# The Fungicidal Mechanisms of Human Monocytes

## I. EVIDENCE FOR MYELOPEROXIDASE-LINKED AND MYELOPEROXIDASE-INDEPENDENT CANDIDACIDAL MECHANISMS

ROBERT I. LEHRER

*From the Department of Medicine, School of Medicine, The Center for the Health Sciences, University of California, Los Angeles, California 90024*

ABSTRACT We tested the ability of human peripheral blood monocytes to kill *Candida albicans* and *Candida parapsilosis*. Evidence that multiple fungicidal mechanisms operate in normal monocytes was found. Normal monocytes ingested and killed viable *C. albicans*, and could iodinate heat-killed *C. albicans*. Both functions were defective in monocytes from subjects with myeloperoxidase deficiency or chronic granulomatous disease. Methimazole, isoniazid, and aminotriazole inhibited iodination by normal monocytes without impairing their ability to kill *C. albicans*, indicating that iodination was not essential to the myeloperoxidase-hydrogen peroxide-mediated fungicidal system of the monocyte. *C. parapsilosis*, an organism killed with supranormal efficacy by monocytes from a patient with hereditary myeloperoxidase deficiency, was selected to examine the myeloperoxidase-independent fungicidal mechanisms of monocytes. Monocytes were obtained from the blood of normal or leukemic subjects and homogenized in 0.34 M sucrose to yield fractions rich in cytoplasmic granules. These fractions were extracted with 0.01 M citric acid and the soluble components were separated by micropreparative polyacrylamide electrophoresis. Monocytes were found to contain cationic proteins, other than myeloperoxidase, that kill *C. parapsilosis* in vitro.

## INTRODUCTION

A microbicidal system that includes myeloperoxidase (MPO)[1] and hydrogen peroxide enhances the ability of human neutrophils to kill certain bacteria and the fungus *C. albicans* (1, 2). A second candidacidal mechanism, independent of these factors, has also been delineated in the human neutrophil (3). In contrast, relatively little is known about the fungicidal mechanisms of mononuclear phagocytes.

We tested the ability of normal human peripheral blood monocytes to kill and iodinate selected *Candida* species and compared these activities with those of monocytes deficient in MPO content or hydrogen peroxide-generating capacity. Our data delineate two fungicidal systems in human monocytes.

## METHODS

*Leukocyte preparation.* Blood was collected in heparinized syringes from healthy young men and women, patients with acute monocytic leukemia, three MPO-deficient persons, and six boys or young men with chronic granulomatous disease (CGD). The clinical histories of C. J. B. (hereditary MPO deficiency) and H. H. (refractory megaloblastic anemia with MPO-deficient leukocytes) have been described (2, 4); M. M., a sister of C. J. B., has hereditary MPO deficiency but is otherwise healthy.

The methods used to prepare mixed leukocytes (5) and purified granulocyte and mononuclear cell fractions by Hypaque-Ficoll sedimentation have been described (3). Before the Hypaque-Ficoll sedimentation, the heparinized blood was centrifuged at 200 *g* for 10 min, the platelet-rich supernatant plasma was removed and centrifuged at 1,500 *g* for 10 min to deposit the platelets, and the blood was reconstituted by adding back the platelet-poor plasma. Granulocyte fractions contained 87–99% neutrophils, 1–8% eosinophils, 1–7% lymphocytes, 0.1–0.8% basophils, and less than 0.2% monocytes. Mononuclear cell fractions contained 62–87% lymphocytes, 10–38% monocytes, 0.4–1.8% basophils, and 0.1–0.5% neutrophils, and were virtually platelet-free. Generally, mononuclear cells were classified according to their morphology in Giemsa-stained preparations. In a few experiments, this was supplemented by histochemical staining for peroxidase and alpha naphthyl butyrate esterase activity (6) to distinguish monocytes (positively stained) from lymphocytes.

This work was presented, in part, at the national meeting of the American Federation for Clinical Research, Atlantic City, N. J., May, 1974.

*Received for publication 8 April 1974 and in revised form 5 August 1974.*

[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; MPO, myeloperoxidase.

In experiments involving subcellular fractionation, the mononuclear cell fraction from normal blood was further enriched in monocytes by suspending the cells in Hanks' balanced salt solution[2] containing 15% vol/vol fetal calf serum[2] and adding them to 150 × 15-mm sterile plastic Petri dishes[3] and incubating for 2 h at 37°C. The nonadherent cells were decanted and the surface of the Petri dish was washed several times with balanced salt solution. Finally, cold phosphate-buffered saline containing 2 mM EDTA was added and, after 10 min, the adherent cells were stripped from the surface by gentle pipetting, collected, and washed with 0.34 M sucrose by centrifugation. Such preparations contained 60–75% typical monocytes, rare granulocytes, and 25–40% small mononuclear cells, presumably lymphocytes. Most of the monocytes were viable as judged by their appearance under light and phase microscopy, phagocytic ability, and trypan blue exclusion. The yield of monocytes from 450 ml of blood was usually $1–2 \times 10^8$ cells. Leukemic blood did not require this additional purification step, as the initial Hypaque-Ficoll mononuclear cell band contained fewer than 5% of contaminating lymphocytes.

Monocyte mixtures were suspended in 0.34 M sucrose and disrupted with a motor-driven Teflon-glass homogenizer until more than 90% cell breakage was achieved (about 5 min, 0°C). Centrifugation (250 *g*, 10 min) deposited unruptured cells and coarse cellular debris, and the opalescent supernate was centrifuged at 27,000 *g* to sediment a lysosome-rich fraction. This sediment was extracted with 0.25–0.5 ml of 0.01 M citric acid for 60 min at 0°C and again subjected to centrifugation at 27,000 g for 20 min. The supernate from this run contained about half of the original pellet's protein and was examined for the presence of candidacidal components by methods that will be described fully elsewhere.[4] Briefly, 200 or 400 μg of protein with a small amount of methyl green to mark the electrophoretic front was applied to polyacrylamide gel columns, 5 mm diameter, and polymerized by light in the presence of riboflavin and tetramethylethylenediamine.[5] Buffers, stacking gel, and sample gels were essentially as described by Reisfeld, Lewis, and Williams (7). After the dye front had migrated 35–45 mm (approximately 60–90 min) in response to a current flow of 4 mA/gel, the gels were removed from their glass tubes and rinsed for 15 min in sterile distilled water to remove the electrophoresis buffers partially. They were then sectioned at 1-mm intervals, and the individual sections were placed into numbered wells in a sterile plastic Microtest II plate.[3] 200 μl of sterile distilled water was added to cover each slice, and the plate was stored at 4°C for 16 h to elute the applied protein. In some experiments the polyacrylamide gel column was first hemisected longitudinally, one half stained for protein with amido black, and the other half sliced and eluted as above. Such gels contained 400 μg of protein. Protein concentrations were measured by the method of Lowry, Rosebrough, Farr, and Randall with hen's egg white lysozyme[5] as standard (8). Protein concentrations in the eluates were determined by a micromodification of the method of Lowry et al. employing a fiberoptic Aminco microfluorocolorimeter.[6]

[2] Grand Island Biologicals, Oakland, Calif.

[3] Falcon Plastics, Oxnard, Calif.

[4] R. I. Lehrer, K. I. Mitchell, and R. B. Hake. Fungicidal mechanisms of mammalian granulocytes. In preparation.

[5] Sigma Chemical Co., St. Louis, Mo.

[6] American Instrument Co., Inc., Silver Spring, Md.

*Fungicidal activity.* The ability of intact neutrophils and monocytes to kill *C. albicans* was tested by the previously described method of "specific staining" (9). Stationary-phase 3–5-day-old *Candida* cultures were washed, counted, and added to leukocytes to achieve a ratio of 1 *Candida* cell/granulocyte in studies with mixed or granulocyte-enriched fractions and 1 *Candida* cell/2–3 monocytes in studies with mononuclear cell preparations. The percentage of *Candida* cells that had the staining characteristics of "ghosts" within a given phagocytic cell type was taken to represent the percentage killed by that cell type within the 2½-h incubation period. The percentage of intracellular *Candida* cells that had developed filamentous "germ tubes" within a given phagocyte type was also determined. Filamentation indicates viability and intraleukocytic growth of a yeast cell. The assumptions underlying these assays have been discussed elsewhere (10).

A number of compounds were tested to determine their effects on the ability of monocytes to kill *C. albicans*. These compounds were dissolved shortly before use in Hank's balanced salt solution at pH 7.4 and preincubated with the leukocyte and serum mixture for 15 min at 37°C before *C. albicans* was added. Paired assays were conducted with untreated but otherwise identical normal leukocyte mixtures serving as controls. The results were expressed as a relative candidacidal index: percent of *C. albicans* within treated monocytes converted to ghosts after 2½ h/percent *C. albicans* ghosts within the paired control. A filamentation ratio was calculated as the percent of *C. albicans* with germ tubes in treated monocytes/percent of *C. albicans* with germ tubes in the control monocytes.

The ability of monocytes and neutrophils to kill *C. parapsilosis* and *C. pseudotropicalis* was studied by a previously described colony-counting procedure (3): purified cell populations were used in these studies. The test organisms were cultured for 48–72 h at 33°C in tryptose phosphate broth[7] and were added at a ratio of 1 colony-forming U/3–5 phagocytic leukocytes. Ingestion was monitored by periodically removing samples from the incubation mixtures and examining them microscopically. Although it was our impression that monocytes ingested the yeast somewhat more slowly than neutrophils, virtually all of the added organisms were ingested within 30 min by monocytes and within 5 min by neutrophils.

The ability of monocyte constituents to kill *C. parapsilosis* in cell-free systems was tested by two methods: dye exclusion and colony counting. Colony count assays were conducted in sterile 12 × 75-mm plastic culture tubes[3]: dye-exclusion assays were conducted in sterile Microtest II culture plates.[3] Assay vessels contained 100 μl of the eluates from the serially sectioned polyacrylamide gel and 25 μl of *C. parapsilosis*, ATCC 22019, at a concentration of $3 \times 10^7$ cells/ml in 0.075 M, pH 5.0, citrate-phosphate buffer. Assay components were incubated at 37°C for 60 min and samples were removed, suitably diluted, and spread on Sabouraud's agar Petri dishes, which were incubated for 48–72 h at 37°C. No additional colony formation developed if the incubation period was extended to 2 wk. Controls included eluates from sections of the gel cathodal to the dye front, distilled water, and in some experiments, eluates prepared from gels to which no sample was applied. The latter eluates lacked intrinsic candidacidal activity.

After removing samples for colony counting, 50 μl of a solution containing 0.3% trypan blue and 0.1% eosin was added to the remainder of the incubation mixtures. After

[7] Difco Laboratories, Detroit, Mich.

TABLE I

*Effect of Sodium Azide on Candicidal Activity*

| White cell donor | Candida ghosts in | | | | Pseudogerm tubes in | | | |
|---|---|---|---|---|---|---|---|---|
| | Neutrophils | | Monocytes | | Neutrophils | | Monocytes | |
| | Control | Azide | Control | Azide | Control | Azide | Control | Azide |
| | % | | | | | | | |
| 1 | 35.0 | 5.0 | 60.0 | 4.0 | 22.0 | 61.0 | 15.0 | 76.0 |
| 2 | 35.0 | 2.0 | 74.0 | 7.0 | 14.0 | 57.0 | 6.0 | 65.0 |
| 3 | 36.0 | 0.0 | 63.0 | 10.0 | 10.0 | 63.0 | 13.0 | 78.0 |
| Mean | 35.3 | 2.3 | 65.7 | 7.0 | 15.3 | 60.3 | 11.3 | 73.0 |

Effect on cellular candidacidal activity of the addition of 2 mM sodium azide to mixed peripheral blood leukocytes 5 min after *C. albicans* had been added to the incubation mixtures.

the contents were mixed and allowed to stand at 37°C for 10 min, the percentage of the yeast cells stained was determined by direct microscopic observation of a drop of the suspension. Preliminary experiments revealed that the staining mixture was not toxic to *C. parapsilosis*, that all of the stained cells were nonviable in terms of subsequent colony formation, and that these proportions of stain afforded optimal contrast between the stained organisms and the background color of the solution.

*Quantitative iodination.* Studies were performed with granulocyte-enriched and mononuclear cell fractions by the method of Pincus and Klebanoff (11) with the following modifications: [$^{125}$I]NaI replaced [$^{131}$I]NaI, heat-killed *C. albicans* was used as the particle, and a total of 700 nmol of glucose (including that provided by the serum component of the mixture) was present. In most experiments, tubes contained $2.5 \times 10^6$ granulocytes or monocytes and $5 \times 10^6$ heat-killed *C. albicans* in a final volume of 0.5 ml. In experiments with CGD leukocytes, tubes contained $1 \times 10^6$ granulocytes or monocytes from the subjects or normal controls. Other components were unchanged.

## RESULTS

Normal monocytes killed 63.4±10.2% of ingested *C. albicans* in 2½ h (91 subjects, mean ±SD). These studies employed mixed leukocyte preparations and killing was equated to the percentage of intramonocytic *C. albicans* cells converted to ghosts during the incubation period. Of the remaining intramonocytic organisms, 10.5±5.9% bore filamentous pseudogerm tubes and 26.4 ±9.2% had maintained their original shape and staining characteristics.

MPO-deficient monocytes from C. J. B. were tested on eight occasions over a period of 3 yr. These monocytes killed 9.5±5.0% of ingested *C. albicans* under the same conditions of testing ($P < 0.001$). Of the remaining intramonocytic *C. albicans* cells, 54.6±21.7% had pseudogerm tubes ($P < 0.001$) and 35.6±23.7% retained their original morphology ($P < 0.025$). MPO-deficient monocytes from M. M. and H. H. were each tested on a single occasion, and each killed 4% of ingested *C. albicans*. Leukocytes from three unrelated boys with CGD were each tested on one or more occasions. The monocytes in these mixtures killed between 1 and 4% of ingested organisms, whereas over 90% of the intramonocytic *C. albicans* cells contained pseudogerm tubes at the conclusion of the incubation period ($P < 0.001$).

As most of the *C. albicans* cells added to mixed leukocyte preparations were ingested by neutrophils rather than monocytes, we wondered if the apparent candidacidal activity of the monocytes in these mixtures was a consequence of preferential uptake of nonviable organisms by these cells. This was excluded by a set of studies wherein 2 mM sodium azide was added to the leukocyte-*C. albicans* mixtures after sufficient time (5 min) had elapsed to permit virtually all of the added organisms to be ingested. As shown by the data in Table I, azide diminished the percentage of ghosts in both neutrophils and monocytes substantially, and most of the ingested organisms went on to develop pseudogerm tubes, denoting viability and growth in their intracellular milieu. The somewhat higher percentage of viable *Candida* cells in azide-treated monocytes effectively excludes the possibility that they phagocytize nonviable organisms preferentially.

To examine the effects of the Hypaque-Ficoll cell purification procedure on the functional integrity of monocytes and neutrophils, we measured the candidacidal efficacy of these cells with *C. albicans*. As shown in Table II, neutrophils killed *C. albicans* with equal effectiveness whether tested in mixed leukocyte suspensions or as purified granulocyte preparations. In contrast, however, normal monocytes consistently demonstrated more candidacidal activity when tested in the presence of neutrophils. In purified monocyte preparations, normal monocytes killed 27.2±2.0% of ingested organisms (mean±SEM, 10 subjects); MPO-deficient monocytes killed 10.0±3.5% (C. J. B., three experiments, $P < 0.001$). To examine whether purified monocytes were less effective because they contained more ingested yeast cells per phagocyte, we challenged purified monocytes with varying numbers of

*C. albicans* cells. Candidacidal activity was equivalent at ratios that ranged from 0.1 to 1.25 *Candida* cells/monocyte and fully encompassed the range encountered in the experiments summarized in Table II. The possibility that the impaired performance of purified normal monocytes resulted from monocyte damage incurred during the cell separation procedure was excluded by recombining the Hypaque-Ficoll-purified mononuclear cells and granulocyte fractions to yield mixed leukocytes. Under these circumstances the ability of monocytes to kill *C. albicans* returned to values approximating those obtained with unfractionated mixed leukocytes.

Although these findings suggest some type of monocyte-granulocyte interaction, we have been unable to clarify its nature. The candidacidal ability of normal monocytes in granulocyte-free fractions was not enhanced by the addition of lysates of intact normal neutrophils, of neutrophil cytoplasmic granules, or of extracellular supernate from phagocytic neutrophils. The possibility of sequential phagocytosis of *C. albicans* cells by neutrophils and monocytes has not been excluded. Although further investigation is warranted to establish the cause of this interesting phenomenon, the data with purified cell preparations confirm that MPO-deficient monocytes are significantly impaired in their ability to kill *C. albicans*.

*Other* Candida *species.* The relative abilities of neutrophils and monocytes from normal subjects and patients with CGD or MPO deficiency to kill other *Candida* species was compared by experiments performed by a colony-counting technique (Table III).

As we have previously reported, neutrophils from the MPO-deficient subject C. J. B. killed *C. parapsilosis* and *C. pseudotropicalis* with normal efficacy.

His MPO-deficient monocytes showed a consistently increased ability to kill both *Candida* species as compared to normal monocytes. Neutrophils from subjects with CGD killed *C. parapsilosis* well but had a diminished ability to kill *C. pseudotropicalis* ($P < 0.05$, 180 min). In contrast to the relatively intact candidacidal activity of their neutrophils, monocytes from the boys with CGD showed impaired killing when tested against these fungal species.

***Inhibition of candidacidal activity.*** 23 compounds were

TABLE II

*Candidacidal Ability of Leukocytes in Various Preparations*

| | *C. Albicans* killed by | | | | | |
|---|---|---|---|---|---|---|
| | Neutrophils | | | Monocytes | | |
| Experiment | Mixed | Purified | Combined | Mixed | Purified | Combined |
| | % | | | | | |
| 1 | 43.5 | 52.5 | 44.4 | 51.0 | 24.5 | 55.0 |
| 2 | 31.2 | 29.7 | 25.8 | 55.6 | 21.5 | 39.4 |
| 3 | 31.0 | 32.3 | 25.3 | 41.0 | 29.0 | 43.3 |
| 4 | 46.0 | 37.0 | 33.5 | 79.8 | 24.3 | 57.5 |
| 5 | 26.7 | 32.5 | 28.0 | 40.0 | 24.0 | 41.5 |
| Mean | 35.7 | 36.8 | 31.4 | 53.5 | 24.7 | 47.4 |
| SEM | 3.8 | 4.1 | 3.6 | 7.2 | 1.2 | 3.7 |

Ability of human leukocytes to kill ingested *C. albicans* when tested in mixed leukocyte preparations, preparations purified by Hypaque-Ficoll sedimentation, and in mixed cell populations prepared by combining neutrophils and monocytes subjected to Hypaque-Ficoll sedimentation. Five experiments, each with different normal donors, are shown.

TABLE III

*Candidacidal Activity of Human Neutrophils and Monocytes*

| | | Reduction from initial colony count levels by | | | | | |
|---|---|---|---|---|---|---|---|
| | | Neutrophils | | | Monocytes | | |
| Organism | Incubation time | Normal | MPO-Deficient | CGD | Normal | MPO-Deficient | CGD |
| | *min* | % | | | | | |
| *C. parapsilosis* | 90 | 60.6±4.1 | 57.9±6.1 | 55.9±3.7 | 35.5±4.6 | 51.6±8.5 | 5.8±8.9* |
| | 180 | 73.5±4.7 | 77.9±5.6 | 65.2±5.7 | 46.5±3.7 | 82.6±7.1‡ | 9.0±4.1‡ |
| *n* | | 12 | 5 | 4 | 12 | 6 | 4, 6§ |
| *C. pseudotropicalis* | 90 | 74.5±7.6 | 71.3±4.1 | 45.5, 48.5 | 50.3±5.2 | 77.2±1.5* | −4.0 |
| | 180 | 90.6±4.0 | 91.0±0.6 | 43.5, 60.1 | 65.7±2.9 | 90.1±0.9‡ | −17.5 |
| *n* | | 6 | 3 | 2 | 6 | 3 | 1 |

Data expressed as mean±SEM, or by individual values if only one or two experiments were done. MPO-deficient leukocytes were obtained from patient C. J. B. Six subjects with CGD were tested. Statistical analysis by unpaired *t* test.

* $P < 0.01$.

‡ $P < 0.001$.

§ Four experiments were analyzed at 90 min, two additional experiments were evaluable at 180 min. Negative numbers signify that the colony count exceeded that of the initial cell-free control specimen.

TABLE IV

*Effect of Various Compounds on the Ability of Normal Human Monocytes to Kill Ingested C. albicans*

| Agent tested | Concentration | Trials | Monocyte candidacidal activity | | | Filamentation ratio |
|---|---|---|---|---|---|---|
| | | | *Mean* | *SEM* | P *value* | *Mean* |
| Metabolic inhibitors | | | | | | |
| Puromycin | 20.0 μg/ml | 6 | 98.0 | 6.7 | NS | 0.6 |
| *p*-fluorophenylalanine | 1.0 mM | 3 | 97.8 | 5.5 | NS | 0.3 |
| Actinomycin D | 5.0 μg/ml | 6 | 101.1 | 4.1 | NS | 0.8 |
| Sodium fluoride | 1.0 mM | 3 | 90.8 | 2.4 | NS | 0.8 |
| Sodium iodoacetate | 0.1 mM | 3 | 101.6 | 1.7 | NS | 1.4 |
| 2,4-Dinitrophenol | 0.25 mM | 3 | 100.8 | 4.5 | NS | 1.1 |
| Sodium azide | 2.0 mM | 3 | 4.3 | 2.1 | <0.001 | 8.4 |
| Potassium cyanide | 1.0 mM | 8 | 31.7 | 5.8 | <0.001 | 0.3 |
| Anti-inflammatory compounds | | | | | | |
| Phenylbutazone | 0.5 mg/ml | 7 | 16.1 | 3.5 | <0.001 | 4.8 |
| Sodium salicylate | 0.5 mg/ml | 3 | 41.7 | 5.0 | <0.001 | 2.4 |
| Colchicine | 40.0 μg/ml | 5 | 61.1 | 5.7 | <0.005 | 2.7 |
| Aminopyrine | 0.5 mg/ml | 7 | 34.6 | 4.6 | <0.001 | 3.5 |
| Antipyrine | 0.5 mg/ml | 7 | 92.8 | 6.9 | NS | 1.0 |
| Hydrocortisone Na succinate | 0.5 mg/ml | 6 | 101.6 | 5.6 | NS | 1.0 |
| Prednisolone 21-phosphate | 0.1 mg/ml | 3 | 98.3 | 6.6 | NS | 1.1 |
| Chloroquine hydrochloride | 10.0 μg/ml | 4 | 94.6 | 2.2 | NS | 1.2 |
| Miscellaneous compounds | | | | | | |
| 3-Amino-1,2,4-triazole | 2.0 mM | 4 | 99.5 | 11.1 | NS | 1.0 |
| Methimazole | 1.0 mM | 4 | 104.3 | 4.9 | NS | 0.6 |
| Isoniazid | 1.0 mM | 3 | 90.4 | 8.3 | NS | 1.5 |
| Theophyllin | 1.0 mM | 5 | 48.7 | 6.4 | <0.001 | 2.8 |
| Dibutyryl cyclic 3′,5′-AMP | 1.0 mM | 4 | 40.8 | 8.8 | <0.01 | 3.7 |
| Ascorbic acid, Na salt | 10.0 mM | 3 | 50.1 | 10.6 | <0.05 | NT |
| Sodium sulfadiazine | 4.0 mM | 4 | 3.3 | 1.2 | <0.001 | 5.6 |

All experiments were conducted with mixed leukocyte populations, and data is expressed as percent of killing achieved relative to the untreated control. Statistical analysis was by paired *t* test. NS signifies $P > 0.05$. NT, not tested.

tested for their effects on the ability of normal monocytes to kill *C. albicans*. The compounds did not impair viability of *C. albicans* at the concentrations employed, and only one (see below) interfered with germ tube formation by the organism in the presence of serum. The results of these experiments, conducted in mixed leukocyte preparations by the specific staining assay, are tabulated in Table IV. All but one of the compounds that inhibited candidacidal activity for *C. albicans* also caused an increased filamentation ratio, providing confirmation that an increased percentage of intramonocytic *C. albicans* cells had remained viable and were able to grow within the monocyte. The sole exception to this correlation was noted in experiments with potassium cyanide, which inhibited killing without causing increased intracellular filamentation. We found potassium cyanide also retarded the germination of viable *C. albicans* cells in cell-free Hanks' solution containing 25% human serum, and believe this accounts for the discrepancy.

Although inhibitors of protein synthesis (puromycin, *p*-fluorophenylalanine), RNA synthesis (actinomycin), glycolysis (fluoride and iodoacetate), and oxidative phosphorylation (2,4-dinitrophenol) did not significantly reduce candidacidal activity, 10 of the other compounds tested did inhibit the ability of normal monocytes to kill *C. albicans*.

Monocyte candidacidal activity was diminished in the presence of the anti-inflammatory compounds phenylbutazone, sodium salicylate, and colchicine. Aminopyrine (4-dimethylamino-1,5-dimethyl-2-phenyl-3-pyrazolone) effectively inhibited candidacidal activity, whereas antipyrine, identical to aminopyrine except for the absence of a substitution on the fourth position of the pyrazolone ring, was ineffective as an inhibitor. Hydrocortisone sodium succinate, prednisolone-21-phosphate, and chloroquine were without effect at the concentrations tested.

Monocyte candidacidal activity was not significantly altered by aminotriazole, methimazole, and isoniazid.

TABLE V

*Iodination of Heat-Killed C. albicans by Normal and MPO-Deficient Leukocytes*

| Incubation conditions | Series I | | | | Series II | | | |
|---|---|---|---|---|---|---|---|---|
| | Normal | | MPO-deficient | | Normal | | CGD | |
| | Neutrophils | Monocytes | Neutrophils | Monocytes | Neutrophils | Monocytes | Neutrophils | Monocytes |
| Basal | 0.51±0.10* | 0.09±0.02 | 0.93 | 0.02 | 0.36 | 0.11 | 0.02 | 0.00 |
| Phagocytic | 3.18±0.16 | 0.57±0.07 | 2.78 | 0.03 | 8.63 | 1.15 | 0.05 | 0.00 |
| Increment | 2.59±0.13 | 0.46±0.06 | 1.85 | 0.01 | 8.27 | 1.04 | 0.03 | 0.00 |
| Experiments | 10 | 10 | 2 | 2 | 2 | 2 | 2 | 2 |

* Data are expressed as mean or mean ±SEM nanomoles iodide converted to an acid precipitable form per $10^7$ neutrophils or monocytes per hour under conditions described in the text. The data for MPO-deficient leukocytes were obtained in two experiments with blood from patient C. J. B. The experiments in series II were performed at a lower leukocyte concentration than was used in series I. and utilized blood from two boys (J. M. and D. D) with CGD.

Theophylline, dibutyryl cyclic AMP, and sodium ascorbate caused moderate reductions in candidacidal activity. Sulfadiazine, sodium azide, and potassium cyanide were potent inhibitors of the ability of monocytes to kill ingested *C. albicans.*

*Iodination.* Normal human monocytes iodinated heat-killed *C. albicans* about 15–20% as effectively as an equal concentration of normal neutrophils tested under parallel conditions (Table V). In two experiments, MPO-deficient monocytes (patient C. J. B.) failed to iodinate these ingested fungi despite intact phagocytic activity. We have previously reported the appreciable, although subnormal, ability of MPO-deficient neutrophils to iodinate heat-killed fungi (3). Neutrophils and monocytes from two subjects with CGD failed to iodinate ingested *C. albicans.*

*Correlation between iodination and killing C. albicans.* Iodination has been suggested as a mechanism of MPO-mediated microbicidal activity in the neutrophil (1). To determine whether iodination was necessary for the killing process in normal monocytes, we compared the ability of 19 compounds to influence iodination and killing of *C. albicans* by these cells (Fig. 1). Nine compounds (azide, cyanide, phenylbutazone, ascorbate, sulfadiazine, aminopyrine, colchicine, theophylline, and salicylate) inhibited both iodination and killing in parallel fashion. Six compounds inhibited neither killing nor iodination. These were antipyrine, prednisolone-21-phosphate, hydrocortisone sodium succinate, 2,4-dinitrophenol, and *p*-fluorophenylalanine. Sodium iodoacetate did not impair candidacidal activity but reduced iodination by approximately half. This effect on iodination may have been artifactual, resulting from a chemical expansion of the nonradioactive iodide pool that effectively reduced the specific activity of the radioactive iodide. The central observation in this series of experiments was that three compounds abolished iodination by monocytes without impairing their ability to kill *C. albicans.* These compounds were aminotriazole, methimazole, and isoniazid. We conclude that iodination is concomitant to rather than a cause of MPO-mediated candidacidal activity.

*MPO-independent mechanism(s).* Although the gran-

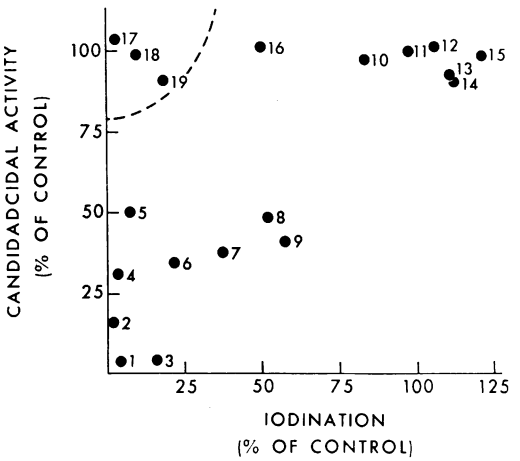


FIGURE 1 Comparative effects of selected compounds on the ability of monocytes to kill and iodinate ingested *C. albicans.* The compounds, tested as described in the text, are indicated by the following numerals: 1, sodium azide; 2, phenylbutazone; 3, sulfadiazine; 4, potassium cyanide; 5, sodium ascorbate; 6, dibutyryl 3′,5′-cyclic AMP; 7, colchicine 8, theophylline; 9, sodium salicylate; 10, *p*-fluorophenylalanine; 11, 2,4-dinitrophenol; 12, hydrocortisone sodium succinate; 13, antipyrine; 14, sodium fluoride; 15, prednisolone 21-phosphate; 16, sodium iodoacetate; 17, methimazole; 18, 3-amino-1,2,4-triazole (aminotriazole); 19, isonicotinic acid hydrazide (isoniazid). Concentrations of agents were as shown in Table IV. Iodination measurements represent mean values from two to four individual experiments with each agent. The dashed line separates three compounds (methimazole, aminotriazole, and isoniazid) that inhibited iodination without impairing killing.

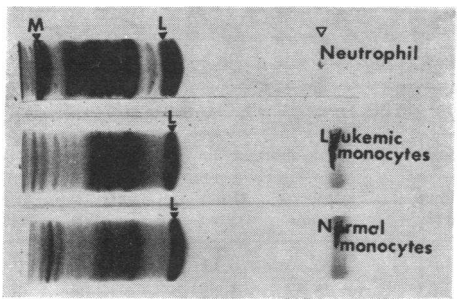


FIGURE 2 Components of human neutrophils and monocytes separated by polyacrylamide electrophoresis. Each gel contained 400 $\mu$g of a 0.01 M citric acid extract of the 27,000 *g* "granule"-fraction, prepared as described in the text. Leukemic monocytes were obtained from the blood of a patient with untreated acute monocytic leukemia. The position of MPO (M) and lysozyme (L) in the gels is indicated on the photograph. The electrophoretic front has been marked by India ink, and is shown by the symbol (▽). The cathode is towards the right. Gels were stained for protein with amido black.

ules of mammalian neutrophils are known to contain components other than MPO that kill fungi or bacteria (12, 13), similar components have not been isolated from blood monocytes.

That MPO-deficient monocytes from patient C. J. B. killed *C. pseudotropicalis* and *C. parapsilosis* with more than the normal efficacy proves that MPO-independent fungicidal systems exist in human monocytes. We have documented the presence of MPO-independent fungicidal mechanisms in human neutrophils (3), and have recently developed methodology for their characterization and identification (13). This methodology was applied to peripheral blood monocytes to seek the presence of fungicidal components among their cellular proteins. A lysosome-rich 27,000 *g* fraction was prepared from normal human neutrophils (97% pure), from normal human monocytes (70% pure, but without granulocyte contamination), and from leukemic monocytes (86% mature-appearing monocytes, 9% immature or atypical mononuclear cells, 4% lymphocytes, and 1% myelocytes), extracted with 0.01 M citric acid, and separated by electrophoresis on polyacrylamide gels (Fig. 2). The pherograms of normal and leukemic monocytes, stained by amido black, were quite similar and differed substantially from that of the neutrophil granules. When the monocyte components were eluted from the gels and tested to determine their ability to kill *C. parapsilosis* in vitro, similar patterns of activity were noted in the normal and leukemic monocyte fractions (Fig. 3). Fungicidal activity was detected by colony-counting and dye exclusion methodology. It was most prominent in the fractions that contained lysozyme activity, but was not restricted to these. It has been our experience that colony-counting methods can discriminate *Candida* populations whose ability to multiply is impaired, yet which have not undergone permeability alterations sufficient to result in dye uptake. It is possible that the apparently greater sensitivity of colony counting is a reflection of the mechanism(s) whereby leukocyte proteins kill *C. parapsilosis*.

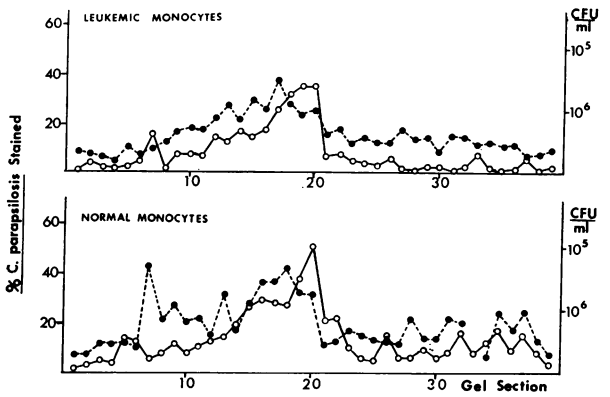


FIGURE 3 Candidacidal activity of human monocyte components. Gels prepared from the leukocytes whose components are illustrated in Fig. 2 were sectioned and eluted, and the eluates were tested for candidacidal activity against *C. parapsilosis* by the method of dye-exclusion (○) and colony-counting (●). Candidacidal components were present, especially among the gel sections containing the more cathodal monocyte proteins. Lysozyme activity was detected in fractions 18–20 from each gel. The cathodal dye front corresponded to fraction 38 (normal monocytes) and 39 (leukemic monocytes). CFU, colony-forming units.

## DISCUSSION

Our knowledge of the actual microbicidal mechanisms employed by mononuclear phagocytes is meager compared to our understanding of this function in mammalian granulocytes.

One microbicidal mechanism of granulocytes results from an interaction between MPO, an enzyme of the primary neutrophil granule, and hydrogen peroxide (14). Heritable or acquired conditions associated with deficiency of MPO or impaired generation of hydrogen peroxide in human neutrophils may increase the affected subject's susceptibility to certain bacterial or fungal infections (1, 4, 15). Neutrophils from such patients do not kill *C. albicans* effectively, although they maintain an ability to kill other *Candida* species, such as *C. parapsilosis* (3).

MPO occurs in the monocytes of many, but not all, mammalian species. It is present in the blood monocytes of man, mouse, rat, and guinea pig, but absent in the rabbit (16–18). In human and guinea pig monocytes, MPO is localized in granules homologous to the azurophil granules of the neutrophil (19). Although human alveolar macrophages and macrophages arising from the growth of blood monocytes in tissue culture lack peroxi-

dase activity, other mammalian macrophages may contain it. For example, rat liver Kupffer cells and guinea pig peritoneal macrophages show peroxidase activity in their perinuclear cisternae, Golgi vesicles, and in some membrane-bounded granules (20–22). The functional significance of this enzymatic activity and its relationship to the enzyme MPO remain to be established.

The metabolic response of human monocytes to phagocytosis includes a marked augmentation of their oxygen consumption and hexose monophosphate shunt activity (23) as well as the iodination of ingested microorganisms. These reactions imply the postphagocytic production of hydrogen peroxide and/or its interaction with MPO within their phagocytic vacuoles. The failure of iodination to occur in MPO-deficient or CGD monocytes is consonant with the assumption that this reaction is catalysed by MPO and $H_2O_2$ in the monocyte, as it is (at least in part) in the neutrophil.

Monocytes from patients with MPO-deficiency or CGD were impaired in their ability to kill *C. albicans,* suggesting that the MPO-$H_2O_2$ fungicidal mechanism operates in the monocyte as well as in the neutrophil. This conclusion is supported by our studies with inhibitors. Azide, cyanide, phenylbutazone, salicylate, colchicine, aminopyrine, theophylline, dibutyryl 3′,5′ cyclic AMP, and sulfadiazine all inhibited the ability of monocytes to kill ingested *C. albicans,* and all caused comparable decreases in the candidacidal activity of normal neutrophils (data not shown). Phenylbutazone has been found to inhibit the ability of human monocytes to kill certain intracellular bacteria (24).

The blood monocytes of patients with CGD (25, 26) or lipochrome histiocytosis, a related disorder (27), have been reported to manifest defective bactericidal activity. These results have recently been challenged on the basis that the antibiotics employed in testing their function might have influenced the results if the monocytes of CGD patients were more avidly phagocytic than normal (24). Our own studies on CGD monocytes were conducted in the absence of antibiotics, and revealed a marked microbicidal defect for two species of *Candida* susceptible to different microbicidal mechanisms.

As MPO-deficient and CGD monocytes also failed to iodinate ingested *C. albicans* normally, it was important to determine whether iodination served as the actual candidacidal event in normal monocytes or whether it was a noncausal concomitant to MPO-$H_2O_2$-mediated candidacidal activity. Although compounds that inhibited candidacidal activity caused a parallel decrease in iodination, the converse was not true. Methimazole, aminotriazole, and isoniazid completely blocked iodination without decreasing the ability of normal monocytes to kill *C. albicans.* We conclude that iodination is a noncausal concomitant of the candidacidal process in monocytes. Baehner and Johnston have also reported that human monocytes can iodinate ingested microorganisms. *Staphylococcus aureus* was used in their studies (28).

We observed that *C. parapsilosis* and *C. pseudotropicalis* were killed more effectively by MPO-deficient monocytes than by normal monocytes, unequivocally demonstrating the presence of MPO-independent fungicidal mechanisms in the monocyte. Diamond, Root, and Bennett investigated the ability of human monocytes to kill *Cryptococcus neoformans,* and found that this could be accomplished effectively, although with altered kinetics, in the presence of azide concentrations sufficient to inhibit MPO activity (29). This is compatible with the presence of MPO-linked and MPO-independent fungicidal systems in the monocyte with activity against this organism.

What is the nature of the MPO-independent fungicidal mechanisms of the monocyte? One possibility is that these cells might contain additional fungicidal constituents in their cytoplasmic granules. We have recently described a number of fungicidal proteins in fractions of normal neutrophils rich in cytoplasmic granules. These components are present in MPO-deficient and CGD neutrophils as well. Such components could explain the maintained ability of neutrophils from patients with these hereditary disorders of leukocyte function to kill *C. parapsilosis.*

We found that human monocytes contained components that kill *C. parapsilosis* in vitro. These components differ in several respects from the fungicidal proteins of the neutrophil, most strikingly by lacking the class of highly cathodal fungicidal esterases. We cannot yet assert that these components participate in monocyte fungicidal processes, but this hypothesis underlies our ongoing attempts to establish the intracellular localization and precise identification of these proteins.

We cannot presently explain why CGD monocytes, unlike CGD neutrophils, are so compromised in their ability to kill *C. parapsilosis.* It will be necessary to establish whether CGD monocytes contain the normal complement of candidacidal proteins, and to examine whether these proteins gain access to the phagocytic vacuole in normal as well as in CGD monocytes. For the moment we must note and underscore this discrepancy. Its explanation may eventually provide a means of validating an identification of the MPO-independent fungicidal mechanisms of the monocyte.

## ACKNOWLEDGMENTS

I thank Kathryn I. Mitchell and Randall B. Hake for skillful technical assistance.

The research was supported in part by grants from the National Institutes of Health (AI 10547 and CA 11067) and by a grant from the Research Corporation (Brown-Hazen Fund).

## REFERENCES


1. Klebanoff, S. J. 1967. Iodination of bacteria: a bactericidal mechanism. *J. Exp. Med.* **126**: 1063–1078.
2. 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.
3. Lehrer, R. I. 1972. Functional aspects of a second mechanism of candidacidal activity by human neutrophils. *J. Clin. Invest.* **51**: 2566–2572.
4. Lehrer, R. I., L. S. Goldberg, N. P. Rosenthal, and M. A. Apple. 1972. Refractory megaloblastic anemia with myeloperoxidase-deficient neutrophils. *Ann. Intern. Med.* **76**: 447–453.
5. Lehrer, R. I., and M. J. Cline. 1969. Interaction of *Candida albicans* with human leukocytes and serum. *J. Bacteriol.* **98**: 996–1004.
6. Ansley, H., and L. Ornstein. 1970. Enzyme histochemistry and differential white cell counts on the Technicon Hemalog D. *Adv. Autom. Anal.* **1**: 437–451.
7. Reisfeld, R. A., U. J. Lewis, and D. E. Williams. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature (Lond.)*. **195**: 281–283.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
9. 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.
10. Lehrer, R. I. 1972. The fungicidal activity of human leukocytes. *In* Phagocytic Mechanisms in Health and Disease, R. C. Williams, Jr. and H. H. Fudenberg, editors. Intercontinental Medical Book Corporation, New York. 151–166.
11. Pincus, S. H., and S. J. Klenbanoff. 1971. Quantitative leukocyte iodination. *N. Engl. J. Med.* **284**: 744–750.
12. Zeya, H. I., and J. K. Spitznagel. 1965. Cationic proteins of polymorphonuclear leukocyte lysosomes. I. Resolution of antibacterial and enzymatic activities. *J. Bacteriol.* **91**: 750–754.
13. Lehrer, R. I., K. I. Mitchell, and R. B. Hake. 1974. The fungicidal proteins of mammalian neutrophils. *J. Clin. Invest.* **53**: 44*a*. (Abstr.)
14. Klebanoff, S. J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J. Bacteriol.* **95**: 2131–2138.
15. Quie, P. G., J. G. White, B. Holmes, and R. A. Good. 1967. In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood. *J. Clin. Invest.* **46**: 668–679.
16. Van Furth, R., J. G. Hirsch, and M. E. Fedorko. 1970. Morphology and peroxidase cytochemistry of mouse promonocytes, monocytes, and macrophages. *J. Exp. Med.* **132**: 794–812.
17. Nichols, B. A., D. F. Bainton, and M. G. Farquhar. 1971. Differentiation of monocytes. Origin, nature, and fate of their azurophil granules. *J. Cell Biol.* **50**: 498–515.
18. Whitelaw, D. M. 1966. The intravascular lifespan of monocytes. *Blood J. Hematol.* **28**: 455–464.
19. Nichols, B. A., and D. F. Bainton. 1973. Differentiation of human monocytes in bone marrow and blood. Sequential formation of two granule populations. *Lab. Invest.* **29**: 27–40.
20. Cotran, R. S., and M. Litt. 1970. Ultrastructural localization of horseradish peroxidase and endogenous peroxidase activity in guinea pig peritoneal macrophages. *J. Immunol.* **105**: 1536–1546.
21. Widmann, J. J., R. S. Cotran, and H. D. Fahimi. 1972. Mononuclear phagocytes (Kupffer cells) and endothelial cells. Identification of two functional cell types in rat liver sinusoids by endogenous peroxidase activity. *J. Cell. Biol.* **52**: 159–170.
22. Daems, W. T., R. E. Poelmann, and P. Brederoo. 1973. Peroxidatic activity in resident peritoneal macrophages and exudate monocytes of the guinea pig after ingestion of latex particles. *J. Histochem. Cytochem.* **21**: 93–95.
23. Cline, M. J., and R. I. Lehrer. 1968. Phagocytosis by human monocytes. *Blood J. Hematol.* **32**: 423–435.
24. Steigbigel, R. T., L. H. Lambert, Jr., and J. S. Remington. 1974. Phagocytic and bactericidal properties of normal human monocytes. *J. Clin. Invest.* **53**: 131–142.
25. Davis, W. C., H. Huber, S. D. Douglas, and H. H. Fudenberg. 1968. A defect in circulating mononuclear phagocytes in chronic granulomatous disease of childhood. *J. Immunol.* **101**: 1093–1095.
26. Rodey, G. E., B. H. Park, D. B. Windhorst, and R. A. Good. 1969. Defective bactericidal activity of monocytes in fatal granulomatous disease. *Blood. J. Hematol.* **33**: 813–820.
27. Rodey, G. E., B. H. Park, D. K. Ford, B. H. Gray, and R. A. Good. 1970. Defective bactericidal activity of peripheral blood leukocytes in lipochrome histiocytosis. *Am. J. Med.* **49**: 322–427.
28. Baehner, R. L., and R. B. Johnston, Jr. 1972. Monocyte function in children with neutropenia and chronic infections. *Blood. J. Hematol.* **40**: 31–41.
29. Diamond, R. D., R. K. Root, and J. E. Bennett. 1972. Factors influencing killing of *Cryptococcus neoformans* by human leukocytes *in vitro*. *J. Infect. Dis.* **125**: 367–376.