Inhibition of Intracellular Growth of *Histoplasma capsulatum* Yeast Cells by Cytokine-Activated Human Monocytes and Macrophages

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Human monocytes/macrophages (Mφ) were infected with *Histoplasma capsulatum* yeast cells, and intracellular growth was quantified after 24 h of incubation in medium alone or in medium containing cytokines. Yeast cells multiplied within freshly isolated monocytes, cultured Mφ, and alveolar Mφ with intracellular generation times of 14.2 ± 1.4, 18.5 ± 2.1, and 19.9 ± 1.9 h (mean ± standard error of the mean), respectively. Monocytes and Mφ inhibited the intracellular growth of yeast cells in response to cytokine supernatant; maximum inhibition was obtained when cytokines were added to cell monolayers immediately after infection. Opsonization of yeast cells in normal serum or in *H. capsulatum*-immune serum did not affect the intracellular generation time of yeast cells in either control Mφ or cytokine-activated Mφ.

*Histoplasma capsulatum* infects the host via deposition of microconidia into the terminal bronchioles and alveoli of the lung. Inhaled microconidia convert into yeast cells and are phagocytized by alveolar macrophages (AM), within which they multiply (5). Presumably, the dividing yeast cells destroy the AM and then are ingested by other resident AM and by macrophages (Mφ) recruited to the loci of infection. Maturation of specific cell-mediated immunity activates Mφ to inhibit yeast cell proliferation; the disease process gradually resolves in most immunocompetent hosts (11, 21, 27).

In vitro studies of the interaction of *H. capsulatum* with Mφ have demonstrated that peritoneal Mφ (PM) from mice immune to *H. capsulatum*, but not from normal mice, restrict intracellular growth of yeast cells and that inhibition of growth is dependent on the presence of immune lymphocytes (13, 15, 16). Furthermore, lymphokines generated from immune splenic T cells stimulated with *H. capsulatum* antigens or recombinant murine gamma interferon (IFN-γ) activate resident mouse PM to inhibit intracellular growth of yeast cells (34, 35). Killing of yeast cells has not been observed.

We have shown that unopsonized *H. capsulatum* yeast cells and conidia bind to the surface of monocytes, cultured monocyte-derived Mφ, AM, and polymorphonuclear neutrophils (PMN) via the CD18 family of adhesion-promoting glycoproteins (1, 22, 30). Attachment of unopsonized yeast cells and conidia to cultured Mφ and AM is followed rapidly by ingestion (22). In contrast, monocytes and PMN ingest few unopsonized yeast cells (22, 30). Phagocytosis of unopsonized yeast cells by Mφ (1) and of opsonized yeast cells by PMN (30) is followed by activation of the respiratory burst and the production of toxic oxygen metabolites.

The current study was designed for the following reasons: (i) to quantify the intracellular growth of *H. capsulatum* yeast cells in human monocytes and Mφ, (ii) to determine whether human Mφ can be activated to inhibit the intracellular growth of these yeast cells and/or to kill them, and (iii) to determine whether opsonization of yeast cells affects their rate of growth within Mφ.

*H. capsulatum* (strain G217B) was grown in HMM media (33) at 37°C for 2 to 3 days and was harvested by centrifugation. Yeast cells were washed in Hanks balanced salt solution containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and 0.25% bovine serum albumin (HBSA) and resuspended to a volume of 30 ml in HBSA, and large aggregates were removed by centrifugation at 200 × g for 5 min. The single-cell suspension obtained was standardized to 1 × 10⁶ cells per ml in HBSA.

Human monocytes were separated from buffy coats by sequential centrifugation on Ficoll-Hypaque and Peroll gradients and were cultured in Teflon beakers at 1 × 10⁶ cells per ml in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) containing 15% human serum and 10 μg of gentamicin per ml (Sigma Chemical Co., St. Louis, Mo.) (22). After 4 to 7 days of culture, Mφ were washed and suspended to 2.5 × 10⁶/ml in HBSA containing 0.3 units of aprotinin per ml (22). When freshly isolated monocytes were studied, mononuclear cells were obtained by dextran sedimentation and Ficoll-Hypaque centrifugation (23). Mononuclear cells were suspended to 3 × 10⁶/ml to 4 × 10⁶/ml in HBSS containing 20 mM HEPES and 0.1% autologous plasma-serum.

Human AM were obtained by bronchoalveolar lavage from healthy volunteers, who had given informed consent to an approved protocol (7). The lavage fluid contained >90% AM on the basis of differential counts of Wright-Giemsa-stained cyt centrifuged preparations, and >95% of AM were viable on the basis of trypan blue dye exclusion. The AM were washed twice in RPMI 1640 containing 2% fetal bovine serum and resuspended to 2.5 × 10⁶/ml.

One-milliliter aliquots of monocytes, cultured Mφ, or AM were added to 12-mm-diameter glass coverslips in 24-well culture plates (Costar, Cambridge, Mass.) for 1 h at 37°C in 5% CO₂-95% air. The cells were washed, 1 × 10⁶ viable yeast cells were added, and the cells were incubated for 30 min at 37°C to allow phagocytosis to proceed. The cells were washed twice in HBSA and incubated for an additional 30 min to ensure that all yeast cells were internalized. Two of the cell monolayers were fixed in 2% glutaraldehyde-1% sucrose in 0.01 M phosphate buffer–0.15 M NaCl, pH 7.2.
(phosphate-buffered saline). These cells provided a baseline for the percentage of MΦ initially infected and for determining the average number of yeast cells per infected MΦ and were designated 1 h infection.

The remaining MΦ were incubated for 24 to 72 h at 37°C in M199 (Grand Island Biological Co.) containing 10% human serum and 10 μg of gentamicin per ml or in medium containing cytokine supernatant. After incubation, medium was removed, the cells were washed, and the monolayers were fixed in glutaraldehyde. Coverslips were mounted cell side down onto microscope slides, and the number of yeast cells per 100 infected MΦ was determined via phase-contrast microscopy at a magnification of ×1,000.

Cytokine supernatant was prepared from mononuclear cells suspended to 1 × 10⁶/ml in RPMI 1640 containing 10% fetal bovine serum and 10 μg of gentamicin per ml. The cells were stimulated with 3 to 5 μg of phytohemagglutinin per ml, and the supernatant was harvested after overnight incubation at 37°C in 5% CO₂-95% air.

**Intracellular growth of H. capsulatum yeast cells in monocytes and MΦ.** After 1 h of infection, 20 to 30% of the monocytes and MΦ were infected with yeast cells. Infected monocytes contained 2.2 ± 0.2 yeast cells per cell (mean ± standard error of the mean [SEM], n = 4), whereas infected MΦ cultured for 4 to 7 days contained 4.0 ± 0.4 yeast cells per cell (range, 3.3 to 5.2 yeast cells per MΦ). Infected AM contained 3.2 ± 0.3 (n = 5) yeast cells per cell.

During 24 h of incubation in medium, yeast cells grew in human monocytes with an average intracellular generation time (IGT) of 14.2 ± 1.4 h. In contrast, the average IGT in MΦ cultured for 4 to 7 days was 18.5 ± 2.1 h (range, 14.2 to 23.8 h). The IGT of yeast cells in infected human AM was 19.9 ± 1.9 h. Although yeast cells multiplied at a consistently lower rate within cultured MΦ than in freshly isolated monocytes, these differences were not statistically significant (P > 0.05, two-tailed Wilcoxon rank sum test [2]).

No yeast cells were observed outside of MΦ at any time during culture, and the IGT was consistently 16 to 19 h through 72 h of incubation. Lysis of MΦ was not observed until 96 h of incubation.

**Activation of MΦ by cytokines.** We next determined whether human MΦ could be activated by cytokines to restrict the intracellular growth of yeast cells or to kill them. MΦ were infected with yeast cells and then were incubated for 24 to 48 h at 37°C in medium alone or in medium containing various concentrations of a phytohemagglutinin-generated cytokine supernatant. Cytokine supernatant activated MΦ to inhibit the intracellular growth of yeast cells in a concentration-dependent fashion. Maximum inhibition was obtained with 40% supernatant. Control medium containing phytohemagglutinin did not activate MΦ to inhibit the intracellular growth of yeast cells. Therefore, 40% supernatant was used in subsequent experiments to quantify the ability of monocytes and cultured MΦ to be activated by cytokines.

Freshly isolated monocytes, or MΦ cultured for 4, 5, or 7 days, were infected with yeast cells and then were incubated for 24 to 48 h in medium alone or in medium containing 40% cytokine supernatant. The data in Fig. 1 show that monocytes and MΦ cultured for various periods responded equally well to cytokines, as evidenced by similar inhibition of the intracellular growth of yeast cells. Inhibition of intracellular growth was maintained through 48 h of culture and did not require additional cytokines (data not shown).

We next sought to determine whether preincubation of MΦ with cytokines would enhance the ability of MΦ to restrict the intracellular growth of yeast cells. MΦ cultured for 4 to 5 days were adhered to coverslips and incubated in medium or in 40% cytokine supernatant. After 24 h, MΦ were washed, infected with yeast cells, and then incubated for a further 24 h in medium alone or in medium containing 40% cytokine supernatant. Preincubation of MΦ with cytokines resulted in considerably less inhibition of intracellular yeast cell growth (Fig. 2) than when MΦ were infected with yeast cells and cytokines were added immediately thereafter (Fig. 1). In fact, preincubation of MΦ with cytokines did not decrease the intracellular generation time at all when medium alone was added to the cells postinfection (Fig. 2b).

When MΦ were preincubated in medium alone and then incubated with cytokines after infection with yeast cells, they inhibited the intracellular growth of yeast cells as well as MΦ that had been incubated with cytokines both before and after infection.

**Effect of opsonization of yeast cells on intracellular growth.** Phagocytosis of H. capsulatum yeast cells by MΦ proceeds rapidly in the absence of serum opsonins (22). However, opsonization could affect the intracellular fate of yeast cells by ligating additional receptors on the MΦ membrane (i.e., CR₁, FcR), thereby more efficiently activating MΦ fungicidal or fungistatic activity. We tested this hypothesis by opsonizing yeast cells in 10% pooled human serum (PHS),

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**FIG. 1.** Cytokines activate both monocytes and cultured MΦ to inhibit the intracellular growth of yeast cells. Freshly isolated monocytes and MΦ cultured for 4, 5, and 7 days were adhered to glass coverslips and infected with yeast cells. Cells were cultured for 24 h in medium alone or in medium containing 40% cytokine supernatant (CK), and the number of yeast cells per infected MΦ was determined via phase-contrast microscopy. Results are the means ± SEM of four experiments performed in duplicate.
10% heat-inactivated PHS (56°C, 30 min), and 10% heat-inactivated human immune serum and then quantified their intracellular growth in MΦ incubated in medium alone or in medium containing cytokines. PHS was prepared from eight individual donors and contained no complement-fixing antibody against H. capsulatum. The immune serum was from a histoplasmosis patient with a complement fixation titer of 1:128 (provided by L. J. Wheat, Indiana University, Indianapolis).

Opsonized and unopsonized yeast cells were added to MΦ monolayers to achieve an average of 2.3 to 2.4 yeast cells per infected MΦ. As shown in Table 1, opsonization of yeast cells with immune or nonimmune serum did not affect their rate of intracellular growth in control MΦ or in MΦ stimulated with cytokines.

These experiments demonstrate that H. capsulatum yeast cells multiply within human monocytes, cultured MΦ, and AM and that cytokines activate human monocytes/MΦ to inhibit the intracellular growth of yeast cells. The pattern of intracellular multiplication of yeast cells in human monocytes/MΦ and the ability of cytokines to activate mono-

![Graph](image)

**FIG. 2.** MΦ preincubated with cytokines (CK) for 24 h minimally restrict the intracellular growth of yeast cells. Cultured MΦ were adhered to glass coverslips and then incubated for 24 h in medium only (a) or in medium containing 40% cytokine supernatant (b). The monolayers were washed, infected with yeast cells, and then incubated for an additional 24 h in medium alone [(-) CK] or in medium containing 40% cytokine supernatant [(+) CK]. Results are the means ± SEM of four experiments performed in duplicate. The numbers adjacent to each line on the graphs are the IGTs of yeast cells.

...cytes/MΦ to inhibit the intracellular growth of yeast cells are different in several respects from those reported for most other intracellular pathogens.

First, H. capsulatum yeast cells grow better in freshly isolated monocytes than in cultured MΦ or in AM. In contrast, previous studies have found that viral (4), fungal (6, 28), bacterial (3, 12, 26), and protozoan (19, 20) intracellular pathogens multiply more rapidly in cultured MΦ than in monocytes. This loss in MΦ antimicrobial activity is temporally concomitant with the loss during culture of the enzyme myeloperoxidase (28) and the decline in the ability of cultured MΦ to be stimulated to release O2- (25, 28), H2O2 (19, 28), and -OH (28).

A second difference between the interaction of H. capsulatum yeast cells with human MΦ and that of other intracellular parasites is that optimal activation of MΦ requires that cytokines be added to the cultures immediately after infection of MΦ by yeast cells. These results suggest that cytokine(s) very rapidly activate MΦ to inhibit the intracellular growth of H. capsulatum yeast cells and that the effect is transient. In contrast, preincubation of cultured MΦ with cytokine-containing supernatants or with IFN-γ for 24 to 48 h prior to infection does not activate MΦ to inhibit the intracellular growth of Trypanosoma cruzi (24), Leishmania donovani (19), Toxoplasma gondii (20), and Chlamydia psittaci (26). Although studies with these organisms indicate that cytokine activation of MΦ microbicidal and microbicidal activity is concomitant with an increase in the production of respiratory burst products (19, 20), they also demonstrate clearly that cytokines activate MΦ oxygen-independent activity as well (19, 20, 26, 28).

A third difference between the interaction of H. capsulatum yeast cells with human MΦ and that of other intracellular parasites is that recombinant human IFN-γ does not activate cultured MΦ to inhibit the intracellular growth of yeast cells (9, 21a). Likewise, IFN-γ does not activate cultured MΦ to kill Cryptococcus neoformans (18). Thus, the specific cytokine(s) required to activate human MΦ antifungal activity may be different from those that activate antibacterial and antiprotozoan activity.

H. capsulatum yeast cells activate the respiratory burst of phagocytes upon phagocytosis (1, 29, 30) but are not killed (9, 29). Furthermore, the intracellular growth of H. capsulatum yeast cells proceeds at a similar rate in freshly isolated monocytes and in cultured MΦ that have no myeloperoxidase and a decreased ability to produce toxic oxygen metabolites. In addition, monocytes and cultured MΦ that have been stimulated by cytokines inhibit the intracellular growth of yeast cells equally well. Therefore, these data suggest that yeast cells are resistant to toxic oxygen metabolites and that inhibition of the intracellular growth of yeast cells...

### TABLE 1. Effect of opsonization of H. capsulatum yeast cells on growth within MΦ

<table>
<thead>
<tr>
<th>Condition*</th>
<th>No. of yeast cells/MΦ (mean ± SEM; n = 4)</th>
<th>IGT (h) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>Medium, 24 h</td>
</tr>
<tr>
<td>Unopsonized</td>
<td>2.3 ± 0.1</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>PHS</td>
<td>2.3 ± 0.0</td>
<td>6.7 ± 0.8</td>
</tr>
<tr>
<td>ΔPHS</td>
<td>2.4 ± 0.1</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>ΔHuImS</td>
<td>2.4 ± 0.1</td>
<td>6.0 ± 0.4</td>
</tr>
</tbody>
</table>

* Unopsonized, Unopsonized yeast cells; PHS, yeast cells opsonized in 10% pooled human serum; ΔPHS, yeast cells opsonized in 10% heat-inactivated pooled human serum; ΔHuImS, yeast cells opsonized in 10% heat-inactivated human immune serum.
cells by cytokine-activated monocytes/M¢ probably is mediated by a non-oxyn-dependent pathway.

Although opsonization of *H. capsulatum* yeast cells in serum is not required for phagocytosis by human M¢ (22), opsonization could influence the rate of intracellular growth of yeast cells. However, yeast cells opsonized in normal or immune serum multiplied within control and cytokine-activated M¢ at similar rates. Thus, opsonization of yeast cells does not enhance M¢ fungistic activity regardless of whether the M¢ are activated. Opsonization of *H. capsulatum* yeast cells in immune serum also does not alter their rate of intracellular growth in PM from mice or guinea pigs immunized by sublethal infection with viable yeast cells or immunized with killed yeast cells (14).

Studies of *H. capsulatum* reveal two interesting differences in the way that this yeast interacts with human M¢ and mouse PM. First, unopsonized yeast cells stimulate the respiratory burst in human M¢ (1) but not in mouse PM (8, 31, 32). Second, IFN-γ activates mouse PM to restrict the intracellular growth of yeast cells (35), but it does not activate human M¢ to do so (9, 21a). Interestingly, IFN-γ also activates mouse PM (10, 17), but not cultured human M¢ (18), to kill C. neoformans. Killing of *H. capsulatum* yeast cells has not been demonstrated in either human M¢ or mouse M¢.

Current efforts are directed towards identifying the cytokine(s) that activates human M¢ to inhibit the intracellular growth of *H. capsulatum* yeast cells and characterizing the mechanism(s) by which inhibition of growth is mediated.

We thank George S. Depeé for critical review of the manuscript. This work was supported by Public Health Service grants AI-17339, AI-23985, and AI-28392 from the National Institute of Allergy and Infectious Diseases.

**REFERENCES**


