INFECTION AND IMMUNITY, June 1980, p. 1001-1008
0019-9567/80/06-1001/08$02.00/0 Vol. 28, No. 3

# Fungicidal Activity of Rabbit Alveolar and Peritoneal Macrophages Against *Candida albicans*†

ROBERT I. LEHRER,* LINDA G. FERRARI, JUDITH PATTERSON-DELAFIELD, AND TANIA SORRELL‡

*Department of Medicine, Division of Hematology and Oncology, UCLA Center for the Health Sciences, Los Angeles, California 90024*

We tested the ability of rabbit macrophages to kill *Candida albicans* in vitro. Resident (unstimulated) alveolar macrophages killed 28.1 ± 1.9% of ingested organisms in 4 h, whereas resident peritoneal macrophages killed only 15.2 ± 1.3% (mean ± standard error of the mean, $P < 0.01$). Peritoneal macrophages obtained from rabbits treated 3 weeks earlier with complete Freund adjuvant showed enhanced candidacidal activity relative to normally resident peritoneal cells (28.2 ± 3.1%, $P < 0.01$). Candidacidal activity by alveolar macrophages recovered from such treated animals was slightly enhanced relative to untreated alveolar macrophages (32.9 ± 2.3%). Candidacidal activity by peritoneal and alveolar macrophages was not decreased by several agents (cyanide, azide, sulfadiazine, and phenylbutazone) that inhibit the ability of human blood monocytes to kill *C. albicans*. In contrast, candidacidal activity by alveolar macrophages was greatly diminished by iodoacetate, an ineffective inhibitor of this function in human monocytes. We conclude that rabbit macrophages kill *C. albicans* by a fungicidal mechanism distinct from the peroxidase-$H_2O_2$ mechanism of human granulocytes and monocytes, and that the fungicidal properties of peritoneal and alveolar macrophage populations are enhanced after nonspecific stimulation with complete Freund adjuvant.

Despite much information dealing with mechanisms used by polymorphonuclear leukocytes to kill fungi such as *Candida albicans* (4, 8, 17, 20), knowledge of the fungicidal properties of mononuclear phagocytes is scant and often contradictory. Several years ago, we demonstrated that human blood monocytes possessed at least two candidacidal mechanisms (18). One of these, effective against *C. albicans*, appeared to be identical to the myeloperoxidase-$H_2O_2$-halide system of neutrophils described by Klebanoff (13) and others. The existence of this mechanism in monocytes, suggested by studies with selected inhibitors, was proven by the functional characteristics of monocytes congenitally deficient in myeloperoxidase (18). The alternative (myeloperoxidase-independent) fungicidal mechanism(s) of monocytes was relatively ineffective against *C. albicans*.

Although blood monocytes can enter the lungs and transform into alveolar macrophages, this maturation is generally attended by a loss of the peroxidase activity in their cytoplasmic granules (3). Accordingly, it seemed worthwhile to ascertain how effectively mature alveolar macrophages might kill *C. albicans* and to compare this function in alveolar and peritoneal macrophages. Although techniques exist for obtaining selected macrophage populations from humans, their routine use is precluded by significant ethical, theoretical, and practical considerations. We selected, instead, an animal model that yielded adequate numbers of alveolar and peritoneal macrophages under controlled experimental conditions. This report describes the ability of rabbit macrophages to kill *C. albicans*. Characterization of their candidacidal mechanisms with inhibitors revealed that they kill *C. albicans* by a peroxidase-independent mechanism.

(A preliminary report of these findings was presented at the 69th Annual Meeting of the American Society for Clinical Investigation, Washington, D.C., May 1977 [Clin. Res. **25:** 490A, 1977].)

## MATERIALS AND METHODS

**Preparation of *C. albicans*.** C. albicans 820 was cultured in Sabouraud 2% dextrose broth (Difco Laboratories, Detroit, Mich.) for 48 to 72 h at 33°C. The organisms, all in yeast phase, were washed twice with sterile distilled water and suspended in Hanks bal-

† Publication no. 2 of the Collaborative California Universities Mycology Research Unit.

‡ Present address: Department of Medicine, Westmead Centre, Westmead, N.S.W. 2145, Australia.

anced salt solution (GIBCO Laboratories, Grand Island, N.Y.). A sample was diluted with 0.01% methylene blue and examined in a hemacytometer to determine the concentration and viability of the yeast cells (21).

Another sample was subjected to a slide filamentation test that provided an independent assessment of the culture's viability. The slide filamentation test was performed by mixing a drop of *Candida* suspension ($1 \times 10^8$ to $3 \times 10^8$ cells per ml) with 4 ml of Hanks solution containing 25% fetal calf serum (GIBCO) and 1% agarose (Sigma Chemical Co., St. Louis, Mo.) at 40°C. Several drops of this mixture were allowed to gel upon a sterile glass microscope slide, and the slide culture was placed in a humidified petri dish and incubated for 4 h at 37°C. Since *C. albicans* responds to serum by sprouting characteristic filaments (35), the percentage of sprouted cells indicates the minimal percentage of viable yeasts in the starting inoculum. Both measures indicated high viability in our test cultures: >97% by methylene blue exclusion, and 95 ± 2% by slide filamentation.

**Preparation of macrophages.** New Zealand white rabbits weighing 2 to 3 kg were used. Some were untreated, and others were injected via a marginal ear vein with 1 ml of complete Freund adjuvant containing *Mycobacterium butyricum* (Difco, 0638-60). Many of the intravenously injected rabbits received a concurrent intraperitoneal injection of 20 ml of heavy paraffin oil (Fisher Chemicals, Fair Lawn, N.J.) with 0.5 ml of complete Freund adjuvant.

Our preliminary experiments indicated that 21 days was optimal between administration of Freund adjuvant and recovery of the macrophage populations. Moreover, virtually all (of more than 50) rabbits that received intraperitoneal and/or intravenous adjuvant survived for 21 days without manifest illness or debility. To recover their macrophages, rabbits were anesthetized with 90 mg of sodium pentobarbital (Diabutal; Diamond Labs, Des Moines, Iowa) and killed by air embolism. After tracheal cannulation, the lungs were lavaged with a total of 300 ml of Dulbecco phosphate-buffered saline (GIBCO) containing 5 IU of sodium heparin (Riker, Northridge, Calif.) per ml, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. To recover peritoneal macrophages, we lavaged the peritoneal cavity with a total of approximately 500 ml of this solution, introducing it via a midline ventral incision.

Macrophages were centrifuged at $200 \times g$ for 10 min at room temperature. Contaminating erythrocytes, if present (rarely), were removed by hypotonic lysis. Cells were counted in a hemacytometer, and slides were prepared with a cytocentrifuge (Shandon, Sewickley, Pa.) and stained with Giemsa or for alpha-naphthyl butyrate esterase activity (1).

**Cell yield and purity.** We recovered 47.3 (±1.2) $\times 10^6$ cells from the alveoli of unstimulated rabbits ($n$ = 22, geometric mean ± standard error of the geometric mean), and 8.9 (±0.1) $\times 10^6$ cells ($n$ = 14) from their peritoneal cavities. Administration of complete Freund adjuvant increased cell yields approximately sevenfold: alveolar, 277.3 (±12.2) $\times 10^6$ ($n$ = 19), and peritoneal, 56.8 (±16.2) $\times 10^6$ ($n$ = 13).

The composition of these populations was as follows. Resident alveolar cells consisted of 94.8 ± 3.4% macrophages (mean ± standard deviation), 1.8 ± 1.6% heterophils, and 3.2 ± 2.4% lymphocytes. Freund adjuvant-elicited alveolar cells were composed of 90.3 ± 5.6% macrophages, 5.0 ± 5.4% heterophils, and 4.7 ± 3.6 lymphocytes. Resident peritoneal cells contained 88.4 ± 7.9% macrophages, 6.8 ± 8.2% heterophils, and 4.8 ± 2.9% lymphocytes. Freund adjuvant-elicited peritoneal cells were comprised of 71.9 ± 12.1% macrophages, 23.1 ± 11.1% heterophils, and 4.6 ± 4.5% lymphocytes. Although the aforementioned differential counts were obtained on Giemsa-stained preparations, virtually identical results were obtained when we used alpha-naphthyl butyrate esterase positivity to identify macrophages (data not shown).

**Phagocytosis assay.** We used a previously described method to measure the phagocytosis of *C. albicans* by macrophages (27). Briefly, heat-killed (95°C, 30 min) and washed *C. albicans* were opsonized by placing them ($10^8$ cells per ml) for 10 min in fresh frozen normal human serum that contained 5% (vol/vol) specific immune rabbit serum. The yeasts were then washed twice with distilled water and suspended in Hanks solution. Uptake was measured in polystyrene tubes (12 by 75 mm; Falcon 2054, Oxnard, Calif.) that contained $2.5 \times 10^6$ macrophages, $1.25 \times 10^7$ yeast cells, 0.9 ml of Hanks solution, and 0.1 ml of fetal calf serum. The tubes were rotated, 30 rpm, in air at 37°C for 60 min. At intervals, a sample was removed, mixed with an equal volume of stain (0.4% trypan blue and 0.2% eosin Y in phosphate-buffered saline), and examined by direct microscopy. Intracellular yeast cells (unstained) were readily distinguished from extracellular or surface-adherent yeasts (stained purple), and viable macrophages (unstained) and nonviable ones (stained) were also easily distinguished. We examined 100 to 200 macrophages to determine the percentage of viable macrophages that was phagocytic and the mean number of ingested yeast cells per viable macrophage. Unless otherwise noted in the text, at least 95% of the macrophages remained viable under the conditions used in these tests.

**Candidacidal assay.** We measured the ability of rabbit macrophages to kill *C. albicans* by another previously described method (15). Polystyrene tubes (12 by 75 mm), containing $2.5 \times 10^6$ macrophages and an equal number of opsonized (see above) viable yeast cells in 1 ml of Hanks solution with 10% (vol/vol) fetal calf serum, were rotated (30 rpm) in air at 37°C. At intervals (0.25, 2.5, and 4 h), small samples were removed and slides were prepared with a cytocentrifuge. These were fixed with methanol, stained with Giemsa, examined by microscopy, and interpreted as previously described (15).

Intracellular *Candida* cells were classified into three mutually exclusive classes: (i) yeast cells that had maintained their initial spherical shape and uniform blue cytoplasmic staining; (ii) yeast cells that had developed filamentous pseudogerm tubes and maintained their blue cytoplasmic staining; and (iii) "ghosts"—*Candida* cells that stained faintly grey to pink, reflecting the extensive digestion of their cytoplasmic contents. Most often spherical in shape, some ghost cells with filaments were also noted, particularly at the 4-h time point. Such filamentous forms repre-

sent *Candida* killed and digested by macrophages after an initial period of cellular growth. In our analysis of candidacidal activity, we consider ghost cells ([iii] above) to be nonviable and filament-bearing yeasts ([ii] above) to be viable organisms manifesting intracellular growth. Unaltered cells ([i] above) are, in the main, viable yeasts that have not commenced to grow intracellularly, perhaps as a consequence of some candidastatic properties of the phagocyte (15). These features are illustrated in Fig. 1.

## RESULTS

**Phagocytosis.** As may be noted in Fig. 2, approximately three-quarters of the alveolar and peritoneal macrophages could ingest heat-killed opsonized *C. albicans* when these organisms were present in modest excess (5:1 ratio). Macrophage populations recovered from rabbits that had received complete Freund adjuvant were not appreciably different in phagocytic rate or capacity from populations recovered from untreated rabbits. The relatively low yield of peritoneal macrophages from untreated rabbits prevented more extensive examination of their phagocytic properties in these experiments.

**Candidacidal activity.** Approximately 30% of the *C. albicans* cells ingested by alveolar and peritoneal macrophages from rabbits pretreated with complete Freund adjuvant were killed and degraded (i.e., converted to ghosts) by 4 h of incubation (Table 1). Resident alveolar macro-

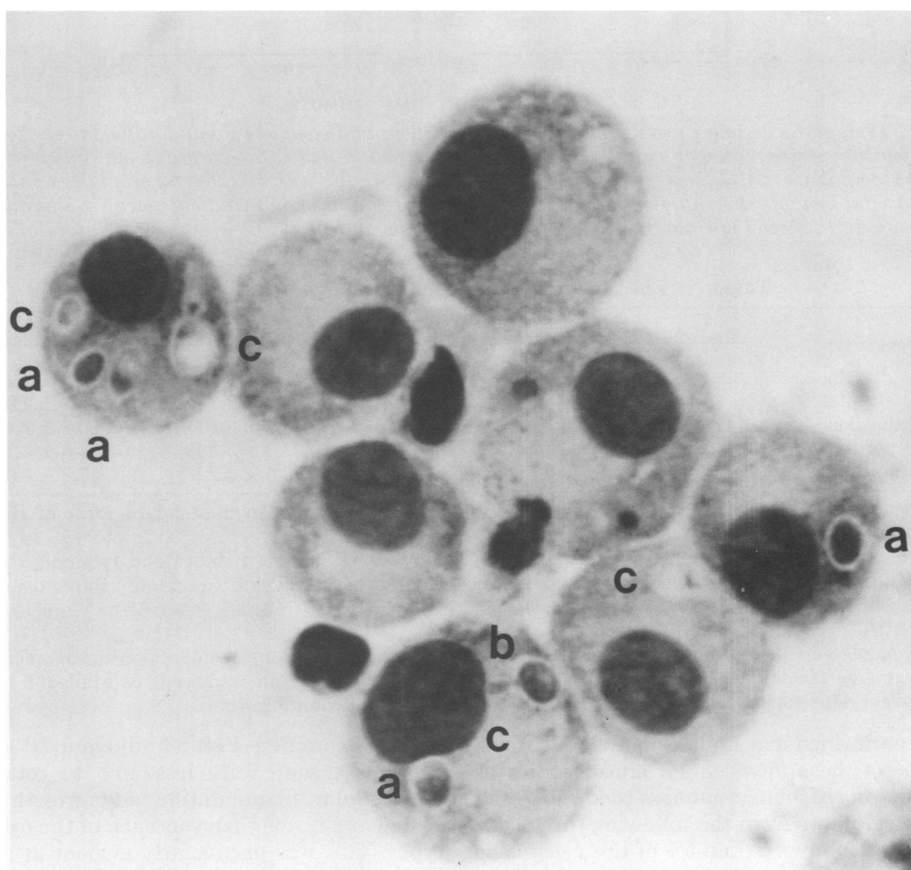


FIG. 1. *Rabbit alveolar macrophages and C. albicans. These complete Freund adjuvant-elicited alveolar macrophages had ingested viable yeast-phase organisms 4 h earlier. Their candidacidal and candidastatic activities were analyzed by examining the morphology and Giemsa-staining properties of the ingested yeasts, which were classified as follows: (a) spherical yeast cells with blue-staining cytoplasm; (b) yeast cells with blue-staining cytoplasm and filamentous pseudo-germ tubes; and (c) fungal cells, with or without pseudo-germ tubes, whose cytoplasm was colorless or took a faint grey or pink stain (ghost cells). Ghosts are nonviable Candida cells whose cytoplasmic contents have undergone extensive degradation after their death within the macrophage. Class (b) cells display the characteristic dimorphism of viable, intracellularly growing C. albicans. Class (a) cells, considered to be viable in this assay, have undergone neither intracellular growth nor degradation.*

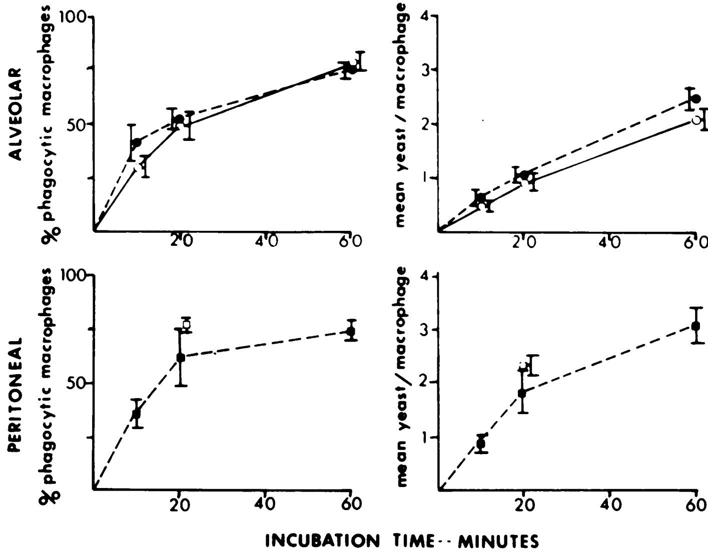


FIG. 2. *Phagocytic activity of rabbit macrophages. Resident and complete Freund adjuvant-elicited alveolar and peritoneal macrophages were exposed to opsonized, heat-killed C. albicans at a multiplicity of 5 yeast cells per macrophage. Solid lines and open symbols represent studies with resident macrophages. Interrupted lines and solid symbols show studies with elicited macrophages. Data points indicate mean ± standard error of the mean and reflect 3 to 6 observations for peritoneal cells and 7 to 21 observations for alveolar cells per data point.*

TABLE 1. *Effects of rabbit macrophages on ingested C. albicans*

| Macrophage type | Candidacidal activity[a] at: | | Intracellular filamentation[b] at: | |
|---|---|---|---|---|
| | 2.5 h | 4 h | 2.5 h | 4 h |
| Resident alveolar | 16.4 ± 2.1 (19)[c] | 28.1 ± 1.9 (19) | 20.0 ± 2.3 (19) | 22.0 ± 2.6 (20) |
| Elicited alveolar | 22.9 ± 2.2 (19) | 32.9 ± 2.3 (22) | 25.1 ± 3.1 (19) | 20.8 ± 2.4 (22) |
| Resident peritoneal | 10.8 ± 2.4 (8)[c] | 15.2 ± 1.3 (9)[d] | 33.3 ± 3.5 (8)[c] | 21.6 ± 3.1 (9)[e] |
| Elicited peritoneal | 24.0 ± 4.2 (8) | 28.2 ± 3.1 (14) | 13.5 ± 3.2 (8) | 17.8 ± 1.6 (14) |

[a] Percentage of intracellular *C. albicans* converted to ghost cells, mean ± standard error of the mean. Parentheses indicate number of rabbits tested.

[b] Percentage of intracellular *C. albicans* bearing filamentous pseudo-germ tubes and retaining their blue cytoplasmic staining, mean ± standard error of the mean. Parentheses indicate number of rabbits tested.

[c] $P < 0.05$.

[d] $P < 0.01$.

[e] This decrease in filamentous forms at 4 h was largely due to rupture of some resident peritoneal macrophages by enlarging intracellular pseudohyphae. Comparisons were made by an unpaired, two-tailed *t* test that compared resident populations with their Freund adjuvant-elicited counterparts.

phages performed in a similar manner. That the presence of these intracellular ghosts indicates the ability of these macrophages to kill ingested organisms follows from the following three considerations. No more than 5% of the yeast cells in the original inoculum were nonviable; virtually all of the added organisms were ingested; and the noncellular components of the assay mixtures (specific immune globulin, fetal calf serum, and balanced salt solution) did not kill *C. albicans* (see Materials and Methods for details).

Resident peritoneal macrophages were significantly less able to kill ingested *C. albicans* than were their counterparts from rabbits that had received complete Freund adjuvant ($P < 0.01$). Moreover, they were less able to retard the intracellular filamentation and growth of ingested organisms than was each of the other cell types. This was particularly evident at 2.5 h of incubation, when only 10.8% of ingested organisms had been converted to ghosts, whereas 33.3% had formed intracellular filamentous pseudogerm tubes (both values differ from elicited peritoneal cells, $P < 0.05$). By 4 h of incubation, many of the resident peritoneal macrophages had been disrupted and killed by the rapidly enlarging filaments, so that the results at 4 h are somewhat biased (tending to overestimate the competence of the peritoneal macro-

phages) by removal of the least competent cohort of macrophages from the population analyzed at that time. Nevertheless, candidacidal activity of these resident macrophages was decidedly inferior to that of the other types tested ($P < 0.01$).

The effect of Freund adjuvant pretreatment on the function of alveolar macrophages was substantially less pronounced than its effects on the functional performance of the peritoneal macrophage populations. Although alveolar macrophages from treated rabbits had converted 6.5% and 4.8% more of their intracellular *C. albicans* to ghost cells at 2.5 and 4 h, respectively, only at the earlier time point did the difference from resident alveolar macrophage populations achieve statistical significance ($P < 0.05$).

**Effect of inhibitors.** We characterized the candidacidal mechanism of rabbit macrophages by testing the effect of previously studied inhibitors on their ability to kill *C. albicans*. We used alveolar and peritoneal cells from rabbits pretreated with Freund adjuvant because of the substantially increased macrophage yield after this procedure.

The effects of these inhibitors on uptake were determined in our standard phagocytic system (Table 2). With few exceptions, the compounds did not perturb uptake of organisms sufficiently to render suspect their use in probing subsequent candidacidal activity. The exceptions included: iodoacetate and puromycin, which inhibited uptake by peritoneal macrophages; cyanide, which impaired uptake by alveolar macrophages, and cytochalasin B, which profoundly inhibited uptake by both macrophage types. Iodoacetate and puromycin also impaired the viability of peritoneal macrophages, killing a majority of the cells by 60 min (trypan blue exclusion). The other compounds did not appear to be cytotoxic to either macrophage type.

The effect of these inhibitors on candidacidal activity is summarized in Table 3. We were particularly interested in observing any differences between the response of alveolar and peritoneal macrophages and in comparing the results obtained with these compounds with those previously obtained when their effects on the ability of human blood monocytes were investigated (18). It was evident that a group of compounds that were effective inhibitors of candidacidal activity by human blood monocytes (cyanide, azide, sulfadiazine, and phenylbutazone) had no inhibitory effect on this function in alveolar or peritoneal macrophages. Indeed, 1 mM cyanide consistently potentiated the ability of peritoneal macrophages to kill *C. albicans* ($P < 0.05$), whereas it had no significant effect on alveolar macrophages. Sulfadiazine (2 mM) had a similar effect on peritoneal macrophages, but this was smaller than the cyanide effect and did not achieve statistical significance.

Iodoacetate (0.1 mM) profoundly diminished the ability of alveolar macrophages to kill ingested *C. albicans*. It had no effect on the ability of such macrophages to degrade heat-killed *C. albicans* to ghosts, suggesting that its inhibition was of killing rather than of the subsequent enzymatic degradation required to yield intracellular *Candida* ghosts. It will be recalled that iodoacetate did not have significant effects on the ability of alveolar macrophages to ingest *C. albicans* (Table 2).

TABLE 2. *Effect of selected agents on phagocytosis of C. albicans by complete Freund adjuvant-elicited rabbit macrophages*[a]

| Inhibitor | Concn | Alveolar macrophages | | | Peritoneal macrophages | | |
|---|---|---|---|---|---|---|---|
| | | *n* | % Phagocytic | Yeasts per cell | *n* | % Phagocytic | Yeasts per cell |
| None | | 4 | 67.9 ± 6.5 | 1.60 ± 0.18 | 3 | 92.5 ± 3.3 | 2.87 ± 0.58 |
| Puromycin | 20 μg/ml | 2 | 78.2 | 1.95 | 3 | 77.0 ± 3.0[b] | 2.36 ± 0.13 |
| Azide | 2 mM | 3 | 74.8 ± 10.9 | 1.99 ± 0.44 | 3 | 79.3 ± 9.8 | 2.42 ± 0.51 |
| Cyanide | 1 mM | 5 | 35.9 ± 11.5[b] | 0.88 ± 0.40 | 3 | 95.0 ± 3.2 | 3.24 ± 0.29 |
| Sulfadiazine | 2 mM | 3 | 74.3 ± 11.0 | 2.12 ± 0.40 | 3 | 87.1 ± 9.6 | 2.81 ± 0.47 |
| Phenylbutazone | 1.6 mM | 2 | 63.7 | 1.63 | 3 | 89.2 ± 4.3 | 2.82 ± 0.22 |
| 2,4-Dinitrophenol | 0.25 mM | 3 | 50.3 ± 11.0 | 1.51 ± 0.34 | 3 | 88.7 ± 7.3 | 2.83 ± 0.41 |
| Iodoacetate | 0.1 mM | 3 | 54.7 ± 2.4 | 1.16 ± 0.13 | 3 | 48.7 ± 20.3 | 0.88 ± 0.46[b] |
| Fluoride | 1 mM | 3 | 75.8 ± 6.3 | 1.68 ± 0.08 | 3 | 83.3 ± 9.0 | 2.78 ± 0.43 |
| Colchicine | 0.1 mM | 4 | 62.6 ± 10.4 | 1.53 ± 0.34 | 3 | 77.0 ± 11.9 | 2.15 ± 0.44 |
| Cycloheximide | 10 μg/ml | 0 | NT | NT | 3 | 78.7 ± 13.0 | 2.57 ± 0.65 |
| Cytochalasin B | 25 μg/ml | 3 | 13.0 ± 4.5[c] | 0.21 ± 0.10[c] | 3 | 8.7 ± 2.4[c] | 0.13 ± 0.04[c] |

[a] Heat-killed *C. albicans* ($1.25 \times 10^7$) and macrophages ($2.5 \times 10^6$) were incubated in 1 ml of medium for 20 min at 37°C, and uptake was determined as described in the text. Data indicate mean ± standard error of the mean; *n*, number of experiments; NT, not tested.

[b] $P < 0.05$, unpaired $t$ test.

[c] $P < 0.01$, unpaired $t$ test.

TABLE 3. *Effect of inhibitors on candidacidal activity by mononuclear phagocytes*[a]

| Inhibitor | Concn | Rabbit alveolar macrophages | Rabbit peritoneal macrophages | Human blood monocytes |
|---|---|---|---|---|
| Puromycin | 20 μg/ml | 99.9 ± 18.8 (3) | Cytotoxic | 98.0 ± 6.7 (6) |
| | 2 μg/ml | NT | 97.6 (2) | NT |
| Azide | 2 mM | 83.8 ± 6.5 (4) | 91.8 ± 32 (4) | 4.3 ± 2.1 (3)[c] |
| Cyanide | 1 mM | 90.6 ± 17.8 (6) | 151.2 ± 15.5[b] (6) | 31.7 ± 5.8 (8)[c] |
| Sulfadiazine | 2 mM | 89.9 ± 23.6 (4) | 124.4 ± 7.7 (3) | 3.3 ± 1.2 (4)[c] |
| Phenylbutazone | 1.6 mM | 105.6 ± 7.2 (3) | 96.1 ± 10.9 (3) | 16.1 ± 3.5 (7)[c] |
| 2,4-Dinitrophenol | 0.25 mM | 97.6 ± 11.8 (4) | 95.7 ± 24.5 (3) | 100.8 ± 4.5 (3) |
| Iodoacetate | 0.1 mM | 38.8 ± 6.0[b] (4) | Cytotoxic | 101.6 ± 1.7 (3) |
| Fluoride | 1 mM | 83.6 ± 10.1[b] (4) | 103 ± 15.8 (3) | 90.8 ± 2.4 (3) |
| Colchicine | 0.1 mM | 91.6 ± 18.6 (5) | 104.3 ± 13.8 (3) | 61.1 ± 5.7 (5) |
| Antimycin A | $10^{-7}$ mM | NT | 80.5 (2) | NT |

[a] The effects of these compounds on candidacidal activity (percentage of intracellular *C. albicans* ghosts at 4 h) is expressed relative to an untreated control. Data for human monocytes have been published previously (20) and are used with permission. Alveolar and peritoneal macrophages were derived from rabbits that had received complete Freund adjuvant. Certain inhibitors, as indicated above, were toxic to peritoneal macrophages, and their effects on candidacidal activity could not be determined. Data represent mean ± standard error of the mean; *n*, number of experiments; NT, not tested. Statistical analysis was done by paired *t* test.

[b] $P < 0.05$.

[c] $P < 0.01$.

Few peritoneal macrophages survived a 2.5-h incubation with 0.1 mM or 0.05 mM iodoacetate. A lower concentration of iodoacetate, 0.01 mM, impaired neither the viability nor the candidacidal activity of the peritoneal macrophages.

## DISCUSSION

That little attention has been paid to the ability of macrophages to ingest and kill *C. albicans* may be a consequence of the disappointing performance of murine peritoneal macrophages in early studies of this phenomenon. Stanley and Hurley reported that resident murine peritoneal macrophages, maintained in tissue culture for 24 h before challenge, could ingest but not kill *C. albicans* (34). Ozato and Uesaka obtained peritoneal macrophages from caseinate-treated mice of a different strain and placed them into tissue culture for 24 to 48 h before testing their candidacidal activity. Although these cells inhibited isotope incorporation and filamentation by the intracellular yeasts for 2 to 3 h, virtually all of the ingested organisms grew thereafter (26). It is unknown whether the period of tissue culture, known to diminish fungicidal (16) and cytotoxic (11, 33) activity of mononuclear phagocytes in other systems, contributed to the functional deficiencies of peritoneal macrophages in these studies.

Two recent studies reported that resident rabbit alveolar macrophages are active against *C. albicans* in vitro. Arai et al. reported that approximately 20% of the organisms ingested by these macrophages appeared nonviable after 2 h of incubation. This contrasted with 45 to 60% nonviability among the *Candida* incubated with guinea pig heterophils (2). Peterson and Calderone measured inhibition of [$^3$H]leucine incorporation by yeast cells to demonstrate antifungal activity and reported substantial inhibitory activity by macrophages (30). Both Arai et al. and Peterson and Calderone used freshly harvested macrophages in their assays, without subjecting them to the unknown consequences of preliminary tissue culture. More recent studies by Peterson and Calderone have shown that lysosomal extracts from rabbit alveolar macrophages inhibit uptake of specific amino acids by *C. albicans* (31).

Our results confirm that rabbit alveolar macrophages kill *C. albicans*. Indeed, their ability to do so is similar in extent to that of human granulocytes (19). In contrast, resident peritoneal macrophages exert significantly less candidacidal activity. Peritoneal macrophages recovered from rabbits that had been stimulated by the intravenous and intraperitoneal injection of complete Freund adjuvant were increased approximately sevenfold in number and twofold in their candidacidal activity. Such alterations could result from recruitment of more efficacious cells from circulating macrophage precursors or from the in situ proliferation and activation of the resident population. Both effects are compatible with the host response to mycobacterial antigens (9, 23, 37). The ability of resident macrophage populations to respond to certain stimuli by increasing their fungicidal efficacy is noteworthy and merits examination in experimental models of *Candida* infection.

Our previous studies have demonstrated that human monocytes and neutrophils kill *C. albicans* primarily by mechanisms that depend on

myeloperoxidase (17, 18, 20). It has been both claimed (29, 32) and denied (3, 5, 10, 28) that rabbit macrophages possess peroxidase activity, and the involvement of this peroxidase (if it exists) in microbicidal reactions (29) remains to be established. Azide is a particularly effective inhibitor of the peroxidase of human neutrophils and monocytes and has been reported to inhibit the putative macrophage peroxidase as well (32). Although we used 2 mM azide in this study, our unpublished data indicate that 0.2 mM azide suffices to block virtually completely the ability of human neutrophils to kill *C. albicans* by their peroxidase-dependent fungicidal mechanism. Human monocytes are completely blocked by 0.4 mM azide. Higher azide concentrations (10 mM, 20 mM) substantially impaired macrophage viability and phagocytic activity, precluding any meaningful testing. That candidacidal activity by rabbit alveolar macrophages was insensitive to 2 mM azide offers strong evidence that, even if peroxidase activity is present in these cells, it does not contribute to their ability to kill *C. albicans*. Cyanide, phenylbutazone, and sulfadiazine also inhibit peroxidase-mediated reactions in human monocytes and neutrophils, yet none inhibited candidacidal activity by rabbit macrophages. Thus, our studies demonstrate that the mechanism used by alveolar and peritoneal macrophages to kill *C. albicans* differs from the peroxidase-mediated candidacidal process of human blood monocytes.

In contrast, iodoacetate greatly inhibited candidacidal activity by alveolar macrophages, although it lacked effect on blood monocytes (Table 3). Altough iodoacetate is a classical glycolytic inhibitor, it also reacts with sulfhydryl compounds and inhibits a myriad of metabolically unrelated enzymes (7). It reportedly ablates the postphagocytic respiratory stimulation of rabbit alveolar macrophages (25) and diminishes the ability of human blood monocytes to kill *Staphylococcus aureus* (12). It may (24) or may not (22) block bactericidal activity by murine peritoneal macrophages. Further study is required to ascertain the mechanism of its effect on macrophage candidacidal activity.

Several investigators have examined the effects of cyanide and related inhibitors on the microbicidal activity of macrophages. Klebanoff and Hamon found that cyanide and azide substantially diminished the ability of resident rabbit alveolar macrophages to kill ingested *S. aureus* (14). The failure of cyanide to inhibit candidacidal activity by these cells, found in the present study, may indicate that their staphylocidal and candidacidal mechanisms differ.

Since the other studies did not employ rabbit macrophages, they are of doubtful relevance to our own. However, Miller found that cyanide (and anaerobiosis, antimycin A, amytal, and certain dyes) substantially reduced the bactericidal activity of murine peritoneal macrophages (22), and suggested that an intact respiratory electron chain was required for the process. In contrast, anaerobic conditions did not diminish the ability of resident murine peritoneal macrophages to kill *Escherichia coli* and *Staphylococcus enteritidis* (36), and cyanide reportedly did not diminish the ability of tissue culture-grown human macrophages to kill *Listeria monocytogenes* (4).

Clearly, the microbicidal mechanisms of mammalian macrophages require further definition. Although studies of human macrophages will ultimately be required, we believe that rabbits afford a fine model for evaluating possible microbicidal mechanisms and delineating potential mechanisms of fungicidal activation.

## ACKNOWLEDGMENTS

This work was supported in part by Public Health Service research grants AI-16252, AI-16005, and CA-15688 from the National Institutes of Health and by a grant from the California Institute for Cancer Research.

## LITERATURE CITED

1. **Ansley, H., and L. Ornstein.** 1970. Enzyme histochemistry and differential white cell counts on the Technicon Hemalog D. Adv. Autom. Anal. 1:5-14.
2. **Arai, T., Y. Mikami, and K. Yokoyama.** 1977. Phagocytosis of *Candida albicans* by rabbit alveolar macrophages and guinea pig neutrophils. Sabouraudia **15**: 171-178.
3. **Biggar, W. D., and J. M. Sturgess.** 1976. Peroxidase activity of alveolar macrophages. Lab. Invest. **34**:31-42.
4. **Cline, M. J.** 1970. Bactericidal activity of human macrophages: analysis of factors influencing the killing of *Listeria monocytogenes*. Infect. Immun. **2**:156-161.
5. **Cohn, Z. A., and E. Wiener.** 1963. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. J. Exp. Med. **118**:991-1008.
6. **Diamond, R. D., and R. Krzesicki.** 1978. Mechanisms of attachment of neutrophils to *Candida albicans* pseudohyphae in the absence of serum, and of subsequent damage to pseudohyphae by microbicidal processes of neutrophils *in vitro*. J. Clin. Invest. **61**:360-369.
7. **Dixon, M., and E. C. Webb.** 1964. Enzymes, 2nd ed., p. 341-343. Longmans, Green and Co., Ltd., London.
8. **Drazin, R. E., and R. I. Lehrer.** 1977. Fungicidal properties of a chymotrypsin-like cationic protein from human neutrophils: adsorption to *Candida parapsilosis*. Infect. Immun. **17**:382-388.
9. **Faulkner, C. S. II, and J. R. Esterly.** 1971. Ultrastructural changes in the alveolar epithelium in response to Freund's adjuvant. Am. J. Pathol. **64**:559-566.
10. **Gee, J. B. L., C. L. Vassallo, P. Bell, J. Kaskin, R. E. Basford, and J. B. Field.** 1970. Catalase-dependent peroxidase metabolism in the alveolar macrophage during phagocytosis. J. Clin. Invest. **49**:1280-1287.
11. **Keller, R., R. Keist, and R. J. Ivatt.** 1974. Functional and biochemical parameters of activation related to macrophage cytostatic effects on tumor cells. Int. J. Cancer **14**:675-683.
12. **King, G. W., G. Bain, and A. F. LoBuglio.** 1975. The effect of tuberculosis and neoplasia on human monocyte staphylocidal activity. Cell. Immunol. **16**:389-395.
13. **Klebanoff, S. J.** 1968. Myeloperoxidase-halide-hydrogen

peroxide antibacterial system. J. Bacteriol. 95:2131-2138.
14. **Klebanoff, S. J., and C. B. Hamon.** 1975. Antimicrobial systems of mononuclear phagocytes, p. 507-529. *In* R. vanFurth (ed.), Mononuclear phagocytes in immunity, infection and pathology. Blackwell Scientific Publications, Oxford.
15. **Lehrer, R. I.** 1970. Measurement of candidacidal activity of specific leukocyte types in mixed cell populations. I. Normal, myeloperoxidase-deficient, and chronic granulomatous disease neutrophils. Infect. Immun. 2:42-47.
16. **Lehrer, R. I.** 1970. The fungicidal activity of human monocytes: a myeloperoxidase-linked mechanism. Clin. Res. 18:408.
17. **Lehrer, R. I.** 1972. Functional aspects of a second mechanism of candidacidal activity in human neutrophils. J. Clin. Invest. 51:2566-2572.
18. **Lehrer, R. I.** 1975. The fungicidal mechanisms of human monocytes. I. Evidence for myeloperoxidase-linked and myeloperoxidase-independent candidacidal mechanisms. J. Clin. Invest. 55:338-346.
19. **Lehrer, R. I.** 1978. Host defense mechanisms against disseminated candidiasis, p. 94-96. *In* Edwards, J. E., Jr. (moderator), Severe candidal infections. Clinical perspective, immune defense mechanisms, and current concepts of therapy. Ann. Intern. Med. 89:91-106.
20. **Lehrer, R. I., and M. J. Cline.** 1969. Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to *Candida* infection. *J. Clin. Invest.* 48:1478-1488.
21. **Lehrer, R. I., and M. J. Cline.** 1969. Interaction of *Candida albicans* with human leukocytes and serum. J. Bacteriol. 98:996-1004.
22. **Miller, T. E.** 1971. Metabolic event involved in the bactericidal activity of normal mouse macrophages. Infect. Immun. 3:390-397.
23. **Myrvik, Q. N., E. S. Leake, and S. Oshima.** 1962. A study of macrophages and epithelioid-like cells from granulomatous (BCG-induced) lungs of rabbits. J. Immunol. 89:745-751.
24. **Nakae, T., M. Nakano, and K. Saito.** 1967. Metabolic study of intracellular killing of bacteria by mouse macrophages. Jpn. J. Microbiol. 11:189-201.
25. **Ouchi, E., R. J. Selvaraz, and A. J. Sbarra.** 1965. The biochemical activities of rabbit alveolar macrophages during phagocytosis. Exp. Cell Res. 40:456-468.
26. **Ozato, K., and I. Uesaka.** 1974. The role of macrophages in *Candida albicans* infection *in vitro*. Jpn. J. Microbiol. 18:29-35.
27. **Patterson-Delafield, J., and R. I. Lehrer.** 1977. A simple microscopic method for identifying and quantitating phagocytic cells in vitro. J. Immunol. Methods 18:377-379.
28. **Paul, B. B., R. R. Strauss, A. A. Jacobs, and A. J. Sbarra.** 1970. Function of $H_2O_2$, myeloperoxidase, and hexose monophosphate shunt enzymes in phagocytizing cells from different species. Infect. Immun. 1:338-344.
29. **Paul, B. B., R. R. Strauss, R. J. Selvaraj, and A. J. Sbarra.** 1973. Peroxidase mediated antimicrobial activities of alveolar macrophage granules. Science 181:849-850.
30. **Peterson, E. M., and R. A. Calderone.** 1977. Growth inhibition of *Candida albicans* by rabbit alveolar macrophages. Infect. Immun. 15:910-915.
31. **Peterson, E. M., and R. A. Calderone.** 1978. Inhibition of specific amino acid uptake in *Candida albicans* by lysosomal extracts from rabbit alveolar macrophages. Infect. Immun. 21:506-513.
32. **Romeo, D., R. Cramer, T. Marzi, R. Soranzo, G. Zabucchi, and F. Rossi.** 1973. Peroxidase activity of alveolar and peritoneal macrophages. RES J. Reticuloendothel. Soc. 13:399-409.
33. **Sorrell, T. C., R. I. Lehrer, and M. J. Cline.** 1977. Mechanism of non-specific macrophage-mediated cytotoxicity: evidence for lack of dependence upon oxygen. J. Immunol. 120:347-352.
34. **Stanley, V. D., and R. Hurley.** 1969. The growth of *Candida* species in cultures of mouse peritoneal macrophages. J. Pathol. 97:357-366.
35. **Taschdjian, C. L., J. J. Burchall, and P. J. Kozinn.** 1960. Rapid identification of *Candida albicans* by filamentation on serum and serum substitutes. Am. J. Dis. Child. 99:212-215.
36. **Thalinger, K. K., and G. L. Mandell.** 1971. Bactericidal activity of macrophages in an anaerobic environment. RES J. Reticuloendothel. Soc. 9:393-396.
37. **Volkman, A.** 1976. Disparity in origin of mononuclear phagocyte population. RES J. Reticuloendothel. Soc. 19:249-268.