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Phagocytosis of Histoplasma capsulatum yeasts and microconidia by human cultured macrophages and alveolar macrophages. Cellular cytoskeleton requirement for attachment and ingestion.

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Research Article

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Phagocytosis of *Histoplasma capsulatum* Yeasts and Microconidia by Human Cultured Macrophages and Alveolar Macrophages

Cellular Cytoskeleton Requirement for Attachment and Ingestion

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Abstract

Phagocytosis of *Histoplasma capsulatum* (Hc) yeasts and microconidia by human macrophages (Mφ) was quantified by a fluorescence quenching technique. Phagocytosis of unopsonized Hc yeasts by monocyte-derived Mφ and human alveolar Mφ (AM) was rapid. After 60 min, 79% of cultured Mφ and 59% of AM had ingested an average of 9.8 and 11 yeasts/Mφ, respectively. In contrast, only 26% of monocytes ingested 4.5 yeasts/cell after 60 min. Phagocytosis of unopsonized microconidia by cultured Mφ and by AM was equivalent.

Monoclonal antibodies specific for the α-chains and β-chain of the CD18 family of adhesion receptors inhibited the binding of Hc yeasts and microconidia to cultured Mφ and AM. Thus, the Mφ CD18 complex mediates recognition of both phases of this dimorphic fungus.

Disruption of actin microfilaments with cytochalasin D inhibited both attachment and ingestion of yeasts by Mφ. In contrast, nocodazole, which prevents polymerization of microtubules, did not inhibit binding or ingestion. Both drugs inhibited ingestion, but neither drug inhibited binding of C3b-and C3bi-coated sheep erythrocytes to complement receptors type one (CR₁) or type three (CR₃), respectively. Therefore, different signal transducing mechanisms for phagocytosis appear to be triggered by the binding of Hc yeasts to CD18, and by the binding of EC3bi to CD11b/CD18, respectively. (*J. Clin. Invest.* 1990. 85:223–230.) alveolar • CD18 • histoplasma capsulatum • macrophage • phagocytosis

Introduction

Histoplasma capsulatum (Hc)[1] is a dimorphic fungus that infects the host by deposition of microconidia and small mycelial fragments into the terminal bronchioles and alveoli of the

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1. *Abbreviations used in this paper:* AM, alveolar macrophage; ASI, association index, the total number of bound plus ingested organisms per 100 Mφ; BAL, bronchoalveolar lavage; CD, cytochalasin D; EC3b, EC3bi, sheep erythrocytes coated with C3b and iC3b; HBSA, HBSS containing 20 mM Hepes and 0.25% BSA; Hc, *Histoplasma capsulatum*; Mφ, macrophage; NOC, nocodazole; PBSA, PBS containing 0.05% azide; PI, phagocytic index, total number of organisms ingested per 100 Mφ; PM, peritoneal macrophage.

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lung. Inhaled microconidia subsequently convert into the yeast form that is responsible for the pathogenesis of histoplasmosis. Hc yeasts are phagocytized by alveolar Mφ (AM), within which they multiply (1–4). The dividing yeasts presumably destroy the AM, and subsequently are ingested by other resident AM and by Mφ recruited to the loci of infection. Repetition of this cycle results in spread of infection to hilar lymph nodes and transient dispersal of yeasts to other organs during the acute phase of primary histoplasmosis (1–2 wk). Thereafter, the maturation of specific cell-mediated immunity (CMI) against Hc activates Mφ to halt yeast proliferation with gradual resolution of the disease process in most immunocompetent hosts (5–7).

In vitro studies on the interaction of Hc with Mφ have focused on the intracellular fate of Hc yeasts in monolayers of peritoneal Mφ (PM) from normal and immune animals (8–12). These demonstrate that PM from Hc-immune mice, but not normal mice, restrict intracellular growth of yeasts, and inhibition of growth is dependent on the presence of immune lymphocytes (8–10). Furthermore, lymphokines generated from cultures of immune splenic T cells stimulated with Hc antigens or recombinant murine γ interferon (γIFN), activate resident mouse PM to inhibit intracellular growth of Hc yeasts (11, 12). Actual killing of yeasts never has been observed.

PM and AM from outbred Swiss albino mice have been reported to phagocytose Hc microconidia and small hyphal fragments at rates comparable to yeasts (13). Compared with normal Mφ, Mφ from Hc immunized mice were better able to restrict the growth of mycelial elements. However, only PM and AM from mice treated with BCG actually reduced the numbers of viable particles (13).

There have been no systematic investigations on the phagocytic and fungicidal capacity of human Mφ for Hc yeasts or conidia. Recently, we reported that unopsonized Hc yeasts attached to the surface of human cultured monocyte-derived Mφ via the CD11a/CD18 (LFA-1), CD11b/CD18 (CR₃), CD11c/CD18 (p150,95) family of adhesion promoting glycoproteins (14). Each receptor molecule of the CD18 family contains a unique α-chain subunit noncovalently linked to a common β-chain subunit (15). Experiments using α- and β-chain–specific MAbs to block the binding of Hc yeasts to Mφ demonstrated that the yeasts could bind independently to each of the three receptors.

The current study was designed to: (*a*) determine if Hc yeasts bind to the CD18 receptor family on human AM; (*b*) determine if Hc microconidia bind to CD18 receptors of Mφ; (*c*) quantify the rate and extent of phagocytosis of yeasts and microconidia by human monocytes and Mφ; (*d*) and determine the role of the cellular cytoskeleton in phagocytosis of Hc by human Mφ.

Methods

Reagents. Cytochalasin D (CD) and nocodazole (R17934) (NOC), a synthetic microtubule inhibitor (*methyl* [5-(2-thienylcarbonyl)-^{1}H-benzimidazole-2-yl] carbamate), were purchased from Sigma Chemical Co. (St. Louis, MO). CD and NOC were dissolved in DMSO at a concentration of 1 mg/ml and stored at $-20°C$. The hybridoma line of MAb MN-41 specific for the α-chain of CD11b/CD18 (CR_3) (16) was provided by Dr. Allison Eddy, University of Minnesota Medical School, Minneapolis, MN. IgG was prepared from ascitic fluid as described previously (17). MAbs OKM1 (18) and 904 (19), also specific for the α-chain of CD11b/CD18; TS-1/22 (20), specific for the α-chain of CD11a/CD18; 1B4 (18), specific for the β-chain of the CD18 glycoproteins; 3C10 (21), specific for a 55-kD protein unique to monocytes and macrophages; and 3G8 (22), specific for the low-affinity FcRIII of PMN were a gift of Dr. Samuel Wright, The Rockefeller University, New York. Purified LeuM5, specific for the α-chain of CD11c/CD18 (23) was a gift of Dr. Louis Lanier, Becton, Dickinson & Co. (Mountain View, CA).

Yeasts. The yeast phase of Hc strain G217B was maintained as described (24). Yeasts were grown in brain-heart infusion broth (Difco Laboratories Inc., Detroit, MI) at 37°C with orbital shaking at 200 rpm. After 3 d, they were harvested by centrifugation, washed three times in 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), and then heat-killed at 65°C for 1 h. After removal of large aggregates by centrifugation, the yeasts were washed twice with PBS, and resuspended to 30 ml. To make a single cell suspension, yeasts were sonicated six times for 15 s at 40 W on a sonic dismembrator (model 300; Fisher Scientific, Pittsburgh, PA). Yeasts were centrifuged once more, resuspended in PBS containing 0.05% azide (PBSA), counted in a hemacytometer, standardized to 2×10^8/ml, and were stored at 4°C. A fresh preparation of heat-killed yeasts was made every 6 wk.

Microconidia. Strain 501 of Hc that predominantly produces microconidia in the mycelial phase was provided by Dr. Dexter Howard, UCLA, Los Angeles, CA. Hc 501 was maintained by monthly passage on Smith's defined medium (25) at 30°C. Conidial suspensions were inoculated on to Smith's defined medium in Fernbach flasks and incubated for 4–5 wk at 30°C (25). The hyphal mat was wetted with sterile distilled water, and the surface growth dislodged with a magnetic stir bar. Harvested conidia were transferred to ground glass tissue grinders and triturated briefly to dislodge conidia from the hyphae. The resultant suspension was filtered twice through four to six layers of gauze. Conidia were washed once, resuspended in distilled water, and heat-killed at 60°C for 1 h. Sterility was confirmed by subculture. Heat-killed conidia were separated further from hyphal fragments by filtration through an 8-μm nuclepore filter (Nuclepore Corp., Pleasanton, CA), counted on a hemacytometer, and stored in PBSA. These suspensions contained $\sim$ 65% microconidia, 30% small mycelial fragments, and 5% macroconidia.

EC3. C3b- and C3bi-coated sheep erythrocytes (EC3b, EC3bi) were prepared as described previously using purified components of the alternative complement pathway (17).

Fluorescein labeling of Hc yeasts and microconidia. For use in phagocytosis assays, heat-killed Hc yeasts were suspended to 2×10^8/ml (microconidia, 8×10^6/ml) in 0.01 mg/ml FITC (Sigma Chemical Co.) in 0.5 M carbonate-bicarbonate buffer, pH 9.5. After incubation for 15 min at 25°C, the yeasts were washed twice in HBSS containing 20 mM Hepes and 0.25% bovine serum albumin (HBSA), and resuspended in HBSA to 2×10^7/ml (microconidia, 1×10^6/ml). For rosette assays, yeasts were suspended to 2×10^8/ml (microconidia, 8×10^6/ml) in 0.1 mg/ml FITC and incubated for 60 min at 37°C. After two washes in HBSA, yeasts were resuspended to 2×10^8/ml (microconidia, 8×10^6/ml) in HBSA.

Cultured human Mϕ. Human monocytes were purified from buffy coats via sequential centrifugation on Ficoll-Hypaque and Percoll gradients (26). The monocytes were cultured in suspension in teflon beakers at 1×10^6/ml in RPMI 1640 (Gibco Laboratories, Grand

Island, NY) containing 12.5% human serum, and 10 μg/ml of gentamicin (Sigma Chemical Co.) (27). After 5–7 d of culture, Mϕ were washed and suspended in HBSA containing 0.3 U/ml of aprotinin. Mϕ were suspended to 0.5–1.0 $\times$ 10^6/ml for rosette assays and to 2.5 $\times$ 10^5/ml for phagocytosis assays.

For adherent cultures, mononuclear cells were obtained by dextran sedimentation and Ficoll-Hypaque centrifugation (28). Mononuclear cells were suspended to 3–4 $\times$ 10^6/ml in HBSS containing 20 mM Hepes and 0.1% autologous plasma-serum, and 1-ml aliquots were added to each well of a 24-well tissue culture plate (Costar, Cambridge, MA) containing a 12-mm diam glass coverslip. Monocytes were adhered for 1 h at 37°C in 5% CO_2-95% air, washed twice, and used immediately (freshly isolated monocytes), or cultured in M199 (Gibco) containing 10% autologous plasma-serum and 10 μg/ml of gentamicin. Medium was replaced on day 3 or 4, and Mϕ were tested on day 7 (28).

Human alveolar Mϕ. Human AM were obtained from normal volunteers who had given informed consent to a protocol approved by the Institutional Review Board of the University of Cincinnati College of Medicine. Bronchoalveolar lavage (BAL) was performed with sequential instillation and immediate withdrawal of four, 60-ml boluses of sterile saline (29). The pooled BAL fluid contained > 90% AM by differential counts of Wright-Giemsa stained cytocentrifuge preparations. The AM were washed twice in RPMI 1640 containing 2% fetal bovine serum, counted in a hemacytometer, and resuspended to 2.5 $\times$ 10^5/ml for phagocytosis assays, and to 5 $\times$ 10^5/ml for rosette assays. The viability of AM was consistently > 95% as determined by trypan blue dye exclusion.

Rosette assays. Terasaki tissue culture plates (Miles Laboratories Inc., Naperville, IL) were coated with 1 mg/ml of HSA (Worthington Biochemical Corp., Freehold, NJ) or MAb (50 μg/ml) by incubating for 90 min at 25°C. The wells were washed, and 5 μl of cultured Mϕ or AM were allowed to adhere for 1 h at 37°C in 5% CO_2-95% air. The monolayers were washed and 5 μl of FITC-labeled yeasts or microconidia were added to each well. Alternatively, Mϕ were adhered to HSA-coated wells, MAb added for 30 min at 4°C, and then yeasts or microconidia added without washing. After incubating for 30 min at 37°C, unattached organisms were washed off, and the monolayers were fixed in 1% paraformaldehyde. Attachment of organisms was quantified by counting 200 cells via fluorescence microscopy on a Nikon diaphot inverted microscope. The data are reported as an attachment index, which is the total number of organisms bound per 100 Mϕ.

Phagocytosis assay. To differentiate between attachment and ingestion of Hc, we employed a modification of the fluorescence quenching technique described by Hed et al. (30). Freshly isolated monocytes, cultured Mϕ or AM were adhered to glass coverslips for 1 h at 37°C in 5% CO_2. The monolayers were washed twice with HBSA, and 1-ml aliquots of FITC-labeled yeasts (2×10^7/ml) or microconidia (1×10^6/ml) were added. After incubating at 37°C for varying periods of time, nonadherent organisms were removed from the monolayers by washing twice with HBSA. Trypan blue (0.3 ml of 1 mg/ml in PBS) was added to each well for 15 min at 25°C to quench the fluorescence of bound but uningested organisms. The monolayers then were washed twice with HBSA and fixed in 1% paraformaldehyde. Coverslips were mounted cell side down onto microscope slides in 90% glycerol in PBS, and sealed with clear nail polish. Phagocytosis was quantified via phase contrast and fluorescence microscopy at ×1,000 on a Zeiss microscope fitted with a IV FL Epi-fluorescence condenser. 200 Mϕ were counted per coverslip, and the number of yeasts or microconidia ingested, or bound but not ingested, were enumerated. Data are presented as percent ingesting (the percentage of Mϕ ingesting one or more organisms); phagocytic index (PI) (the total number of organisms ingested per 100 Mϕ); and the association index (ASI) (the total number of bound plus ingested organisms per 100 Mϕ).

To quantify binding and ingestion of EC3b(i), Mϕ adherent to glass coverslips were incubated with 2 $\times$ 10^7 EC3b(i) for 60 min at 37°C. To quantify attachment, nonadherent EC3b(i) were removed by washing the monolayers twice with HBSA. To quantify ingestion, bound but

uningested E were lysed with NH_4Cl. Mϕ then were fixed in 2% glutaraldehyde-1% sucrose in PBS, and coverslips were mounted onto microscope slides as described above. Attachment and ingestion was quantified via phase contrast microscopy at $\times 1,000$. 200 Mϕ were counted on each coverslip, and scored for percent rosettes, the percentage of Mϕ with five or more EC3b(i) attached to their surface; and the phagocytic index, the total number of E ingested per 100 Mϕ.

Statistics. Data were analyzed by the Wilcoxon rank sum test (31). Results were considered significant at P values of < 0.05.

Results

Binding of Hc yeasts to CD18 receptors on AM. Previous studies from this laboratory demonstrated that Hc yeasts bound to CD18 receptors on human cultured monocyte-derived Mϕ (14). Therefore, we sought to determine if Hc yeasts bound to this receptor complex on freshly adherent human AM. AM were adhered to surfaces precoated with HSA or MAbs specific for the α-chains or β-chain of the CD18 receptors, and then incubated with FITC-labeled yeasts for 30 min at 37°C. Table I shows that MAbs specific for the CD18 complex, but not MAbs to other Mϕ receptors inhibited the binding of Hc yeasts. MAbs specific for CD11a and CD11b inhibited binding by $\sim 35\%$, whereas a MAb specific for CD11c inhibited binding by 48%. As was found previously, MAb specific for the β-chain of CD18 was the most inhibitory.

Binding of Hc microconidia to CD18 receptors on cultured Mϕ and AM. To determine if Hc microconidia also are recognized by Mϕ CD18 receptors, cultured Mϕ or AM were adhered to HSA- or MAb-coated surfaces, and the attachment of FITC-labeled microconidia quantified. The mean±SEM of the attachment indices for cultured Mϕ and AM was 386 ± 42 ($n = 9$) and 356 ± 35 ($n = 6$), respectively. As was found for yeasts, MAbs specific for the CD18 complex reduced the binding of microconidia to cultured Mϕ and AM. Antibodies specific for other Mϕ membrane receptors failed to reduce binding of microconidia. The percent inhibition obtained with each of the MAbs was similar for both populations of Mϕ (Table II).

Phagocytosis of Hc yeasts by human monocytes, cultured Mϕ and AM. To quantify the rate and extent of phagocytosis of Hc yeasts by human monocyte/Mϕ, we established a fluo-

Table I. Inhibition of Hc Yeast Binding to AM by Monoclonal Antibodies

Antibody	Specificity	Percent inhibition*
Buffer	—	‡
TS-1/22	CD11a	38±6.0
MN-41	CD11b	36±3.9
OKM1	CD11b	35±2.2
904	CD11b	34±7.0
LeuM5	CD11c	48±3.5
1B4	CD18	58±3.7
3C10	Mϕ	3±2.8
3G8	FcR	2±5.0

AM were adhered for 60 min at 37°C to surfaces precoated with HSA (1 mg/ml) or MAbs (50 μg/ml), and then were incubated with FITC-labeled Hc yeasts for 30 min at 37°C.
* Mean±SEM ($n = 3–8$).
‡ The attachment index in the buffer control was $2,115\pm185$ (mean±SEM, $n = 8$).

Table II. Inhibition of Hc Microconidia Binding to Cultured Mϕ and to AM by Monoclonal Antibodies

Antibody	Percent inhibition	
	Cultured Mϕ	Alveolar Mϕ
Buffer	*	*
TS-1/22	43±4.9‡	43±3.2‡
MN-41	46±2.3	41±7.1
OKM1	44±4.8	52±6.1
904	34±1.0	27±8.8
LeuM5	46±1.0	40±11
1B4	54±3.2	56±1.8
3C10	3±8	8±9
3G8	1±7	6±5

Mϕ were adhered for 60 min at 37°C to surfaces precoated with HSA (1 mg/ml) or monoclonal antibodies (50 μg/ml), and then were incubated with FITC-labeled Hc microconidia for 30 min at 37°C.
* The mean±SEM of the attachment index in the buffer control was 386 ± 42 ($n = 9$) for cultured Mϕ, and 356 ± 35 ($n = 6$) for AM.
‡ Mean±SEM ($n = 3–9$).

rescence quenching assay that distinguished ingested vs. bound but uningested organisms. Using this procedure, internalized yeasts appeared bright green, while bound uningested organisms appeared either dark green or reddish brown in color. To verify the validity of the assay, yeasts were opsonized in 10% human serum and incubated with Mϕ monolayers that had been preincubated with 5 μg/ml of CD. CD disrupts actin microfilaments and, therefore, prevents ingestion of bound yeasts. Under these conditions, addition of trypan blue extinguished the fluorescence of all Mϕ-associated yeasts. Opsonized yeasts were used in this experiment since CD was found to inhibit the binding of unopsonized yeasts to Mϕ (see below).

Monocytes, Mϕ cultured for 5–7 d in teflon beakers, and AM were adhered to glass coverslips, and the respective cell preparations then incubated with FITC-labeled Hc yeasts for varying periods of time at 37°C. The data presented in Fig. 1 show the percent of phagocytes ingesting one or more yeasts (percent ingesting, *A*), and the total number of yeasts ingested per 100 Mϕ (phagocytic index, *B*). Phagocytosis of yeasts by Mϕ cultured for 5–7 days in teflon beakers was rapid, with the number of ingested yeasts increasing linearly for 40 min. After 10 min, 44% of adherent Mϕ had ingested an average of 4.5 yeasts/Mϕ. By 60 min, 79% of Mϕ had ingested an average of 9.8 yeasts/Mϕ.

AM were adhered to glass coverslips and studied immediately. After a lag phase of 20 min, phagocytosis of Hc yeasts by AM was rapid, and similar to the rate of phagocytosis observed with cultured Mϕ (Fig. 1 *B*). By 60 min, 59% of Mϕ had ingested an average of 11 yeasts/Mϕ.

In contrast to Mϕ, the rate and extent of phagocytosis of Hc yeasts by freshly isolated monocytes was markedly lower. No yeasts were observed in monocytes until 20 min (Fig. 1 *B*), and after incubation for 60 min, only 26% of monocytes had ingested an average of 5.5 yeasts/Mϕ. Table III compares the number of yeasts ingested by monocytes, cultured Mϕ, and AM after 60 min of incubation. The average number of yeasts ingested/Mϕ by cultured Mϕ and AM was equivalent, and about twice the number of yeasts ingested by monocytes. In
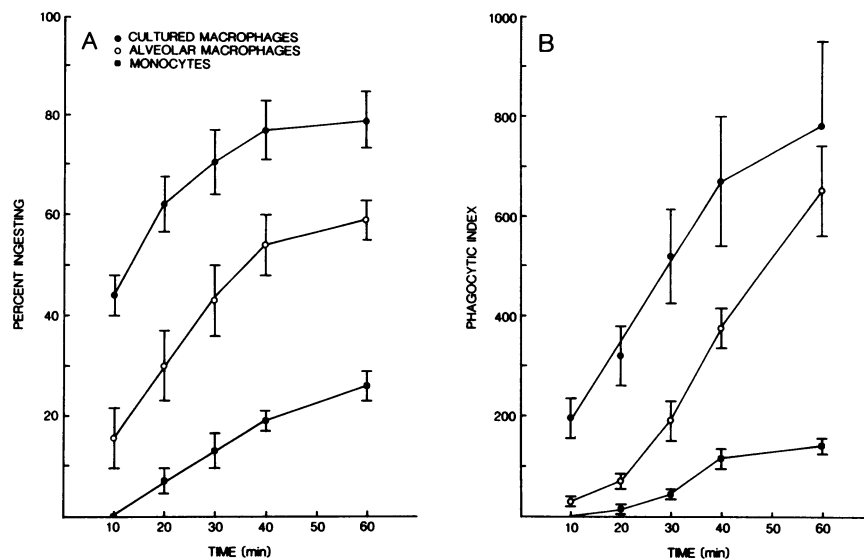
Figure 1. Kinetics of phagocytosis of unopsonized Hc yeasts by human monocyte/Mφ and AM. Freshly isolated peripheral blood monocytes, 5–7 d cultured Mφ, and AM were adhered to glass coverslips in 24-well tissue culture plates, and then were incubated with FITC-labeled Hc yeasts for varying periods of time at 37°C. The fluorescence of bound, but uningested yeasts, was quenched with trypan blue, and the Mφ were fixed in 1% paraformaldehyde. 200 cells were scored for binding and ingestion via fluorescence and phase contrast microscopy. (*A*) Percent ingesting (the percent of Mφ ingesting one or more yeasts; (*B*) phagocytic index (the total number of ingested yeasts per 100 Mφ). Data are the mean±SEM of eight experiments with cultured Mφ from different donors, four experiments with monocytes, and five experiments with AM.

addition, the phagocytic indices for cultured Mφ and AM were 5.5- and 4.6-fold greater than the index obtained with monocytes ($P < 0.02$). However, the percentage of attached yeasts that were internalized (phagocytic index/association index) by both monocytes and Mφ was ~ 80%. This high percentage of internalized vs. bound yeasts was evident at all time points, and indicates that binding of yeasts to the Mφ membrane was followed by rapid ingestion.

Phagocytosis of Hc microconidia by cultured Mφ and AM. Phagocytosis of FITC-labeled Hc microconidia by Mφ was quantified as described for yeasts. Hc strain 501 was chosen for these studies because the mycelia produce mostly microconidia at 30°C, and it is these small conidia (3–5 μm) that enter the alveolar spaces and presumably are ingested by AM. In these experiments, it was not feasible to examine the kinetics of phagocytosis because of the low numbers of conidia that were obtained. Therefore, we compared the phagocytosis of 1 × 10⁶ microconidia and the same number of yeasts. After 1 h at 37°C, cultured Mφ and AM phagocytosed equivalent numbers of microconidia (Fig. 2). Although the percent of cultured Mφ that ingested yeasts or microconidia was approximately the same (44% vs. 50%, respectively), Mφ ingested significantly greater numbers of yeasts than of microconidia (PI = 121±13.8 vs. 70±7.7; $P < 0.05$). AM also ingested greater numbers of yeasts than microconidia but this difference was not statistically significant. Thus, Mφ can recognize and phagocytose unopsonized microconidia almost as rapidly as unopsonized yeasts. We also observed that cultured Mφ and AM ingested macroconidia and small hyphal fragments that

were present with the microconidia, but phagocytosis of these particles was not quantified.

Requirements for cellular cytoskeleton in binding and ingestion of Hc yeasts by Mφ. Ingestion of EC3b, EC3bi, and EIgG by 7-d adherently cultured Mφ via CR₁, CR₃, and FcR, respectively, requires intact actin microfilaments. Microtubule polymerization also is required for ingestion of EC3b and EC3bi, but not for ingestion of EIgG. However, neither microfilaments nor microtubules are required for attachment of ligand-coated E to complement receptors (CR) or FcR[2] (32, 33). Hc yeasts bind to Mφ CD11a/CD18 and CD11c/CD18 as well as CD11b/CD18 (CR₃). Therefore, we performed experiments to determine if the requirement for functional actin microfilaments and microtubules to phagocytose EC3bi was similar for phagocytosis of Hc yeasts.

Mφ monolayers were preincubated with 5 μg/ml of cytochalasin D (CD) for 5 min at 37°C, and then yeasts were added for 1 h at 37°C. Unexpectedly, preincubating Mφ with CD completely abolished the binding of yeasts to cultured Mφ. This effect of CD was concentration dependent as shown in Fig. 3. The percent rosettes, phagocytic index (PI), and ASI, all decreased in parallel with increasing concentrations of CD from 0.05 to 1.0 μg/ml.

Mφ cultured nonadherently in teflon beakers for 5–7 d require a second stimulus such as PMA (32) to mediate phagocytosis of EC3b and EC3bi, whereas Mφ cultured adherently to glass or tissue culture plastic spontaneously activate CR for phagocytosis (17, 28). To determine if the effect of CD on Mφ binding of Hc yeasts might be related to the conditions of

Table III. Comparison of Phagocytosis of Hc Yeasts by Human Monocytes, Cultured Mφ, and AM

Cell	Percent ingesting	Phagocytic index	Association index	Percent attached yeasts ingested	Yeasts/macrophage
Monocyte	26±3.1 (4)*	142±13*	179±18*	79	5.5
Cultured Mφ	79±5.9 (8)	777±172	993±202	78	9.8
Alveolar Mφ	59±4.3 (5)	649±86	759±90	85	11.0

* Mean±SEM (*n*). Phagocytic index, the number of ingested yeasts per 100 Mφ. Association index, the number of bound plus ingested yeasts per 100 Mφ.
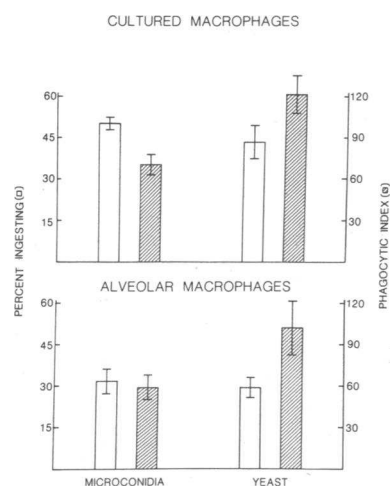
CULTURED MACROPHAGES

ALVEOLAR MACROPHAGES

MICROCONIDIA   YEAST

Figure 2. Phagocytosis of microconidia and yeasts by cultured Mϕ and AM. 1 ml of FITC-labeled microconidia or yeasts (1 × 10^6/ml) was added to Mϕ monolayers, and phagocytosis allowed to proceed for 1 h at 37°C. Binding and ingestion were quantified as described in the legend to Fig. 1. (*Open bars*) Percent ingesting; (*hatched bars*) phagocytic index. Data are the mean±SEM of six experiments with cultured Mϕ (*top*) and five experiments with AM (*bottom*).

culture, Mϕ were cultured adherently on glass coverslips. In parallel, Mϕ were cultured in teflon beakers and then adhered to glass coverslips. Both Mϕ preparations were pretreated with 1 μg/ml of CD for 5 min at 37°C, and binding and ingestion of Hc yeasts was quantified after 60 min. Table IV shows that Mϕ cultured under either condition bound and ingested equivalent numbers of Hc yeasts. In addition, binding, as well as ingestion, was abrogated by preincubating the cells with CD.

To determine if the effect of CD was specific for yeast binding to CD18 on Mϕ, suspension cultured Mϕ were treated with CD, and then were incubated with EC3b and EC3bi for 60 min at 37°C. As shown in Table V, CD did not inhibit EC3b binding to CR$_1$, or EC3bi binding to CR$_3$. Thus, Hc yeast binding to CD18 receptors is inhibited by disruption of actin microfilaments, but ligand binding to either CR$_1$ or CR$_3$ (CD11b/CD18) is not.

The requirement for cellular cytoskeleton function was explored further by determining if polymerization of microtubules is required for phagocytosis of Hc yeasts by human Mϕ. Cultured Mϕ were adhered to glass coverslips, preincubated with varying concentrations of nocodazole (NOC) (34) for 30 min at 37°C, and then were incubated with Hc yeasts for 60 min. Concentrations of NOC from 0.1 to 2.5 μg/ml had no effect on the percentage of Mϕ ingesting or the PI (Fig. 4). The ASI also was not changed (data not shown), indicating that unlike CD, NOC had no effect on the binding of Hc yeasts to Mϕ. Likewise, NOC did not inhibit binding of EC3b or EC3bi to suspension cultured Mϕ, but did inhibit ingestion of EC3b(i) by adherently cultured Mϕ, confirming our previous experiments[2] and those of others (32, 33) (Table V). Thus, although microtubule polymerization is required for phagocytosis of EC3b and EC3bi, it is not required for ingestion of Hc yeasts.

Discussion

In this study, we examined the binding and ingestion of Hc yeasts and microconidia by human monocytes, cultured Mϕ,
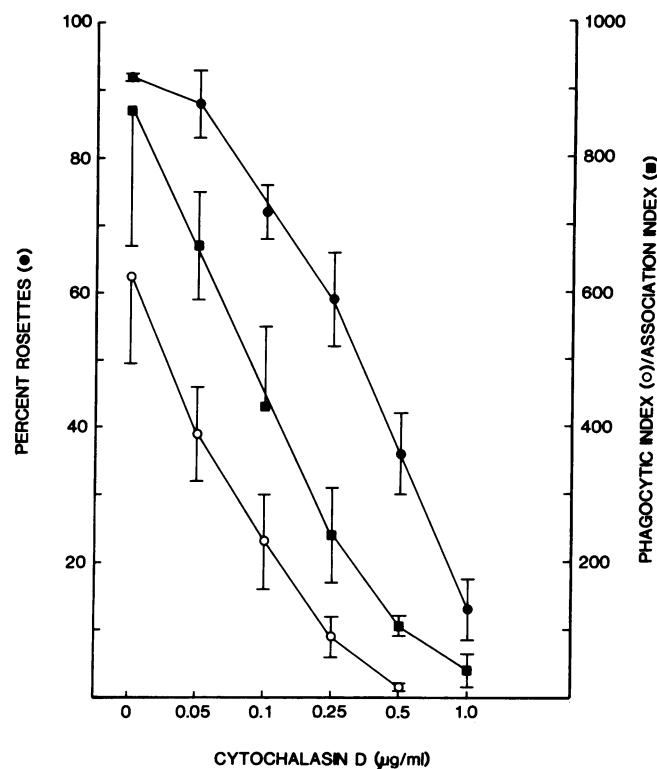
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Figure 3. Concentration-dependent inhibition by cytochalasin D of binding and ingestion of Hc yeasts by cultured Mϕ. Adherent Mϕ were preincubated with various concentrations of CD for 5 min at 37°C, and then were incubated with FITC-labeled yeasts for 60 min at 37°C. Phagocytosis was quantified as described in the legend to Fig. 1. •, Percent rosettes; o, phagocytic index; ■, association index. Data are the mean±SEM of three experiments.

and AM. As was found for cultured Mϕ (14), AM also bind Hc yeasts via the CD18 receptor complex. Thus, MAbs specific for the α-chains of each member of the CD18 family all partially blocked the binding of Hc yeasts to AM, and maximum inhibition was obtained with a MAb specific for the β-chain. Antibodies that recognized Mϕ membrane components not associated with the CD18 complex did not block yeast binding to AM. Similarly, MAbs specific for CD18 receptors, but not control MAbs, inhibited the binding of Hc microconidia to cultured Mϕ and AM. These data indicate that both microconidia of the mycelial phase and yeasts of Hc are recognized by the same receptor complex on human Mϕ.

Table IV. Effect of CD on Binding and Ingestion of Hc Yeasts by Macrophages Cultured Adherently vs. Nonadherently

	Adherent macrophages*		Nonadherent macrophages*	
	Control	CD	Control	CD
% Ingestion	91±4.5	1.0±0.6	83±2.4	1.0±0.6
PI	1,081±66	2.7±1.7	1,010±43	2.3±1.4
ASI	1,318±48	165±39	1,208±18	86±32

Mϕ were preincubated with 1 μg/ml of CD for 5 min at 37°C, and then incubated with Hc yeasts for 60 min at 37°C.
* Mean±SEM (n = 3).

Table V. Effect of CD and NOC on EC3b and EC3bi Binding to Suspension Cultured Mφ and on Ingestion of EC3b and EC3bi by Adherently Cultured Mφ

Addition	Percent Rosettes*		Phagocytic index[‡]	
	EC3b	EC3bi	EC3b	EC3bi
Buffer	96±2.0[§]	91±3.4	232±84[§]	723±186
CD	89±1.5	84±13	ND	ND
NOC	85±2.6	88±10	47±19	273±98

* Mφ cultured in suspension were adhered to glass coverslips and preincubated with 1 μg/ml CD for 5 min, or 2.5 μg/ml NOC for 30 min at 37°C, and rosette formation with EC3b and EC3bi quantified after 60 min at 37°C.
[‡] Mφ cultured adherently were preincubated with 2.5 μg/ml NOC for 30 min at 37°C, and phagocytosis of EC3b and EC3bi quantified after 60 min at 37°C.
[§] Mean±SEM ($n = 3$).

The three MAbs to CD11b/CD18 used in these studies recognize distinct epitopes on the α-chain. The unique specificity of these MAbs has made it possible to demonstrate that specific sites on the α-chain mediate phagocyte binding to different target particles or substrates. Thus, MAb MN-41 inhibits EC3bi binding to PMN (35) and monocytes (17), and inhibits zymosan binding to PMN (35), whereas OKM1 inhibits neither EC3bi nor zymosan binding to PMN (35). MAb 904 inhibits PMN adherence to protein-coated plastic (19) and LPS binding to PMN (36), but does not inhibit EC3bi binding to PMN (36).

The attachment of Hc to Mφ CD18 receptors is a more complex phenomenon. As proposed previously (14), Hc might bind independently to each of the α-chains of the CD18 family, or the binding site for Hc might be located on the common β-chain. In our original experiments (14), OKM1, a MAb specific for CD11b/CD18, inhibited the attachment of yeasts to cultured Mφ to a somewhat greater degree than MAbs specific for CD11a/CD18 and CD11c/CD18. In the experiments reported here, each of the three anti-CD11b MAbs (MN-41, OKM1, and 904) inhibited attachment of yeasts to AM by 34–36%, whereas MAb LeuM5, specific for the α chain of
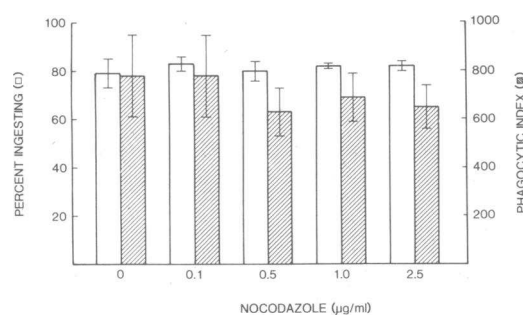

Figure 4. Lack of inhibition by nocodazole of phagocytosis of Hc yeasts by cultured Mφ. Adherent Mφ were preincubated with various concentrations of NOC for 30 min at 37°C, and then were incubated with FITC-labeled yeasts for 60 min at 37°C. Phagocytosis was quantified as described in the legend to Fig. 1. (*Open bars*) Percent ingesting; (*hatched bars*) phagocytic index. Data are the mean±SEM of five experiments.

CD11c/CD18, inhibited yeast binding to AM by 48%. These data, in conjunction with the previously published data (14), are consistent with previous observations that cultured Mφ express greater numbers of CD11b/CD18 than CD11c/CD18, whereas AM express greater numbers CD11c/CD18 than CD11b/CD18 (37).

In contrast, inhibition of the binding of microconidia to cultured Mφ and AM by MAb does not follow the inhibition pattern of yeasts. With the exception of MAb 904, MAbs specific for CD18 α chains all inhibited microconidia binding to cultured Mφ and AM to a similar degree. One explanation for this finding is that microconidia may bind preferentially to the Mφ CD18 β-chain. An alternative explanation is that each of the Mφ CD18 α-chains bind microconidia with differing affinities. A third possibility is that the individual CD18 receptors on cultured Mφ and AM are not all equally mobile within the plasma membrane. Thus, a MAb specific for a particular CD18 receptor could cause an equal amount of inhibition of microconidia binding to both cultured Mφ and AM, even though these two Mφ populations possess different numbers of that receptor. Finally, it is possible that ligands on the surface of microconidia may promote binding to other as yet unidentified receptors on the Mφ surface, as well as binding to Mφ CD18 receptors. Currently, we cannot distinguish between these possibilities. To address these questions, we are attempting to purify and characterize the ligand(s) on Hc yeasts and conidia, which mediates attachment to Mφ. Once purified, the ligand(s) will be employed in affinity columns to isolate the specific Mφ receptor(s) to which the ligand(s) binds.

Phagocytosis of unopsonized yeasts by cultured Mφ and freshly adherent AM was rapid, and both cell populations ingested an equivalent number of yeasts. By comparison, monocytes ingested significantly fewer yeasts than Mφ. However, in each of these three cell populations, 75%–80% of attached yeasts were ingested at all time points (Table III and data not shown). Thus, the rate of phagocytosis of yeasts by monocyte/Mφ is determined by the rate of attachment of the yeasts to the phagocyte membrane. It is possible, therefore, that differences in the rates of phagocytosis demonstrated by monocytes, cultured Mφ, and AM may relate to the different numbers of CD11a/CD18, CD11b/CD18, and CD11c/CD18 receptors on these cells. As mentioned above, quantitation of CD18 receptors by RIA has shown that cultured Mφ express twice the amount of CD11b/CD18 as CD11c/CD18, whereas AM express greater numbers of CD11c/CD18 than CD11b/CD18. In addition, cultured Mφ express five times more CD11b/CD18 and eight times more CD11c/CD18 than do freshly isolated monocytes (37).

An alternative explanation for the different rates of phagocytosis exhibited by monocytes and Mφ is that individual receptors may differ in their binding affinity for yeasts, and/or in their ability to trigger a phagocytic signal. Another possibility is that attachment could be mediated by one receptor, and ingestion triggered by another receptor. In this regard, it has been suggested that CD11b/CD18 receptors on U937 Mφ stimulated by phorbol ester mediate binding of EC3bi, whereas CD11c/CD18 triggers ingestion of this particle (38).

Cultured Mφ and AM phagocytosed unopsonized microconidia to a similar extent, and Mφ phagocytosed microconidia almost as efficiently as yeasts. This observation may be relevant with regard to the transformation of microconidia into yeasts in vivo. Since acute infection of humans with Hc

frequently is asymptomatic and self-limited, the sequence of events in the early inflammatory response to inhaled microconidia is unknown (1). Furthermore, the fate of microconidia in the lungs of experimental animals has not been described (2). Thus, the events that are initiated once microconidia enter the pulmonary alveoli are unknown. However, our data indicate that microconidia are ingested rapidly by AM.

It is likely, therefore, that upon exposure to Hc in nature, microconidia are inhaled into the pulmonary alveoli and phagocytosed by resident AM within which an unknown proportion transform into yeasts. This event initiates primary infection, which in turn stimulates the development of Hc-specific CMI with eventual resolution of the infection. Some support for this hypothetical sequence of events is provided by observations that in regions where histoplasmosis is endemic, up to 90% of sampled adult populations demonstrate CMI to Hc as demonstrated by delayed type skin test reactions or by blastogenic transformation of their peripheral blood lymphocytes to histoplasmin (1). In addition, it has been reported that following in vitro infection of AM from naive mice by microconidia and hyphal fragments of Hc, the number of intracellular fungi increased over 48 h, whereas there was no increase in fungi within AM from mice that had been immunized with Hc (13). Currently, we are evaluating the fungicidal capacity of human monocyte/Mφ and AM against viable microconidia to determine their fate in human phagocytes.

Several aspects of our work suggest that the phagocytic signal initiated by the binding of yeasts to CD18 receptors of Mφ differs from that initiated by the binding of EC3bi to CD11b/CD18. First, binding of EC3bi to monocytes, to Mφ cultured in teflon beakers, or to freshly adherent AM, does not initiate a phagocytic signal (17, 28, 32, 39). That is, EC3bi bind to these phagocytes but are not ingested. In contrast, all three cell populations phagocytose Hc yeasts. Second, Mφ cultured adherently can phagocytose EC3bi, (17), whereas Mφ cultured in suspension can not (32). Conversely, Mφ obtained by either culture method phagocytosed equivalent numbers of unopsonized Hc yeasts.

Additional evidence that different phagocytic signals are triggered by binding of Hc and EC3bi is provided by the disparate effects of cytochalasin D and nocodazole on binding and ingestion of these respective particles. Disruption of actin microfilaments prevented binding, as well as the ingestion of yeasts, whereas inhibition of microtubule polymerization did not inhibit either yeast binding or ingestion by Mφ. In contrast, prior studies have shown that phagocytosis of EC3b and EC3bi by adherently cultured Mφ via CR$_1$ and CR$_3$, respectively, requires intact actin microfilaments and microtubules; neither cytoskeletal element is required for binding of EC3b or EC3bi[2] (32, 33). Lastly, phagocytosis of EC3b or EC3bi by cultured human Mφ does not stimulate the production of H$_2$O$_2$ (40), whereas phagocytosis of Hc yeasts does trigger it (14). Characterization of the biochemical pathway by which Hc triggers phagocytosis may provide insight into the ability of this microorganism to survive within the hostile environment of the Mφ phagolysosome. In addition, Hc yeasts and EC3bi provide useful probes for exploring the different Mφ activation pathways initiated by ligand-binding to CD18 receptors.

Acknowledgments

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