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Growth Inhibition of *Cryptococcus neoformans* by Cultured Human Monocytes: Role of the Capsule, Opsonins, the Culture Surface, and Cytokines

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Despite a presumed critical role of macrophages in the host response to cryptococcal infections, previous studies have failed to show growth inhibition of encapsulated *Cryptococcus neoformans* by human peripheral blood cultured monocyte-derived macrophages (MO-Mϕ). Here, we examined whether MO-Mϕ could be induced to inhibit growth of an encapsulated strain and an isogenic acapsular mutant strain of *C. neoformans*. MO-Mϕ were cultured in microwells, and inhibition was measured by comparing CFU at 0 and 24 h after fungal challenge. MO-Mϕ cultured on plastic surfaces failed to inhibit growth of the encapsulated strain, even in the presence of pooled human serum and/or anticapsular antibody. Moreover, the presence of anticapsular antibody significantly enhanced fungal growth. However, if MO-Mϕ were cultured on surfaces coated with fibronectin or poly-L-lysine (but not laminin or collagen) and yeast cells were opsonized with pooled human serum, then complete growth inhibition occurred. Preincubation with various concentrations of tumor necrosis factor, granulocyte macrophage colony-stimulating factor, 1,25-dihydroxycholecalciferol, or supernatants from *C. neoformans*-stimulated lymphocytes failed to activate macrophages for enhanced antifungal activity. The addition of gamma interferon resulted in a significant loss of growth inhibition. For the acapsular strain, complete growth inhibition was observed regardless of the choice of culture surface, opsonins, or cytokines. Fungicidal activity, as measured by a significant decrement in CFU compared with the initial inoculum, was not observed under any conditions tested. These data demonstrate that macrophages are capable of inhibiting cryptococcal growth but that this capacity is markedly influenced by the culture surface, opsonins, cytokines, and the fungal capsule.

Infections caused by the encapsulated yeast *Cryptococcus neoformans* are major causes of morbidity and mortality in patients with impaired cell-mediated immunity, including those with acquired immunodeficiency syndrome (10). While an adequate cell-mediated immune response appears essential for controlling infections caused by *C. neoformans*, it remains unclear which cell type(s) is ultimately responsible for mediating antifungal activity. In murine models of infection, several lines of evidence point to the importance of macrophages as anticryptococcal effector cells. First, the in vivo treatment of mice with silica to incapacitate the reticuloendothelial system has resulted in decreased resistance to cryptococcosis (40), whereas nonspecific macrophage activation has been protective (19, 40). Second, fungistasis mediated both by *Mycobacterium bovis* BCG-stimulated, endotoxin-primed murine peritoneal macrophages and by murine macrophage cell lines has been demonstrated (22, 26). Third, murine resident peritoneal cells and bone marrow macrophages can be activated by the lymphokine gamma interferon (IFN-γ) to kill *C. neoformans* (17, 32). Finally, bronchoalveolar macrophages, even in the absence of activation, efficiently kill *C. neoformans* in vivo and in vitro (33). In addition to macrophages, murine splenic natural killer and antibody-dependent killer cells have been shown to inhibit cryptococcal growth (41, 44).

Given the important differences between the immune systems of mice and humans, results obtained with murine models are not necessarily applicable to humans. Indeed, Diamond and Bennett demonstrated not only that human peripheral blood cultured monocyte-derived macrophages (MO-Mϕ), obtained by culturing peripheral blood monocytes on a plastic surface, failed to kill *C. neoformans*, but also that the yeast cells grew more rapidly than a corresponding inoculum in culture medium alone (12). This does not preclude a role for macrophages as effector cells in cryptococcosis, as evidence exists that MO-Mϕ cultured on plastic surfaces may differ in important respects from tissue macrophages (25). Macrophages possess receptors for extracellular matrix proteins, such as fibronectin, collagen, and laminin, and MO-Mϕ differentiated on surfaces coated with these proteins (as opposed to glass or plastic) appear to more closely resemble tissue macrophages (4, 5, 25). Moreover, cytokines released both locally and systemically in the course of the cell-mediated immune response to cryptococcal infection could substantially alter the ability of macrophages to mediate anticryptococcal activity by affecting the state of activation of the cell. Furthermore, opsonization of yeast cells with anticapsular immunoglobulins produced during a humoral response to infection could alter how the macrophage is stimulated. The effects of cytokines and anticapsular antibody on macrophage interactions with *C. neoformans* have added clinical significance with the availability of pharmacological quantities of recombinant cytokines and the potential availability of human monoclonal antibodies against *C. neoformans*. Therefore, in the present study, we examined whether such host variables as matrix proteins, serum opsonins, and macrophage-activating cytokines would affect the anticryptococcal ability of MO-Mϕ. By comparing macrophage responses to organisms containing capsules with isogenic mutants lacking capsules, we

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were also able to study the role the capsule plays in helping *C. neoformans* elude macrophage defenses. We found that the ability of MO-Mϕ to mediate fungistasis of *C. neoformans* was markedly affected by the culture surface, opsonization, cytokine treatment, and the capsule.

MATERIALS AND METHODS

Materials. All reagents used, except where noted otherwise, were obtained in the highest quality available from Sigma Chemical Co., St. Louis, Mo. The medium used in all experiments, unless stated otherwise, was RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with L-glutamine, penicillin, streptomycin, and 10% human AB male serum. Human recombinant IFN-γ and tumor necrosis factor (TNF) were gifts of Genentech, Inc., South San Francisco, Calif. IFN-γ and TNF had specific activities of 2.5×10^7 U/ml and 5.6×10^7 U/mg, respectively. Both IFN-γ and TNF contained less than 0.1 pg of endotoxin per mg, as measured by the *Limulus* lysate assay. Human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) was a gift of Genetics Institute, Cambridge, Mass. 1,25-Dihydroxycholecalciferol (1,25-D_3) was a gift of Milan R. Uskokovic, Hoffmann-La Roche Inc., Nutley, N.J., and stored in ethanol at 2.4×10^{-3} M at −70°C until use. Preliminary experiments demonstrated that the cytokines were biologically active.

C. neoformans. Encapsulated, serotype D strain MP415 (also known as strain B3501) and acapsular, isogenic mutant strain CAP67 (ATCC 52817) (18, 24, 33) were obtained from Eric S. Jacobson, Richmond, Va. In mouse models of infection, MP415 is highly virulent whereas CAP67 is avirulent (18, 24). The yeast cells were maintained by serial passage at 30°C on asparagine minimal agar medium (30) and harvested from 3- to 5-day-old cultures by suspending a loopful from a single colony in phosphate-buffered saline, as in our previous studies (32, 33). The organisms were washed three times with phosphate-buffered saline, counted on a hemacytometer, and suspended at the desired concentration in phosphate-buffered saline or other medium. Under these conditions, greater than 95% of the organisms were present as single cells, viability of the cells as measured by a microcolony assay was greater than 98% (33), and clumping of organisms was not observed. The encapsulated strain averaged 7.48 μm in diameter, with an average capsule thickness of 1.47 μm, whereas the acapsular strain had an average diameter of 4.36 μm.

Human MO-Mϕ. Human peripheral blood was obtained by venipuncture from 10 normal volunteers. For each set of experiments, the same blood donor was not used more than once. Blood was anticoagulated with 10 U of heparin per ml, and the monocytes were purified by two sequential centrifugations with Sepracell-MN (Sepratech Corp., Oklahoma City, Okla.), according to the directions of the manufacturer. This procedure routinely yields a population of leukocytes containing approximately 90% monocytes and 10% lymphocytes. The cells were washed three times and suspended in medium at 5×10^6/ml, and 100 μl was added per well of 96-well flat-bottom polystyrene tissue culture plates (no. 25860, Corning Glass Works, Corning, N.Y.). Preliminary experiments established that this concentration of cells resulted in a confluent monolayer. Plates were incubated in a 37°C, 5% CO_2 humidified environment and washed after 24 h to remove nonadherent cells, and MO-Mϕ were cultured for 6 to 8 days before challenge with *C. neoformans*. In a single experiment, MO-Mϕ were cultured for 21 days before challenge. Following the washing step, the remaining cells in the well were greater than 99% monocytes, as assessed by nonspecific esterase staining. In selected experiments, MO-Mϕ were cultured in suspension at 10^6/ml in medium in Teflon beakers (Savillex Corp., Minnetonka, Minn.), exactly as described previously (52). These MO-Mϕ were used for experiments in which (i) monolayers were built up to confluence by repeatedly washing wells of nonadherent cells and then adding more MO-Mϕ, and (ii) the effects of differentiation in suspension, rather than on plastic, were studied.

Protein-coated wells. In some experiments, before the addition of cells, wells were coated with human plasma fibronectin (Chemicon, El Segundo, Calif.), laminin, type 1 rat tail collagen, or poly-L-lysine, according to the directions of the manufacturer. Briefly, fibronectin-coated plates were prepared by adding 80 μl of a solution (20 μg/ml) of fibronectin in distilled water to each well, allowing the wells to air dry, and then washing the wells with distilled water. Collagen-coated wells were prepared in the same manner, except 100 μl of a 0.01% solution of collagen in 0.1 M acetic acid was added. Laminin-coated wells were prepared by incubating 32 μl per well of a solution (20 μg/ml) of laminin in medium 45 min before the addition of cells. Wells were coated with poly-L-lysine by adding 50 μl per well of a solution (0.1 mg/ml) of poly-L-lysine in PBS for 30 min and then washing the wells with distilled water.

Inhibition and killing of *C. neoformans*. MO-Mϕ were challenged with approximately 5×10^3 to 10×10^3 CFU of *C. neoformans* per well. In some experiments, a final concentration of 10% pooled human serum (PHS; obtained by pooling sera from 10 healthy volunteers and stored in aliquots at −70°C until use), 10% heat-inactivated (56°C, 30 min) PHS, and/or a subagglutinating concentration (1:250) of rabbit anticapsular antibody (prepared by immunizing rabbits with a methylated bovine serum albumin conjugate of purified serotype D capsular polysaccharide and kindly supplied by Thomas Kozel, Reno, Nevada [27]) were added prior to fungal challenge. Clumping of organisms was never observed following the addition of PHS or antibody. Cells and fungi were incubated 2 to 144 h (usually 24 h) in a 37°C, 5% CO_2 atmosphere. The plates were then chilled to 4°C, and the contents of the wells were gently sonicated with the microtip attachment of a sonicator (VibraCell model; Sonics and Materials, Danbury, Conn.) for 10 0.2-s pulses at a setting of one. These conditions completely disrupted the MO-Mϕ and broke up the *C. neoformans* into single cells, as determined by visual inspection under an inverted microscope, without affecting its viability (32, 33). The contents of the wells were then completely transferred to tubes containing 10 ml of distilled water, samples were spread onto Sabouraud agar plates, and CFU were counted after 5 days of incubation at 23°C. For each experiment, two sets of controls were included containing yeast cells, medium, and PHS but without MO-Mϕ. The first set was processed and plated on agar immediately. The CFU observed were used to calculate the inoculum (0-h control). The second set was incubated at 37°C for the same amount of time as the experimental groups before being processed and plated. The CFU observed for this set of controls were used to calculate fungal growth in medium not containing MO-Mϕ (24-h control). Killing of *C. neoformans* by MO-Mϕ was defined as a significant reduction in CFU compared with the inoculum. Complete growth inhibition was defined as the lack of a significant increase in CFU compared with the inoculum. Partial growth inhibition was defined as a significant decrease in CFU compared with the 24-h control. None of the

following variables had a significant effect on the growth of *C. neoformans* in medium not containing MO-Mϕ: heat inactivation of serum, anticapsular antibody, culture surfaces, and cytokines.

Binding and phagocytosis assays. The binding assay, measuring the ability of MO-Mϕ to bind *C. neoformans* under the same conditions as those used in the inhibition and killing assays, was performed essentially as previously described (33). Briefly, wells containing MO-Mϕ monolayers were challenged for 2 h with 5×10^3 fluorescein isothiocyanate-labeled, heat-killed yeast cells. Preliminary experiments and previous studies have established that the use of fluorescein isothiocyanate-labeled, heat-killed (as opposed to unlabeled, live) organisms does not affect the binding of *C. neoformans* to phagocytes (32, 33). The total numbers of organisms per 16 randomly selected microscope fields were determined with an inverted microscope at 200× magnification under epifluorescence. This number ranged between 296 and 388. The wells were then washed vigorously five times, and the organisms in 16 fields were again counted. The percentage of the inoculum bound to MO-Mϕ was calculated as (total number of organisms after washing/total number of organisms before washing) × 100. Under the same conditions, but in wells containing no MO-Mϕ, fewer than 1% of the organisms remained in the wells after washing. To assay whether organisms bound to MO-Mϕ were completely phagocytosed (internalized), as opposed to being attached only to the cell surface, our previously described Fungiqual (diaethanol or Uvitex; Ciba-Corning, Fernwald, German Democratic Republic) assay was used (32–34). Briefly, MO-Mϕ were challenged with *C. neoformans* for 30 min, stained for 1 min with 0.01% Fungiqual, washed, and fixed. Fungi attached to the cell surface fluoresce blue following UV excitation, whereas internalized fungi do not (34).

Mononuclear cell supernatants. Supernatants from *C. neoformans*-stimulated mononuclear cells were prepared under conditions previously shown to induce a maximal T-cell proliferative response (39). Human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll-Hypaque sedimentation. To each well of a six-well plate (Falcon 3046; Becton Dickinson Labware, Oxnard, Calif.) was added 5×10^7 mononuclear cells and 5×10^7 heat-killed *C. neoformans* encapsulated strain MP415 cells in a final volume of 2.2 ml of medium. The plates were incubated for 9 days at 37°C, 5% CO_2. Supernatants were then collected, passed through a 0.2-μm filter to remove cellular debris, and stored in portions at −70°C until use.

Presentation of data and statistics. Except for Fig. 2, results are expressed as means ± standard error of the mean (SEM) of number of organisms. The data in Fig. 2 are expressed as percent CFU compared with the inoculum and were calculated as (CFU in experimental group/CFU in inoculum) × 100. This method of calculation was necessary to correct for variations in the inocula between separate experiments, since each experiment did not include all culture surfaces. Means and SEM were compared by using the two-sample, two-tailed Student *t* test for independent means.

RESULTS

Inhibition on plastic versus fibronectin-coated surfaces. Initial experiments examined the antifungal activity of human MO-Mϕ against PHS-opsonized *C. neoformans* over a 24-h period (Fig. 1). In agreement with results reported previously (12), MO-Mϕ cultured on plastic surfaces failed

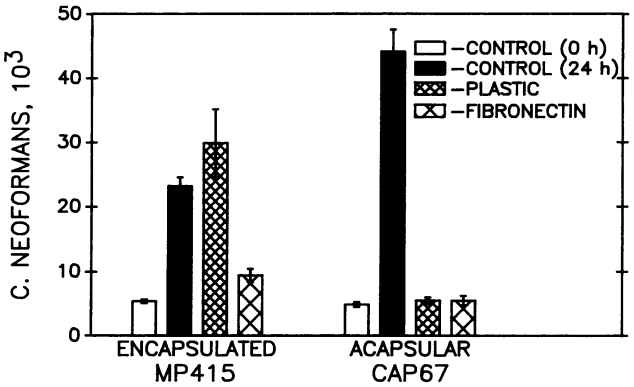

FIG. 1. Effect of culture surface on inhibition of cryptococcal strains by cultured human monocytes. MO-Mϕ (5×10^5) were cultured on either a plastic or fibronectin-coated surface and incubated for 24 h with strains of *C. neoformans* in the presence of medium containing 10% PHS, and CFU were counted. Control (0 h) represents CFU in the initial inoculum whereas control (24 h) represents CFU following the growth of organisms in wells without MO-Mϕ. Data represent the means ± SEM of five triplicate experiments. All experimental groups showed significant inhibition ($P < 0.0001$) compared with 24-h controls, except for MO-Mϕ cultured on plastic and challenged with encapsulated organisms. A significant increment in CFU compared with 0-h controls occurred only when the encapsulated strain was added to MO-Mϕ cultured on plastic ($P < 0.000001$).

to show inhibitory activity against the growth of encapsulated yeast cells. However, if MO-Mϕ were cultured on fibronectin-coated surfaces, then complete inhibition of growth was seen. Fibronectin added to the wells in the fluid phase, at a concentration of 20 μg/ml, had no effect on cryptococcal growth, both in wells containing and lacking MO-Mϕ (data not shown). In contrast to the results obtained with encapsulated organisms, MO-Mϕ completely inhibited the growth of acapsular yeast cells regardless of whether they were cultured on plastic or fibronectin surfaces. Nearly identical results were obtained when inhibition of both cryptococcal strains by MO-Mϕ cultured for 7 versus 21 days was compared (data not shown).

Complete inhibition of the acapsular strain was seen for up to 144 h, whereas the ability of MO-Mϕ cultured on fibronectin to completely inhibit the encapsulated strain lasted only 48 to 72 h. If the number of MO-Mϕ per fibronectin-coated well was decreased fivefold to 10^5, then inhibition of the encapsulated strain was no longer seen. In contrast, inhibition of the acapsular strain was seen with as few as 2×10^4 MO-Mϕ per well. The inability of MO-Mϕ cultured on plastic to inhibit the encapsulated strain was not secondary to the lack of binding of the organisms to the MO-Mϕ, as 94 ± 6% (mean ± SEM of three experiments) of *C. neoformans* organisms remained attached to the monolayer after five vigorous washes. Moreover, microscopic examination of the monolayers 2 h postchallenge revealed that greater than 99% of the yeast cells were physically associated with MO-Mϕ. Solid-phase fibronectin has been shown to enhance complete phagocytosis (internalization) of certain particles bound to MO-Mϕ complement receptors (3, 4, 51, 53). However, for encapsulated *C. neoformans*, there were only small differences between the mean percentages of organisms internalized by MO-Mϕ cultured on plastic versus fibronectin-coated surfaces (27 versus 38%, respectively).

Effects of collagen, laminin, and poly-L-lysine. We next examined whether the extracellular matrix proteins collagen

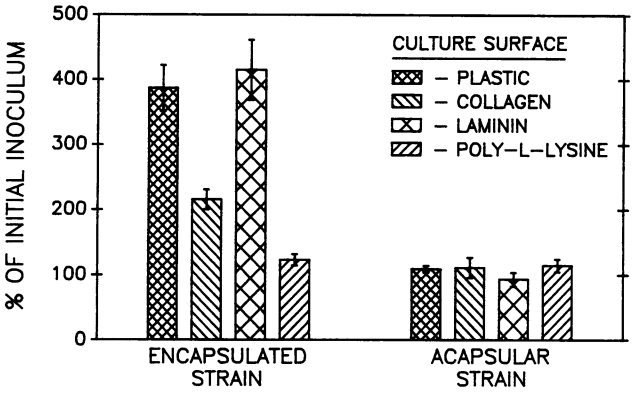

FIG. 2. Effect of culture surface on inhibition of cryptococcal strains by cultured human monocytes. MO-Mϕ (5×10^5) were cultured on a plastic or collagen-, laminin-, or poly-L-lysine-coated surface and incubated for 24 h with strains of *C. neoformans* in the presence of medium containing 10% PHS, and CFU were quantitated. To correct for variations in the initial inoculum between separate experiments (which ranged from 4.4×10^3 to 9.5×10^3), results are expressed as the percentage of the value for the initial inoculum. Similar results were obtained regardless of the inoculum. Data represent means ± SEM of two to four triplicate experiments. A significant increment in CFU compared with the initial inoculum was seen when the encapsulated strain was added to MO-Mϕ cultured on plastic ($P = 0.000002$), collagen ($P = 0.0008$), and laminin ($P = 0.000001$).

and laminin, as well as the cationic protein poly-L-lysine, which has been shown to enhance cell adhesion, would have effects similar to those of fibronectin (Fig. 2). Following 24-h incubation, the encapsulated strain was completely inhibited by MO-Mϕ plated on poly-L-lysine. Partial inhibition was effected by MO-Mϕ plated on collagen (215 ± 16 versus 291 ± 11% of initial inoculum for wells with versus without MO-Mϕ; $P = 0.0007$). No growth inhibition was seen with laminin (415 ± 46 versus 359 ± 21% of initial inoculum for wells with versus without MO-Mϕ; $P > 0.05$). Complete inhibition of the acapsular strain was again seen regardless of the culture surface. The inhibition was MO-Mϕ mediated and not secondary to a fungistatic effect of certain culture surfaces, since in the absence of MO-Mϕ nearly identical growth curves were obtained whether the yeast cells were cultured on plastic surfaces or surfaces coated with fibronectin, poly-L-lysine, collagen, or laminin (data not shown).

No killing of either strain was seen, as assessed by decreased CFU compared with the inoculum. However, it remained possible that a subpopulation of organisms was in fact killed but that the killing was not detected because of subsequent growth of the subpopulation of yeast cells not killed. Two sets of experiments were done to help exclude (although not entirely eliminate) this possibility. First, the quantitative results obtained by counting CFU were confirmed qualitatively by examination of wells under an inverted microscope. Clusters of budding encapsulated *C. neoformans* organisms were seen in wells containing MO-Mϕ cultured on plastic or laminin-coated surfaces (Fig. 3A). Eventually the monolayer was destroyed by MO-Mϕ migrating to these clusters of organisms in an unsuccessful attempt to control fungal growth. In contrast, a confluent monolayer was maintained with MO-Mϕ cultured on fibronectin- or poly-L-lysine-coated surfaces and growth of organisms (as assessed by the presence of buds or clusters of organisms) was not observed (Fig. 3B). Second, the incubation time of MO-Mϕ with *C. neoformans* was decreased to 2 h. Since this incubation time is too short to allow for fungal replication, fungicidal activity, if it occurs early, should be detected. However, regardless of the culture surface or the cryptococcal strain, significant killing was not seen at 2 h (data not shown).

MO-Mϕ adhere to matrix-coated surfaces better than to plastic. Thus, fibronectin or poly-L-lysine surfaces may have been promoting MO-Mϕ fungistasis simply by virtue of increasing the cell density in the wells to a critical number. However, MO-Mϕ cultured on plastic did not inhibit encapsulated *C. neoformans* even if the monolayer was built to confluence by the sequential addition of 2.5×10^6 MO-Mϕ or if the nonadherent cells were not washed away (Fig. 4). Moreover, culture of MO-Mϕ in suspension for 7 days, followed by their addition to wells coated with poly-L-lysine 8 h before fungal challenge, did not result in fungistasis. Thus, it appeared that MO-Mϕ differentiation (and not just plating) on poly-L-lysine was a prerequisite for fungistatic competence.

Effects of opsonins. We next studied the opsonic requirements for MO-Mϕ-mediated fungistasis of encapsulated and acapsular *C. neoformans* by comparing organisms opsonized with PHS versus those treated with heat-inactivated (56°C, 30 min) PHS. For these experiments, the AB serum in the medium was heat inactivated, and the medium was changed prior to fungal challenge so as to inactivate or dispose of complement derived from MO-Mϕ and AB serum. As in previous experiments (Fig. 1 to 4), MO-Mϕ plated on plastic failed to inhibit encapsulated yeast cells even in the presence of PHS (Fig. 5). When heat-inactivated PHS was substituted, there was a trend toward increased fungal growth, although this did not achieve statistical significance. MO-Mϕ cultured on fibronectin were unable to inhibit the growth of encapsulated yeast cells if the PHS was heat inactivated, suggesting that heat-labile component(s) in PHS were required for fungistasis. In contrast, the acapsular strain was inhibited by MO-Mϕ regardless of whether the PHS was heat inactivated.

In view of the data of previous investigators (12), as well as our observation of trends toward increased growth of encapsulated organisms in plastic wells containing (as opposed to lacking) MO-Mϕ, experiments were performed to see whether MO-Mϕ contained a factor enhancing cryptococcal growth. However, there were no significant differences between fungal growth in wells containing medium alone versus wells containing medium plus sonicated MO-Mϕ (data not shown).

Anticapsular immunoglobulin is protective in mouse models of cryptococcosis (16), and its presence in serum has been associated with a favorable outcome in human cryptococcosis (13). Therefore, we next looked at the effect of opsonization of encapsulated *C. neoformans* with PHS and rabbit anticapsular immunoglobulin on inhibition by MO-Mϕ (Fig. 6). When MO-Mϕ were cultured on plastic, there was a paradoxical 43% increase in CFU when anticapsular immunoglobulin was added to the wells ($P = 0.03$). In contrast, for the MO-Mϕ cultured on fibronectin, similar degrees of fungistasis were seen whether or not immunoglobulin was present.

Effects of cytokines. In the final set of experiments, the effect of selected cytokines on the capacity of MO-Mϕ to inhibit or kill *C. neoformans* was studied (Fig. 7). For these experiments MO-Mϕ were cultured on either fibronectin or poly-L-lysine, with similar results. Twenty-four hours before fungal challenge, MO-Mϕ were exposed to a final concentration of 100 U of IFN-γ per ml, 100 U of TNF per ml, 100

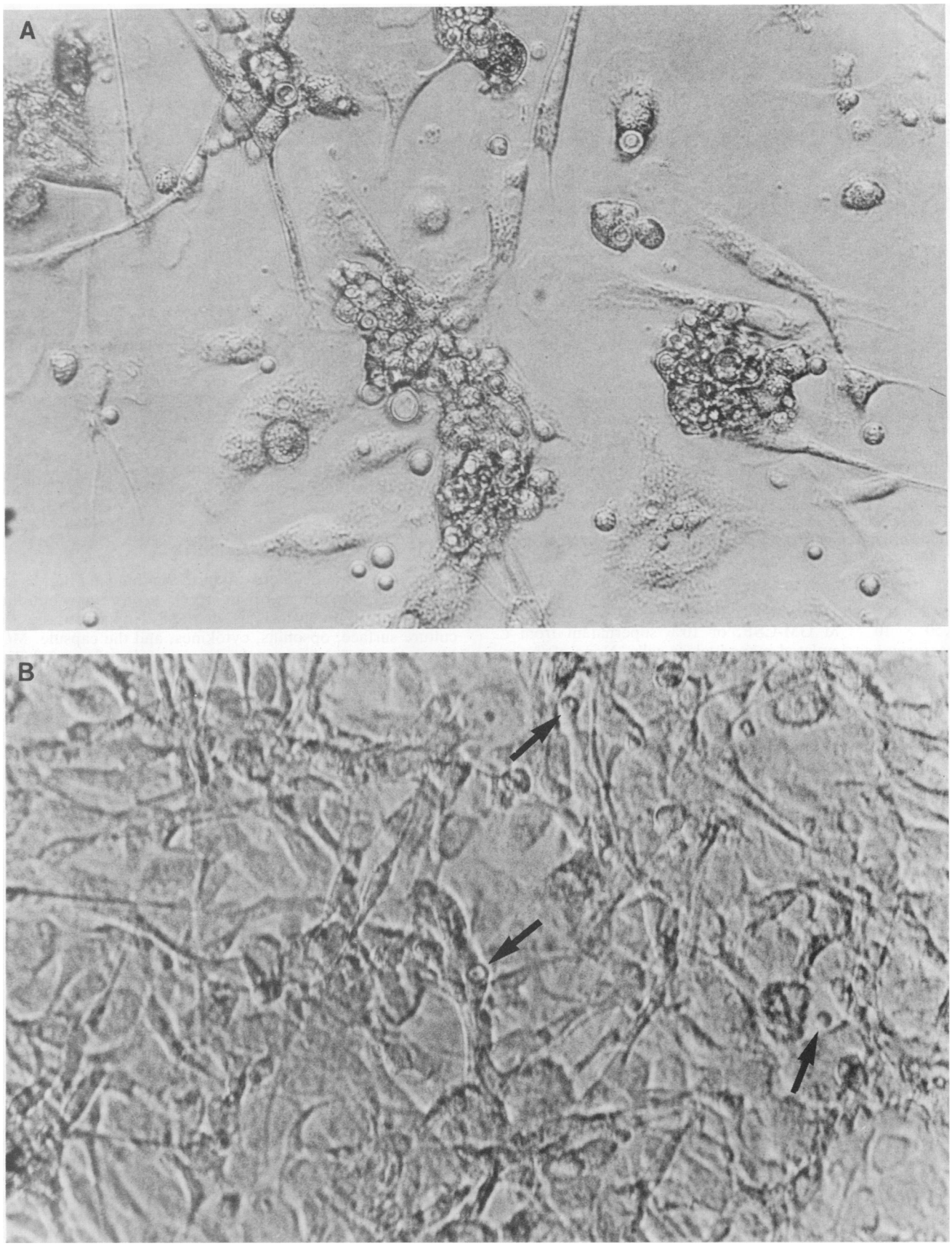

FIG. 3. Phase-contrast photomicrographs of MO-Mϕ 24 h following challenge with encapsulated *C. neoformans* (original magnification, ×200). (A) MO-Mϕ cultured on plastic surfaces. Heavy growth of *C. neoformans* is evident, with disruption of the monolayer. (B) MO-Mϕ cultured on poly-L-lysine. The integrity of the monolayer is preserved. The yeast cells (arrows), many of which are difficult to see, have not divided.

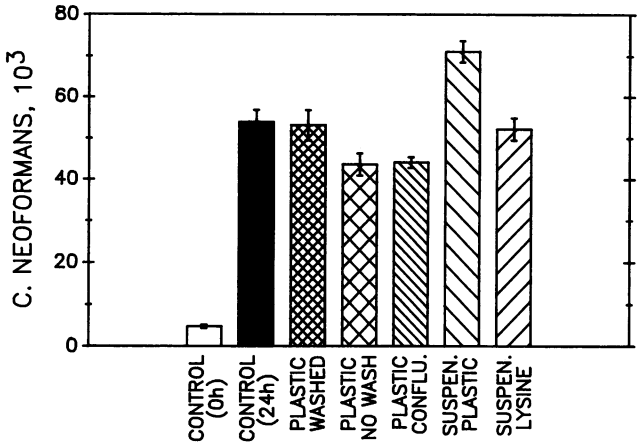

FIG. 4. Effect of various culture conditions on inhibition of encapsulated *C. neoformans* by MO-Mϕ. MO-Mϕ were cultured for 7 days under the indicated conditions and challenged for 24 h with encapsulated *C. neoformans* in the presence of 10% PHS, and CFU were counted. Control (0 h) and control (24 h) are as described for Fig. 1. Bars: Plastic washed, MO-Mϕ (5×10^5) cultured on plastic and washed prior to fungal challenge; plastic unwashed, MO-Mϕ (2×10^6) cultured on plastic and not washed prior to challenge; plastic conflu., confluent monolayer built by the sequential addition of 2.5×10^6 MO-Mϕ; Suspen. plastic and suspen. lysine, MO-Mϕ cultured in suspension and plated on plastic and poly-L-lysine, respectively, 8 h before fungal challenge. Data represent means ± SEM of two triplicate experiments.

U each of both IFN-γ and TNF per ml, 5×10^{-7} M 1,25-D_3, 2.50×10^{-10} M GM-CSF, or 10% supernatant from *C. neoformans*-stimulated mononuclear cells. None of the cytokines tested made MO-Mϕ competent to kill either strain of *C. neoformans*. Moreover, a significantly detrimental effect on MO-Mϕ-mediated inhibition of encapsulated yeast cells was seen when IFN-γ was added to the wells ($P = 0.0003$). However, if the concentration of IFN-γ was reduced to 10 U/ml, or if the IFN-γ was added 1 h before fungal challenge rather than 24 h, then neither a detrimental nor an enhancing effect was observed (data not shown).

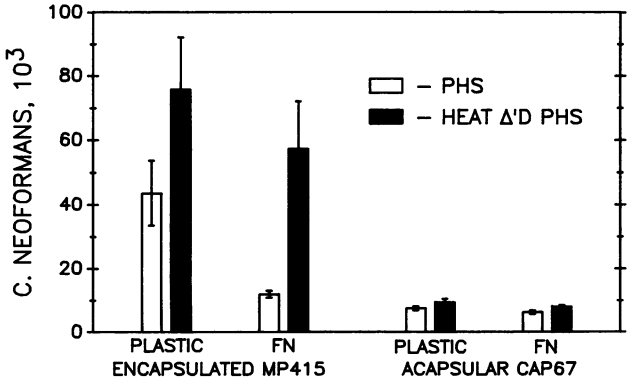

FIG. 5. Effect of heat inactivating PHS on the ability of MO-Mϕ to inhibit *C. neoformans*. MO-Mϕ (5×10^5) were cultured on either a plastic or fibronectin (FN)-coated surface and incubated for 24 h with strains of *C. neoformans* in the presence of either PHS or heat-inactivated PHS, and CFU were counted. Results represent means ± SEM of two triplicate experiments. $P = 0.006$ for encapsulated organisms on fibronectin plates opsonized with PHS versus heat-inactivated PHS.

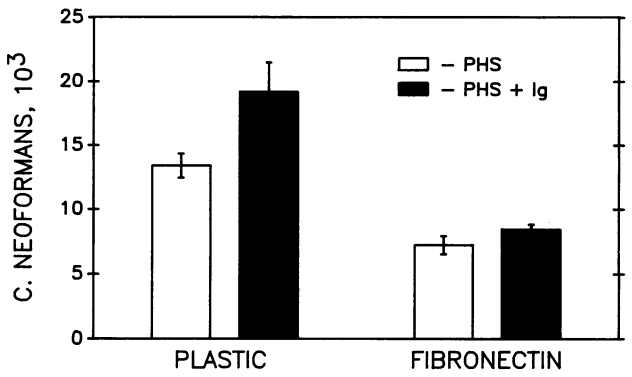

FIG. 6. Effect of anticapsular immunoglobulin (Ig) on inhibition of encapsulated strain MP415 by MO-Mϕ. MO-Mϕ (5×10^5) were cultured on either a plastic or fibronectin-coated surface and incubated for 24 h with strains of *C. neoformans* opsonized with PHS plus or minus rabbit anticapsular immunoglobulin. Results represent means ± SEM of three triplicate experiments. $P = 0.03$ for inhibition with versus without immunoglobulin by MO-Mϕ cultured on plastic.

There was a trend, although not significant ($P = 0.08$), toward increased growth when the combination of TNF and IFN-γ was added.

DISCUSSION

The data presented here establish that human MO-Mϕ are capable of inhibiting growth of *C. neoformans* but that attainment of fungistasis is markedly influenced by the culture surface, opsonins, cytokines, and the capsule. MO-Mϕ cultured on plastic or laminin-coated surfaces failed to inhibit the growth of encapsulated *C. neoformans*, whereas complete inhibition was obtained on fibronectin- and poly-L-lysine-coated surfaces. Partial inhibition was seen for collagen (Fig. 1 and 2). To our knowledge, this is the first demonstration of the ability of fibronectin-coated surfaces to influence the antimicrobial activity of phagocytes. The addition of soluble fibronectin to the culture medium has previ-

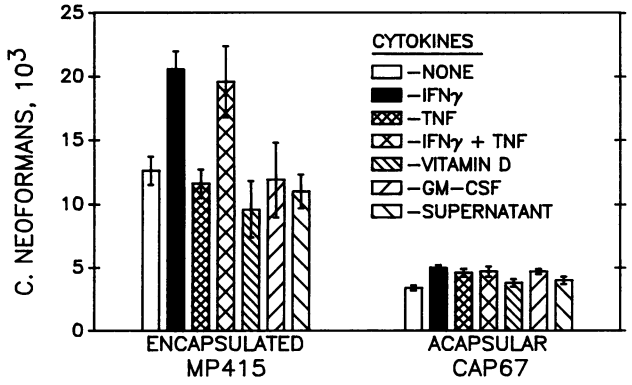

FIG. 7. Effect of cytokines on inhibition of cryptococcal strains by MO-Mϕ. MO-Mϕ (5×10^5) were exposed to cytokines 24 h before challenge with *C. neoformans*. MO-Mϕ and fungi were then incubated an additional 24 h in the presence of PHS, and CFU were counted. Results are the means ± SEM of three triplicate experiments, two of which used MO-Mϕ cultured on fibronectin, one of which used MO-Mϕ cultured on poly-L-lysine, and all of which had similar results. $P < 0.0003$ for CFU of the encapsulated strain with IFN-γ versus without cytokines.

ously been shown to enhance the ability of MO-Mϕ cultured on plastic to kill *Staphylococcus aureus* (47). In our experiments, soluble fibronectin had no effect on the inhibition of *C. neoformans* by MO-Mϕ. Moreover, as opposed to other microbes such as *S. aureus* and *Candida albicans* (6, 47), for which fibronectin has been shown to bind directly to the organism and act as an opsonin, preliminary studies in our laboratory have not shown such interactions between encapsulated *C. neoformans* and fibronectin (S. M. Levitz, unpublished observations).

The biochemical and functional profiles of MO-Mϕ have been shown to be influenced by the surface on which the MO-Mϕ are cultured. For instance, MO-Mϕ cultured on glass surfaces give rise to cells resembling foreign body granuloma macrophages, whereas those cultured on collagen matrices give rise to highly phagocytic cells resembling resident tissue macrophages (25). Fibronectin and laminin have been shown to enhance phagocytosis of certain particulate stimuli via both complement and Fc receptors (3, 4, 51, 53). However, our studies showed similar phagocytosis of serum-opsonized encapsulated *C. neoformans* by MO-Mϕ cultured on fibronectin as opposed to plastic. This finding, combined with our data demonstrating the high efficiency (94%) of binding of *C. neoformans* to MO-Mϕ cultured on plastic, makes it unlikely that the inhibitory effect of fibronectin was simply secondary to the enhanced ability of MO-Mϕ cultured on fibronectin to bind or internalize the organisms. Although the MO-Mϕ are similar in many respects, important differences between MO-Mϕ cultured on collagen, laminin, and fibronectin have recently been reported. For instance, there are distinct membrane receptors on mononuclear phagocytes for these proteins and disparate effects on the attachment of monocytes to surfaces coated with these proteins have been noted (4, 5, 49). Culture on poly-L-lysine, a synthetic cationic polypeptide which binds nonspecifically to cell membranes, has been shown to induce differentiation of several cell lines (7, 48). Moreover, recent evidence suggests that lysine may play an important role in leukocyte adherence to endothelium (35). Studies in our laboratory are under way to better define the mechanisms by which MO-Mϕ mediate inhibition when differentiated on fibronectin or poly-L-lysine but not plastic, collagen, or laminin surfaces.

MO-Mϕ adhere better to fibronectin and poly-L-lysine than to plastic. Therefore, it is possible that the fungistasis-promoting properties of these proteins seen in our experiments are simply secondary to greater numbers of MO-Mϕ in the wells, rather than being due to effects on cellular differentiation. However, taken together, several pieces of evidence strongly argue against this possibility. First, collagen and laminin, which have similar adherence-promoting properties, did not promote fungistasis. Second, increasing the numbers of MO-Mϕ in the plastic wells by sequentially adding MO-Mϕ to the monolayer did not result in fungistasis. Third, even if nonadherent cells were not washed away, fungistasis in the plastic wells was not obtained. Fourth, culture of MO-Mϕ in suspension followed by adherence to poly-L-lysine 8 h before fungal challenge did not result in inhibition. The MO-Mϕ must mature and differentiate on the poly-L-lysine in order to become competent for fungistasis. Finally, MO-Mϕ cultured on plastic were still able to bind nearly all of the *C. neoformans* inoculum.

The ability of extracellular matrix proteins to influence MO-Mϕ inhibition of *C. neoformans* is of potential relevance since, in vivo, macrophages differentiate from monocytes attached to the extracellular matrix (25). Moreover, the amounts of fibronectin and other matrix proteins may vary under inflammatory conditions, further influencing macrophage differentiation as well as the subsequent recruitment of phagocytic cells (46). The marked dependence of MO-Mϕ-mediated inhibition of *C. neoformans* on the culture surface emphasizes the importance of studying these cells under various culture conditions before judgments are made as to their functional abilities. Although MO-Mϕ cultured on surfaces coated with matrix proteins resemble tissue macrophages in many respects, it should be noted that the construction of the extracellular matrix is quite complex (37). Significant differences between tissue macrophages and MO-Mϕ cultured on matrix proteins are likely to exist which may have important functional implications. Moreover, tissue macrophages have discrete properties depending on anatomic location. Human alveolar macrophages have previously been shown capable of inhibition of growth of a thin-capsuled clone of *C. neoformans* (50).

MO-Mϕ cultured on fibronectin required heat-labile factor(s) in serum for effective fungistasis of encapsulated *C. neoformans* (Fig. 5). The cryptococcal capsule is a potent activator of the alternative complement pathway, with large amounts of iC3b deposited on the surface of encapsulated organisms after incubation in PHS (28, 29). PHS promotes binding of *C. neoformans* by different phagocyte populations, including macrophages, and this opsonic activity is lost if the alternative complement pathway is inhibited (9, 14, 28, 29, 33). Moreover, studies in our laboratory have demonstrated that monoclonal antibodies directed at complement receptors powerfully inhibit binding of PHS-opsonized *C. neoformans* to MO-Mϕ (S. M. Levitz and A. Tabuni, submitted for publication). Taken collectively, these data suggest that MO-Mϕ-mediated inhibition of encapsulated *C. neoformans* requires a functional complement system to opsonize the yeast cells so that they bind to the phagocytes and effectively trigger the fungistatic mechanisms of the phagocytes. Human serum has also been shown to contain heat-stabile component(s) capable of slowing down the growth of *C. neoformans* in the absence of leukocytes (1, 23). This effect of serum was confirmed in our studies, as the encapsulated strain underwent two to three replications in 24 h when incubated in medium containing 20% human serum at 37°C (Fig. 1, 2, and 4), compared with more than six replications if serum was omitted from the medium (S. M. Levitz and T. P. Farrell, unpublished data). This variation in growth in the presence of serum appeared to be dependent on the lots of AB serum and PHS used in each set of experiments. Bicarbonate ion, at the concentrations present in our medium, has also been shown to slow the cell generation time of *C. neoformans* (21).

Opsonization of yeast cells with specific anticapsular antibody failed to promote cryptococcal inhibition by MO-Mϕ cultured on either plastic or fibronectin (Fig. 6). In fact, the addition of specific antibody resulted in significantly greater cryptococcal growth in wells containing MO-Mϕ differentiated on plastic. Disparate biochemical and functional responses result when MO-Mϕ are stimulated via complement versus Fc receptors (4, 51, 54). Of note, when particulate stimuli are coated with immunoglobulin G, they are generally bound and completely internalized by macrophages. In contrast, except under certain activating conditions, sensitization of the stimulus with complement normally results in binding but not internalization (34, 36, 51, 54). Thus, the enhanced growth of *C. neoformans* opsonized with anticapsular antibody could result from a more favorable growth environment inside the MO-Mϕ compared with

outside, where inhibitors in serum of cryptococcal growth are present (1, 23). Our data failing to show increased activity of MO-Mϕ against antibody-opsonized *C. neoformans* contrast with previous observations for other phagocyte populations, including mouse resident peritoneal macrophages, human neutrophils, and human nonadherent mononuclear cells, in which antibody-dependent fungicidal activity was observed (11, 32, 38).

The release of cytokines from lymphocytes, natural killer cells, and, in an autocrine manner, macrophages themselves during the course of a cell-mediated immune response is thought to greatly influence the ability of macrophages to defend against microbial challenge (42). Several cytokines, including IFN-γ, TNF, GM-CSF, and 1,25-D_3, alone or in combination, have been shown in some systems capable of activating human MO-Mϕ to inhibit or kill a variety of bacterial, parasitic, and tumor targets (2, 8, 42, 45). The depletion and dysfunction of T4 lymphocytes in patients with acquired immunodeficiency syndrome and other disorders of cell-mediated immunity result in impaired production of IFN-γ, and in vivo administration of IFN-γ appears capable of enhancing MO-Mϕ killing of certain pathogens in patients with acquired immunodeficiency syndrome and other disorders (31, 42, 43). Our studies failed to demonstrate enhanced antifungal activity of MO-Mϕ when exposed to IFN-γ, TNF, GM-CSF, 1,25-D_3, or supernatants from *C. neoformans*-stimulated mononuclear cells (Fig. 7). Moreover, as opposed to studies with murine macrophages in which IFN-γ stimulated cryptococcal killing (17, 32), replication of encapsulated *C. neoformans* was significantly enhanced following treatment of human MO-Mϕ with IFN-γ (Fig. 7). A similar detrimental effect of IFN-γ on the inhibition of *Mycobacterium tuberculosis* by human MO-Mϕ has been reported (15). Although it remains possible that alternative dosing schedules or combinations will prove protective, our data do not lend support to the use of these cytokines as immunoadjuvant therapy in patients with cryptococcosis.

In contrast to the special conditions required to achieve inhibition of encapsulated organisms, acapsular *C. neoformans* cells were inhibited by MO-Mϕ regardless of the culture surface or whether a source of complement was present (Fig. 1, 2, and 5). Moreover, inhibition persisted for as long as 6 days. Nevertheless, neither encapsulated nor acapsular *C. neoformans* was killed in any of our experiments. It appears then that a virulence factor(s) other than the capsule accounts for the inability of MO-Mϕ to kill *C. neoformans* but that the capsule imparts upon the organism the ability to resist fungistasis when conditions are favorable. The mechanisms by which inhibition occurs are presently under investigation in our laboratory. An L-arginine-dependent system with nitrite as an end product has been associated with the inhibition (20) and killing (32) of *C. neoformans* by activated murine macrophages. However, such a system has not been demonstrated in human macrophages, which may help explain the disparate results obtained when anticapsular antibody or IFN-γ is used in the mouse, compared with the human, system.

The epidemiology of cryptococcosis is incompletely understood, and it is unknown whether some cases result from reactivation of a latent focus of infection, as has been demonstrated for other diseases associated with impaired cell-mediated immunity, such as histoplasmosis, tuberculosis, and toxoplasmosis. Our data showing inhibition, but not killing, of *C. neoformans* by MO-Mϕ support the speculation that some cases of cryptococcosis may arise when latently infected macrophages lose their ability to inhibit yeast cells.

In summary, our data establish the ability of MO-Mϕ to inhibit the growth of a virulent, encapsulated strain and an avirulent, isogenic acapsular mutant strain of *C. neoformans*. Whereas inhibition of the acapsular strain was seen regardless of the culture conditions employed, MO-Mϕ inhibited encapsulated organisms only when cultured on fibronectin or poly-L-lysine in the presence of PHS. A panel of cytokines failed to improve MO-Mϕ defenses against either strain, and one of the cytokines, IFN-γ, had a significantly detrimental effect. Decreased fungistasis was also seen when MO-Mϕ cultured on plastic were challenged with encapsulated yeast cells opsonized with anticapsular antibody. Thus, the inhibition of *C. neoformans* by MO-Mϕ is influenced by the culture surface on which the cells differentiate, cytokine treatment, and the presence of opsonins and a capsule on the surface of the organism.

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