate receptors and only low amounts of cathepsin D (Garcia-del Portillo and Finlay, 1995). Similarly, *Mycobacterium tuberculosis* phagosomes (MPs) have properties of early endosomes in that they contain low amounts of LAMP-1 and CD63 and are not acidic (Sturgill-Koszycki et al., 1994). However, MPs are still fusion competent as demonstrated by their ability to acquire exogenously added markers such as transferrin (Clemens and Horwitz, 1996).

In contrast to the *Salmonella* or *Mycobacteria* phagosome, *A. fumigatus* phagosomes (APs) in J774 and A549 cells are not arrested and acquire the lysosomal membrane proteins CD63 and LAMP-1 as well as cathepsin D. Although staining of APs with the cathepsin D antibody was weak (Fig. 2A), both the co-localization and LysoTracker experiments were consistent with the idea that conidia were trafficking to late endosomes/lysosomes. Furthermore, since APs were acidic, CD63 and LAMP-1 positive, it was not unexpected that cathepsin D would also be present within the lumen.

A. fumigatus phagosomes (APs) fused quickly with late endosome markers after internalization by A549 and J774 cells. Within 30 minutes after internalization, APs in A549 cells contained only low levels (3% were positive) of transferrin receptor (CD71) at the early time points compared to high levels (51% and 38%) of CD63 and LAMP-1. This rapid movement into lysosomes resembles a lysosomal recruitment strategy that has been reported to occur in several other microorganisms. For example, *Salmonella enteritis* Serovar Typhimurium fuses with lysosomal membrane proteins 15 minutes after bacterial uptake by HeLa cells (Garcia-del Portillo and Finlay, 1995) while lysosomes migrate to the entry site of *Trypanosoma cruzi* into non-phagocytic cells and actively provide a source of membrane for the parasite phagosome (Andrews, 1995). *Candida albicans* also rapidly attracts late endosomes following phagocytosis by mouse macrophages (Káposzta et al., 1999).

The kinetics of transport through the endosomal network was more rapid in J774 cells than A549 cells. This discrepancy was most likely due to the fact that J774 cells are professional

phagocytes, whereas A549 cells are not. For example, Oh et al. (Oh et al., 1996) reported that in mouse bone marrow-derived macrophages, *Salmonella enteritis* Serovar Typhimurium fused with lysosomes within 20 minutes of phagocytosis (Oh et al., 1996). Moreover, Desjardins et al. (Desjardins et al., 1994) reported that latex beads merged with lysosomal compartments of J774 cells within 60-90 minutes following uptake. Similarly, movement of *A. fumigatus* conidia through the J774 endosomal work was rapid; 75% of APs were LAMP-1 positive in J774 cells after 120 min, whereas only 55% were LAMP-1 positive in A549 cells after 24 hours.

In addition to these differences in transport rates within the endosomal network, the viability of conidia within J774 and A549 cells was also markedly different. After 12 hours post-infection, there was a 10-fold difference in viability between the conidia in J774 and A549 cells; 99% of the conidia added to J774 cells had been washed off or killed, whereas significant amounts of viable conidia remained in the A549 cells after 24 hours (3% of the initial inoculum). The oxidative metabolism of alveolar macrophages during phagocytosis of *A. fumigatus* conidia has been well documented (Gil-Lamaignere et al., 2001; Nessa et al., 1997). In both cell lines, conidia resided in acidic organelles containing lysosomal proteins, therefore, the difference in killing rates between the two cell lines was most likely due to the respiratory burst present in J774 cells. The finding that the acidic conditions of the AP phagosome did not inhibit survival or germination of the conidia within A549 cells was not unexpected as most fungi (including aspergilli) can tolerate low pH (≥4) (Carlile and Watkinson, 1994).

The composition of the AP phagosome is similar to the intracellular compartments of other pathogens. The *C. albicans* phagosome within murine macrophages is a LAMP-enriched late endosomal vacuole (Káposzta et al., 1999). Similarly, during the growth phase of *Legionella pneumophila* in murine macrophages (more than 8 hours post-infection), *Legionella* reside in LAMP-1-cathepsin/positive phagosomes with an average pH of 5.6 (Sturgill-Koszycki and Swanson, 2000). Inhibition of phagosome acidification decreased the intracellular growth of the bacteria within the cell (Sturgill-Koszycki and Swanson, 2000).

Others have seen that Amphotericin B, which is structurally related to nystatin, increased the killing *A. fumigatus* conidia and *C. albicans* yeast cells by macrophages (Jahn et al., 1998; Martin et al., 1994). Jahn et al. (Jahn et al., 1998) reported a killing rate of only 10-15% after a 12 hour incubation of conidia with monocyte-derived macrophages. Preincubation of the cells for 16 hours with 1 μg/ml Amphotericin B increased the kill rate to 57%. Martin et al. (Martin et al., 1994) observed a synergistic effect of Amphotericin B with monocyte killing of *C. albicans*. However, this effect was not due to amphotericin B-dependent monocyte activation as the respiratory burst and expression of human leukocyte antigen-DR was unaltered (Martin et al., 1994). In contrast, we determined that 99% of the added conidia were washed off or killed by 12 hours. Our values were reported as percent survival relative to the initial inoculum added. Previous experiments in our lab have demonstrated that 65-80% of the bound conidia are killed by J774 cells in 6 hours (Wasylnka and Moore, 2002). These values are more in line with those reported by Schnaffer et al. (Schnaffer et al., 1983); they found that up to 50% of the conidia internalized by alveolar macrophages were killed after 12 hours. The differences between our kill rates and those reported by Schaffner et al. (Schaffner et al., 1983) might be dependent on the anatomical source of the macrophage. Schaffner et al. found that alveolar macrophages effectively killed 90% of the ingested conidia by 30 hours, however 85% of conidia internalized by peritoneal macrophages transformed into mycelia by 24 hours. Alternatively, the higher kill rates we observed with J774 cells may have been due to accumulation of nystatin within the cells. However, though the structure is similar to Amphotericin B, nystatin has different properties in vitro. Nystatin is more selective than Amphotericin B in binding to ergosterol over cholesterol (Walker-Caprioglio et al., 1989) and a critical nystatin concentration must be reached before any lipid membrane permeability is observed (Bolard et al., 1991). Furthermore, if some leakage of nystatin did occur during the nystatin-kill step, then it was likely permeating both A549 and J774 cell lines. Therefore, the percent survival of conidia in A549 cells estimated from our experiments may actually underestimate the survival in the cells in vivo.

Germination within phagolysosomes has also been observed during phagocytosis of *C. albicans* by murine macrophages (Káposzta et al., 1999). In addition, germ tube formation resulted in the escape of the fungus from the macrophage, and the frequency of germination was significantly reduced by neutralization of lysosomal pH (Káposzta et al., 1999). We observed similar results during internalization of *A. fumigatus* conidia by A549 cells. By 6 hours post-infection, we observed that some intracellular conidia had germinated, and after 24 hours, more than 30% had formed germ tubes. Immunofluorescence labelling using an extracellular antibody against fungal cell walls showed that some of the germlings had penetrated the plasma membrane from within. By 36 hours, extensive hyphal formation suggested that these germ tubes had elongated. Interestingly, the data suggested that the hyphal penetration was not accompanied by the concomitant release of large amounts of cytosolic enzymes such as LDH. Therefore, we hypothesize that the germlings emerge from the A549 plasma membrane with minimal disruption to the host cell and that hyphal extension continues once the germling has access to extracellular nutrients. It is possible that the hyphae are released from the cell membrane at a later time point.

In summary, our data suggests that *A. fumigatus* conidia fuse with lysosomes once internalized by A549 and J774 cells; however, their fate within these cells differs markedly. Conidia are rapidly destroyed by murine macrophages but a significant percentage of internalized conidia persist and germinate in A549 epithelial cells. Future experiments will investigate the mechanism of hyphal escape from A549 cells.

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