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Antifungal Effects of Peroxidase Systems

R. I. LEHRER

Cancer Research Institute and Department of Medicine, University of California Medical Center, San Francisco, California 94122

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In the presence of hydrogen peroxide and either potassium iodide, sodium chloride, or potassium bromide, purified human myeloperoxidase was rapidly lethal to several species of *Candida*. Its candidacidal activity was inhibited by cyanide, fluoride, and azide, and by heat inactivation of the enzyme. A hydrogen peroxide-generating system consisting of D-amino acid oxidase, flavine-adenine dinucleotide, and D-alanine could replace hydrogen peroxide in the candidacidal system. Horseradish peroxidase and human eosinophil granules also exerted candidacidal activity in the presence of iodide and hydrogen peroxide; however, unlike myeloperoxidase or neutrophil granules, these peroxidase sources were inactive when chloride replaced iodide. Cells of *Saccharomyces*, *Geotrichum*, and *Rhodotorula* species, and spores of *Aspergillus fumigatus* and *A. niger* were also killed by the combination of myeloperoxidase, iodide, and hydrogen peroxide. Peroxidases, functionally linked to hydrogen peroxide-generating systems, could provide phagocytic cells with the ability to kill many fungal species.

Recent studies on a patient with systemic candidiasis showed that his neutrophils, genetically deficient in the lysosomal enzyme myeloperoxidase (MPO), could phagocytize several *Candida* species in a normal manner but could not kill them (R. I. Lehrer and M. J. Cline, Clin. Res. **16**:331, 1968). Myeloperoxidase, under certain conditions, exerts bactericidal activity in vitro (11-13), and the participation of the enzyme in the antibacterial actions of intact leukocytes has been proposed (2, 11, 15, 16). The effects of peroxidase systems on fungi have not been previously reported. This study was undertaken to investigate the actions of highly purified MPO and other peroxidase preparations on several species of fungi, with special attention to their effects on *Candida* species.

MATERIALS AND METHODS

Fungi. *Candida* cultures, obtained from the mycology collection of the University of California Department of Clinical Pathology, included strains of *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. parapsilosis*, *C. krusei*, and *C. pseudotropicalis*. Strains of *Saccharomyces cerevisiae*, *Geotrichum candidum*, and a *Rhodotorula* species were obtained from the same source. Stock cultures were maintained at room temperature on Sabouraud 2% dextrose agar slants with bimonthly transfers. Before experimental use, cultures were inoculated into Sabouraud 2% dextrose broth and incubated at 33 C (*Candida* species) or room temperature (other species) until sufficient growth occurred (24 to 96 hr). The concentration of fungal "units" (14) was determined by hemocytometer count. The fungi were then washed twice with 0.1 M citrate-phosphate buffer and suspended in buffer at a concentration of about 10^7 units/ml. The *p*H of the citrate-phosphate buffer used in washing and in the final assay mixture was 5.0 unless otherwise stated.

Aspergillus fumigatus (strain 14-7) and *A. niger* (strain 14-5), kindly provided by Carlyn Halde, were grown on Sabouraud dextrose-agar slants for 2 months. The spores were harvested by flooding the surface of the slants gently with Hanks balanced salt solution containing 0.01% Triton X 100 (BSS-T). After two washings with BSS-T, the preparations consisted principally of single spores free from mycelial contamination. Before they were added to the incubation mixtures, the spores were washed twice with citrate-phosphate buffer and suspended in buffer at appropriate concentrations. In experiments with *A. fumigatus*, the buffer and final incubation medium contained 0.01% Triton X 100.

Components of fungicidal assay system. Highly purified human MPO was generously provided by J. Schultz. Its preparation and characteristics have been described previously (18, 19). D-Amino acid oxidase (DAAO), prepared and crystallized from hog kidney, was purchased from Calbiochem, Los Angeles, Calif. Its activity, determined at 30 C with D-alanine as substrate, was 57 international units (IU)/mg. Chromatographically homogeneous D-alanine and horseradish peroxidase (A grade, RZ 3.0) were obtained from the same source. The disodium salt of flavine-adenine dinucleotide (FAD; grade III) was purchased from Sigma, St. Louis, Mo.

Erythrocyte-free leukocyte preparations were prepared from 70 ml of heparinized normal venous blood

(8). A granule fraction was isolated by the method of Williams (20), washed twice with 0.34 M sucrose, resuspended in 0.34 M sucrose, and stored at −20 C until used. Eosinophils were isolated from a patient with tropical eosinophilia (leukocyte count, 18,000 per mm^3 with 50% eosinophils) by the method of Archer and Hirsch (4). After initial dextran sedimentation of the heparinized blood (8), leukocytes were resuspended in 36% bovine serum albumin (BSA; Pentex Laboratories, Kankakee, Mich.), layered over BSA, and centrifuged at 200 × *g* for 25 min at room temperature. Over 97% of the leukocytes below the cell layer at the original interface were eosinophils; the remaining 3% consisted almost entirely of lymphocytes. Contaminating erythrocytes were removed by hypotonic lysis (8). A 40-ml sample of the patient's blood yielded 5×10^7 eosinophils. The cells were disrupted in 0.1 M sodium citrate (4), and the resultant granule fraction was stored at −20 C in 0.2 M sucrose until used.

Fungicidal assay technique. The assay components, in a final volume of 0.5 ml, were placed in sterile, disposable plastic tubes (12 by 75 mm) and incubated for 30 min at 37 C with rotation at 30 rev/min. Unless otherwise noted, the components were added in the following amounts: citrate-phosphate buffer, 50 μmoles; fungi, 10^6 units; MPO, 0.6 μg; potassium iodide, 0.05 μmole; and hydrogen peroxide, 0.5 μmole, or a hydrogen peroxide-generating system composed of DAAO, 25 μg; D-alanine, 25 μmoles; and FAD, 36 μmoles. Any omitted reactants were replaced by appropriate volumes of buffer or water.

After 30 min, triplicate samples were diluted with water, spread on Sabouraud dextrose-agar plates, and incubated for 24 to 72 hr at 33 C for colony counting. Pour plates prepared with Sabouraud dextrose-agar were used for studies of *Aspergillus*. In additional studies with *C. albicans*, samples were removed at intervals and used to prepare unstained wet mounts for direct microscopic examination and to determine the percentage of cells that excluded 2×10^{-4} M methylene blue (14). To investigate germinative ability, 0.1 ml of fetal calf serum was added to each tube after the 30-min incubation period. After further incubation at 33 C for 3 to 4 hr, samples were examined microscopically, and the percentage of units showing evidence of serum-induced germination was determined.

RESULTS

Candidacidal systems. Although human MPO, potassium iodide, and hydrogen peroxide failed to depress the colony count significantly when they were added individually or in combinations of two, the addition of all three killed the entire *Candida* inoculum within 2 min (Table 1). When the concentrations of two of the components were maintained at customary levels and the third was varied, complete candidacidal activity was observed with as little as 0.28 μg of MPO per ml or 10^{-5} M hydrogen peroxide. All of the above studies were performed at *p*H 5.0; however, the basic system was also lethal to *Candida* cells at *p*H 6.0 and 7.0.

TABLE 1. *Myeloperoxidase-iodide-hydrogen peroxide candidacidal system*[a]

MPO	Iodide	H_2O_2	*p*H	Candida albicans (colonies/ml)
−	−	−	5.0	1.9×10^6
+	−	−	5.0	1.9×10^6
+	+	−	5.0	2.2×10^6
−	+	+	5.0	2.2×10^6
+	+	+	5.0	0
−	−	−	6.0	2.7×10^6
−	+	+	6.0	3.0×10^6
+	+	+	6.0	0
−	−	−	7.0	2.3×10^6
−	+	+	7.0	3.5×10^6
+	+	+	7.0	0

[a] The presence of a reactant (+), its absence (−).

The candidacidal activity of MPO, iodide, and hydrogen peroxide was completely inhibited by the presence of 2 mM cyanide, 2 mM azide, or 10 mM fluoride, or by boiling the enzyme for 10 min. When hydrogen peroxide was replaced by a hydrogen peroxide-generating system composed of DAAO, D-alanine, and FAD, complete activity was retained.

Horseradish peroxidase could be substituted for MPO in the basic system, and, on a weight basis, its candidacidal activity approximated that of MPO. In assays with MPO, chloride in concentrations exceeding 6×10^{-4} M or 10^{-4} M bromide could be substituted for iodide without decreasing candidacidal activity. In contrast, in assays with horseradish peroxidase (20 μg/ml), no fungicidal effect was observed when chloride (5×10^{-2} M) replaced iodide in the system.

When *C. albicans* cells were exposed to the basic system or to any modification in which candidacidal activity was retained, a characteristic series of events occurred. Almost immediately, the morphology of the yeast cell showed striking alterations, which were apparent on light or phase microscopy of unstained preparations. The cytoplasm became highly refractile and granular, and often contained multiple small vacuoles. These vacuoles sometimes had irregular contours, suggesting that the cytoplasm had formed a rigid gel. Such yeast cells did not germinate when serum was added to the assay system, and, although initially they might not take up 2×10^{-4} M methylene blue, most or all showed extensive cytoplasmic staining within a few hours.

In contrast, when *Candida* cells were exposed only to buffer or to various combinations of MPO, halide, and hydrogen peroxide short of the complete basic system, their cytoplasm remained clear and nonrefractile, and vacuoles, when present, were usually single, large, and regular in contour. Such *Candida* cells remained unstained by 2×10^{-4} M methylene blue, and within a few hours after the addition of serum most had budded or formed germ tubes.

Effects of leukocyte granules on C. albicans. The effects of a granule preparation obtained from a mixed population of human peripheral blood leukocytes is shown in Table 2. Although the granules alone or hydrogen peroxide and iodide together lacked candidacidal activity, the combination of the three was highly lethal to *C. albicans*. The lethal effect was inhibited by the presence of cyanide or methimazole. Sublethal granule concentrations exhibited candidastatic activity (in the presence of iodide and hydrogen peroxide) as evidenced by stepwise inhibition of serum-induced germination (Table 2). The granule fraction was also active with chloride. Eosinophil granules could be substituted for the neutrophil-predominant preparation, and were candidacidal at a concentration of 2.4 μg of total granule protein per ml of incubation mixture. The eosinophil granule fraction was active with iodide, but not with chloride.

Spectrum of activity. The complete MPO-iodide-hydrogen peroxide system was effective against all the fungi studied, including six *Candida* species, *S. cerevisiae*, *G. candidum*, a *Rhodotorula* species, and the spores of *A. niger* and *A. fumigatus* (Table 3). Myeloperoxidase alone, or the combination of iodide and hydrogen peroxide, had little or no fungicidal effect in these assays.

TABLE 2. *Effects of leukocyte granules on Candida albicans*[a]

Granule preparation	H_2O_2	Iodide	Addition	Cytopathic effect	Germination	Colonies/ml
μg					%	
	−	−	−	None	78	1.36×10^6
	+	+	−	None	74	1.93×10^6
5.3	−	−	−	None	76	1.72×10^6
5.3	+	+	−	Marked	0	5.00×10^4
2.6	+	+	−	Trace	34	1.16×10^6
1.3	+	+	−	None	44	1.02×10^6
0.7	+	+	−	None	54	1.98×10^6
0.3	+	+	−	None	74	1.48×10^6
5.3	+	+	KCN	None	23	1.04×10^6
5.3	+	+	Methimazole	None	67	1.96×10^6

[a] The presence of a reactant (+), its absence (−). Where indicated, 0.05 μmole of potassium cyanide or 0.05 mmole of methimazole was added to the incubation mixture.

TABLE 3. *Susceptibility of fungi to myeloperoxidase, iodide, and hydrogen peroxide*[a]

Organism	Colonies/ml after incubation with:			
	Buffer only	H_2O_2 + iodide	MPO only	MPO + H_2O_2 + iodide
Candida albicans	1.8×10^6	1.5×10^6	2.0×10^6	0
C. parapsilosis	3.0×10^6	3.0×10^6	2.8×10^6	0
C. krusei	2.1×10^6	1.8×10^6	1.5×10^6	0
C. tropicalis	1.7×10^6	1.7×10^6	1.6×10^6	0
C. stellatoidea	1.8×10^6	1.5×10^6	9.3×10^5	0
C. pseudotropicalis	8.7×10^5	5.0×10^5	7.6×10^5	0
Saccharomyces cerevisiae	2.2×10^6	2.4×10^6	3.0×10^6	0
Rhodotorula species	3.8×10^5	4.0×10^5	2.2×10^5	0
Geotrichum candidum	1.4×10^5	2.4×10^5	1.1×10^5	0
Aspergillus fumigatus	3.9×10^5	4.2×10^5	6.7×10^5	0
A. niger	8.6×10^5	8.4×10^5	5.4×10^5	0

[a] All concentrations of reactants were as described in the text, except that in studies with *Saccharomyces*, *Rhodotorula*, and *Geotrichum*, 0.05 μmole of hydrogen peroxide was used. Assays were performed at *p*H 5.0.

DISCUSSION

The phagocytic cells of human blood all contain peroxidase. The enzyme is especially abundant in the neutrophil, where, as MPO, it comprises between 1 and 5% of the total dry weight of the cell (1, 18). Eosinophil peroxidase, also plentiful (4), differs structurally from MPO (3). Monocytes contain considerably less peroxidase than either neutrophils or eosinophils, and their enzyme is structurally related to MPO (Salmon et al., *submitted for publication*).

Three mammalian peroxidases—MPO, lactoperoxidase, and salivary peroxidase—have previously been shown to be capable of participating in antibacterial systems under appropriate in vitro conditions (10–13, 22); each requires the presence of hydrogen peroxide and a halide or pseudohalide (such as thiocyanate) to exert its maximal effect. Although all are effective with iodide, chloride was found to be an active cofactor only with MPO (12), and was relatively ineffective with lactoperoxidase (12) or salivary peroxidase (22). The peroxidase sources that we studied could exert candidacidal activity only in the presence of hydrogen peroxide and an appropriate halide. Although all were active with hydrogen peroxide and iodide, only MPO and the preparation rich in neutrophil granules were candidacidal when chloride was substituted.

Studies with human neutrophils support the contention that MPO and hydrogen peroxide generation are involved in leukocyte candidacidal activity. The neutrophils of a patient with hereditary MPO deficiency could phagocytize *Candida* species normally, but were unable to kill them (R. I. Lehrer and M. J. Cline, Clin. Res. **16**:331, 1968). Neutrophils of children with chronic granulomatous disease also fail to kill ingested *Candida* species (14). The neutrophils of such children possess MPO and form normal MPO-containing phagocytic vacuoles around ingested yeast particles (6). Chronic granulomatous disease neutrophils, however, lack the ability of normal neutrophils to generate hydrogen peroxide after particle ingestion (9). The inhibitory effects of cyanide, colchicine, and anaerobiosis on the candidacidal activity of normal leukocytes (14) are also compatible with the thesis that MPO and hydrogen peroxide participate in the candidacidal processes of leukocytes.

Salivary peroxidase was not examined in this study; however, it has previously been shown to have bactericidal activity, suggesting that it would also be active against *Candida* species. Salivary glands trap and concentrate iodide ion and thiocyanate (7), both active as cofactors in bactericidal studies with salivary peroxidase (22). Austin and Zeldow (5), in studies of salivary peroxidase activity in neonates, reported a physiological absence of the enzyme during the first few days of life. Perhaps this transient deficiency facilitates the development of neonatal thrush.

It is noteworthy that iodides, although lacking direct fungicidal effects, are useful in the therapy of several fungal infections (17, 21). These data suggest the possibility that their in vivo effectiveness results from an interaction with host peroxidase enzymes, either extracellularly or within a peroxidase-containing phagocyte.

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