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In Vitro Study of Contact-Mediated Killing of *Candida albicans* Hyphae by Activated Murine Peritoneal Macrophages in a Serum-Free Medium

TADAYO HASHIMOTO

Department of Microbiology and Immunology, Loyola University Stritch School of Medicine, Maywood, Illinois 60153

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Activated peritoneal macrophages obtained from *Listeria*-immune mice were demonstrated to kill nonphagocytosable *Candida albicans* hyphae by contact-mediated mechanisms in a serum-free synthetic medium. The actual killing of hyphae was confirmed by a microculture technique utilizing the dimorphic nature of the fungus. The most efficient candidacidal activity was demonstrated by the macrophages obtained from mice first immunized with live *Listeria monocytogenes* and then elicited with heat-killed *L. monocytogenes* cells. Resident macrophages from control mice showed only low candidacidal activity against *C. albicans* hyphae and yeast cells. Direct physical contact appeared to be required for macrophages to efficiently kill oversized *C. albicans* hyphae. Efficient in vitro killing of hyphae also required relatively high effector/target cell ratios (50 or higher). The contact-mediated candidacidal activity of activated macrophages was not significantly abrogated by oxygen-radical scavengers, suggesting the involvement of oxygen-independent mechanisms. These results suggest that the enhanced nonspecific immunity to candidiasis seen in *Listeria*-immune hosts can be attributed, at least in part, to activated fungicidal macrophages. The ability of macrophages to detect and destroy both yeast and hyphal *C. albicans* cells is clearly an important element of the host defense against candidiasis.

Human candidiasis is most frequently caused by *Candida albicans*, a fungus indigenous to the skin and mucous membrane of the host (25). Depending on the surrounding environment, *C. albicans* can grow in either yeast or hyphal form. In infected host tissues, the fungus predominantly proliferates in the hyphal form, often reaching a size that individual phagocytes cannot completely engulf (11, 12, 14). This fact suggests that host resistance to candidiasis depends to some extent on the ability of the host to detect, destroy, and eliminate both yeast and hyphal forms of *C. albicans*.

In candidiasis, phagocytes are believed to provide the primary line of defense for the host (25, 29). Among phagocytes, neutrophils are generally regarded as the predominant effector cells, especially in the early phase of candidiasis (30). Although both monocytes and macrophages have been shown to possess anti-*C. albicans* activity, their relative contribution to the overall host resistance against candidiasis is less clearly defined (30, 31). Some have observed that macrophages are mobilized to infected sites very early, even before neutrophils (32). Others have concluded that macrophages are important in the later stage of *C. albicans* infection (30).

Neutrophils have been shown to kill *C. albicans* yeast cells (1, 9, 19) and to damage hyphae (1, 9, 14). Although the hyphae of *C. albicans* (12) and *Aspergillus fumigatus* (13) have been reported to be damaged by human blood monocytes, the actual killing of hyphae has not been confirmed (12, 14). Thus, it is of interest to examine whether naturally activated macrophages from animals resistant to candidiasis can indeed kill both the yeast and hyphal forms of *C. albicans* in the absence of other host defense mechanisms.

It has been known for years that the development of specific immunity to certain intracellular bacteria, such as *Listeria monocytogenes* and *Mycobacterium bovis* BCG, often transiently enhances nonspecific resistance to *C. albicans* (2, 21), parasitic and bacterial agents (8, 20), and neoplastic cells (15). Immunization with BCG, for example, has been shown to enhance the long-term survival of mice when they were challenged with viable *C. albicans* (2). Mice immunized with live *L. monocytogenes* and then challenged with *C. albicans* intravenously were able to reduce the candidal population in the kidneys after being boosted with *L. monocytogenes* (21, 26). Since the immunity to these intracellular pathogens is primarily mediated by activated macrophages, it seems reasonable to suspect that such effector cells contribute to the elevated nonspecific immunity to candidiasis. Activated macrophages obtained from BCG-immune animals were fungicidal against *C. albicans* yeast cells (2). However, it is not known whether such activated macrophages can kill *C. albicans* hyphae.

To better understand the cellular basis of the nonspecific immunity to *C. albicans* observed in animals that are immune to intracellular bacteria, peritoneal macrophages obtained from mice previously immunized with live *L. monocytogenes* were tested for their ability to kill *C. albicans* cells. In view of the relative lack of information concerning the fate of nonphagocytosable hyphae in *Candida*-immune hosts, the main focus of this study was the interaction of activated macrophages with oversized *C. albicans* hyphae. By assessing the viability of candidal hyphae with the microculture technique (18), it was possible in the present study to demonstrate unequivocally that activated macrophages can indeed kill *C. albicans* hyphae by using oxygen-independent contact-mediated mechanisms.

MATERIALS AND METHODS

Fungal strain and cultures. *C. albicans* serotype A (ATCC 58716) was used throughout this investigation. This strain was originally isolated from a patient with disseminated candidiasis. The fungus has been maintained in the laboratory on Sabouraud-glucose agar (SGA; BBL) slants. Yeast

cells were cultured aerobically on SGA for 24 h at 37°C. *C. albicans* hyphae, uniformly 30 to 40 μm long, were produced in a synthetic tissue culture medium (Dulbecco's modified Eagle Medium [DMEM]; GIBCO) (18). Briefly, homogeneous suspensions of yeast cells were prepared in Tris-maleate buffer (0.05 M, pH 7.2) by vortexing and ultrasonic treatment (Branson ultrasonic cleaner, model 220). After the cells were washed three times in the same buffer, they were resuspended in DMEM (10^6 cells per ml) and incubated at 37°C. During incubation, cultures were gently rotated (30 rotations per min) to minimize the adherence of cells to the glass wall. Short hyphae developed from yeast cells were washed three times in Hanks balanced salt solution (GIBCO) and resuspended in DMEM at appropriate concentrations before the macrophages were infected. This procedure minimized clumping of short hyphae.

Macrophages. Peritoneal macrophages from outbred female mice (strain CD-1; Charles River) weighing 20 to 25 g were used in this investigation. Peritoneal exudate cells from mice were harvested with DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells harvested from several animals were pooled and washed twice with DMEM by centrifugation (500 × *g*, 10 min). The washed cells were resuspended in antibiotic-free DMEM, and the cell concentration was determined with a hemocytometer. Each flat-bottomed well in a culture chamber (Costar 3524) was seeded with 2×10^6 cells. In most experiments, a sterile circular coverslip (12-mm diameter, no. 1 thickness; Rochester Scientific Co.) had been placed in each well before the seeding. The determination of hyphal viability was greatly facilitated by using these coverslips. Cells were allowed to attach to coverslips or to the plastic bottoms of each well for 2 h at 37°C in 10% CO_2–90% humidified air. Then nonadherent cells were removed from the wells by aspiration, and the adherent cells were washed three times with DMEM. At this stage, the average number of adherent cells per well was approximately 10^6. Unless otherwise specified, the adherent cells were further incubated at 37°C for 18 to 24 h in 10% CO_2–90% humidified air. This additional day of incubation eliminated essentially all neutrophils remaining in macrophage preparations. Before infection with *C. albicans* cells, macrophages were washed once with fresh DMEM. When macrophages required further culturing in vitro, the medium was changed at 24-h intervals. The macrophage viability was routinely determined by trypan blue dye (0.3%) exclusion, and the purity of the macrophage population was examined microscopically after the coverslips were stained with the Diff-Quick-system (Harleco). The procedure used in this study consistently yielded highly pure macrophage populations (96 to 98%). The majority of contaminating cells were fibroblasts, and very few, if any, neutrophils (<1.0%) were detected in macrophages preparations.

Preparation of *L. monocytogenes*-immune macrophages. Mice were immunized with *L. monocytogenes* as described by Harrington-Fowler et al. (17). Briefly, mice were injected intraperitoneally with a single sublethal dose (10^4 cells) of viable *L. monocytogenes* (strain A4413, serotype 4b), obtained from M. Wilder, and elicited 6 to 9 days later by the intraperitoneal injection of 10^7 heat-killed *L. monocytogenes* cells. Macrophages were harvested from the peritoneal cavity 18 h after elicitation as described above. In some experiments, macrophages were obtained from *Listeria*-immune mice by eliciting with DMEM instead of heat-killed bacteria. Where specified, macrophages were obtained from control (*Listeria*-nonimmune) mice by eliciting with heat-killed *L. monocytogenes* (10^7 cells). Resident macrophages were routinely obtained from animals that had not been subjected to any prior immunization.

In vitro interaction of *C. albicans* hyphae with macrophages. Tissue culture wells containing approximately 10^6 macrophages per well were inoculated with the predetermined numbers of freshly prepared *C. albicans* hyphae or yeast cells and incubated at 37°C in 10% CO_2–90% moist air for specified periods. Based on our initial data (see Result), an effector/target cell (E/T) ratio of 50 was routinely used in most experiments. To test whether direct physical contact of macrophages was required for killing target cells extracellularly, microchambers consisting of two Millipore filters (pore size, 0.2 μm) sandwiching a plastic ring (11 mm in diameter, 2 mm thick, Millipore catalog no. PR0001401) were used. Through a minute hole on the side of the plastic ring, *C. albicans* hyphae were injected into the microchamber, and the inoculation hole was immediately sealed with plastic cement. The whole microchamber was submerged into a macrophage culture well and incubated as described above. Hyphae placed inside this microchamber had free access to the outside medium during incubation, but direct contact with macrophages was prevented because of the pore size. Spent media were obtained by filter sterilizing macrophage culture (24 h) and *Candida*-macrophage coculture (4 h) supernatants. The candidacidal activity of spent media was tested by incubating 10^4 *C. albicans* hyphal cells in each well containing 1 ml of test medium.

Effects of oxygen-radical scavengers on contact-mediated killing of *C. albicans* hyphae by macrophages. To test whether contact-mediated killing of *C. albicans* hyphae by macrophages depends on oxygen metabolites, mixed cultures were incubated in the presence of bovine liver catalase (Miles Laboratories) and bovine erythrocyte superoxide dismutase (Miles). The enzymes were used without further purification. The specified amounts of each enzyme were supplemented to macrophage cultures 10 min before or at the time of inoculation of *C. albicans* hyphae.

Quantitative determination of the viability of *C. albicans* hyphae damaged by macrophages. The viability of individual *C. albicans* hyphae exposed to macrophages was determined by the microculture method described earlier (18, 22). With this method the ability of viable hyphae to develop buds and form microcolonies could be determined. Briefly, coverslips retrieved from culture wells were first soaked in Sabouraud-glucose broth supplemented with 0.25% Nonidet P-40 detergent (Sigma) for 10 s and then rinsed gently in Sabouraud-glucose broth. This procedure does not wash away any significant amount (<0.01%) of hyphae from the coverslips (18). After the excess liquid was removed, each coverslip was placed on the surface of microculture agar (SGA) so that *C. albicans* hyphae adhering to the glass surface were sandwiched between the coverslip and the agar. Millipore filters carefully recovered from microchambers were similarly treated. Microcultures were placed in the moist chamber and incubated aerobically at 25 to 28°C for 6 to 8 h. These cultural conditions were highly conducive to yeast-form growth of *C. albicans*. Any hypha not initiating progeny yeast cell formation within 6 h of incubation on SGA almost always failed to reproduce even when the incubation time was extended to 24 h. The ability of each hypha to form a yeast microcolony was determined microscopically. All tests were performed in duplicate, and 200 hyphae were routinely examined for each sample.

Determination of viability of *C. albicans* yeast cells phagocytosed by macrophages. To test the ability of activated macrophages to kill *C. albicans* yeast cells by phagocytosis,

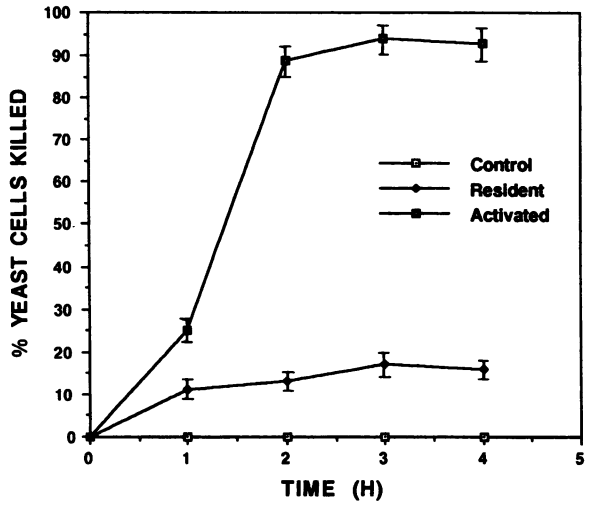

FIG. 1. Killing of *C. albicans* yeast cells by resident and activated murine peritoneal macrophages (E/T ratio of 50). The viability of *C. albicans* cells was determined by the conventional colony count method. Essentially all *C. albicans* yeast cells were phagocytosed within the first hour. No *C. albicans* cells were killed in the absence of macrophages (control). Each data point represents the mean ± SEM for six samples (duplicate samples in three independent experiments).

2×10^4 yeast cells were inoculated to each well containing 1×10^6 macrophages (E/T ratio of 50) and incubated at 37°C in 10% CO_2–90% humidified air. After predetermined intervals, mixed cultures were harvested by aspiration and culture wells were repeatedly washed with sterile distilled water. Each well was made to a final volume of 10 ml. The number of viable *C. albicans* cells remaining in each well was determined by plating 0.1 ml of appropriately diluted samples on SGA plates. All plates were incubated aerobically at 30°C for 48 h before colonies were counted.

Microscopy. Macrophages interacting with *C. albicans* hyphae in Costar wells were observed under a phase-contrast microscope. Micrographs were taken with a Nikon camera and Tri-X film (Kodak).

Statistical analysis. Data are presented as the means ± standard error of the means (SEM). Where appropriate, the statistical significance between means was assessed by using the Student *t* test.

RESULTS

Activity of activated macrophages against *L. monocytogenes* and yeast-form *C. albicans*. As previously reported (17), peritoneal macrophages obtained from mice immunized first with live *L. monocytogenes* and then elicited with heat-killed *L. monocytogenes* cells (referred to as activated macrophages) exhibited all of the morphological characteristics associated with the activated state: increased size, ruffles, abundant lysosomal granules, and vacuoles. The macrophages activated by live *Listeria* immunization followed by heat-killed *Listeria* elicitation have been shown to be highly listericidal (17). The listericidal activity of the activated macrophages was confirmed in our preliminary experiments (data not shown). These activated macrophages were found to phagocytose and kill *C. albicans* yeast cells very efficiently (Fig. 1). The majority of *C. albicans* yeast cells phagocytosed by activated macrophages appeared to be killed before even developing germ tubes. Resident macrophages, on the other hand, phagocytosed yeast cells but could not kill them as effectively as activated macrophages (Fig. 1). Most *C. albicans* yeast cells phagocytosed by resident macrophages transformed to hyphae, eventually disrupting phagocytes from the inside (data not shown). These observations confirmed that activated macrophages, but not resident macrophages, are highly efficient effector cells against yeast-form cells of *C. albicans*.

Microscopic observation of surface interactions between macrophages and *C. albicans* hyphae. To assess the morphological changes occurring in macrophages and *C. albicans* hyphae during interaction, samples taken at various time intervals were observed under the phase-contrast microscope. Macrophages maintained in DMEM formed a monolayer and were uniformly dispersed. Upon infection with *C. albicans*, several or more macrophages migrating from adjacent areas established contact with a target hypha and rapidly spread over the hyphal surface, usually within 30 min (Fig. 2). When the E/T ratios were high, visualization of entire *C. albicans* hyphae with a microscope was often hampered by macrophages clustering over single hyphae. Such hyphae almost always ceased to elongate, eventually showing some irregularity or deformation of cell shape (Fig. 2). At lower E/T ratios, which were not fungicidal (see below), *C. albicans* hyphae attracted a few macrophages but continued to elongate, eventually overwhelming the macrophages. Although capable of migration and adherence, resident macrophages could suppress the elongation of only 10 to 20% of *C. albicans* hyphae even at high E/T ratios (data not shown).

Effect of E/T ratio on extracellular killing of *C. albicans* by activated macrophages. Activated macrophages were found to kill *C. albicans* hyphae very effectively. Since all hyphae infecting macrophages were nonphagocytosable because of their size, they are believed to have been killed by nonphagocytic mechanisms. The efficiency of such extracellular killing was heavily dependent on the E/T ratios used in the experiments (Fig. 3). At E/T ratios of 50 or higher, the majority of *C. albicans* hyphae were killed within 3 h of interaction. Although some hyphae were killed at lower E/T ratios (less than 50), surviving *C. albicans* cells rapidly overwhelmed and killed the surrounding macrophages. Only 10 to 20% of *C. albicans* hyphae were killed by resident macrophages even at high E/T ratios (Fig. 3). No natural death of *C. albicans* hyphae occurred during this incubation time in the absence of effector cells. Based on this observation, all subsequent experiments were performed with an E/T ratio of 50.

Kinetics of extracellular killing of *C. albicans* hyphae by activated macrophages. As shown in Fig. 4, activated macrophages could damage and kill *C. albicans* hyphae maximally within a few hours provided sufficiently high E/T ratios were used. Resident macrophages were far less inefficient in extracellular candidacidal activity under the identical experimental conditions (Fig. 4). Although the kinetic data indicate that maximal killing of *C. albicans* hyphae by activated macrophages required 3 h, there were indications that some nonlethal damage may have occurred in hyphae much earlier. When examined under the microscope, hyphae exposed to resident macrophages for 60 min showed the first sign of bud formation within 2 h of incubation on the viability test medium. The majority of hyphae sampled at the early stage of interaction (60 min) required 3 to 4 h longer than control hyphae to initiate progeny yeast formation on the same medium. This strongly suggests that some incipient early cell

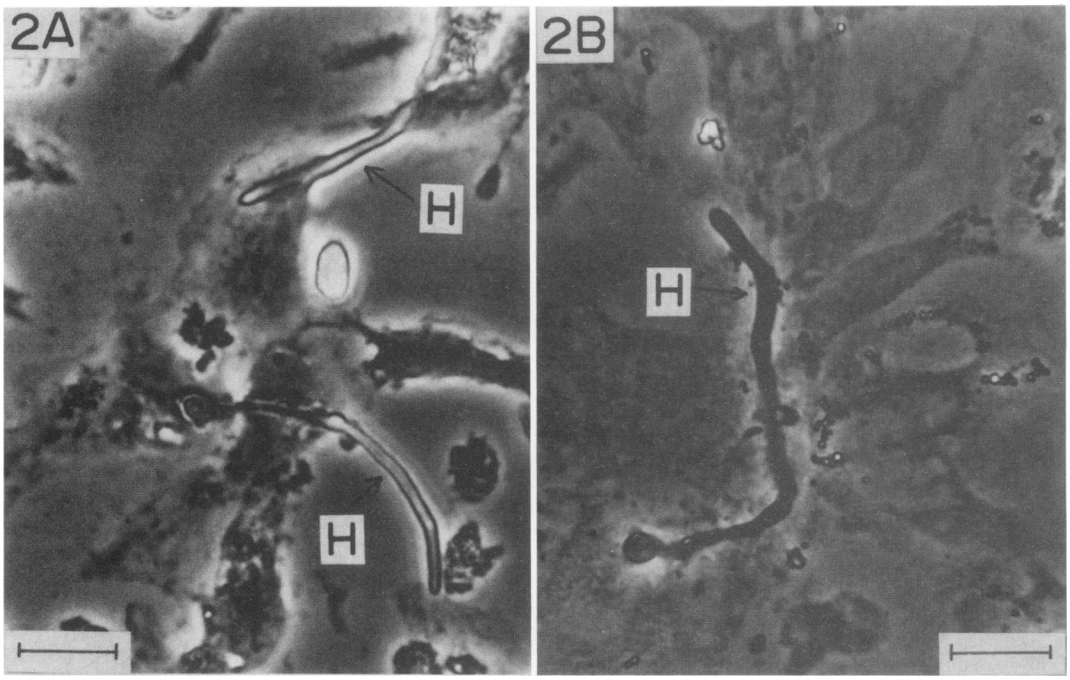

FIG. 2. Phase-contrast micrographs of *C. albicans* hyphae cocultured with activated macrophages (E/T ratio of 50) for 30 min (A) and 3 h (B). Note some changes in refractility and shape of hyphae (H) due to interaction with adhered activated macrophages. Control hyphae continued to elongate, showing no such morphological changes (data not shown). Bars, 10 μm.

injuries had been sustained by hyphae as a result of their interaction with activated macrophages. However, this nonlethal damage of hyphae was not systematically assessed in this study.

Effect of elicitation methods on extracellular candidacidal activity of macrophages. As summarized in Fig. 5, the highest candidacidal activity was demonstrated by the macrophages obtained from *Listeria*-immune mice elicited with listerial antigens (heat-killed *L. monocytogenes* cells). When *Listeria*-immune mice were elicited with DMEM instead of listerial antigens, the resultant macrophages were significantly less candidacidal than were those elicited with heat-killed *L. monocytogenes* cells (bar C in Fig. 5). Regardless of the elicitation methods used, a low degree of anticandidal activity was detected in the resident macrophages obtained from nonimmune control mice (bars A and B in Fig. 5). The capacity of activated macrophages to kill *C. albicans* hyphae waned rather rapidly when the cells were cultured in the synthetic medium (Fig. 6).

Requirement for physical contact for extracellular killing of hyphae by activated macrophages. When *C. albicans* hyphae were physically separated from activated macrophages during interaction, no killing of hyphae was noted regardless of the E/T ratio (1 to 100) used. The spent medium obtained from pure macrophage cultures or cocultures of *Candida* and macrophage showed no discernible candidacidal activities. These observations suggest that direct physical contact is required for extracellular killing of *C. albicans* hyphae by activated macrophages. However, this does not preclude the possibility that under some circumstances direct contact is not required.

Effects of oxygen-radical scavengers on extracellular killing of *C. albicans* hyphae by activated macrophages. To test whether oxygen metabolites are involved in the contact-mediated killing of *C. albicans* hyphae by activated macro-

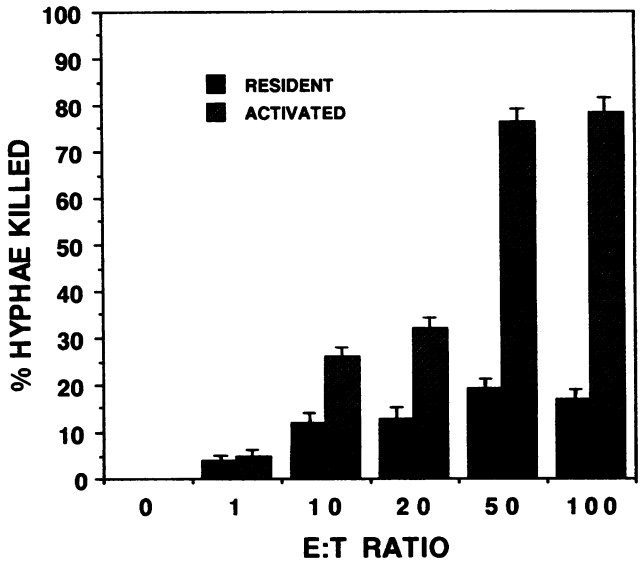

FIG. 3. Effects of E/T ratios on the contact-mediated killing of *C. albicans* hyphae. To a constant number of resident or activated macrophages (10^6 cells), various numbers of *C. albicans* hyphae were added and incubated for 3 h as described in Materials and Methods. Killing rates were calculated from the values obtained by the microculture method (18). Each bar represents the mean ± SEM for five to six samples (duplicate samples in three independent experiments, except for the resident control in the experiment with an E/T ratio of 100, where duplicates of two separate experiments and a single sample of one experiment were used).

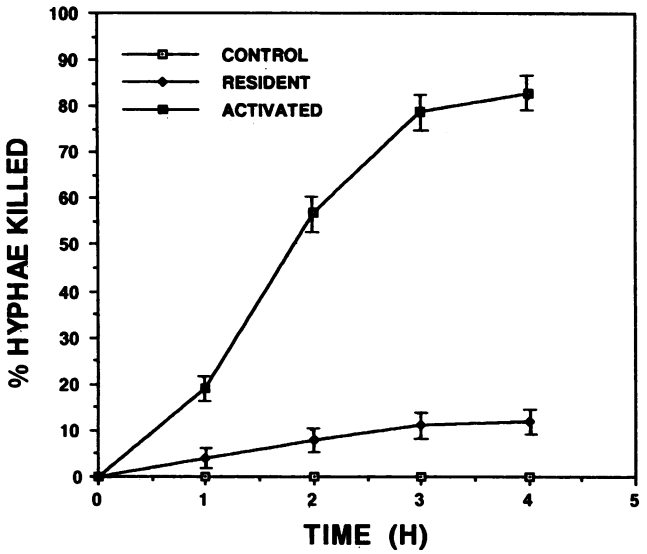

FIG. 4. Kinetics of contact-mediated killing of *C. albicans* hyphae by resident and activated macrophages (E/T ratio of 50). The mixed cultures were allowed to interact in vitro for 4 h as specified in Materials and Methods. Maximum killing was consistently achieved after 3 h of incubation. Each data point represents the mean ± SEM for six samples (duplicate samples in three independent experiments).

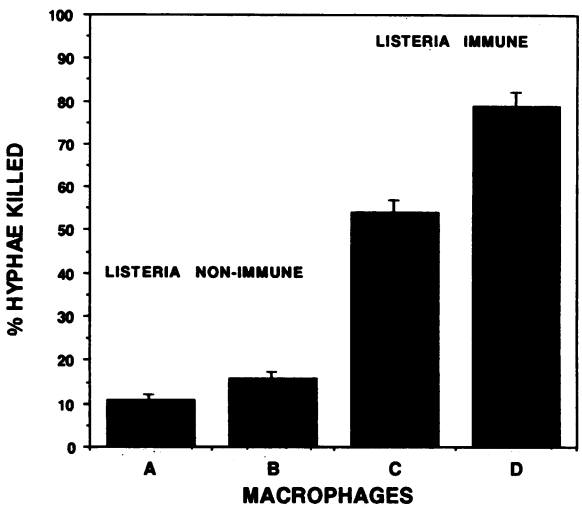

FIG. 5. Killing of *C. albicans* hyphae by peritoneal macrophages (E/T ratio of 50) obtained from control (nonimmune) and *Listeria*-immune mice with different elicitation methods. Bars: A, resident macrophages from control mice elicited with DMEM; B, resident macrophages from nonimmune mice elicited with heat-killed *L. monocytogenes* cells; C, activated macrophages from *Listeria*-immune mice elicited with DMEM; D, activated macrophages from *Listeria*-immune mice elicited with heat-killed *L. monocytogenes* cells. The interaction time was 3 h as specified in Materials and Methods. The data are expressed as the means ± SEM for five or six samples (duplicate samples in three independent experiments except for B, in which duplicate samples in two separate experiments and a single sample in one experiment were used).

phages, relatively high doses of both catalase and superoxide dismutase were added to the *Candida*-macrophage interaction system. When both of these oxygen-radical scavengers were added to the macrophage cultures before (10 min) or simultaneously with the inoculation of *C. albicans* hyphae, they had no drastic effect on extracellular candidacidal activity (Fig. 7). Active and heat-inactivated enzymes had no discernible effects on *C. albicans* growth in vitro (data not shown).

DISCUSSION

The focus of this study was to examine the extracellular candidacidal capability of macrophages to assess their role in host resistance to candidiasis. The results clearly show that activated macrophages derived from the peritoneal cavity of mice immunized with live *L. monocytogenes* can kill both the yeast and hyphal forms of *C. albicans* very effectively. Since no comparably high candidacidal activity was demonstrated by resident macrophages even at high E/T ratios (Fig. 1 and 4), it may be concluded that the augmented candidacidal activity demonstrated by activated macrophages is an expression of nonspecific immunity resulting from the specific immunization against *L. monocytogenes*. These results may account for the elevated immunity to *C. albicans* infections reported earlier for *Listeria*-immune animals (21, 26). The transient nature of the nonspecific immunity to candidiasis in *Listeria*-immune animals (20) is also consistent with the results obtained in this study (Fig. 6).

The present results are probably the first unequivocal demonstration of contact-mediated killing of *C. albicans* hyphae by macrophages. Previously, *C. albicans* pseudohyphae and hyphae were shown to be damaged by neutrophils (1, 9, 14) and human monocytes (12). The death of the damaged hyphae was inferred from the electron micrographs (13, 14), although the actual killing of hyphae could not be confirmed. Radiolabeling techniques also show that other fungi (5, 6, 13, 16) are damaged by extracellular mechanisms of phagocytes. The propensity of hyphae to clump in suspensions has rendered the routine use of the conventional colony count technique unfeasible for hyphal viability deter-

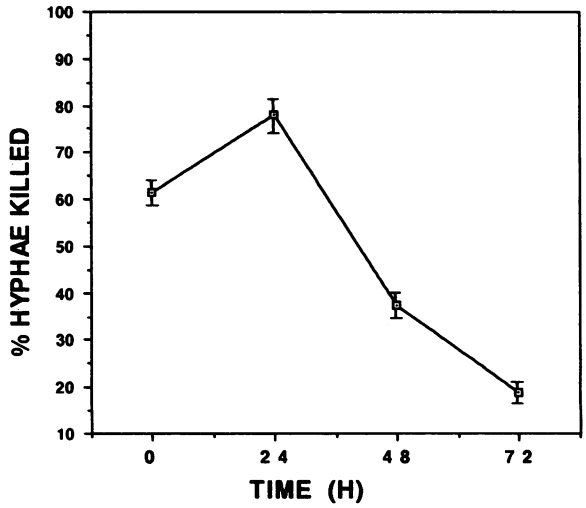

FIG. 6. Effect of in vitro cultivation of activated macrophages on the extracellular killing activity against *C. albicans* hyphae. Serum-free medium (DMEM) was changed at 24-h intervals. In all experiments, an E/T ratio of approximately 50 and interaction time of 3 h were used. The data for each group represent the means ± SEM for six samples (duplicate samples from three separate experiments).

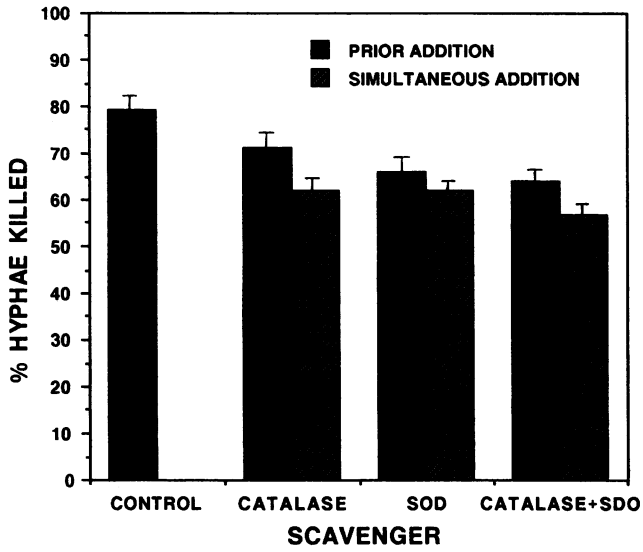

FIG. 7. Effects of selected oxygen-radical scavengers on the contact-mediated killing of *C. albicans* hyphae by activated macrophages (E/T ratio of 50). Catalase (final concentration, 27,000 U/ml) and superoxide dismutase (final concentration, 5,500 U/ml) were added to activated macrophage cultures 10 min before or simultaneously with the inoculation of *C. albicans* hyphae and incubated for 3 h. No scavengers were included in the control cultures. Data are expressed as the means ± SEM of six samples (duplicate samples in three independent experiments).

mination (1, 12–14). Radiolabeling techniques, although convenient and readily accessible, do not allow viability determination. Although the microculture technique is somewhat tedious and time consuming, it can be used to determine hyphal viability (18).

The demonstration in this study that activated macrophages from animals with elevated resistance to candidiasis (21, 26) can kill candidal yeast and hyphal cells strongly argues for their active role in resistance against *C. albicans* infection. This argument is further supported by a recent study showing activation of macrophages for candidacidal activity in animals infected with an avirulent strain of *C. albicans* (4). As to the exact timing of involvement of activated macrophages, it may all depend on the site of *C. albicans* infection. Some reported early involvement (32) and the others observed later involvement (19) of macrophages in *C. albicans* infections. These apparent discrepancies can be reconciled when the existence of two different macrophage activation systems is recognized. Recent evidence indicates that macrophage activation can occur in distinctly different routes during *L. monocytogenes* infection. One activation route mediated by T cell-independent mechanisms occurs early (24), whereas the other depends on T cell-dependent mechanisms and comes into play at a later stage of the infection (3). It is quite probable that both of the activation mechanisms also work in animals infected with *C. albicans*. If infection occurs at a site where the T cell-independent activation system is available, macrophages could become the primary effector cells even in the early phase of candidiasis. So far, the candidacidal activity of macrophages activated at an early stage of listeriosis has not been tested.

It is remarkable that chemotactic migration, adherence, and subsequent contact-mediated killing of *C. albicans* hyphae by activated macrophages could occur effectively in the serum-free synthetic medium (DMEM). Human neutrophils (14) and murine macrophages (23) do not require serum for their interaction with *C. albicans* hyphae or yeast cells. In the presence of serum, however, attachment and phagocytosis are significantly augmented (23). The fact that the effective contact-mediated killing of *C. albicans* hyphae in vitro required relatively high E/T ratios (Fig. 3) may argue against the active role of macrophages in the control of candidal hyphae in vivo. One possible reason why high E/T ratios were required in vitro is that massive early assault on hyphae by macrophages is necessary to arrest fungal growth (9) and prevent accumulation of cytotoxic metabolites or cell constituents (10) in the medium. Another reason may be that macrophages may function more efficiently in vivo, where serum and other ingredients of host defense machineries are readily available.

In vitro killing of nonphagocytosable *C. albicans* hyphae by activated macrophages appears to require direct physical contact between effector and target cells. Since spent media obtained from the supernatants of macrophage cultures or *Candida*-macrophage cocultures failed to kill any *C. albicans* hyphae, the candidacidal principle(s) released by activated macrophages is mostly confined to poorly diffusible interface spaces between target and effector cells. An electron microscopic study is in progress to study this aspect of *Candida* hypha-macrophage interactions.

The apparent inability of oxygen-radical scavengers to fully abrogate the candidacidal activity (Fig. 7) is consistent with the idea that oxygen-independent mechanisms are predominantly responsible for the contact-mediated killing of *C. albicans* hyphae by activated macrophages. Since the active enzymes were added to macrophage cultures 10 min before or simultaneously with hyphal infection, it is very unlikely that the scavengers were excluded from the interface space between target and effector cells. Thus, it appears that macrophages use both oxygen-dependent mechanisms (7, 27, 28) and oxygen-independent mechanisms (31; this study) in killing *C. albicans* cells.

Gamma interferon plays a major role in the activation of macrophages. In mice infected with *L. monocytogenes*, the development of activated macrophages is attributed to gamma interferon produced by both early T cell-independent (24) and late T cell-dependent (3) mechanisms. Judging from the protocols used in present study, it is most likely that gamma interferon produced by a T cell-dependent mechanism is primarily responsible for the development of candidacidal macrophages. Recently, researchers in several laboratories have reported successful in vitro activation by gamma interferon of macrophages for candidacidal activity (5, 7, 30, 31), although the ability of these macrophages to kill oversized *C. albicans* hyphae has not been tested.

Much still remains to be learned about the nature of the nonspecific candidacidal mechanisms of activated macrophages. The elucidation of such mechanisms is undoubtedly important for clearer understanding host resistance to candidiasis and better management of infected patients.

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