

MACROPHAGE MICROBICIDAL ACTIVITY

Correlation between Phagocytosis-associated Oxidative Metabolism and the Killing of *Candida* by Macrophages*

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The mechanism by which polymorphonuclear neutrophils kill most microorganisms includes conversion of oxygen by the cell to superoxide anion (O_2^-),¹ hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and, possibly, singlet oxygen (1). These toxic species kill ingested organisms, presumably through oxidation reactions and in conjunction with the contents of lysosomal granules. The microbicidal mechanisms of macrophages are still obscure (2). However, mouse peritoneal macrophages have been shown to respond to phagocytosis or plasma membrane perturbation with the vigorous release of H_2O_2 (3) and O_2^- (4), and it has been suggested that the microbicidal mechanisms of macrophages may be similar to those of the neutrophil, at least for some organisms (2-5). We report here that macrophages elicited by injection of lipopolysaccharide (LPS) or obtained from animals infected with bacillus Calmette-Guérin (BCG), shown previously to release greater amounts of O_2^- and H_2O_2 when stimulated, killed *Candida* better than did resident cells. The killing of both *Candida albicans* and *Candida parapsilosis* was inhibited by scavengers of oxygen radicals, suggesting that oxygen metabolites play an important role in macrophage candidicidal activity. We also report that *C. parapsilosis*, which was killed more effectively than *C. albicans*, elicited a greater oxidative metabolic response from macrophages.

Materials and Methods

Macrophages. Mouse peritoneal macrophages were harvested as previously described (4). Approximately 10^6 washed cells in 1 ml of medium supplemented with 20% heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.) (4) were plated on 16-mm diameter tissue culture dishes (Costar, Data Packaging, Cambridge, Mass.) or, in phagocytosis experiments, on 13-mm diameter glass cover slips. After incubation for 120 min at 37°C in 5% CO_2 -95% air, plated cells were washed vigorously with medium twice, then cultured in medium with 20% FCS, penicillin, and streptomycin (4). After overnight in culture, adherent cells were washed with vigorous swirling with Hanks' balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, N. Y.) before assay. Cultures of the murine cell line J774.1 and peritoneal macrophages elicited with LPS or removed from BCG-infected mice were obtained and processed as previously described. (4)

* Supported by U. S. Public Health Service grant AI 14148 from the National Institute of Allergy and Infectious Diseases.

¹ *Abbreviations used in this paper:* BCG, bacille Calmette-Guérin; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; H_2O_2 , hydrogen peroxide; LPS, lipopolysaccharide; O_2^- , superoxide anion; $\cdot OH$, hydroxyl radical; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

With all types of peritoneal exudates, cells adherent after overnight culture were >97% macrophages and <1% granulocytes, as estimated by differential counts of stained cells (4) and by phagocytic capacity for candida. The number of macrophages adherent at the beginning of all assays was $3-4 \times 10^6$, as estimated by counting adherent cells in at least five representative fields (4). For candidacidal assays, different numbers of peritoneal cells were plated, and the number of adherent cells was determined by subtraction of the cells that were nonadherent after 2 h and overnight in culture from the total cells added to the dish. This result was then checked by counting cells in representative fields, and dishes with equivalent numbers of adherent cells were chosen for the assay.

Candida. C. albicans (ATCC 18804) and *C. parapsilosis* (ATCC 22019) were cultured in 2% Sabouraud's dextrose broth (Difco Laboratories, Detroit, Mich.) at 30°C for 4-5 d for *C. albicans* and 2-3 days for *C. parapsilosis*. The fungi were centrifuged, washed twice with Dulbecco's phosphate-buffered saline (PBS) (Grand Island Biological Co.), suspended to a concentration of $2-4 \times 10^7$ /ml, and sonicated for 25 s at a power setting of 1 (microtip; Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) in order to disperse aggregated organisms. Viability was determined by exclusion of methylene blue (Fisher Scientific Co., Pittsburgh, Pa.), 0.005% (6), and the number of candida was determined by counting in a hemocytometer. Viability was always >98%.

Phagocytosis of Candida. Phagocytosis was studied using macrophages plated overnight on cover slips. The assay was started by the addition to washed cells of suspensions of 1×10^5 or 1×10^6 candida in 1 ml HBSS, with or without 20% heated or fresh FCS. After incubation at 37°C in 5% CO₂-95% air, cover slips were washed in HBSS, dried, and stained with Wright's and May-Giemsa stains. Approximately 95% of cell-associated candida could be seen within a detectable phagocytic vacuole with any macrophage type. The macrophage density on the culture dishes was low. Therefore, all candida fully within the macrophage cell boundaries were considered to be engulfed. At least 200 macrophages were studied with each variable in each experiment.

Phagocytosis was also studied using candida stained with fluorescein isothiocyanate (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) at pH 9.5 (7). The number of fluorescent fungi was counted immediately before and after addition of crystal violet (Fisher Scientific Co.), 0.5 mg/ml, which extinguished fluorescence of extracellular but not phagocytosed organisms. Results with this and the histochemical assay were equivalent.

Phagocytic Candidacidal Activity. Macrophages that had been cultured overnight were washed vigorously, and the wash medium was replaced with a 1 ml suspension of 1×10^5 candida in HBSS-20% fresh FCS. In some experiments superoxide dismutase (SOD), (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.), catalase, sodium azide, or mannitol (all from Sigma Chemical Co., St. Louis, Mo.), or sodium benzoate (Fisher Scientific Co.) were added at the beginning of incubation. Catalase was further purified by gel filtration (8); after filtration its activity was 61,000 Sigma U/mg (9). SOD was free of catalase activity (9).

After incubation at 37°C in 5% CO₂-95% air, the culture plates were placed on ice and promptly sonicated for 30 s at power setting 1. The sonicated suspensions were diluted 1:100 with PBS, and 0.1 ml was incubated at 37°C in a pour plate made with 1% purified agar-2% Sabouraud's dextrose broth. The number of colonies formed (generally 20-100/100-mm diameter plate) was counted after incubation for 48 h. Each reaction was run in triplicate. For each candidacidal experiment it was determined with stained cell preparations that all candida were ingested by 60 min of incubation. In early comparative experiments, counts of viable organisms obtained by dye exclusion (6) were consistently about 15% higher than those obtained by counting colonies, as previously reported (6).

At a ratio of macrophages to candida of 3-4:1, used in candidacidal assays, >90% of *C. albicans* and >95% *C. parapsilosis* remained in the yeast form after 120 min in culture (means of four experiments). At a ratio of macrophages to candida of 0.3-0.4:1, used as noted in Results, >97% of either species remained as yeasts for 60 min. After 120 min, 4% of *C. parapsilosis* and approximately 18% of *C. albicans* had formed incipient buds or pseudohyphae in cultures with LPS-elicited and resident macrophages ($n = 4$).

O₂⁻ Release. This assay was performed as previously described (4), except that candida were used as the stimulus for O₂⁻ release, and the 80 μM ferricytochrome *c* and candida were added

in a volume of 1 ml. Each reaction was run in duplicate. Cytochrome reduction in the system was consistently inhibited completely by SOD, 30 $\mu\text{g}/\text{ml}$, but not by autoclaved SOD. There was no significant release of O_2^- in the absence of candida; candida alone did not reduce cytochrome within 60 min, by which time all were phagocytosed. The extent of O_2^- release was expressed in relationship to the mean protein content of three culture plates incubated without candida, using the method of Lowry et al. (10) with albumin as standard (4).

Oxygen Consumption. LPS-elicited peritoneal cells were washed with PBS twice, after contaminating erythrocytes were eliminated by hypotonic lysis. Oxygen consumption during phagocytosis by suspended cells was measured at 37°C by a polarographic technique employing a Clark membrane electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The 3-ml phagocytic mixture contained 1×10^7 macrophages, 2×10^7 candida, and 2 mM KCN (Fisher Scientific Co.) in Krebs-Ringer phosphate buffer with 11 mM dextrose. Counts of Wright's and May-Giemsa-stained smears of the cell suspensions used showed a mean of 54% macrophages, 35% lymphocytes, and 11% granulocytes. Mouse granulocyte preparations obtained by peritoneal lavage 24 h after intraperitoneal injection of LPS contained a mean of 88% granulocytes, 5% macrophages, and 7% lymphocytes.

SOD and Catalase Activity in Candida. Suspensions of 10^9 candida in 10 ml of 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA (Sigma Chemical Co.), were kept in an ethanol-ice bath and sonicated four times at power setting 1 for 5 min each, with cooling for 3 min in-between. This lysed virtually all organisms, as determined by stained smears. The supernate after centrifugation at 1,000 g was centrifuged for 60 min at 100,000 g . This second supernate was assayed for SOD activity in the presence of 10^{-5} M KCN to eliminate cytochrome c oxidase activity (11), and for catalase (9), cytochrome c oxidase (12), and protein (10). Cytochrome c oxidase activities in extracts of *C. albicans* and *C. parapsilosis* were 8.1 and 4.6 nmol cytochrome c oxidized/mg/min, respectively. This activity was abolished by 10^{-5} M KCN.

Candidacidal Activity of Oxygen Metabolites. Candida were suspended in 0.05 M potassium phosphate buffer with 0.1 mM EDTA, pH 7.0, or in 0.05 M sodium carbonate buffer with EDTA, pH 10.0 (13, 14). The 1 ml reaction mixture contained 10^6 candida and 25 mM acetaldehyde (Eastman Kodak Co., Rochester, N. Y.) that had been distilled previously. The pH 7 reaction mixture contained 25 $\mu\text{g}/\text{ml}$ xanthine oxidase (grade I; Sigma Chemical Co.), and the incubation time with candida was 2.5 h at 37°C in a shaking water bath. The pH 10 mixture contained 5 $\mu\text{g}/\text{ml}$ xanthine oxidase, and the incubation time was 60 min. The killing of candida by H_2O_2 was studied with fungi suspended in 0.05 M potassium phosphate buffer, pH 7.0, and incubated at 37°C in a shaking water bath for 60 min. The number of viable candida was determined by the pour plate technique (above).

Data Presentation. Unless otherwise noted, data is expressed as mean \pm SEM, and statistical analysis used a paired t test.

Results

Candidacidal Activity of Macrophages. Macrophage killing of candida was studied using *C. albicans* and *C. parapsilosis* because the mechanisms by which these two species are killed by neutrophils and monocytes have been studied extensively (6, 15, 16). As shown in Fig. 1, *C. albicans* was not easily killed by the macrophages, in agreement with the demonstration of only 25–36% killing of *C. albicans* by isolated human monocytes and neutrophils (16, 17). However, LPS-elicited and BCG-activated macrophages killed two to three times more *C. albicans* than did resident cells at each time point studied ($P < 0.001$ for each at both 2 and 3 h). All three groups of macrophages killed *C. parapsilosis* better than *C. albicans*. Differences in the killing of *C. parapsilosis* by the different macrophage types were small, and obvious only after incubation for 3 h.

Cells of the macrophage-like line J774.1, adherent to culture dishes, ingested both species of candida completely by 60 min at a ratio of cells to candida of 5:1. Although

MACROPHAGE CANDIDACIDAL ACTIVITY

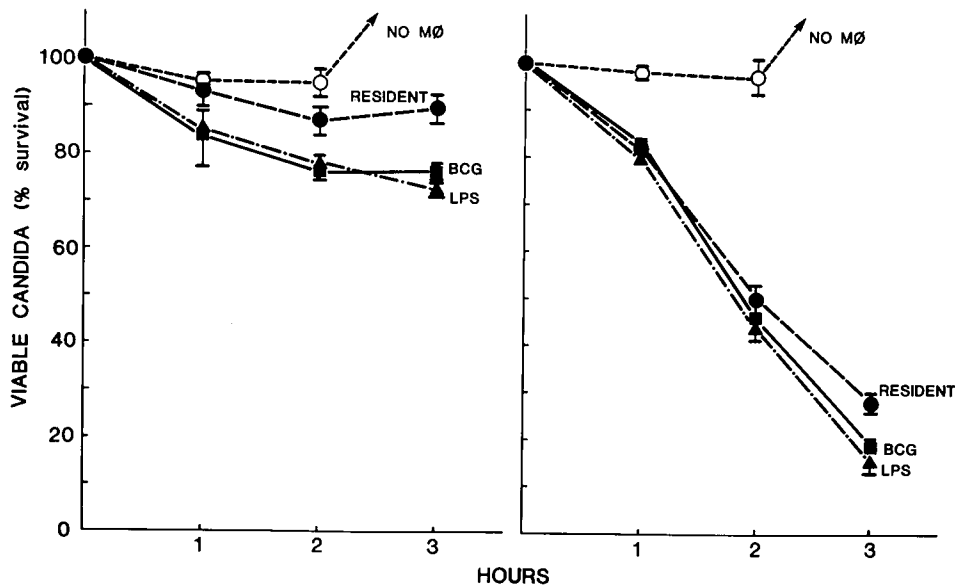


FIG. 1. The killing of candida by resident, LPS-elicited, and BCG-activated macrophages. The number of viable candida, expressed as a percentage of the colony count at time 0, is plotted as a function of incubation time. The points represent means, and the bars indicate the SEM. In a single experiment, killing by resident macrophages was compared with that of either LPS or BCG macrophages using both candida species. The macrophage to candida ratio was 3-4:1; 20% fresh FCS was present. In experiments with *C. albicans* (left panel) resident cells killed $10 \pm 3\%$ of fungi in 3 h ($n = 16$), LPS cells killed $27 \pm 1\%$ (9), and BCG cells killed $23 \pm 2\%$ (7). With *C. parapsilosis* (right panel) resident cells killed $72 \pm 2\%$ (16), LPS cells killed $84 \pm 2\%$ (9), and BCG cells killed $81 \pm 2\%$ (7) of fungi. The greater killing of *C. parapsilosis* by LPS and BCG macrophages at 3 h was significant ($P < 0.001$ and $P < 0.01$, respectively). The survival of fungi in the absence of macrophages (No M ϕ) is shown for 16 experiments with both candida species.

the J774.1 cells prevented the pronounced growth of fungi that was seen after 3 h in the absence of macrophages (Fig. 1), there was no reduction in the original inoculum of *C. parapsilosis* after 3 h ($100 \pm 4\%$ of original colony count, $n = 8$), and the number of *C. albicans* increased by 23% ($n = 2$). When stimulated by phorbol myristate acetate (PMA), 0.5 $\mu\text{g}/\text{ml}$, cells of this line released O_2^- weakly ($9 \pm 1 \text{ nmol}/\text{mg}/90 \text{ min}$) compared with resident cells (95 ± 11) or LPS-elicited cells (606 ± 45) (seven experiments with each).

Effects of Scavengers of Oxygen Metabolites on Macrophage Candidacidal Activity. The role of oxidative metabolism in the killing of candida by macrophages was explored using scavengers of toxic oxygen metabolites. The results with *C. parapsilosis* are shown in Fig. 2. Candidacidal activity of macrophages was inhibited effectively by SOD, which removes O_2^- , and less effectively but significantly by either concentration of catalase, which removes H_2O_2 ($P < 0.001$ and $P < 0.01$, respectively). Inhibition was also achieved with benzoate, which is believed to scavenge $\cdot\text{OH}$ (8, 18), and with azide, which is thought to remove singlet oxygen (18) ($P < 0.02$ with the lower concentrations of each). Equivalent inhibition was achieved with SOD and catalase using LPS-elicited and BCG-activated cells (Fig. 2). Autoclaved SOD or heat-inactivated catalase did not affect candidacidal activity of macrophages. None of the inhibitors at the concentrations used in these experiments affected macrophage morphology (by phase-

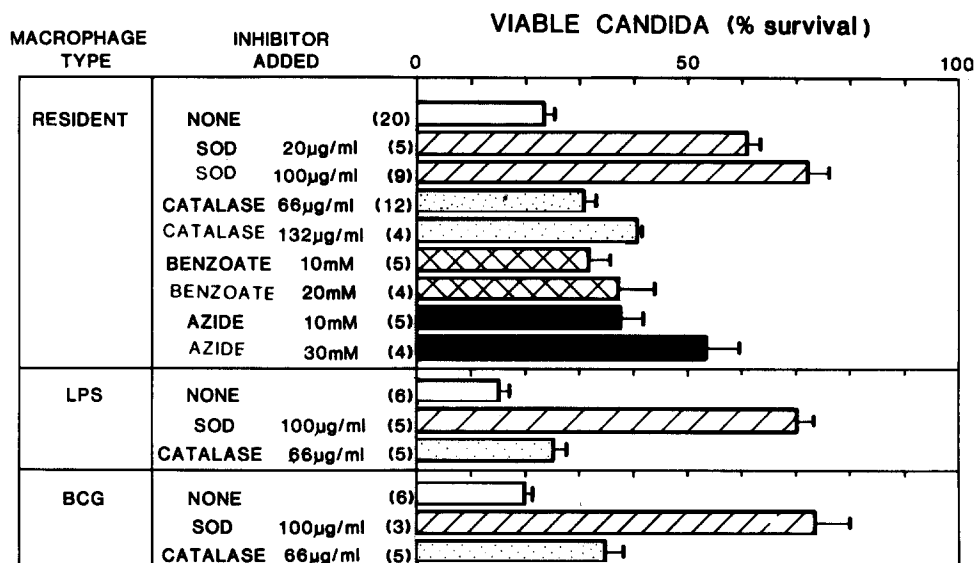


FIG. 2. The effects of scavengers of oxygen metabolites on the killing of *C. parapsilosis* by macrophages. Scavengers were added with the candida at time 0. The number of viable candida was determined after 2.5 h incubation. The columns indicate the means, and the bars the SEM. The number of experiments is given in parentheses. Final concentrations of inhibitors are shown.

contrast microscopy), viability (by trypan blue dye exclusion), or capacity to ingest candida; and azide did not affect O_2^- release from macrophages stimulated by PMA, 0.5 µg/ml. None of the inhibitors affected growth of candida in the absence of macrophages. For reasons that are not clear, mannitol (100 and 200 mM), a second scavenger of $\cdot OH$, inhibited candidacidal activity only slightly.

SOD and catalase also decreased the killing activity of macrophages for *C. albicans*. However, because the killing was not as pronounced as that of *C. parapsilosis*, differences were smaller. The surviving fraction of *C. albicans* in resident macrophages without any inhibitors was $84 \pm 2\%$, compared with $95 \pm 4\%$ with SOD and $94 \pm 3\%$ with catalase ($n = 7$; $P < 0.01$ and $P < 0.02$, respectively). The killing of *C. albicans* by macrophages from LPS-treated or BCG-infected mice was also significantly inhibited by SOD and catalase.

Effect of Serum. The greater killing of *C. parapsilosis* than *C. albicans* was striking with each of the three macrophage types (Fig. 1). Because of the possibility that this difference might be caused by more efficient interaction of *C. parapsilosis* with opsonins in fresh (unheated) FCS, we tested the candidacidal activity of LPS-elicited macrophages in the presence of heat-inactivated FCS and in the absence of serum. Except for less killing of *C. parapsilosis* after 1 h compared with the results with fresh FCS, the results of five experiments with heat-inactivated FCS were almost identical with those with fresh FCS. Increased killing of *C. parapsilosis* was also noted in the absence of serum ($P < 0.01$ at 3 h, $n = 5$).

Release of O_2^- . In a further attempt to understand the basis for the relatively enhanced macrophage killing of *C. parapsilosis*, we compared the capacity of the two candida species to stimulate macrophage O_2^- release. As summarized in Table I, all three macrophage types released more O_2^- when exposed to *C. parapsilosis* than to *C. albicans*. Release of O_2^- when stimulated by either species was greater in LPS-elicited

TABLE I
Release of O_2^- by Macrophages during Stimulation by *Candida*

Stimulus	O_2^- released* by macrophage type		
	Resident	LPS-elicited	BCG-activated
	<i>nmol/mg protein</i>		
<i>C. albicans</i>	76 ± 13 (11)	90 ± 10 (10)	112 ± 23 (3)
<i>C. parapsilosis</i>	150 ± 13 (11)	224 ± 23 (8)	204 ± 12 (3)

* The extent of O_2^- release from each of the three macrophage types is compared for *C. albicans* and *C. parapsilosis* as stimuli. Incubation was for 120 min. The macrophage:candida ratio was 1:3. Values represent mean ± SEM (*n*). The difference in stimulation by the two candida was significant with each macrophage type ($P < 0.001$ with resident and LPS cells; $P < 0.02$ with BCG cells). O_2^- release was significantly greater by BCG cells than by resident cells with *C. albicans* as stimulus, and by LPS cells than by resident cells with *C. parapsilosis* as stimulus ($P < 0.02$ for both, *t* test).

and BCG-activated macrophages than in resident cells, but the difference was less than that seen in previous studies using stimuli that elicited a more vigorous O_2^- response (4).

The kinetics of the difference in O_2^- release with the two candida species are shown in Fig. 3. LPS-elicited macrophages incubated with *C. parapsilosis* were stimulated to release more O_2^- at each time point studied from 20 to 120 min. Because added candida had to settle onto adherent macrophages, it was difficult to study the initial effects on O_2^- release of contact with candida. Therefore, the release of O_2^- was studied using macrophages in suspension. As shown in the inset for Fig. 3, the greater O_2^- response to *C. parapsilosis* was detectable within the first few minutes of the reaction. Greater stimulation of O_2^- release by *C. parapsilosis* than by *C. albicans* was also demonstrable with varying numbers of candida in the reaction mixture, as shown in Fig. 4.

Because variations in the rate or extent of phagocytosis of the two candida species might affect the generation of O_2^- , we compared O_2^- release by LPS-elicited macrophages exposed to the candida after pretreatment for 10 min with cytochalasin B, 10 μ g/ml, to depress ingestion. The number of candida of either species ingested by 200 macrophages was reduced in cytochalasin-treated cells to 4% of the number ingested by untreated cells after 60 min of incubation and to 7% after 120 min (four experiments). *C. parapsilosis* stimulated more O_2^- release than did *C. albicans* also in the presence of cytochalasin B, and the difference was almost identical with that shown in Fig. 3 ($n = 4$, data not shown). The extent of O_2^- release from cytochalasin-treated macrophages was slightly but consistently increased at the late incubation times, especially with *C. albicans* as stimulus, perhaps because of the enhanced viability (trypan blue exclusion) of macrophages pretreated with cytochalasin B (97% with *C. albicans* and 99% with *C. parapsilosis* after 120 min) compared with the viability of untreated cells (82 and 90%, respectively, $n = 3$).

Oxygen Consumption. Because of the possibility that the difference in macrophage O_2^- release when exposed to the two candida species might be caused by greater removal of O_2^- by *C. albicans*, the effect of the candida on the macrophage respiratory burst was explored further by studying macrophage oxygen consumption. There was a much more prompt and vigorous increase in oxygen consumption when the cells were incubated with *C. parapsilosis* than with *C. albicans* (Fig. 5). Oxygen uptake in the

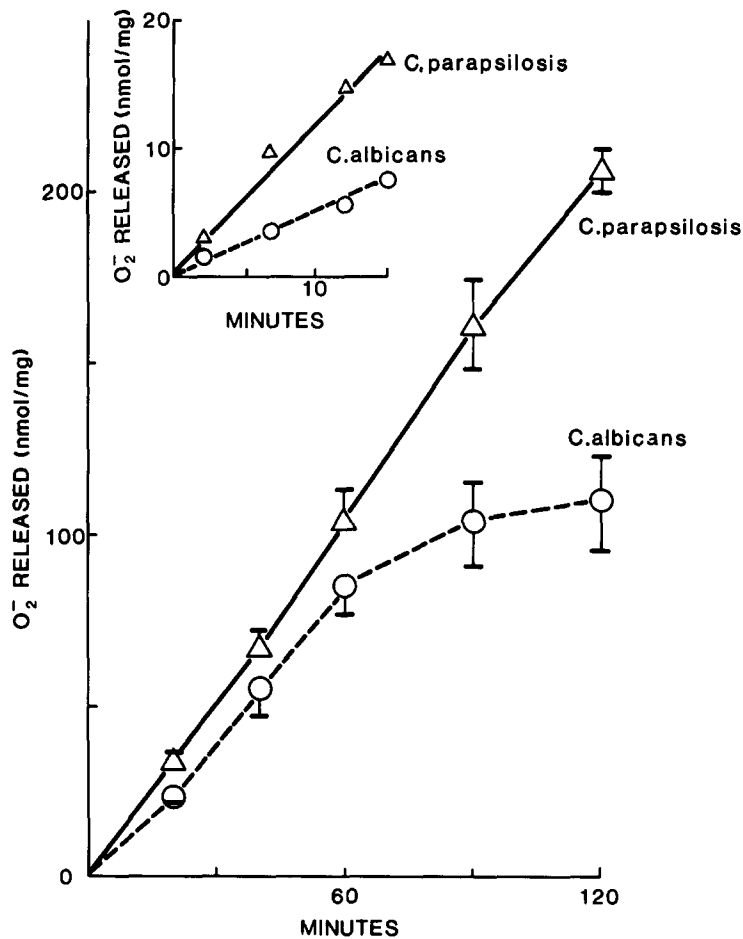


FIG. 3. Comparison of the capacity of *C. albicans* and *C. parapsilosis* to stimulate macrophage O_2^- release. Means \pm SEM of six experiments with adherent LPS-elicited macrophages are plotted. In the inset, O_2^- release was studied with suspended peritoneal cells obtained 4 d after intraperitoneal injection of LPS. The suspension contained 81% macrophages, 15% lymphocytes, and 4% granulocytes. The macrophage to candida ratio was 1:3 in all experiments.

presence of autoclaved ($121^\circ C$, 20 min) *C. parapsilosis* was also between two and three times greater than that with heat-killed *C. albicans* at 5, 10, and 15 min of incubation.

Of the phagocytic cells present in the preparations studied, 80% were macrophages and 20% were granulocytes. Oxygen uptake by preparations in which 95% of the phagocytes were granulocytes was 41 nmol/15 min with *C. albicans* and 43 nmol/15 min with *C. parapsilosis*. Thus, the greater stimulation by *C. parapsilosis* noted with total peritoneal cells cannot be attributed to an effect on granulocytes. Approximately equivalent respiratory responses by murine peritoneal macrophages and granulocytes has been reported previously with other stimuli (3, 19).

Phagocytosis. As indicated in Fig. 6, resident and LPS-elicited macrophages ingested *C. parapsilosis* slightly more quickly than *C. albicans*, whether 10^5 fungi (lower tracings) or 10^6 fungi (upper tracings) were added. However, phagocytosis of both strains was equivalent by 60 min of incubation. With 10^5 candida (the number used in the

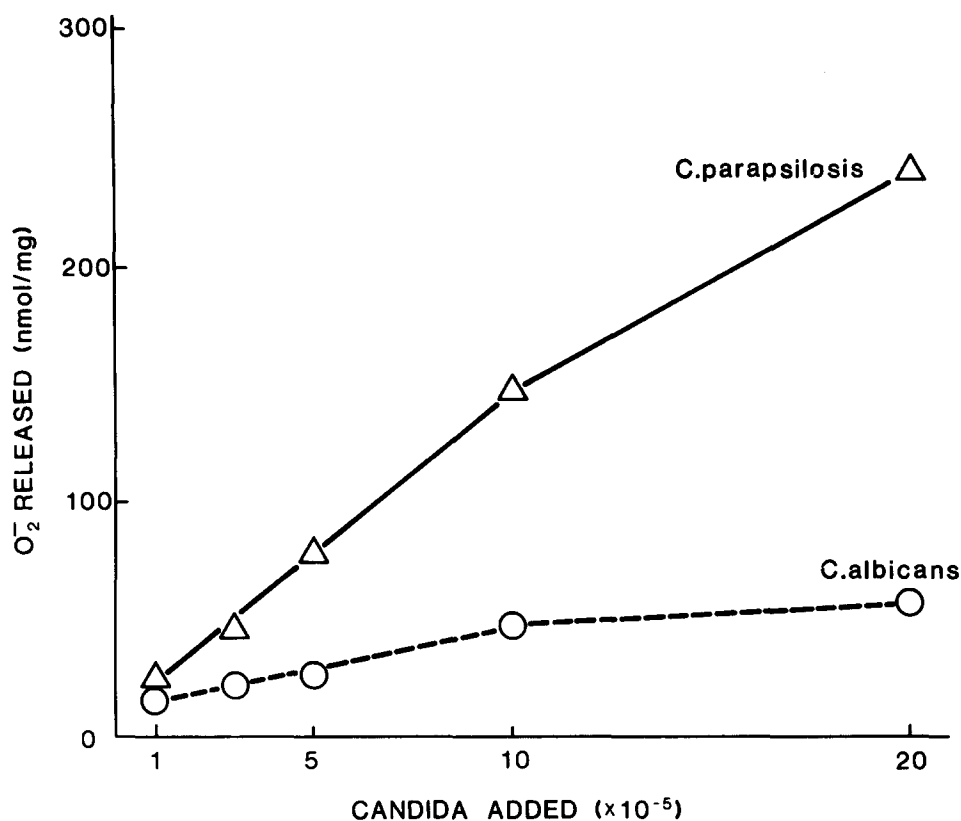


FIG. 4. Comparison of the capacity of varying numbers of *C. albicans* and *C. parapsilosis* to stimulate macrophage O₂⁻ release. Approximately 3×10^5 adherent LPS-elicited macrophages were present. Incubation time was 2 h. The means of triplicate values are plotted.

candidacidal assay), over 99% of the organisms on stained cover slips were engulfed by both resident and LPS-elicited macrophages after 60 min, and the number of candida in the supernate was <0.01% of the inoculated number. Fluorescein-stained candida did not lose fluorescence on the addition of crystal violet (7), indicating full ingestion. Almost all candida were also ingested by 60 min with 10^6 fungi in the system. When FCS was omitted from the incubation medium, phagocytosis of both species was decreased moderately, but equally, at 20 min of incubation, but by 60 min reached the level achieved with FCS (data not shown). Macrophages from LPS-treated mice ingested both kinds of candida slightly better at the 20-min time point than did macrophages from untreated mice (Fig. 6).

SOD and Catalase Activities of Candida. Extracts of *C. albicans*, prepared by sonication of viable fungi, contained approximately 10 times more SOD (21.8 ± 3.8 U/mg protein) and catalase (320 ± 85 U/mg) than did extracts of *C. parapsilosis* (2.4 ± 0.6 and 30 ± 5 U/mg, respectively, $n = 3$). However, 10^6 intact viable candida of either species showed no activity of SOD or catalase, suggesting that intrafungal SOD did not affect the level of detectable O₂⁻ in reaction mixtures with macrophages (Figs. 3 and 4). In agreement with this interpretation, the extent of O₂⁻ release by LPS macrophages stimulated by 5×10^5 each of *C. parapsilosis* and *C. albicans* mixed

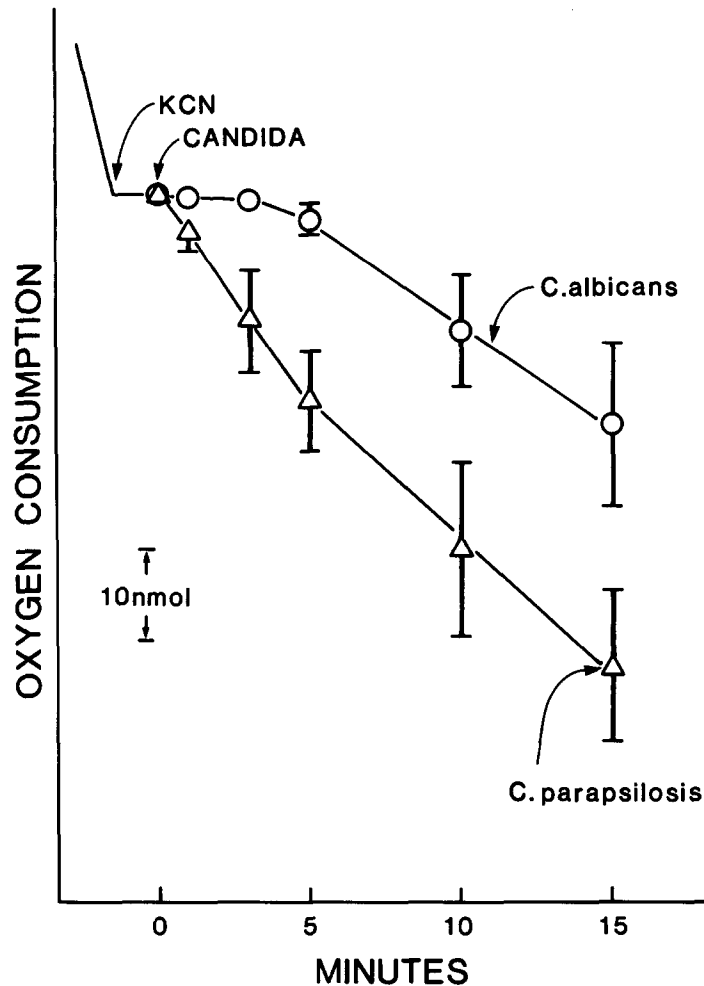


FIG. 5. Comparison of the capacity of *C. albicans* and *C. parapsilosis* to stimulate macrophage oxygen uptake. Peritoneal cells obtained 4 d after intraperitoneal injection of LPS were studied in suspensions containing 1×10^7 macrophages. KCN, 2 mM, and 2×10^7 candida were added as indicated. The curves reproduce mean values taken from polarographic tracings, after correction for oxygen uptake by candida in the presence of 2 mM KCN, which was equivalent for the two species. The means \pm SEM of six experiments are shown; values for the SEM of <2 nmol are not plotted. Uptake at 15 min was 25.6 ± 9.0 nmol with *C. albicans* and 51.7 ± 8.3 nmol with *C. parapsilosis* ($n = 6$).

together was intermediate (140 ± 3 nmol/mg) between release with 1×10^6 *C. parapsilosis* (213 ± 10 nmol/mg) and release with 1×10^6 *C. albicans* (85 ± 10 nmol/mg) ($n = 3$).

Susceptibility of Candida to Oxygen Metabolites. The oxygen radical-generating system xanthine oxidase-acetaldehyde was used to explore the possibility that the greater content of SOD and catalase in *C. albicans* might protect this species from oxygen metabolites. When this system is run at pH 10, the generation of O_2^- is favored; at pH 7, only about 20% of the oxygen undergoes univalent reduction, and the production of H_2O_2 is favored (14). As summarized in Table II, acetaldehyde itself

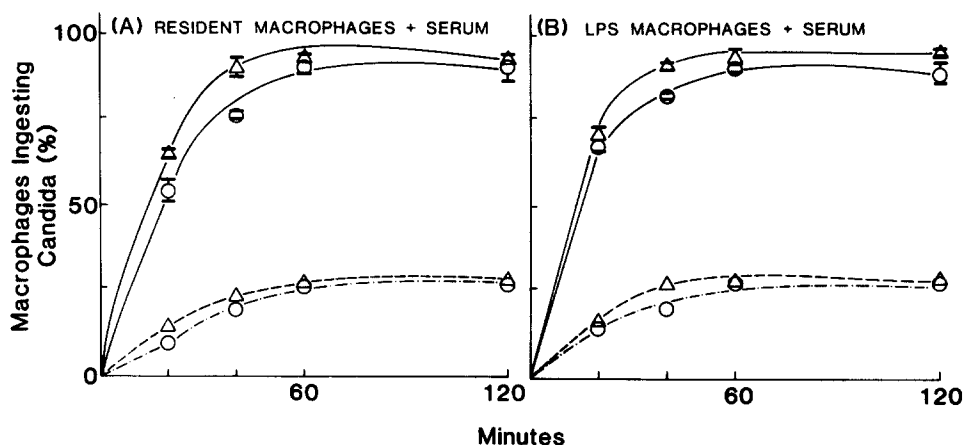


FIG. 6. Phagocytosis of the two candida species by resident (A) and LPS-elicited (B) macrophages. FCS (20%) was present; fresh and heated FCS gave equivalent results. The percentage of macrophages ingesting at least one organism is plotted. If the extent of phagocytosis was plotted as the number of candida ingested per 100 macrophages, the results were equivalent to those shown here. The upper tracings of both panels illustrate results of four experiments performed at a macrophage to candida ratio of 1:3. Means \pm SEM are shown. The lower tracings represent averages obtained in two experiments at a macrophage to candida ratio of 3:1.

TABLE II
Candidacidal Activity of Acetaldehyde-Xanthine Oxidase System and of H_2O_2

Constituents added	Candidacidal activity*			
	pH 7		pH 10	
	<i>C. albicans</i>	<i>C. parapsilosis</i>	<i>C. albicans</i>	<i>C. parapsilosis</i>
	% survival			
Acetaldehyde	68 \pm 6 (6)	73 \pm 4 (6)	68 \pm 12 (4)	74 \pm 5 (4)
Acetaldehyde plus xanthine oxidase	38 \pm 4 (6)	63 \pm 3 (6)	5 \pm 4 (4)	38 \pm 9 (4)
Acetaldehyde plus xanthine oxidase plus SOD, 20 μ g/ml	32 \pm 8 (4)	62 \pm 7 (4)	69 \pm 17 (3)	63 \pm 10 (3)
Acetaldehyde plus xanthine oxidase plus catalase, 20 μ g/ml	81 \pm 15 (3)	93 \pm 11 (3)	12 \pm 6 (3)	47 \pm 10 (2)
H_2O_2 , 10^{-4} M	100 \pm 11 (4)	72 \pm 4 (3)		
H_2O_2 , 10^{-3} M	77 \pm 8 (4)	60 \pm 10 (3)		
H_2O_2 , 10^{-2} M	10 \pm 5 (4)	30 \pm 2 (3)		
H_2O_2 , 10^{-1} M	0 (4)	5 \pm 1 (3)		

* The killing of candida by the acetaldehyde-xanthine oxidase system was conducted at pH 7 and pH 10, as described in Methods. The effect of H_2O_2 was tested only at pH 7. Results are expressed as the mean \pm SEM of the percentage of the original (time 0) inoculum that survived after incubation with the constituents shown. The number of experiments is given in parentheses.

was mildly candidacidal, but the addition of xanthine oxidase at either pH further enhanced killing, especially of *C. albicans*. Killing was inhibited effectively at pH 10 by SOD and only slightly by catalase; at pH 7 catalase was completely inhibitory, and SOD had no effect. Autoclaving the SOD removed over 90% of its inhibitory activity; boiling the catalase removed about 85% of its inhibitory effect. Catalase

activity at pH 10 was about 60% of that at pH 7; however, increasing the catalase concentration fivefold did not further enhance the slight inhibition shown in Table II at pH 10. *C. albicans* was more resistant than *C. parapsilosis* to killing by H₂O₂ at lower concentrations but more sensitive at higher concentrations (Table II).

In order to reproduce the exposure of phagocytosed candida to other cell constituents in addition to oxygen metabolites, resident macrophages were allowed to phagocytose candida for 60 min before disruption by sonication. Approximately 85% of either species remained viable after this exposure. The released candida were washed, then cultured for viability (100% control) or exposed to the xanthine oxidase system at pH 10. In three paired experiments, $1 \pm 1\%$ of released *C. albicans* survived exposure to the oxygen radical-generating system, compared with $26 \pm 16\%$ of *C. parapsilosis*. Therefore, *C. albicans* appears to be at least as sensitive as *C. parapsilosis* to death from exogenous oxygen metabolites, with or without additional prior exposure to macrophages.

Discussion

Macrophages obtained from animals that have recently survived infection by intracellular parasites exhibit a state of morphologic and biochemical activation and an enhanced capacity to kill certain bacteria, viruses, and protozoa (20). The data presented here indicate that infection-activated, as well as LPS-elicited, macrophages also kill certain fungi better than do normal (resident) cells. The effect was best demonstrated with *C. albicans*, but was demonstrable with *C. parapsilosis* after incubation for 3 h. The enhanced candidacidal effect, like an enhanced capacity to release O₂⁻ during phagocytosis (4), was equivalent for LPS-elicited and BCG-activated macrophages.

The molecular basis for the enhanced killing capacity of activated macrophages has not been completely defined. The higher content of hydrolytic enzymes could play a role, and it has seemed likely that the greater phagocytosis-associated oxidative metabolic response of activated macrophages (2-4) is important. The importance of oxygen-dependent mechanisms to macrophage microbicidal activity has been emphasized by the finding that killing of toxoplasma by activated macrophages can be inhibited by SOD, catalase, and agents that remove ·OH or singlet oxygen (5).

The data reported here indicate that phagocytosis-associated oxidative metabolism is required for effective killing of fungi by resident, elicited, and activated macrophages: (a) The greater candidacidal activity of LPS-elicited and BCG-activated macrophages is associated with a greater capacity to release O₂⁻ on exposure to PMA (4), opsonized zymosan (4), or candida. (b) Cells of the line J774.1, which produced <10% as much O₂⁻ as did resident macrophages, could ingest the candida normally but not kill them. (c) Killing of both candida species was inhibited effectively by SOD, catalase, and agents believed to scavenge ·OH or singlet oxygen. Although this inhibition was incomplete, it is likely that the capacity of the inhibitors to enter the phagocytic vacuole with the candida is limited in this system.

The particularly efficient inhibition of candidacidal activity by SOD raises the possibility that O₂⁻ is an especially important oxygen species in macrophage candidacidal activity. However, SOD is also the most effective inhibitor of phagocytosis-associated chemiluminescence (2) and ethylene formation (21) by neutrophils, and available data do not indicate that these oxygen-dependent phenomena are caused

primarily by O_2^- . Moreover, other variables in this system might influence the outcome, e.g., differences in penetration of the scavengers into the phagocytic vacuole, differences in macrophage inactivation of the agents, or a lack of specificity in the action of the $\cdot OH$ and singlet oxygen scavengers. Therefore, it would seem more appropriate at the present time to leave open the question of whether certain oxygen species are more important than others in macrophage candidacidal activity and to emphasize the conclusion that oxidative metabolism is an essential component of this process.

Also compatible with an important role for oxidative metabolism in macrophage candidacidal activity is the finding that *C. albicans*, which was killed less easily than *C. parapsilosis* by all macrophage types, was much less stimulatory of macrophage oxidative metabolism. Miller et al. reported a similar relationship with *Salmonella typhi*: phagocytosis of the virulent Quail's strain by neutrophils was not accompanied by increased oxygen uptake, whereas ingestion of the less virulent 0901 strain stimulated the predicted burst in oxygen consumption (22). In our studies, oxygen uptake by mouse neutrophils, as contrasted with macrophages, was stimulated to an equal extent by the two candida.

The greater killing of *C. parapsilosis* than *C. albicans* could not be attributed to greater activation of the complement system or more interaction with antibody by *C. parapsilosis* because the enhanced killing of this species occurred with heated serum or in the absence of serum. *C. parapsilosis* was ingested slightly more readily than *C. albicans*, but the difference was small and present only through 40 min of incubation. Moreover, inhibition of phagocytosis by pretreatment of the macrophages with cytochalasin B did not affect the greater stimulation of the respiratory burst by *C. parapsilosis*.

The content of SOD and catalase was much higher in *C. albicans* than in *C. parapsilosis*. Mandell has reported that neutrophils killed a low-catalase strain of *S. aureus* better than a high-catalase strain (23). On the other hand, Gregory et al. found that induction of increased concentrations of SOD in *Escherichia coli* by growth in oxygenated medium protected bacteria against endogenous O_2^- resulting from further increases in pO_2 , but offered no protection against exogenous O_2^- generated by photochemical or xanthine oxidase systems (24). In experiments reported here, neither species of candida scavenged H_2O_2 or O_2^- derived from chemical sources or macrophages, and *C. albicans* was killed at least as easily as *C. parapsilosis* by exogenously generated oxygen radicals. Lehrer found *C. albicans* to be more sensitive than *C. parapsilosis* to H_2O_2 at all concentrations tested (15). Thus, it is not clear at the present time that intramicrobial SOD and catalase play any role in protecting candida from the damaging effects of external oxygen metabolites.

Taken together, these findings suggest that the greater resistance of *C. albicans* to macrophage killing depends primarily on its relatively decreased stimulation of macrophage oxidative metabolism. This property, in turn, presumably depends upon an interaction between *C. albicans* and the macrophage plasma membrane that is less favorable for stimulation of the enzyme responsible for the respiratory burst. A similar relationship has been reported with salmonella, and it is possible that this mechanism has general relevance to microbial pathogenicity.

Summary

The mechanisms by which macrophages kill ingested microorganisms were explored using *Candida albicans* and *Candida parapsilosis*. The results indicate that efficient

macrophage candidacidal activity depends upon the generation of oxygen metabolites by the phagocytic cell: (a) peritoneal macrophages from mice infected with bacillus Calmette-Guérin (BCG) or injected intraperitoneally with lipopolysaccharide (LPS) released more superoxide anion (O_2^-) during phagocytosis of candida and killed candida better than did resident macrophages; (b) cells of the macrophage-like line J774.1, which released negligible amounts of O_2^- , could ingest the candida normally but not kill them; (c) killing of candida by resident, LPS-elicited, and BCG-activated macrophages was inhibited by agents that scavenge O_2^- , hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen; and (d) all three macrophage types killed *C. parapsilosis* more effectively than *C. albicans*, and *C. parapsilosis* stimulated a more prompt and vigorous burst of macrophage oxygen consumption and O_2^- release than did *C. albicans*. Macrophages ingested *C. parapsilosis* slightly more quickly than *C. albicans*, but phagocytosis of both strains was equivalent by 60 min of incubation. Although *C. albicans* contained higher concentrations of the oxygen-metabolite scavengers superoxide dismutase and catalase, neither fungal species scavenged O_2^- or H_2O_2 effectively; and *C. albicans* was killed more easily than *C. parapsilosis* by a xanthine oxidase system that generates primarily H_2O_2 at pH 7, or O_2^- and $\cdot OH$ at pH 10. Thus, the decreased killing of *C. albicans* appears to result primarily from the capability of this species to elicit less vigorous stimulation of macrophage oxidative metabolism. This capability may have general relevance to the pathogenicity of microorganisms.

We are grateful to Doctors Samuel C. Silverstein and Seymour J. Klebanoff for helpful suggestions, to Philip A. Kemp for guidance in the statistical analyses, and to Dr. Peter Ralph for his gift of the original J774.1 cells.

Received for publication 11 February 1980 and in revised form 9 April 1980.

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