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Human Macrophages Do Not Require Phagosome Acidification to Mediate Fungistatic/Fungicidal Activity against *Histoplasma capsulatum*¹

Simon L. Newman,²* Lisa Gootee,*, Jeremy Hilty,† and Randall E. Morris†

*Histoplasma capsulatum* (Hc) is a facultative intracellular fungus that modulates the intraphagosomal environment to survive within macrophages (Mφ). In the present study, we sought to quantify the intraphagosomal pH under conditions in which Hc yeasts replicated or were killed. Human Mφ that had ingested both viable and heat-killed or fixed yeasts maintained an intraphagosomal pH of ~6.4–6.5 over a period of several hours. These results were obtained using a fluorescent ratio technique and by electron microscopy using the 3-(2,4-dinitroanilo)-3’-amino-N-methylpropylamine reagent. Mφ that had ingested *Saccharomyces cerevisiae*, a nonpathogenic yeast that is rapidly killed and degraded by Mφ, also maintained an intraphagosomal pH of ~6.5 over a period of several hours. Stimulation of human Mφ fungicidal activity by coculture with chloroquine or by adherence to type 1 collagen matrices was not reversed by bafilomycin, an inhibitor of the vacuolar ATPase. Human Mφ cultured in the presence of bafilomycin also completely degraded heat-killed Hc yeasts, whereas mouse peritoneal Mφ digestion of yeasts was completely reversed in the presence of bafilomycin. However, bafilomycin did not inhibit mouse Mφ fungistatic activity induced by IFN-γ. Thus, human Mφ do not require phagosomal acidification to kill and degrade Hc yeasts, whereas mouse Mφ do require acidification for fungicidal but not fungistatic activity. *The Journal of Immunology*, 2006, 176: 1806–1813.

*Histoplasma capsulatum* (Hc)³ is a dimorphic fungal pathogen that causes a broad spectrum of disease activity. In most cases, immunocompetent individuals develop a mild flulike illness, and only a small number of individuals actually exhibit acute clinical symptoms that require medical attention. However, if the inoculum is large enough, some individuals may develop a chronic cavitary pulmonary infection or a progressive disseminated infection that is life threatening (1). This latter disease progression is commonly observed in individuals whose immune system is compromised either by immunosuppressive agents or infection with HIV (2).

Infection with Hc occurs when conidia living in the soil are aerosolized and inhaled into the terminal bronchioles and alveoli of the lung. Inhaled conidia then convert into the yeast form that are aerosolized and inhaled into the terminal bronchioles and alveoli of the lung. Inhaled conidia then convert into the yeast form that is responsible for the pathogenesis of histoplasmosis, and the yeasts are phagocytized by the alveolar macrophages (Mφ) within which they multiply (1). The replicating yeasts destroy the alveolar Mφ, and subsequently they are ingested by other resident alveolar Mφ and by inflammatory phagocytes recruited into the lung. Replication of this cycle leads to lymphohematogenous spread of infection to lymph nodes and other organs rich in mononuclear phagocytes. The hallmark of host defense against Hc is the development of specific cell-mediated immunity that leads to activation of Mφ and resolution of the infection (3, 4).

The interaction of Hc with Mφ is the key event in the pathogenesis of histoplasmosis. Mφ first provide an environment for fungal replication and dissemination and then subsequently act as the final effector cells to remove the organism from the host. The strategies that Hc yeasts use to survive in Mφ are only partly known, but it is clear that, in both human and murine Mφ, the yeasts survive by controlling the intraphagosomal environment (5, 6). Upon ingestion, Hc yeasts activate the respiratory burst of human Mφ (7) but do not stimulate a respiratory burst when phagocytosed by resident (8, 9) or IFN-γ-activated (10) mouse peritoneal Mφ (PM). Furthermore, phagocytosis of Hc yeasts by the mouse P388D1 Mφ cell line (11) and resident PM (12) leads to normal phagolysosomal (PL) fusion. In contrast, there is minimal PL fusion after ingestion of Hc yeasts by human Mφ (13, 14). As Hc yeasts multiply readily within all of these Mφ populations (15–17), the yeasts apparently are able to evade the normally destructive effects of Mφ lysosomal hydrolases.

The mechanism by which this is accomplished in mouse Mφ is that Hc yeasts maintain an intraphagosomal pH of ~6.5 (18). In the RAW 264.7 mouse Mφ cell line, normal acidification of the phagosome is prevented by inhibition of the insertion of the Mφ vacuolar ATPase (V-ATPase; proton pump) into the phagosomal membrane (18). These data suggest that the yeasts themselves must acidify the phagosome to pH 6.5. This strategy presumably allows the yeasts to avoid killing by lysosomal acid hydrolases and still acquire iron from transferrin that would be half-saturated at pH 6.5 (19), leaving some free iron available for yeast survival and growth.

In the present study, we sought to determine the intraphagosomal pH in human Mφ under conditions in which Hc yeasts multiply or are killed. To our surprise, we found that, under both

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3 Abbreviations used in this paper: Hc, *Histoplasma capsulatum*; Mφ, macrophage; PM, peritoneal Mφ; PL, phagolysosomal; V-ATPase, vacuolar ATPase; DAMP, 3-(2,4-dinitroanilo)-3’-amino-N-methylpropylamine; HK, heat-killed; Sc, *Saccharomyces cerevisiae*.
conditions, the intraphagosomal pH remains relatively neutral at pH ~6.4–6.5. Thus, human Mϕ lysosomal hydrolases do not require an acid pH to kill and degrade Hc yeasts.

Materials and Methods

Reagents

Bafilomycin was a generous gift from K. Altendorf (Universitat Osnabruck, Osnabruck, Germany). Type I collagen from rat tails (Sigma-Aldrich) was dissolved in 0.1% acetic acid at 1 mg/ml and dialyzed overnight at 4°C in distilled water. Collagen (50 μl) was dispensed into the wells of a 96-well tissue culture plate and exposed to ammonia fumes for 3 min at 25°C. The gels were washed four times with RPMI 1640 before adherence of Mϕ. 3-(2,4-Dinitroanilino)-3’-amino-N-methylpropyamine (DAMP) was obtained from Oxford Biomedical Research. Zymosan (Sigma-Aldrich) was prepared as described previously (20).

Yeasts

Hc strain G217B was maintained as described previously (21). Yeasts were grown in histoplasma Mϕ medium (22) at 37°C with orbital shaking at 150 rpm. For the digestion assay, log-phase yeasts were heat-killed (HK) at 65°C for 1 h and stored at 4°C in PBS containing 0.05% sodium azide (21). HK yeasts were labeled with FITC (0.1 mg/ml in 0.5 M carbonate-bicarbonate buffer; pH 9.0), and washed, and resuspended in HBSS containing 20 mM HEPES and 0.25% BSA (HBBSA) as described previously (21). For studies with viable yeasts, 48-h, log-phase yeasts were harvested by centrifugation, washed three times in HBSSA, and resuspended to 50 ml in HBSSA. Large aggregates were removed by centrifugation at 200 x g for 7 min at 4°C. The top 10 ml was removed, and the single-cell suspension obtained was standardized to the appropriate concentration in RPMI 1640 medium containing 5% FCS and 10 g/ml gentamicin. Saccharomyces cerevisiae (Sc) was inoculated into 50 ml of yeast extract-peptone-dextrose broth and cultured for 24 h at 37°C with orbital shaking at 150 rpm. The yeasts were harvested, washed, and standardized following the same procedure as for Hc yeasts.

Preparation of human Mϕ

Human monocytes were purified from buffy coats obtained from the Hoxworth Blood Center (Cincinnati, OH). After partial purification via Ficol-Hypaque centrifugation, monocytes were separated from lymphocytes by positive selection with anti-CD14 and EasySep magnetic nanoparticles (StemCell Technologies) according to the manufacturer’s instructions. Mϕ were obtained by culture of monocytes at 1 x 10^6/ml in Teflon beakers in RPMI 1640 medium containing 15% human serum, 10 μg/ml gentamicin (Sigma-Aldrich) and 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) for 5–7 days (21). The acquisition and use of human blood cells for these studies has been approved by the Internal Review Board of the University of Cincinnati College of Medicine.

Preparation of mouse PM

Mice were sacrificed by CO2 asphyxiation and 10 ml of HBSSA injected into the peritoneal cavity. After abdominal massage, PM were harvested by withdrawal of the medium. PM were washed twice and standardized to 1 x 10^6/ml in RPMI 1640 medium containing 10% FCS and 10 μg/ml gentamicin. Depending on the experiment, either 1 ml of PM was seeded into the wells of a 24-well tissue culture plate containing a 12-mm-diameter round coverglass, or 0.1 ml of PM was seeded into the wells of a 96-well tissue culture plate. PM were cultured for 24 h at 37°C in 5% CO2/95% air before assay. In some experiments, the PM were cultured for 24 h with 5 ng/ml murine IFN-γ. The acquisition and use of mice in these studies has been approved by the Institutional Animal Care and Use Committee of the University of Cincinnati College of Medicine.

Quantitation of intraphagosomal pH by fluorescence ratio

Viable, HK, and paraformaldehyde (1% in PBS)-fixed yeasts (10^6/ml) were labeled by incubation at 37°C for 1 h in 0.1 mg/ml FTTC in 0.5 M carbonate-bicarbonate buffer (pH 9.0). Zymosan (10^6/ml) was labeled by incubation at 25°C for 30 min in 0.1 mg/ml FTTC. All particles were washed twice in HBSSA and then resuspended to 10^6/ml in HBSSA. Mϕ were adhered to 10 x 23-mm glass coverslips, and then incubated with 10^6 FTTC-labeled yeasts or zymosan particles at 37°C for 1 h. After removal of nonadherent yeasts or zymosan particles were plated on a cuvette containing 0.1 M phosphate buffer (pH 7.0), and the cuvette was placed into a thermostatted SPEX spectrophotofluorometer (Jobin Yvon Horiba) set at 37°C. Intracellular pH then was calculated by linear regression analysis from the fluorescence intensity ratio at Ex/Em = 495/450 (23) by comparison to a standard curve. Standard curves were generated with yeasts or zymosan alone, and with yeast and zymosan-infected Mϕ. In the former, yeasts or zymosan were suspended at 10^6/ml in 0.1 M phosphate buffer ranging in pH from 4 to 7.5 in half pH increments. In the latter, adherent Mϕ were infected with yeasts or zymosan as described above. After washing, the monolayers were inserted into the cuvette, and buffer of varying pH was added. The monolayers then were permeabilized by adding 0.1 ml of 3 mM monensin, and the fluorescence intensity ratio at Ex/Em=495/450 was determined.

Quantitation of intraphagosomal pH by immunoelectron microscopy

As an alternative method, we quantified Mϕ intraphagosomal pH using DCF, a weak base that accumulates exclusively in the acidic compartments of cells (24, 25). Mϕ adhered in six-well tissue culture plates were incubated with 30 μM DCF at 37°C for 30 min. The monolayers were washed with HBSS and then incubated for 1 h with 5 x 10^6 viable or HK Hc or Sc yeasts to allow for phagocytosis. After removal of unbound yeasts by washing, one set of monolayers was fixed in 4% paraformaldehyde/0.5% glutaraldehyde. The remaining monolayers were incubated in RPMI 1640 medium for an additional 2, 4, 8, and 24 h. At each time point, the monolayers were washed and then fixed in 4% paraformaldehyde/0.5% glutaraldehyde. The monolayers then were washed with 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed in 2% osmium tetroxide. After embedding in Epon, ultrathin sections were cut and put on a nickel grid. The sections then were incubated with a rabbit anti-DNP Ab (Sigma-Aldrich), followed by a biotinylated goat anti-rabbit IgG. After further washing, the sections were developed with streptavidin colloidal gold (5 nm). Pictures were made at a total magnification of ×81,000, overlaid with a grid containing 12.5-mm squares, and the number of the gold particles per square micrometer in yeast-containing phagosomes and the cell nucleus (as the neutral control) was quantified. The pH of yeast-containing phagosomes then was calculated by using the formula: pH = 7.0 – log D1/D2, where D1 is the density of DCF-specific gold particles in yeast-containing phagosomes, and D2 is the density of gold particles in the nucleus (24).

Quantitation of PL fusion

Mϕ were adhered in 24-well tissue culture plates to 12-mm round glass coverslips. Monolayers then were incubated with 200 nM LysoTracker Red (Invitrogen Life Technologies) in RPMI 1640 medium containing 5% FCS for 2 h to label lysosomes. After two washes, the Mϕ were incubated with HK and viable Hc yeasts (2 x 10^6) for 1 h at 37°C. HK Hc was labeled with FTTC as described previously (26). After an additional two washes, the Mϕ were cultured in 200 nM LysoTracker for an additional 2 h. After washing, the monolayers were fixed in 3.75% paraformaldehyde for 20 min at 4°C. The Mϕ then were covered in Dubecco’s PBS containing 5% glucose. Coverslips were mounted cell-side down in 90% glycerol in PBS onto a microscope slides, and 100 yeasts-containing phagosomes were counted and scored for lysosomal fusion or no fusion. The data are expressed as the mean ± SEM of the percent PL fusion in three or more experiments performed in duplicate.

Quantitation of intracellular growth of Hc yeasts in Mϕ

Intracellular growth of Hc yeasts in human and mouse Mϕ was quantified by the incorporation of [3H]leucine as described previously (27). Human Mϕ cultured in Teflon beakers were harvested, washed, and adhered (1 x 10^6/ml in a 96-well plate for 1 h at 37°C in HBSSA containing 2% aprotonin (Sigma-Aldrich). After adherence, Mϕ were washed twice in RPMI and incubated for 24 h with 1 x 10^6 viable yeasts. Mouse PM cultured for 24 h in 96-well plates were infected with Hc yeasts in an identical manner. All plates then were centrifuged and supernatants were carefully aspirated through a 27-gauge needle. Fifty microtiter (1.0 μlci) of [3H]leucine (specific activity, 153 Ci/mmol; DuPont/New England Nuclear) in sterile water and 5 μl of a 10 μM yeast nitrogen broth (Difco Laboratories) were added to each well. After further incubation for 24 h at 37°C, 50 μl of 1-leucine (10 mg/ml) and 50 μl of sodium hyochlorite were added to each well. The contents of the wells were harvested onto glass fiber filters using an automated harvester (Molecular Devices). The filters were placed into scintillation vials, scintillation mixture was added, and the vials were counted in a Beckman LS 7000 liquid scintillation spectrometer (Beckman Coulter). The results are expressed as the mean ± SEM of the cpm incorporated by remaining viable Hc yeasts. Experiments were performed in triplicate, and three to five experiments were performed with cells from different donors.
well tissue culture plate were infected with 5 × 10⁶ FITC-labeled viable and HK Hc yeasts. Mϕ that had ingested FITC-labeled yeasts were placed in buffer of varying pH and permeabilized with monensin so that the pH of the phagosome was the same as the medium. The ratio of fluorescence emissions after excitation at 495 and 450 nm then was determined. The fluorescence intensity ratio of yeasts alone also was determined for comparison. The data are the mean ± SEM of three to four individual experiments.

Yeast digestion assay

Human or mouse Mϕ adherent to 12-mm round glass coverslips in a 24-well tissue culture plate were infected with 5 × 10⁶ FITC-labeled HK yeasts for 1 h at 37°C. The monolayers were washed twice to remove noningested yeasts. One pair of coverslips then was fixed in 1% paraformaldehyde, and the remaining monolayers were cultured for 24 h at 37°C in the absence or presence of 25 nM bafilomycin. At the end of the incubation period, the Mϕ were washed and then fixed in 1% paraformaldehyde. Coverslips were mounted cell-side down onto microscope slides, and 100 Mϕ per coverslip were counted via phase and fluorescent microscopy. The data are presented as the association index that is defined as the total number of yeasts bound or ingested per 100 Mϕ. Although we did not specifically discriminate bound from ingested yeasts in these experiments, under the conditions of this assay, >90% of yeasts reside in Mϕ phagosomes after 1 h of phagocytosis, and after additional culture, all yeasts are internalized (21).

Statistics

Statistical analysis of the data was performed using SigmaStat (Jandel). Student’s t test or the Mann-Whitney rank sum test was used to analyze the data, and the results were considered significant at p < 0.05.

Results

Intraphagosomal pH of Hc-infected human Mϕ

To quantify intraphagosomal pH, we took advantage of the fact that excitation of FITC is sensitive to pH (23). Viable and HK Hc yeasts were labeled with FITC, and the relationship of fluorescence intensity to pH was used to generate standard curves (Fig. 1). Separate standard curves were generated with Mϕ infected with viable or HK yeasts. In addition, standard curves were generated with viable or HK yeasts alone. In Fig. 1, it is clear that the curves generated by Hc-infected Mϕ and Hc alone were almost identical, confirming that the Mϕ were effectively permeabilized with monensin. FITC labeling of live yeasts did not affect their viability as determined by comparison of their growth to unlabeled yeasts in tissue culture medium (data not shown). A similar standard curve was generated for experiments using paraformaldehyde-fixed Hc yeasts (data not shown).

Mϕ were infected with viable or HK Hc yeasts, and then pH was determined at various times postinfection. After 1 h of phagocytosis, the pH in Mϕ phagosomes containing viable Hc was 6.5 (Table I). These data confirm the original observation of Eissenberg et al. (6) with the P388D1 mouse Mϕ cell line, and our own studies with the RAW 264.7 mouse Mϕ cell line (18). Unexpectedly, we found that the pH in phagosomes containing HK Hc was 6.4, and the pH did not decrease over a 4-h period. Likewise, the pH in Mϕ phagosomes containing paraformaldehyde-fixed Hc yeasts was ≈6.5, and again there was no acidification over a 4-h period (Table I). These data contrast with previous studies with P388D1 Mϕ (6) and RAW Mϕ (18) in which the intraphagosomal pH with methanol-fixed yeasts or HK yeasts were reported to be 4.7 and 4.8, respectively.

Intraphagosomal pH of human Mϕ ingesting zymosan

We next sought to determine whether the lack of phagosome acidification was unique to Hc yeasts. Mϕ were allowed to ingest FITC-labeled zymosan particles for 1 h and were pH quantified immediately or after culture for a further 2–4 h. The pH standard curve for monocytes or Mϕ that had ingested FITC-labeled zymosan is shown in Fig. 2. The data in Table II show that Mϕ phagosomes containing zymosan slowly acidified over a 4-h period, dropping from an initial pH of 6.6 to 5.2. By comparison,

Table I. Intraphagosomal pH of human Mϕ containing Hc yeasts

<table>
<thead>
<tr>
<th>pH</th>
<th>Viable</th>
<th>HK</th>
<th>Paraformaldehyde fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>6.5 ± 0.2(3)b</td>
<td>6.4 ± 0.4(4)</td>
<td>6.7 ± 0.4(7)</td>
</tr>
<tr>
<td>2 h</td>
<td>6.8</td>
<td>6.5 ± 0.3(7)</td>
<td>7.1 ± 0.3(7)</td>
</tr>
<tr>
<td>3 h</td>
<td>6.9</td>
<td>7.1 ± 0.3(7)</td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>6.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Mϕ were infected with FITC-labeled Hc yeasts for 1 h, and then pH was quantified immediately or after an additional 2–4 h of culture, as described in Materials and Methods.

b The data are the mean ± SEM (number of experiments).
freshly isolated monocytes acidified phagosomes containing zymosan after 1 h. Thus, the lack of acidification of Mφ phagosomes containing HK or paraformaldehyde-fixed yeasts was not an artifact of the assay system.

**Intraphagosomal pH in human Mφ quantified by immunoelectron microscopy**

To confirm that human Mφ do not acidify phagosomes containing HK Hc yeasts, we quantified intraphagosomal pH by immunoelectron microscopy using the DAMP reagent (24, 25). Mφ were labeled with DAMP and then allowed to phagocytose viable or HK Hc yeasts for 1 h. Mφ monolayers were fixed and immediately processed for immunoelectron microscopy or were cultured for 2–24 h before fixation. Fig. 3 shows a yeast-containing phagocytic vacuole at 1 h postinfection. The gold particles indicate the localization of DAMP in the phagosome. Fig. 4A shows that from 2 to 8 h postingestion, Mφ phagosomes containing viable Hc yeasts maintained a pH of ~6.4. Likewise, Mφ phagosomes containing HK Hc yeasts maintained a similar pH up to 24 h postingestion. These data confirm the results obtained with the fluorescent ratio technique.

Human Mφ rapidly kill and degrade the nonpathogenic yeast Sc. Therefore, we sought to determine whether human Mφ phagosomes would acidify after ingestion of Sc. Fig. 4B shows that, 2 and 4 h after phagocytosis of viable or HK Sc, the pH in yeast-containing phagosomes again was ~6.4.

**Bafilomycin does not reverse the fungicidal activity of human Mφ**

Overall, these data suggested that human Mφ did not require phagosomal acidification to digest HK Hc yeasts or to kill nonpathogenic Sc yeasts. Previously, we identified two conditions under which human Mφ could be induced to kill viable Hc yeasts. In the first, Hc-infected Mφ were incubated in the presence of chloroquine (28). As a weak base, chloroquine raises intraphagosomal pH, thereby restricting the availability of iron from transferrin, which is pH dependent (29). Our original interpretation of the data was that after the yeasts became metabolically inactive, at some point thereafter, yeast-containing phagosomes were acidified, and the yeasts then were killed and degraded by lysosomal enzymes that presumably required an acid pH to be active. As the current data suggested otherwise, we tested this hypothesis by culturing Hc-infected Mφ in the absence and presence of chloroquine and bafilomycin A1, an inhibitor of the V-ATPase (30). As originally reported, culture of Hc-infected Mφ in the presence of chloroquine caused significant inhibition of the intracellular growth of Hc yeasts (Fig. 5A). The addition of bafilomycin to chloroquine-treated Mφ did not reverse inhibition of Hc replication, and bafilomycin alone had no effect on the replication of yeasts in control Mφ.

The second condition known to induce Mφ fungicidal activity against Hc is by adherence of the Mφ to type 1 collagen matrices (13). Under these conditions, Mφ override the yeasts’ ability to

### Table II. Intraphagosomal pH in human monocytes and Mφ containing zymosan

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocyte</td>
<td>5.6 ± 0.3 (5)</td>
<td>5.8 ± 0.1 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>6.6 ± 0.1 (3)</td>
<td>6.2 ± 0.2 (3)</td>
<td>5.6 ± 0.2 (3)</td>
<td>5.2 ± 0.4 (3)</td>
</tr>
</tbody>
</table>

* means monocytes or Mφ were allowed to phagocytose FITC-labeled zymosan particles for 1 h, and then pH was quantified immediately or after an additional 2–4 h of culture, as described in Materials and Methods.

* The data are the mean ± SEM (number of experiments).
inhibit PL fusion. Therefore, Mφ adhered to collagen gels were infected with viable or HK Hc yeasts (A) or viable or HK Sc for 2 h (B). Either immediately or after further culture for up to 24 h, Mφ monolayers were processed as described in Materials and Methods, and the pH was quantified by comparing the number of gold particles in yeast-containing phagosomes with the number of gold particles in the nucleus. The data represent the mean of 15–22 individual phagosomes as shown over each bar.

Bafilomycin inhibits the capacity of mouse PM, but not human Mφ, to digest HK Hc yeasts

Mφ easily degrade ingested HK Hc yeasts (5). Therefore, to confirm that phagosomal acidification was not required for this process, Mφ were allowed to phagocytose FITC-labeled HK Hc yeasts for 1 h. One set of monolayers then was fixed and served as a baseline for the total number of yeasts ingested. The remaining Mφ were cultured overnight in the absence and presence of bafilomycin. After 1 h of phagocytosis, Mφ had ingested an average of ~3.5 yeasts/Mφ (Fig. 6A). After culture for 24 h, virtually all of the yeasts had been digested, and bafilomycin did not inhibit this process. Fig. 6B shows the identical experiment performed with resident mouse PM. Like human Mφ, PM ingested an average of 3.5 yeasts/Mφ after 1 h of phagocytosis. PM also completely digested the internalized yeasts after further culture for 24 h. However, the addition of bafilomycin completely inhibited the capacity of mouse PM to digest HK Hc yeasts.

Bafilomycin does not inhibit the IFN-γ-induced fungistatic activity of mouse PM

IFN-γ activates mouse PM (31), but not human Mφ (16, 27), to inhibit the replication of Hc yeasts. The mechanism of this inhibition is through the production of NO (32) and the restriction of iron (33). Although Hc yeast replication is completely inhibited in IFN-γ-activated mouse PM, none of the yeasts is killed and degraded (31). This observation is paradoxical, considering that PL fusion occurs even in unactivated mouse PM (12). We have confirmed this previous study using LysoTracker Red to label the
lysosomes. After 1 h of phagocytosis, >90% of mouse PM phagosomes containing viable or HK yeasts demonstrated PL fusion (Fig. 7). In contrast, and as reported previously (13, 14), only 8% of human Mφ phagosomes containing viable Hc yeasts demonstrated PL fusion, whereas >95% of phagosomes containing HK yeasts showed PL fusion (Fig. 7).

Based on the above observations, we hypothesized that the lack of killing of Hc yeasts by IFN-γ-activated mouse PM might be caused by an absence of phagosome acidification. To determine whether this was a viable hypothesis, mouse PM were cultured for 24 h in the absence or presence of bafilomycin. As reported previously (31), PM preincubated with IFN-γ completely inhibited the replication of Hc yeasts (Fig. 8). The addition of bafilomycin did not abrogate the IFN-γ-induced inhibition, and the addition of bafilomycin to control PM was without effect (Fig. 8).

Discussion

The interaction between Hc yeasts and human and murine Mφ differs considerably (reviewed in Ref. 34). Phagocytosis of Hc yeasts by murine Mφ does not stimulate the production of toxic oxygen radicals (8–10), whereas uptake of yeasts by human Mφ does stimulate the respiratory burst (7). Regardless, Hc yeasts multiply readily in both human and murine Mφ. More provocatively, PL fusion occurs normally in some murine Mφ (11, 12), but not others (18), and PL fusion is inhibited in human Mφ (Refs. 13 and 14 and present paper). The reason(s) for these differences are unclear.

In the P388D1 mouse Mφ cell line (6) and in the RAW mouse Mφ cell line (18), phagosomes containing viable Hc yeasts maintain an intraphagosomal pH of ~6.5, whereas phagosomes containing HK or fixed yeasts are acidified. A pH of 6.5 presumably allows Hc yeasts to avoid killing by lysosomal acid hydrolases but still acquire free ferric from transferrin that would be half-saturated at that pH (19). Indeed, culture of Hc-infected human Mφ...
with the weak base chloroquine (to raise intraphagosomal pH) induces Mφ to kill and degrade the yeasts. The effect of chloroquine is reversed by iron nitroacetate, an iron compound that is soluble at neutral to alkaline pH (35), but not by holottransferrin, which releases iron only in an acidic environment. Furthermore, chloroquine given i.p. to Hc-infected C57BL/6 mice significantly reduces the growth of Hc in spleens and livers and even protects mice from a lethal inoculum of Hc (28). Thus, iron is of critical importance to the survival of Hc yeasts in vitro in human Mφ and in vivo in a murine model of histoplasmosis.

In the present study, we sought to determine the intraphagosomal pH in human Mφ infected with viable or HK Hc yeasts and then to determine the temporal acidification of yeast-containing phagosomes after treatment with chloroquine or upon adherence of Mφ to type 1 collagen gels, conditions that lead to the killing and digestion of intracellular Hc (13, 28). As was found for P388D1 and RAW Mφ (6, 18), the intraphagosomal pH in human Mφ that had phagocytosed viable Hc yeasts was ~6.5. However, to our surprise, we never observed the expected acidification in phagosomes containing HK or fixed Hc yeasts. Rather, we consistently observed an intraphagosomal pH of 6.5, the same as for viable yeasts. This finding was verified using two different techniques to quantify intraphagosomal pH. In addition, human Mφ failed to acidify phagosomes containing the nonpathogenic yeast Sc, which is easily killed and degraded by Mφ. These results were not due to a technical complication, because we observed phagosomal acidification in monocytes and Mφ that had ingested zymosan. Thus, it appears that the acidification of phagosomes in human Mφ depends either on the target particle or microorganism that is ingested or the specific receptor-initiated signaling pathway that is engaged by the target organism/particle. Additional experiments with other microorganisms and/or inert target particles will be required to sort out this question.

PL fusion with concomitant acidification of the phagosome is considered to be a critical requirement for effective Mφ antimicrobial activity regardless of whether the encounter is with an extracellular pathogen or an intracellular pathogen. Indeed, a major strategy for survival in Mφ by intracellular pathogens centers on their ability to inhibit PL fusion and/or phagosome acidification. Thus, Mycobacterium tuberculosis (36–40), Mycobacterium avium (41–43), Toxoplasma gondii (44, 45), and Hc (6, 13, 14) all inhibit both PL fusion and acidification. Interestingly, Coccidioides burnetti inhibits PL fusion but permits phagosome acidification, because it apparently prefers this low pH (46–49). Likewise, Listeria monocytogenes and Salmonella typhimurium inhibit PL fusion with concomitant acidification of the phagosome is presumed to be a critical requirement for effective Mφ antimicrobial activity. In fact, the acidification of the phagosome is required for a successful host response in both naive and Hc-immune animals (57–61). Furthermore, the secretion of IL-12 is required for the production of IFN-γ (58–61), which is probably required for activation of Mφ to destroy the yeasts. Although TNF-α production is required for a successful host response in both naive and Hc-immune animals (57–59, 61, 62), the mechanism of action of TNF-α is unknown. However, TNF-α does not appear to directly activate Mφ (55, 61). Based on the data in the present studies, we hypothesize that there is at least one as-yet-unidentified cytokine required for Mφ activation, and this cytokine induces the Mφ to acidify Hc yeast-containing phagosomes.

Disclosures
The authors have no financial conflict of interest.

References