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Enhanced Killing of *Candida albicans* by Human Macrophages Adherent to Type 1 Collagen Matrices via Induction of Phagolysosomal Fusion

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***Candida albicans*, a component of the normal flora of the alimentary tract and mucocutaneous membranes, is the leading cause of invasive fungal disease in premature infants, diabetics, and surgical patients and of oropharyngeal disease in AIDS patients. As little is known about the regulation of monocyte/macrophage anti-*Candida* activity, we sought to determine if fungicidal activity might be regulated by extracellular matrix proteins to which monocytes/macrophages are adherent in vivo. Compared to monocyte/macrophages that adhered to plastic, human monocytes and monocyte-derived macrophages that adhered to type 1 collagen matrices, but not to fibronectin, vitronectin, or laminin, demonstrated a significant increase in candidacidal activity. The enhancement of monocyte fungicidal activity was maintained over a 4-h period, whereas macrophage fungicidal activity was maximum at 1 h. Although adherence of monocytes and macrophages to collagen matrices concomitantly enhanced the production of superoxide anion, only the fungicidal activity of collagen-adherent monocytes was partially blocked by superoxide dismutase and catalase. Remarkably, we found that only 10% of the phagosomes in *C. albicans*-infected macrophages that adhered to plastic fused with lysosomes. In contrast, 80% of yeast-containing phagosomes of collagen-adherent macrophages fused with lysosomes. These data suggest that nonoxidative mechanisms are critical for human macrophage anti-*Candida* activity and that *C. albicans* pathogenicity is mediated, in part, by its ability to inhibit phagolysosomal fusion in macrophages.**

Candida albicans is part of the normal microbial flora that colonizes mucocutaneous surfaces of the oral cavity, gastrointestinal tract, and vagina of the healthy human host. Although *Candida* does not normally cause disease, when immune defenses are compromised or the normal microflora balance is disrupted, *C. albicans* transforms itself into an opportunistic pathogenic killer. Indeed, *Candida* is the leading cause of invasive fungal disease in premature infants, diabetics, and surgical patients and of oropharyngeal disease in AIDS patients (1, 5, 7, 9, 13, 17, 23, 34, 45).

Host resistance against infections with *C. albicans* is mediated predominantly by neutrophils and monocytes/macrophages (Mϕ). In the early stages of *Candida* infection microabscesses contain neutrophils surrounded by a small number of mononuclear cells. As the fungus is eliminated from the lesions, mononuclear cells predominate (32). The importance of neutrophils in host defense against *Candida* infections is underscored by the high incidence of disseminated disease in individuals with neutropenia (1, 40) and the fact that depletion of neutrophils in mice leads to systemic disease (2, 12). Further, numerous in vitro studies have documented the potent ability of neutrophils from mice, humans, and guinea pigs to kill *C. albicans* (3, 6, 37), and killing is mediated by both oxidative and nonoxidative mechanisms (8, 21, 22, 38, 46).

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While neutrophils clearly are important in host defense against *Candida* invasion and exhibit potent killing activity against the fungus, the relatively poor candidacidal activity of Mϕ is perplexing. Thus, in a comparison of human neutrophils, monocytes, and monocyte-derived Mϕ, Mϕ clearly were inferior to neutrophils and monocytes in their candidacidal activity. However, unlike neutrophils and monocytes, Mϕ killed *Candida* equally well under aerobic and anaerobic conditions (42). Indeed the loss of candidacidal activity that occurs as monocytes differentiate into Mϕ in vitro has been shown to correlate with the loss of the enzyme myeloperoxidase and a decrease in the ability to produce hydroxyl radicals (35). As monocytes and Mϕ produced equivalent amounts of superoxide anion, the data suggest that the candidacidal activity of monocytes is mediated by products distal to the superoxide anion. Further, relative to bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus*, *C. albicans* is resistant to toxic oxygen metabolites. Thus, the killing of *Candida* required 10 times more H_2O_2, NaI, and Fe_2SO_4 than the killing of these bacteria (51). Thus, optimum killing by Mϕ must rely more on nonoxidative killing mechanisms than on oxidative mechanisms.

Numerous studies suggest that for optimal host protection against *C. albicans* Mϕ are essential, despite their tepid candidacidal activity in vitro, and that, most likely, they require activation by cytokines (2, 4, 33). However, very little is known in this regard. With respect to human cells, granulocyte-Mϕ colony stimulating factor and interleukin 3 (IL-3), but not

gamma interferon (IFN-γ) have been reported to enhance the growth inhibition and/or killing of *C. albicans* by monocytes (39, 47). In contrast, IFN-γ modestly boosts the candidacidal activity of monocyte-derived Mϕ (25, 26) and alveolar Mϕ (44). In addition, IL-1α has been shown to enhance the anti-*Candida* activity of monocytes and alveolar Mϕ (44).

In vivo, monocytes emigrating from the peripheral circulation enter into an extravascular area rich in extracellular matrix (ECM) proteins. It is in this milieu that phagocytes function in host defense against pathogenic microorganisms. In addition, tissue Mϕ reside in areas that contain ECM proteins. Previously, we demonstrated that, compared to plastic-adherent monocytes, monocytes adherent to type 1 collagen matrices demonstrated significantly enhanced phagocytic capacity for serum-opsonized *E. coli*, *S. aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* and that phagocytic capacity was mediated by activation of complement receptor type 1 (CR1) and CR3 for phagocytosis and augmentation of Fc receptor-mediated phagocytosis (31). Although both collagen- and plastic-adherent monocytes were bactericidal for these microbial pathogens, more bacteria were killed by collagen-adherent monocytes by virtue of their enhanced phagocytic capacity.

More recently, we demonstrated that collagen-adherent Mϕ are fungicidal for the dimorphic fungal pathogen *Histoplasma capsulatum*, whereas plastic-adherent Mϕ are permissive for intracellular growth. The mechanism of this activation is the capacity of collagen-adherent Mϕ to induce massive phagolysosomal (PL) fusion, whereas minimal PL fusion occurs in plastic-adherent Mϕ (29). These results suggest that monocytes/Mϕ adherent to type 1 collagen may express a previously unrecognized microbicidal activity that proceeds in the absence of exogenous cytokines. This microbicidal activity may be important with regard to the capacity of Mϕ to participate in the inflammatory response and in the induction of cell-mediated immunity in the nonimmune host.

The present study was designed to determine if adherence of human monocytes/Mϕ to type 1 collagen matrices or other ECM proteins might augment candidacidal activity and, if so, to determine whether augmentation was caused by oxidative or nonoxidative mechanisms.

MATERIALS AND METHODS

Reagents. Superoxide dismutase (SOD), catalase, and ferricytochrome *c* (type 3) were purchased from Sigma-Aldrich, St. Louis, Mo. Cytochrome *c* (1 mM) was dissolved in Krebs-Ringer phosphate buffer containing 0.2% dextrose and stored at −20°C. All other reagents were prepared in RPMI 1640 and sterile filtered. Human serum prepared from six to eight individual donors was pooled and stored in 200-μl aliquots at −80°C until used.

Preparation of human monocytes and Mϕ. Monocytes were isolated by sequential centrifugation on Ficoll-Hypaque and Percoll gradients (Amersham Pharmacia LKB, Piscataway, N.J.) from buffy coats obtained from the Hoxworth Blood Center, Cincinnati, Ohio, or from blood drawn from healthy adult donors in our laboratory (28). The monocytes were washed, suspended in Hanks balanced salt solution (HBSS) containing 20 mM HEPES and 0.1% autologous serum, and then allowed to adhere to various substrates for 1 h at 37°C in 5% CO_2-95% air. The adherent monocytes were washed and then were studied immediately or cultured in M199 (Bio-Whittaker, Walkersville, Md.) containing 10% autologous serum and 10 μg of gentamicin (Sigma)/ml. Medium was replaced on day 3, and Mϕ were studied after 7 days of culture.

Alternatively, Mϕ were obtained by culture of monocytes at 10^6/ml in Teflon beakers in RPMI 1640 (Bio-Whittaker) containing 15% human serum, 10 μg of gentamicin/ml, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (Sigma) (28). Mϕ were studied after 5 to 7 days in culture.

Yeasts. *C. albicans* yeasts (ATCC 18804) were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.). Yeasts were grown to log phase in Sabouraud dextrose broth at 30°C with orbital shaking at 150 rpm. For superoxide anion production assays, yeasts 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 (HK) at 65°C for 1 h. Yeasts were stored at 4°C in PBS containing 0.05% sodium azide.

For studies with viable *Candida* cells, log-phase yeasts were harvested by centrifugation, washed three times in HBSS containing 0.25% bovine serum albumin (HBSA), and resuspended to 50 ml in HBSA. The yeasts were counted on a hemacytometer and standardized to the appropriate concentration according to the assay protocol.

H. capsulatum strain G217B was maintained as described previously (28). Yeasts were grown in HMM medium (50) at 37°C with orbital shaking at 150 rpm. After 48 h, log-phase yeasts were harvested by centrifugation, washed three times in HBSA, and resuspended to 50 ml in HBSA. Large aggregates were removed by centrifugation at 200 × *g* for 7 min at 4°C. The top 10 ml was removed, and the single-cell suspension obtained was standardized to the appropriate concentration according to the assay protocol. HK yeasts were prepared as described above for *Candida*.

S. cerevisiae was inoculated into 50 ml of yeast extract-peptone-dextrose broth and cultured for 24 h at 37°C with orbital shaking at 150 rpm. The yeasts were harvested, washed, and standardized by following the same procedure as that for *Candida* yeasts.

Preparation of tissue culture plates coated with ECM proteins. (i) Collagen gels. Type I collagen from rat tails (Sigma) was dissolved in 0.1% acetic acid at 1 mg/ml and dialyzed overnight at 4°C in distilled water. Three hundred microliters of collagen was dispensed into 24-well tissue culture plates and exposed to ammonia fumes for 30 min at 25°C. The gels were washed four times with HBSS prior to adherence of monocytes or Mϕ. Alternatively, collagen-coated wells were prepared by dispensing collagen (50 μg/ml in RPMI) into the wells to adhere directly to the plastic for 2 h at 25°C.

(ii) Fibronectin, vitronectin, and laminin. Fibronectin, vitronectin, and laminin (Sigma) were suspended to 50 μg/ml in RPMI 1640, and 250 μl was aliquoted into the wells of a 24-well tissue culture plate. After 2 h at 25°C, the wells were aspirated and monocytes/Mϕ were allowed to adhere for 1 h at 37°C.

Quantitation of monocyte/Mϕ fungicidal activity. Adherent monocytes and Mϕ (~2×10^5) were incubated with *C. albicans* yeasts (2×10^4) in the presence of 10% pooled human serum for 1, 2, and 4 h at 37°C in 5% CO_2-95% air. At each time point, the supernatant was aspirated and the mixtures were resuspended in 1 ml of sterile water to lyse the cells. The contents were vortexed vigorously and then were serially diluted and plated on Sabouraud dextrose agar plates. Inspection of the initial lysate revealed only single colonies, 98% of which were still in the yeast phase. After incubation at 30°C for 48 h, CFU were counted and the percentage of *C. albicans* cells that were killed was calculated by comparison to the CFU obtained from the original inoculum, which also was quantified by serial dilution and plating.

Quantitation of superoxide anion (O_2^-) production. O_2^- generation by monocytes/Mϕ was quantified as the SOD-inhibitable reduction of ferricytochrome *c* (type 3; Sigma) as described previously (36). Monocytes/Mϕ (2×10^5) were incubated for 1 h at 37°C with serum-opsonized *Candida* yeasts (10^7) in Krebs-Ringer phosphate buffer with 0.2% dextrose containing 80 μM cytochrome *c*. Control wells contained cytochrome *c* without *Candida* (resting cells) or *Candida* plus 40 μg of SOD/ml. At the end of the incubation, the supernatants were collected by centrifugation at 4°C. The absorbance of the supernatants at 550 nm was measured in a Spectronic Genesys 5 (Milton Roy, Rochester, N.Y.), and the background absorbance in control tubes containing only buffer and cytochrome *c* was subtracted. All experiments were performed in duplicate, and results were calculated as nanomoles of O_2^- from the equation $E_{550} = 2.1 \times 10^4$ M^{-1} cm^{-1}. Results are expressed as nanomoles of cytochrome *c* reduced per 2×10^5 cells per hour.

Quantitation of PL fusion. Mϕ were allowed to adhere in 24-well tissue culture plates to 12-mm-diameter round glass coverslips or to coverslips coated with collagen gels. Monolayers then were incubated with 200 nM Lysotracker red (Molecular Probes, Eugene, Oreg.) in RPMI 1640 containing 5% fetal calf serum for 2 h to label lysosomes. After two washes, the Mϕ were infected with viable *S. cerevisiae* (2×10^6 cells) or HK and viable *C. albicans* (1×10^5 cells) or *H. capsulatum* (2×10^6 cells) for 1 h at 37°C. HK *C. albicans* and *H. capsulatum* were labeled with fluorescein isothiocyanate as described previously (30). After an additional two washes, the Mϕ were cultured in 200 nM Lysotracker red for an additional 2 h. After being washed, the monolayers were fixed in 3.75% paraformaldehyde for 20 min at 25°C. The Mϕ then were covered in Dulbecco's PBS (DPBS) containing 5% glucose. Mϕ that adhered to collagen matrices were

incubated overnight at 4°C. The buffer was aspirated, and the gels were allowed to desiccate at 30°C for 24 h. The monolayers then were covered in DPBS-glucose. Mϕ that adhered to the coverslips were counted immediately. Coverslips were mounted cell side down in 90% glycerol in phosphate-buffered saline onto microscope slides. One hundred yeast-containing phagosomes were scored for lysosomal fusion or no fusion. The data are expressed as the means ± standard errors of the means (SEM) of the percentages of PL fusion in three or more experiments performed in duplicate.

Alternatively, Mϕ adhered to plastic or collagen gels in six-well plates were incubated for 2 h at 37°C in M199 containing 10% human serum, 10 μg of gentamicin/ml, and 18-nm colloidal gold stabilized with horseradish peroxidase (HRP-Au18) (29). The Mϕ were washed three times with medium and then incubated for an additional 2 h at 37°C to insure that the HRP-Au18 entered the lysosomal compartments. After the second incubation, 10^6 viable or HK *Candida* yeasts were added, and phagocytosis was allowed to proceed for 1 h at 37°C. After 1 h the Mϕ were washed three times with ice-cold 0.1 M sodium cacodylate buffer, pH 7.4, and then processed for electron microscopy. Mϕ PL fusion was quantified by counting the 18-nm gold particles in phagosomes containing *Candida* yeasts (29). The data are expressed as the means ± SEM of the average numbers of gold particles per yeast-containing phagosome.

Statistics. Statistical analysis of the data was performed using Sigma Stat (Jandel Scientific, San Rafael, Calif.). Student's *t* test or the Mann-Whitney rank sum test was used to analyze the data for statistical significance, and results were considered significant at P values <0.05.

RESULTS

Fungicidal activity of monocytes/Mϕ adherent to collagen matrices, fibronectin, vitronectin, and laminin. In initial experiments we sought to determine if adherence of freshly isolated monocytes or monocyte-derived Mϕ to ECM proteins regulated the phagocytes' capacity to kill *C. albicans* yeasts. Therefore, monocytes and suspension-cultured Mϕ were allowed to adhere to plastic or the various substrates for 1 h and were then incubated with 2×10^4 yeasts for an additional 1 h. Candidacidal activity then was quantified by determining the remaining CFU as described in Materials and Methods. After 1 h, monocytes killed an average of 48% of the initial inoculum and Mϕ killed 57% (Fig. 1A and B). In contrast, monocytes and Mϕ that adhered to collagen matrices killed 70 and 79%, respectively, of the *C. albicans* yeasts. This significant enhancement of monocyte/Mϕ candidacidal activity was not observed when the phagocytes were allowed to adhere to fibronectin, vitronectin, or laminin (Fig. 1A and B). In addition, adherence of monocytes/Mϕ to collagen-coated wells did not enhance fungicidal activity (data not shown), indicating that the enhanced killing required that the collagen be in the form of a three-dimensional matrix, as is found in vivo.

We also sought to determine if monocyte differentiation into Mϕ on the different ECM proteins would regulate phagocyte fungicidal activity. Figure 1C shows that, when monocytes were cultured for 7 days on ECM proteins, enhanced candidacidal activity was observed with vitronectin and laminin, as well as collagen, matrices. The enhanced fungicidal activity observed after 7 days of culture on fibronectin was consistent but did not reach statistical significance.

Enhanced fungicidal activity of monocytes/Mϕ adherent to type 1 collagen matrices. We next sought to determine the kinetics of the enhanced fungicidal activity exhibited by monocytes/Mϕ adherent to collagen matrices. Therefore, monocytes/Mϕ were allowed to adhere to plastic or collagen gels for 1 h and candidacidal activity was quantified over a 4-h period. Figure 2A shows that collagen-adherent monocytes continued to kill *C. albicans* yeasts over the 4-h incubation period,

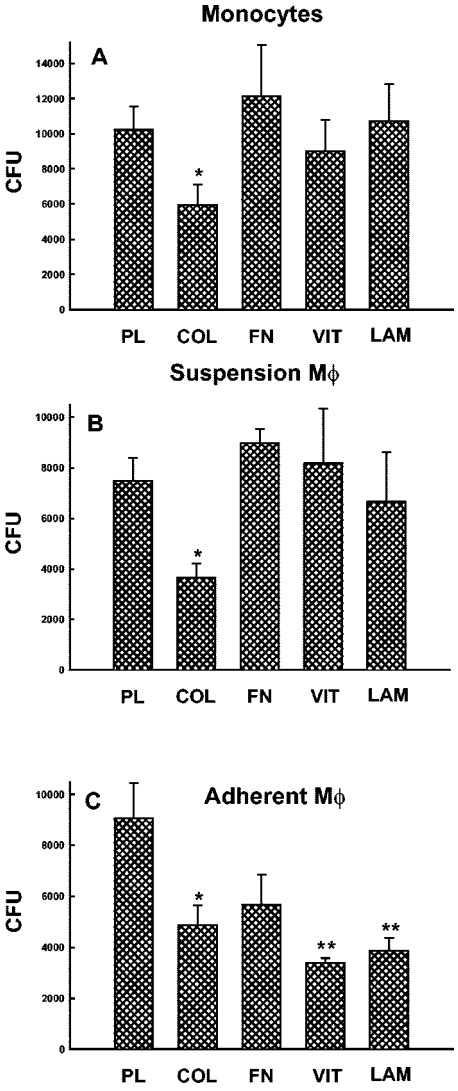

FIG. 1. Collagen-adherent monocytes and Mϕ have enhanced fungicidal activity against *C. albicans*. Freshly isolated monocytes and suspension-cultured Mϕ were allowed to adhere to plastic (PL), type 1 collagen matrices (COL), fibronectin (FN), vitronectin (VIT), or laminin (LAM) for 1 h at 37°C. Alternatively, monocytes were cultured for 7 days on the various ECM components (adherent Mϕ). After the monolayers were washed, the cells were incubated with 2×10^4 *C. albicans* yeasts in the presence of 10% pooled human serum for 1 h at 37°C. At the end of the incubation period, the remaining CFU were quantified as described in Materials and Methods. The data are the means ± SEM of the remaining CFU from five experiments with monocytes and suspension-cultured Mϕ and seven experiments with adherently cultured Mϕ. *, $P < 0.05$ compared to plastic (*t* test); **, $P < 0.05$ compared to plastic (Mann-Whitney rank sum test).

whereas there was an increase in *Candida* CFU at 2 and 4 h with plastic-adherent monocytes. In contrast, with both suspension-cultured Mϕ adherent to collagen gels for 1 h and Mϕ cultured for 7 days on a collagen matrix, maximum fungal killing was observed at 1 h. Thereafter, there was a slight increase in CFU, as was observed with Mϕ adherent to plastic.

Mechanism of monocyte/Mϕ fungicidal activity against *C. albicans*. Data from numerous laboratories suggest that neutrophils kill *C. albicans* predominantly via the production of

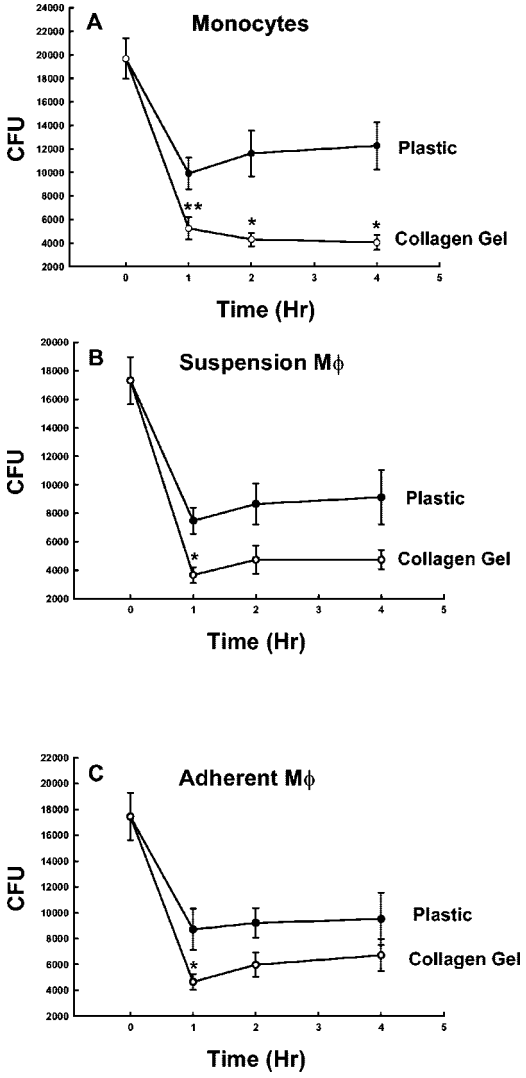

FIG. 2. Time course of monocyte/Mϕ fungicidal activity on plastic versus collagen matrices. Freshly isolated monocytes and suspension-cultured Mϕ were allowed to adhere to plastic or type 1 collagen matrices for 1 h at 37°C, or monocytes were cultured for 7 days on plastic or collagen. After the monolayers were washed, the cells were incubated with 2×10^4 *C. albicans* yeasts in the presence of 10% pooled human serum for 1, 2, and 4 h at 37°C. Remaining CFU then were quantified as described in Materials and Methods. The data are the means ± SEM of the remaining CFU from eight experiments with monocytes and adherently cultured Mϕ and five experiments with suspension-cultured Mϕ. * and **, $P < 0.01$ and < 0.05, respectively, compared to plastic (*t* test).

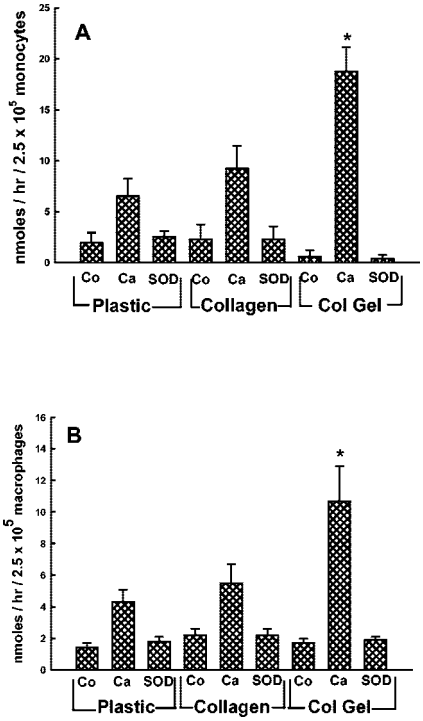

FIG. 3. Adherence of monocytes/Mϕ to collagen matrices enhances the production of superoxide anion. Monocytes (A) or Mϕ (B) adherent to plastic, collagen, or collagen gels (Col Gel) were incubated in buffer alone (Co) or with opsonized *C. albicans* (Ca) (10^7 yeasts) for 1 h at 37°C in the absence or presence of SOD (40 μg/ml), and the production of superoxide anion was quantified by measuring the reduction of cytochrome *c*. The data are the means ± SEM of three experiments with monocytes (A) and six experiments with Mϕ (B). *, $P < 0.05$, compared to plastic (*t* test).

toxic oxygen metabolites (8, 42, 46), whereas Mϕ fungicidal activity is mediated by both oxidative and nonoxidative mechanisms. Therefore, we next sought to determine if collagen-adherent monocytes and Mϕ stimulated the production of greater amounts of superoxide anion than plastic-adherent cells. Monocytes and Mϕ were incubated for 1 h with opsonized *C. albicans* yeasts, and superoxide anion production was quantified by measuring the SOD-inhibitable reduction of cytochrome *c*. The data in Fig. 3A show that monocytes that adhered to collagen matrices, but not nongelled collagen, produced significantly more SOD-inhibitable superoxide anion than plastic-adherent monocytes. Similar results were obtained with Mϕ adherent to collagen matrices (Fig. 3B).

To determine if an enhanced respiratory burst was responsible for the increased candidacidal activity of collagen-adherent monocytes/Mϕ, the effect of respiratory burst inhibitors on monocyte/Mϕ killing of *C. albicans* was quantified. Monocytes and Mϕ adherent to plastic, collagen or collagen matrices were incubated with *Candida* yeasts in the presence or absence of SOD or catalase, and the remaining CFU were quantified after 1 h at 37°C. The fungicidal activity of plastic-adherent monocytes was partially reversed by catalase, but not SOD, but the effect of catalase was not statistically significant (Fig. 4). Likewise, the fungicidal activity of collagen-adherent monocytes was partially reversed by both SOD and catalase, but these results also were not statistically significant (Fig. 4). In identical experiments performed with Mϕ, neither SOD nor catalase had any effect on the fungicidal activity of Mϕ regardless of the substrate to which the cells adhered (data not shown).

As the production of toxic oxygen radicals did not appear to be involved in Mϕ fungicidal activity, we sought to compare the amounts of PL fusion mediated by plastic- versus collagen-adherent Mϕ upon phagocytosis of *C. albicans*. Mϕ were allowed to adhere to plastic or collagen matrices, loaded with Lysotracker red, and then incubated with viable or HK *Candida* yeasts for 1 h at 37°C. For comparison, we quantified Mϕ

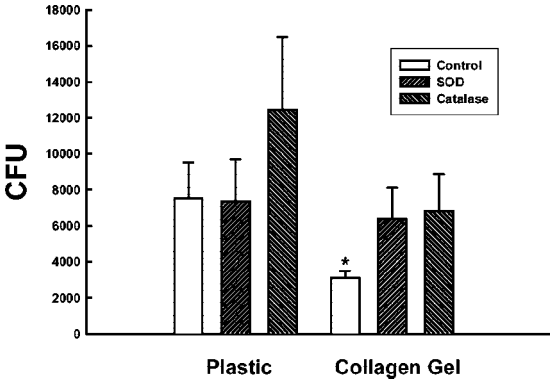

FIG. 4. Reversal of monocyte fungicidal activity by SOD and catalase. Monocytes were allowed to adhere to plastic or type 1 collagen matrices for 1 h at 37°C. After being washed, the monocytes were incubated with 2×10^4 *C. albicans* yeasts for 1 h at 37°C in the presence or absence of SOD or catalase. Remaining CFU then were quantified as described in the legend to Fig. 1. The data are the means ± SEM of five experiments. *, $P < 0.05$ compared to plastic control (Mann-Whitney rank sum test).

PL fusion after phagocytosis of viable *H. capsulatum* and *S. cerevisiae* yeasts and HK *H. capsulatum* yeasts. In Mϕ that ingested either HK *Candida* or HK *Histoplasma*, over 80% of yeast-containing phagosomes demonstrated PL fusion (Fig. 5A). Mϕ phagosomes that contained *H. capsulatum* showed about 5% PL fusion, confirming our previous data (29). Remarkably, only 10% of phagosomes containing *C. albicans* demonstrated PL fusion. There was about 70% PL fusion in Mϕ that had phagocytosed viable *S. cerevisiae*.

When Mϕ were allowed to adhere to collagen matrices, phagosomes containing either viable *C. albicans* or viable *H. capsulatum* demonstrated about 80% PL fusion (Fig. 5B). These results were confirmed for *C. albicans* with HRP-Au18. Figure 5C shows that, after 1 h of phagocytosis, plastic-adherent Mϕ containing *C. albicans* had an average of 0.58 gold particles per phagosome, whereas collagen-adherent Mϕ had an average of 2.3 gold particles per phagosome, a fourfold increase. In contrast, Mϕ that had ingested HK yeasts had an average of about 12 gold particles per phagosome, regardless of the substrate to which the cells were adherent (data not shown). These results are illustrated in Fig. 6. The top panel of Fig. 6 shows that, despite numerous gold particles nearby in the cytoplasm, no gold particles are in the yeast-containing phagosome of a Mϕ adherent to plastic. In contrast, in the bottom panel, numerous gold particles can be seen adjacent to the yeast in a Mϕ adherent to collagen.

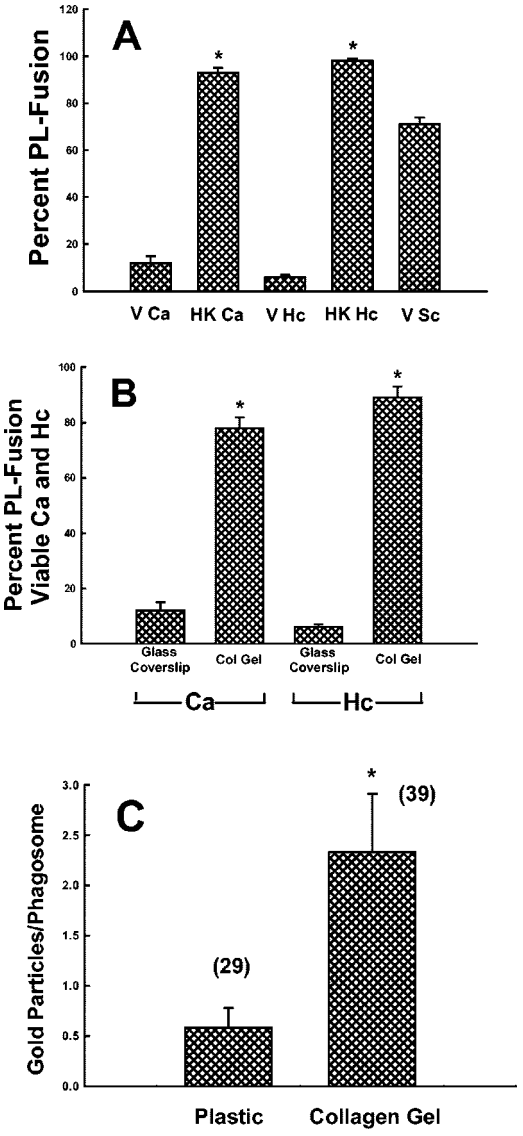

FIG. 5. Adherence of Mϕ to collagen matrices enhances PL fusion. Glass- or collagen-adherent Mϕ were loaded with Lysotracker red and then incubated for 1 h with viable (V) or HK *C. albicans* (Ca) or *H. capsulatum* (Hc) or viable *S. cerevisiae* (V Sc). The percentage of lysosomal fusion was quantified as described in Materials and Methods. The data are the means ± SEM of four to nine experiments with *C. albicans* and *H. capsulatum* and seven experiments with *S. cerevisiae*. (A) Data comparing levels of PL fusion for viable and HK *C. albicans* and *H. capsulatum* and viable *S. cerevisiae* when the Mϕ were allowed to adhere to glass coverslips. *, $P < 0.001$ compared to viable fungi (*t* test). (B) Data comparing levels of PL fusion with viable fungi for Mϕ adherent to glass coverslips versus collagen gels. *, $P < 0.001$ compared to glass-adherent Mϕ (*t* test). (C) Plastic- or collagen-adherent Mϕ were labeled with HRP-Au18 and then incubated with *C. albicans* for 1 h at 37°C. After fixation and processing for electron microscopy, the number of gold particles in yeast-containing phagosomes was quantified. The data are the means ± SEM of the numbers of gold particles per phagosome. The number above each bar on the graph denotes the number of phagosomes counted for each fungus. *, $P < 0.001$ compared to plastic (Mann-Whitney rank sum test).

DISCUSSION

Studies of murine models of disseminated candidiasis demonstrate that Mϕ play an important role in host resistance (2, 4, 33). However, in vitro, Mϕ candidacidal activity is underwhelming, particularly compared to that of neutrophils (reviewed in reference 43). Part of the explanation for the low killing capacity lies in the fact that, as monocytes differentiate into mature Mϕ, they lose the enzyme myeloperoxidase and, therefore, are not able to generate potent toxic oxygen radicals distal to H_2O_2 (24, 35). Thus, for protection of the host, Mϕ

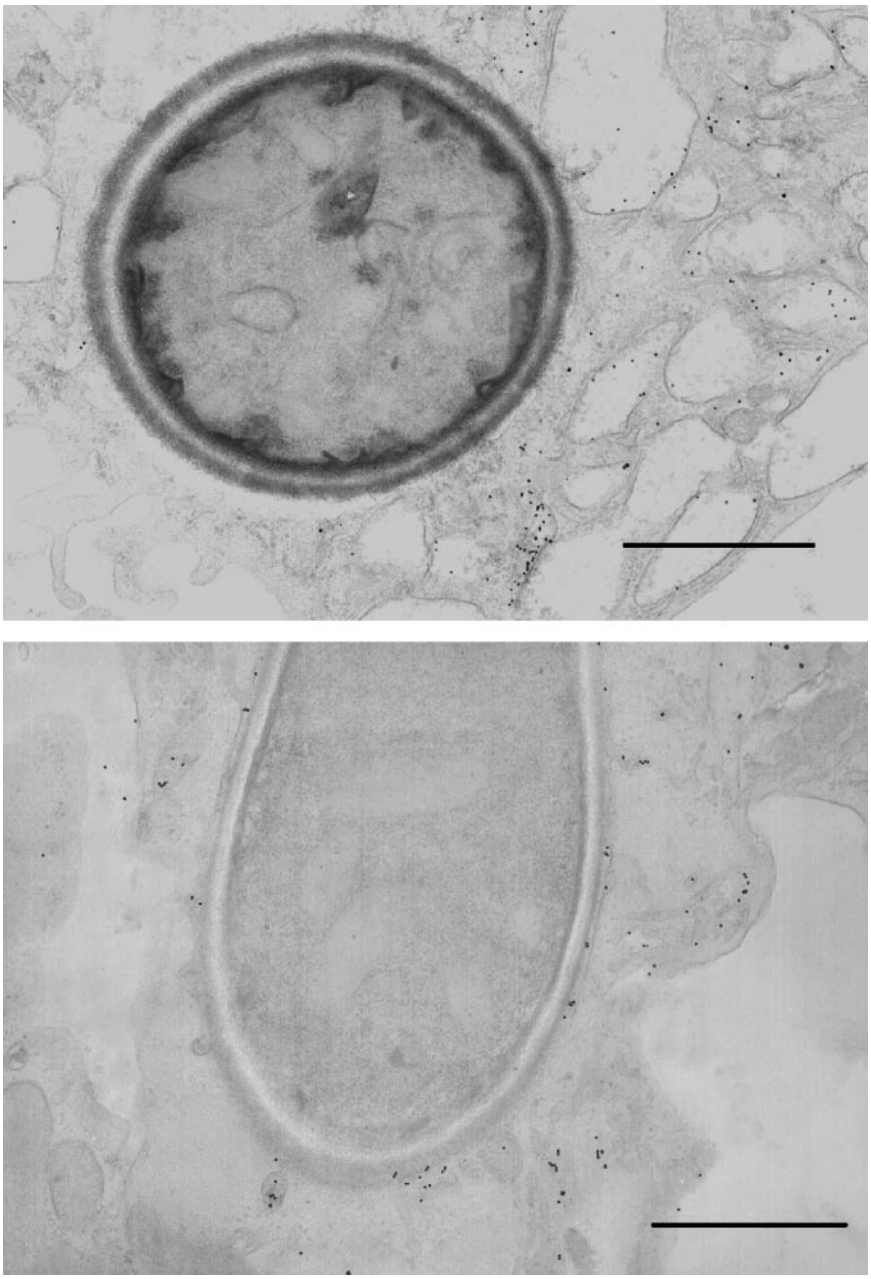

FIG. 6. Adherence of Mϕ to collagen matrices enhances PL fusion. Plastic- or collagen-adherent Mϕ were labeled with HRP-Au18 and then incubated with *C. albicans* for 1 h at 37°C and then fixed and processed for electron microscopy. (Top) Electron micrograph showing a *C. albicans* yeast within the phagosome of a Mϕ adherent to plastic. Note that there are numerous gold particles in the surrounding cytoplasm but none in the phagosome. (Bottom) Collagen-adherent Mϕ with numerous gold particles within the yeast-containing phagosome. Bars, 1 μm.

must either rely on PL fusion and lysosomal enzymes or be activated by T-cell cytokines, such as IFN-γ. However, IFN-γ alone does not turn Mϕ into potent killers of *C. albicans* (25, 26, 47).

We hypothesized that part of the inability of Mϕ to efficiently kill *C. albicans* might be caused by a deficit in PL fusion. Previously we had demonstrated that adherence of human Mϕ to type 1 collagen gels stimulated the Mϕ to kill *H. capsulatum* cells via the induction of PL fusion. Therefore, we sought to determine if adherence to collagen or other ECM proteins might enhance Mϕ candidacidal activity by the same mechanism.

Indeed, we found that both monocytes and monocyte-derived Mϕ demonstrated significantly increased candidacidal activity in cells that adhered to type 1 collagen matrices compared to cells that adhered to plastic. Activation of Mϕ occurred immediately upon adherence and did not require additional time in culture. Enhanced candidacidal activity was not observed when monocytes or Mϕ adhered to fibronectin, vitronectin, laminin, or nongelled collagen. However, when

monocytes were allowed to differentiate into Mϕ while adherent to ECM proteins, culture on vitronectin and laminin, but not fibronectin, also induced a significant increase in Mϕ candidacidal activity. We have focused here on collagen, as it was the most consistent ECM protein in enhancing Mϕ fungicidal activity.

We hypothesize that optimum Mϕ anti-*Candida* activity probably requires some combination of ECM proteins and cytokines and occurs only upon the induction of CMI. However, it also is possible that monocytes emigrating into an inflammatory site where type 1 collagen and other ECM proteins are present may exhibit sufficient fungicidal activity to help prevent or slow the dissemination of *Candida*, particularly when neutrophils are present (16). Thus, we suggest that, in the healthy human host, during the inflammatory response to *Candida* the influx of neutrophils and monocytes, in conjunction with the ECM, might be sufficient to prevent *Candida* from causing disease. Clearly, when neutrophils are removed from the equation, inflammatory monocytes alone are insufficient to prevent dissemination and disease (1, 12, 18, 20, 40).

It is generally accepted that the respiratory burst accounts for the majority of the candidacidal activity of human neutrophils, and both oxygen-dependent and -independent mechanisms have been promoted for monocyte/Mϕ candidacidal activity (reviewed in reference 43). In a previous study with human Mϕ and dendritic cells (DC), we were unable to obtain any evidence that either cell type killed *C. albicans* through the generation of toxic oxygen metabolites (30). In fact, Mϕ killing and DC killing of *Candida* were equivalent under aerobic and anaerobic conditions, confirming earlier studies with Mϕ by Thompson and Wilton (42).

The data in the present study also do not support a significant role for toxic oxygen radicals in the candidacidal activity of monocytes or Mϕ. Remarkably, we found that *Candida*-infected plastic-adherent Mϕ demonstrated very little PL fusion compared to the amount of PL fusion induced by phagocytosis of the nonpathogenic yeast *S. cerevisiae* or HK *Candida* or *Histoplasma*. In contrast, when Mϕ were allowed to adhere to collagen matrices and then infected with *C. albicans*, 80% of yeast-containing phagosomes demonstrated PL fusion, similar to that seen when the Mϕ were infected with *H. capsulatum* (29). Interestingly, and in contrast to our observations with human Mϕ, PL fusion has been reported to occur normally in mouse peritoneal Mϕ (19, 27, 48).

The fact that PL fusion is inhibited upon ingestion of *Candida* cells by Mϕ and the fact that Mϕ do not generate toxic oxygen radicals distal to H_2O_2 (24, 35) may explain why human Mϕ mediate such poor candidacidal activity. The data are surprising considering the facts that *Candida* is an opportunistic pathogen and that the capacity to inhibit PL fusion generally is considered to be a property of intracellular pathogens.

The mechanism by which *C. albicans* inhibits PL fusion is unknown. However, it is not likely to be related to signal transduction through the mannose receptor, as PL fusion occurs normally after ingestion of *S. cerevisiae* and HK *Candida*. It also is not clear if filamentation is involved in inhibition of PL fusion. Most likely, the yeasts respond to phagocytosis by secreting an unknown agent that inhibits the fusion process.

It also is unknown how Mϕ adherence to a collagen matrix stimulates the Mϕ to undergo PL fusion, overriding the negative signal from ingested *C. albicans* cells. The "very late antigen" (VLA) receptor family are cell surface glycoproteins that promote cell-matrix adhesion and belong to the integrin family of adhesion molecules (14). Of the VLA molecules that have been described, VLA-2 has been shown to be specific for collagen and VLA-3 has been shown to be specific for collagen, laminin, and fibronectin (41, 49). Of these, small amounts of only VLA-2 have been detected on human monocytes/Mϕ (15). Thus, we hypothesize that adherence to collagen provides an additional signal to the Mϕ through VLA-2 that promotes PL fusion that enhances Mϕ candidacidal activity.

However, inhibition of PL fusion seems only to be part of the strategy by which *C. albicans* survives in Mϕ. Adherence of Mϕ to collagen induced PL fusion in 80% of phagosomes containing *Candida* cells. Therefore, it is curious that Mϕ candidacidal activity was not more robust. Indeed, adherence of human Mϕ to collagen matrices leads to significant killing of *H. capsulatum* cells, which also survive intracellularly, in part, by inhibiting PL fusion (29). It is possible that the reason that Mϕ *Candida* killing on collagen is not more efficient is because the yeasts also regulate intraphagosomal pH. Thus, *C. albicans* may modulate the pH in the phagosome so that the pH is suboptimal for killing by lysosomal hydrolases, even in the presence of PL fusion. This is similar to the strategy utilized by *H. capsulatum* to survive in murine Mϕ (10, 11).

This idea is supported by studies of murine resident peritoneal Mϕ (48). In these studies, PL fusion in unactivated Mϕ was equivalent to that in IFN-γ-activated Mϕ. However, there was a major difference in levels of phagosomal acidification. Only 3% of *Candida*-infected resident peritoneal Mϕ acidified phagolysosomes to a pH of <4.0, whereas in IFN-γ-activated Mϕ, 42% of infected Mϕ demonstrated acidified phagolysosomes. Further, the enhanced candidacidal activity of IFN-γ-activated murine peritoneal Mϕ directly correlated with phagosomal acidification, but not the production of toxic oxygen radicals. Thus, *C. albicans* clearly uses more than a single strategy for intracellular survival in Mϕ and appears to use slightly different strategies to survive within human versus murine Mϕ. A better understanding of these survival strategies should help clarify the pathogenic properties of this dimorphic fungus and could possibly lead to the discovery of novel therapies.

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