

***Histoplasma capsulatum* Modulates the Acidification of Phagolysosomes**

By Linda Groppe Eissenberg,* William E. Goldman,*
and Paul H. Schlesinger†

From the Departments of *Molecular Microbiology, and †Cell Biology and Physiology,
Washington University School of Medicine, St. Louis, Missouri 63110

Summary

The phagolysosome is perhaps the most effective antimicrobial site within macrophages due both to its acidity and to its variety of hydrolytic enzymes. Few species of pathogens survive and multiply in these vesicles. However, one strategy for microbial survival would be to induce a higher pH within these organelles, thus interfering with the activity of many lysosomal enzymes. Altering the intravesicular milieu might also profoundly influence antigen processing, antimicrobial drug delivery, and drug activity. Here we report the first example of an organism proliferating within phagolysosomes that maintain a relatively neutral pH for a sustained period of time. We inoculated P388D1 macrophages with fluorescein isothiocyanate (FITC)-labeled *Histoplasma capsulatum* or zymosan. Using the ratio of fluorescence excitations at 495 and 450 nm, we determined that vesicles containing either virulent or avirulent FITC-labeled *H. capsulatum* yeasts had a pH one to two units higher than vesicles containing either zymosan or methanol-killed *H. capsulatum*. The difference in pH remained stable for at least 5.5 h postinoculation. Longer-term studies using cells preincubated with acridine orange indicated that phagolysosomes containing live *Histoplasma* continued to maintain a relatively neutral pH for at least 30 h. Many agents raise the pH of multiple vesicles within the same cell. In contrast, *H. capsulatum* affects only the phagolysosome in which it is located; during coinoculation of cells with unlabeled *Histoplasma* and labeled zymosan, organelles containing zymosan still acidified normally. Similarly, unlabeled zymosan had no influence on the elevated pH of vesicles housing labeled *Histoplasma*. Thus, zymosan and *Histoplasma* were segregated into separate phagolysosomes that responded independently to their phagocytized contents. This localized effect might reflect an intrinsic difference between phagosomes housing the two particle types, active buffering by the microbe, or altered ion transport across the phagolysosomal membrane such that acidification is inhibited.

Within professional phagocytes the most hostile environment for ingested microorganisms is the phagolysosome. This organelle is highly acidic and contains a variety of hydrolytic enzymes, many of which are active only at low pH. The acidity within this compartment is believed to be necessary for normal intracellular trafficking, which could affect the delivery of antimicrobial drugs to the vesicle, and for proper antigen processing. Vesicle acidity may also affect the activity of therapeutic drugs. Thus, a microorganism that is able to influence pH in the phagolysosome might have a marked advantage for survival.

Histoplasma capsulatum is one of the few intracellular pathogens known to survive and multiply within phagolysosomes of infected macrophages (1–6). The results presented here suggest that vesicles containing *H. capsulatum* fail to acidify normally. While *Salmonella typhimurium* transiently impedes phagolysosome acidification (7), *Histoplasma* is the only organism of which we are aware that sustains a pH near neutrality in this compartment.

The altered pH within organelles containing *Histoplasma* could either be localized to its hosting vesicle or extend to all vesicles within the cell. Our studies demonstrate that only the pH of the phagolysosome containing *H. capsulatum* is affected. This finding provides the first demonstration of a novel mechanism by which a microbe might circumvent acidification of phagolysosomes to promote intracellular survival.

Materials and Methods

Yeast Strains. *H. capsulatum* strain G186A was obtained from the American Type Culture Collection (Rockville, MD). A previously described avirulent variant, G186AS (8), has a smooth colony morphology on HMM agarose plates (9) and grows as dispersed yeasts in HMM broth culture. The parental strain, which tends to clump in broth culture and has a rough colony morphology, is designated G186AR.

Fluorescent Labeling of Particles. To reduce the size of yeast clumps to one to five organisms, we dispersed *H. capsulatum* G186AR, G186AS, or zymosan (Sigma Chemical Co., St. Louis, MO) as pre-

viously described (10). FITC isomer I (Molecular Probes, Inc., Eugene, OR) was initially dissolved in DMSO (Hybri-Max; Sigma Chemical Co.) at 100 mg/ml using brief sonication. This stock was subsequently diluted 1:100 in HMM broth for labeling particles. Zymosan or log-phase yeasts were resuspended in this solution at 2×10^7 particles/ml and shaken in the dark for 30 min at 37°C in a 95% air, 5% CO₂ atmosphere. After pelleting the particles at 800 g and washing them once with broth, they were reincubated overnight in the dark at 10^7 particles/ml broth.

After this overnight incubation, we treated some yeasts with methanol for 5 min and washed them twice in HMM-M complete (see below). We washed all labeled yeasts and zymosan once more and removed clumps before using the particles as inocula. HMM-M was prepared as in an earlier report (11), but with 10 mM Hepes substituted for sodium bicarbonate. Since pH was found to be critical, HMM-M complete was adjusted to pH 7.05 at 37°C or 4°C, as appropriate.

All labeled particles had a green rim when inspected by fluorescence microscopy. To test the viability of the labeled yeasts, we stained them with 0.5 µg/ml fluorescein diacetate and 12.5 µg/ml ethidium bromide for 15 min at 37°C (12). In this assay, live yeasts appear solid green and dead yeasts are red by fluorescence microscopy; FITC-labeled yeasts that had been killed by a 5-min methanol treatment were included to verify the color discrimination.

Measurement of pH within Phagolysosomes. P388D1.D2 is a randomly selected subclone of P388D1 cells (3) that we maintained in F-12 complete (11). These macrophage-like cells were seeded onto 1-cm² glass chips (1.2×10^5 /chip) for spectrofluorimetric measurements or onto 12-mm-diameter round glass coverslips (6.8×10^4 /coverslip) for microscopic evaluation. The following day one chip or five coverslips were placed in 35-mm wells and inoculated with 5.4×10^7 yeasts in HMM-M complete.

To achieve synchronous uptake, we precooled the monolayers and particle suspensions to 4°C and then coincubated the inocula with the macrophages for 100 min at 4°C. The supernatant was then immediately replaced with ice-cold HMM-M complete containing 50% FCS to prevent nonspecific binding of any free fluorescein to the macrophage. Our rigorous washing also included seven rinses in ice-cold HMM-M complete, followed by dipping individual chips or coverslips into ice-cold medium. Samples were then transferred to fresh HMM-M complete in 35-mm wells for transport to the spectrofluorimeter or examined via fluorescence microscopy.

In studies that did not require synchronous uptake, we inoculated cells at 37°C for 15 min and then rigorously washed the samples at 4°C as described above. Samples were examined in the spectrofluorimeter 20 min later. In some experiments zymosan and *H. capsulatum* yeasts were used to coinoculate cells. The macrophages were first inoculated with 1.4×10^7 yeasts at 37°C, washed as described above, and then reincubated at 37°C for 30 min before adding 1.4×10^7 zymosan particles at 37°C. After 15 min of uptake, the macrophages were again rigorously washed as just described and examined in a spectrofluorimeter 20 min later.

Monolayers on chips were transferred to HBSS (without phenol red) in an SPF-500 spectrofluorimeter. The ratio of fluorescence excitations at 495 and 450 nm was used to establish pH titration curves (13, 14) of labeled yeasts or zymosan in suspension. We also made separate titration curves for P388D1.D2 cells inoculated with FITC-labeled yeasts or zymosan. Cells with internalized particles were pretreated in 120 mM KCl containing 28 µM nigericin (14). This ionophore equilibrated the intravesicular and medium pH. Each lot of FITC required recalibration of fluorescence versus pH. Background from light scattered by cells was minimized by illuminating the samples at a 49° angle. By measuring the fluorescence of mono-

layers on a spectrofluorimeter, we acquired an average pH value derived from hundreds of phagolysosomes. Each experiment was performed in triplicate and repeated two to four times; representative experiments are shown.

Monolayers on coverslips were incubated at 37°C and examined periodically after inoculation. Both synchronous and asynchronous inoculation techniques resulted in infection of an estimated 95% or more of the macrophages with 5–20 yeasts/cell. In experiments in which yeasts and zymosan were added to the same cells, the presence of both particles within single cells was verified by simultaneously examining the coverslips with phase-contrast and fluorescence microscopy. Studies using acridine orange to monitor acidity were performed as previously described (3).

Results

Labeling of the Yeasts. Because excitation of FITC is sensitive to pH, this dye can be used to monitor the acidity of intracellular vesicles (13). We hoped to obtain maximal fluorescence of *H. capsulatum* yeasts while minimally affecting their biological properties. However, covalent linking of FITC to the surface of yeasts profoundly affected their multiplication without decreasing their viability, as measured by staining with fluorescein diacetate and ethidium bromide. Furthermore, when used immediately after labeling, G186AR yeasts no longer killed P388D1.D2 macrophages in our established assay (11). Mock treatment with DMSO, which was used to dissolve the FITC, did not adversely affect the health of the yeasts.

We found that an overnight incubation in broth at 37°C restored the proliferative capabilities of the FITC-labeled organisms: growth rates in broth became normal (doubling time, ~7 h) and colonies could again be formed on HMM-agarose plates. In addition, FITC-labeled G186AR yeasts given this overnight incubation regained their virulence for macrophages (11; data not shown).

Measurement of Intraphagolysosomal pH. To calibrate the intracellular response of FITC to changes in pH, we treated infected P388D1.D2 cells with nigericin-KCl. This treatment enabled us to equilibrate the pH in the phagolysosome with the pH in the medium. The response of FITC to pH differed noticeably depending on whether it was covalently bound to zymosan or to *H. capsulatum* yeasts (Fig. 1). These responses probably reflect the fact that the surfaces of these particles are innately different in their biochemical constituents. FITC responded slightly differently when attached to methanol-treated yeasts or when labeled particles were in suspension rather than inside macrophages (not shown). Consequently, we used curves specific for each sample type to determine pH values in our experiments.

After a 100-min attachment period at 4°C, infected monolayers were placed in a spectrofluorimeter cuvette containing medium prewarmed to 37°C. The observed pH for FITC-zymosan rapidly dropped over 10–15 min from just under 7.0 to 5.5 (Fig. 2) or lower in some experiments. In contrast, vesicles containing either virulent or avirulent *H. capsulatum* yeasts were at pH 6.6. In other experiments the pH of vesicles containing zymosan sometimes fell as low as 4.7, while the pH of those containing live *Histoplasma* ranged from 6.2 to

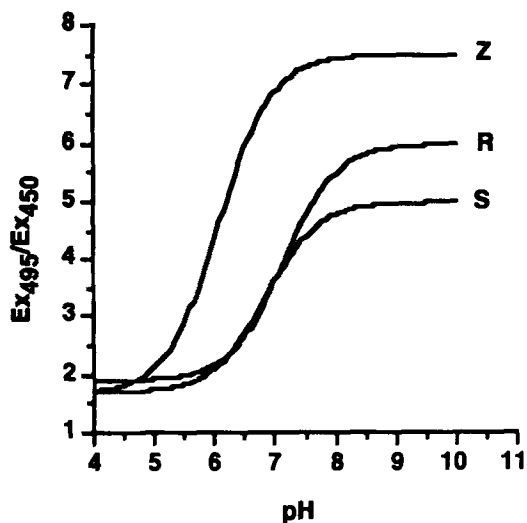


Figure 1. The relationship between pH and fluorescence intensity when FITC is attached to zymosan (Z), *H. capsulatum* strain G186AR (R), or variant G186AS (S). After ingesting individual particles, P388D1.D2 macrophages were treated with nigericin-KCl so that the pH of the phagolysosomes would be the same as that of the extracellular medium. Subsequently, the ratio of fluorescence emissions upon excitation at 495 and 450 nm was monitored as the pH of the samples was changed. Fresh lots of FITC necessitated minor recalibrations of these titration curves as did methanol treatment of yeasts.

7.0 and was never on the flat portion of the titration curve shown in Fig. 1. In general, *Histoplasma* were at a pH one to two units higher than zymosan within 15 min of engulfment. Since the pH scale is logarithmic, our data indicate that there is a substantial difference in the hydrogen ion con-

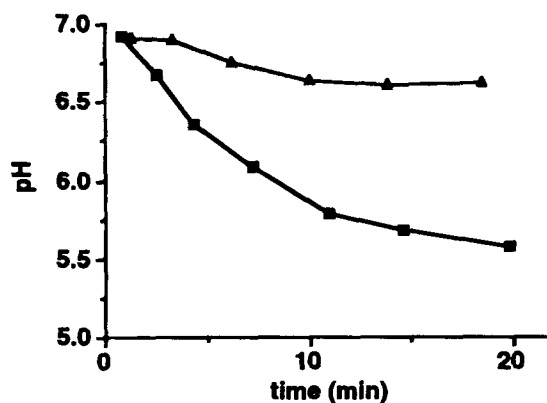


Figure 2. The effect of time on the pH of phagolysosomes containing zymosan (squares) or virulent *H. capsulatum* strain G186AR (triangles). Phagolysosomes containing avirulent variant G186AS behaved similarly to those containing G186AR in this and all experiments shown in subsequent figures. Monolayers were inoculated at 4°C, washed, and then warmed to 37°C in the spectrofluorimeter. The pH of phagolysosomes containing individual particle types was determined from the ratio of fluorescence at 495 and 450 nm and the curves shown in Fig. 1. Experiments presented in this and all subsequent figures were repeated two to four times, and representative data are presented.

centration in phagolysosomes containing the two types of particles.

Phase-contrast microscopy suggested that virtually all yeasts or zymosan particles were internalized. Accordingly, ammonium chloride at pH 7.0 (25 mM) increased the pH of all samples (Fig. 3 A) immediately upon addition, indicating that both zymosan and yeasts were within membrane-bound structures (for a review, see reference 15). The pH of organelles housing live *Histoplasma* was increased to a lesser extent by ammonia, consistent with the higher initial pH detected in these vesicles. As would be expected in healthy cells, washing away the ammonia restored the pH close to the original values. Extended incubations revealed that the pH of both particle types remained constant for at least 5.5 h (Fig. 3 B). Methanol-treated *H. capsulatum* yeasts were found in vesicles that behaved similarly to those containing zymosan; i.e., they acidified to pH 4.7 (Fig. 3 C).

Additional support for the idea that *H. capsulatum* resides in a phagolysosome of relatively neutral pH is indirect. In cells preincubated in 1 mg/ml acridine orange before infection (as in reference 3), *H. capsulatum* phagolysosomes are visible as faint green lines of fluorescence around the yeasts even as late as 30 h postinoculation. In contrast, bright green, orange, or red lines surround *Saccharomyces cerevisiae* throughout this time period (data not shown). Acridine orange is a weak base that becomes membrane impermeable when it is protonated, thus trapping it in acid environments. Brightness and color are related to the intravesicular concentration of this dye, which is a consequence of acidity (16). Our observations with *H. capsulatum* are consistent with long-term maintenance of a weakly acidic phagolysosome.

Local Inhibition of pH Drop. The more neutral pH observed in phagolysosomes housing *H. capsulatum* could be a result of a localized effect on this vesicle or the result of a global change occurring within the infected cell. To discriminate between these two possibilities, we sequentially inoculated P388D1.D2 cells with unlabeled *H. capsulatum* and FITC-zymosan. To enhance the probability that the two particle types would be located in separate phagolysosomes, macrophages were first incubated with *H. capsulatum*, washed, and then inoculated with labeled zymosan 30 min later. By combining phase-contrast and fluorescence microscopy, we estimated that at least 80% of the cells contained both particles (Fig. 4). The presence of unlabeled *H. capsulatum* had no effect on the pH of vesicles containing FITC-zymosan (Fig. 3 D). Under the reverse condition, in which labeled *H. capsulatum* yeasts were used to coinfect cells with unlabeled zymosan, we likewise saw no effect on the pH of phagolysosomes containing the yeasts (Fig. 3 D). Thus it appears that *H. capsulatum* influences only the pH of the phagolysosome in which it is located.

Discussion

Intracellular pathogens have evolved a variety of mechanisms to avoid such host cell defenses as phagosome acidification and exposure to lysosomal contents. Some organisms

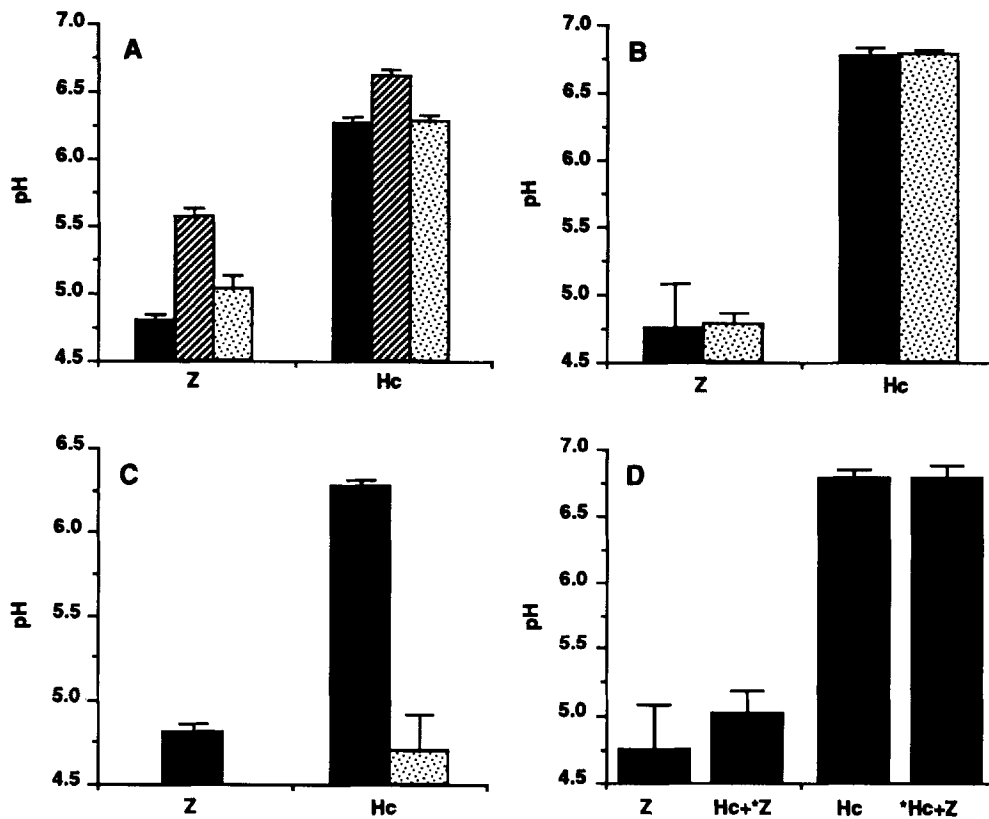


Figure 3. The effect of various conditions on the pH of phagolysosomes containing *H. capsulatum* or zymosan. (A) 15 min after inoculating monolayers with zymosan (Z) or *H. capsulatum* (Hc) at 37°C, extracellular particles were removed, and the pH of internalized particles was monitored 20 min later (filled bars). The cells were then transferred to 25 mM ammonium chloride, and the pH was immediately measured (hatched bars). The pH was measured again immediately after washing away the ammonia (stippled bars). (B) Cells were inoculated with zymosan or *H. capsulatum* at 37°C for 15 min, washed, and examined 20 min (filled bars) and 5.5 h (stippled bars) later in a spectrofluorimeter. (C) A comparison of pH in phagolysosomes containing zymosan, live *H. capsulatum* (filled bar), and methanol-treated yeasts (stippled bar). (D) Monolayers were inoculated with *Histoplasma*, washed, reincubated for 30 min, and then inoculated with zymosan. In coinoculated samples, the particle labeled with an asterisk was fluorescently labeled, while the other particle was not. The standard error determined from triplicate samples is indicated in all panels.

reside in vesicles that fail to acidify but reportedly do not fuse with lysosomes (*Legionella pneumophila*, 17; *Toxoplasma gondii*, 18; *Glugea hertwigi*, 19; *Nocardia asteroides*, 20; and *Mycobacterium tuberculosis* and *M. avium*, 21). *H. capsulatum* uses a strategy that is distinct from any that has previously been described: it exerts a prolonged influence on the pH of phagosomes that have fused with lysosomes.

H. capsulatum most likely resides in a late-stage phagolysosome, a compartment generally characterized by a low pH (i.e., ≤ 5.0). Secondary lysosomes pre-labeled with FITC-dextran (22, 23) begin to fuse with *H. capsulatum*-laden phagosomes as early as 15 min postinoculation, and by 3 h lysosomes have fused with 95% of the phagosomes (3). Whereas the pH of phagosomes containing other organisms may in part be influenced by an inability to fuse with acidic vesicles, the organelle containing *H. capsulatum* fails to acidify despite exhibiting fusion competence.

The intracellular location of *Salmonella typhimurium* is not so clearly defined (for a discussion, see reference 24), perhaps because phagosomes are dynamic organelles that fuse with multiple vesicles of the endocytic/lysosomal system (25). *S. typhimurium* may be an example of an organism that resides in vesicles of intermediate maturity. It was recently reported that this bacterium impedes the acidification of its hosting phagolysosome for at least 4–5 h (7). As the pH slowly drops, certain virulence-associated genes become expressed. This delay in acidification may be sufficient to induce the “acid

tolerance response” (26), which enables the bacterium to adapt to increasingly acidic conditions and eventually to survive at a pH as low as 3.0.

In contrast, there was no drop in pH between 20 min and 5.5 h postinoculation in phagolysosomes containing *H. capsulatum*. Furthermore, studies with acridine orange suggest that the pH remains relatively neutral even 30 h postinoculation. Thus, we believe this to be the first report of a microbe capable of inducing a sustained local pH change within late stage phagolysosomes. It is also the first example of a fungal pathogen that can influence the pH of any intracellular compartment.

It is unclear how microbes interfere with the normal acidification of their hosting vesicles. In measurements of lysosomal pH in uninfected cells, a protein extract from an *M. tuberculosis* culture filtrate reportedly induces a transient cyclical alkalinization of these organelles (27). The mechanism involved, as well as the significance of this observation, is unresolved. However, it has long been known (28) that this bacterium also releases large quantities of ammonia (20 mM; 29) into its culture medium. Because ammonia is often used in vitro to manipulate vesicle pH, some investigators have inferred that the pH of the *M. tuberculosis* phagosome must also be raised. Crowle et al. (21) presented indirect evidence supporting this inference; however, we know of no direct measurements of the pH of organelles containing this bacterium. Nonetheless, ammonia is membrane permeable and

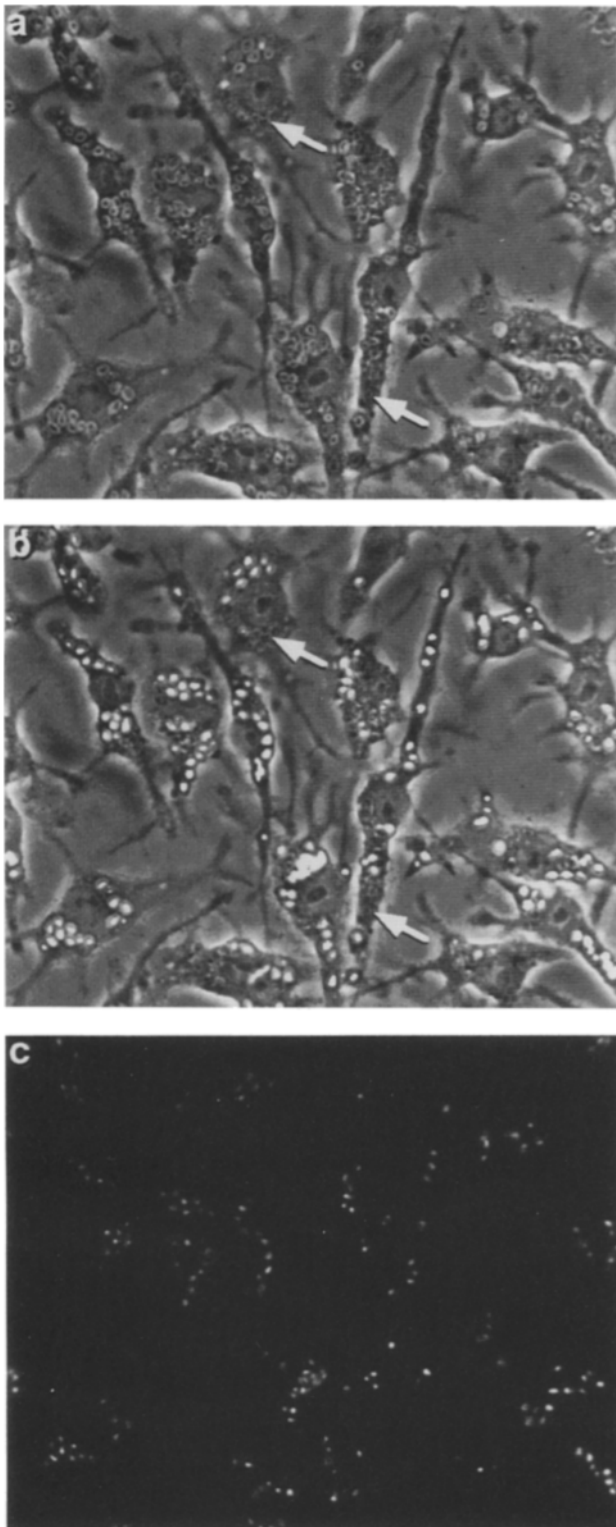


Figure 4. Monolayers preinfected with unlabeled *H. capsulatum* (arrows), were washed, reincubated for 30 min, and then inoculated with FITC-zymosan. To demonstrate that the two particles are found within the same cell, we combined the phase-contrast image (a) and the fluorescence image (c) into the computer-generated composite shown here (b). The *Histoplasma* yeasts are about two-thirds the size of zymosan and appear dark by phase-contrast microscopy.

should affect all the acidic vesicles within the cell. We found only insignificant amounts of ammonia (<2 mM) in *H. capsulatum* culture filtrates using an enzyme-based diagnostic kit (Sigma Chemical Co.) for detection (data not shown).

The anticipated global disturbance in pH by *M. tuberculosis* is in contrast to what we have seen with *H. capsulatum*. We sequentially inoculated cells with *H. capsulatum* and zymosan and found that there is independent control of pH in the two types of phagolysosomes when they exist within the same cell. This result indicates that *H. capsulatum* is not simply rendering its host cell nonfunctional and that the organism affects only the pH of its own phagolysosome. This result also implies that unlike the multiple fusion events seen with endosomes (for a review, see reference 30), phagosomes containing *Histoplasma* and those containing zymosan remain separate.

The localized effect on the pH of phagolysosomes hosting *H. capsulatum* could be mediated by any of several mechanisms. Since relatively little is known about the trafficking of material internalized by phagocytosis, it is possible that *Histoplasma* enters the cell via an as yet undescribed pathway. Thus the phagosome carrying zymosan into the cell may be intrinsically different from the one bearing *H. capsulatum*. Likewise, the H^+ -ATPase may be selectively excluded from the organelle membrane or the enzyme's activity may be neutralized by an increased permeability of the membrane to protons. Finally, the organism may release a factor that can either neutralize the acid in the lumen of its hosting vesicle or inhibit vacuolar ATPases. Bafilomycin is an example of a microbial product (released from *Streptomyces* species) that specifically inhibits vacuolar ATPases (31). However, its potency and membrane permeability should have a global effect on the pH of all vesicles in a cell rather than a local effect as seen with *H. capsulatum*.

The more neutral pH of organelles containing *H. capsulatum* may have multiple biological consequences. One of these resolves an apparent paradox: at pH 5.6, the organism is known to be sensitive to lysosomal components (32), yet the yeast thrives inside phagosomes that have fused with lysosomes. Presumably by limiting the acidification of these organelles, both the virulent and avirulent strains examined here may avoid many of the fungicidal activities within macrophages. The avirulent variant thus survives in macrophages (11) but apparently lacks some undefined additional characteristic(s) necessary for killing the phagocytes. A more thorough study of strains will reveal whether the ability to influence the pH of phagolysosomes has been evolutionarily preserved within this species.

Other consequences of a more neutral pH include effects on the transport of molecules between intracellular organelles (33). Consequently, a more neutral pH may restrict or enhance the availability of various molecules to the yeast. Altered transport of various antimicrobial drugs into the phagolysosome as well as alterations in their activity should therefore be considered during drug design. Finally, since a low pH is critical for proper processing and presentation of antigens

to T cells by macrophages (34), we anticipate that the immune response to this fungus may be aberrant. Thus, the discovery that *Histoplasma* modulates the pH of phagolysosomes

may yield clues to its intracellular survival. *Histoplasma* may also become a useful tool with which to gain insights into the biology of the cell and immune system.

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Address correspondence to Linda Eissenberg, Department of Molecular Microbiology, Box 8230, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110.

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