Damage to *Candida albicans* Hyphae and Pseudohyphae by the Myeloperoxidase System and Oxidative Products of Neutrophil Metabolism In Vitro

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ABSTRACT In previous studies, we noted that *Candida* hyphae and pseudohyphae could be damaged and probably killed by neutrophils, primarily by oxygen-dependent nonphagocytic mechanisms. In extending these studies, amount of damage to hyphae again was measured by inhibition of [^{14}C]cytosine uptake. Neutrophils from only one of four patients with chronic granulomatous disease damaged hyphae at all, and neutrophils from this single patient damaged hyphae far less efficiently than simultaneously tested neutrophils from normal control subjects. Neutrophils from neither of two subjects with hereditary myeloperoxidase deficiency damaged the hyphae. This confirmed the importance of oxidative mechanisms in general and myeloperoxidase-mediated systems in particular in damaging *Candida* hyphae.

Several potentially fungicidal oxidative intermediates are produced by metabolic pathways of normal neutrophils, but their relative toxicity for *Candida* hyphae was previously unknown. To help determine this, cell-free in vitro systems were used to generate these potentially microbicidal products. Myeloperoxidase with hydrogen peroxide, iodide, and chloride resulted in 91.2% damage to hyphal inocula in 11 experiments. There was less damage when either chloride or iodide was omitted, and no damage when myeloperoxidase was omitted or inactivated by heating. Azide, cyanide, and catalase (but not heated catalase) inhibited the damage. Systems for generation of hydrogen peroxide could replace reagent hydrogen peroxide in the myeloperoxidase system. These included glucose oxidase, in the presence of glucose, and xanthine oxidase, in the presence of either hypoxanthine or acetaldehyde. In the presence of myeloperoxidase and a halide, the toxicity of the xanthine oxidase system was not inhibited by superoxide dismutase and, under some conditions, was marginally increased by this enzyme. This suggested that superoxide radical did not damage hyphae directly but served primarily as an intermediate in the production of hydrogen peroxide. The possible damage to hyphae by singlet oxygen was examined using photoactivation of rose bengal. This dye damaged hyphae in the presence of light and oxygen. The effect was almost completely inhibited by putative quenchers of singlet oxygen: histidine, tryptophan, and 1,4-diazobicyclo[2.2.2]octane. These agents also inhibited damage to hyphae by myeloperoxidase, halide, and either hydrogen peroxide or a peroxide source (xanthine oxidase plus acetaldehyde). Myeloperoxidase-mediated damage to hyphae was also inhibited by dimethyl sulfoxide, an antioxidant and scavenger of the hydroxyl radical.

These data support the involvement of oxidative mechanisms and the myeloperoxidase-H_2O_2-halide system, in particular in damaging hyphae in vitro and perhaps in vivo as well.

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INTRODUCTION

In previous studies in our laboratory, it was established that pseudohyphal and hyphal forms of *Candida albicans* could be damaged and probably killed by nonphagocytic mechanisms of human neutrophils in vitro (1). Experimental data indicated that *Candida* hyphae, though too large to be ingested, activated microbicidal mechanisms of neutrophils and caused specific release of neutrophil lysosomal enzymes (2). Indirect evidence supported the impression that oxidative mechanisms of neutrophils were more important than nonoxidative mechanisms in damaging hyphae. *Candida*-neutrophil interactions induced chemiluminescence from the neutrophils, and hyphal proteins were iodinated by

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neutrophils, indicating activation of the myeloperoxidase–hydrogen peroxide–halide system (1). Furthermore, damage to *Candida* by neutrophils was inhibited by agents known to affect neutrophil oxidative microbicidal mechanisms. These include sodium cyanide and sodium azide (inhibitors of myeloperoxidase and of cell respiration in general), catalase (which degrades hydrogen peroxide), superoxide dismutase (which decomposes superoxide anion), and 1,4-diazobicyclo[2.2.2]octane (DABCO),[1] a singlet oxygen quencher (2). In chronic granulomatous disease, neutrophils are deficient in microbicidal function because they are unable to generate normal amounts of hydrogen peroxide and other oxidative intermediates (3, 4). In preliminary experiments, neutrophils from a single patient with chronic granulomatous disease failed to damage *Candida* hyphae (2). Moreover, inhibitors of potential nonoxidative microbicidal mechanisms, such as cationic proteins and lactoferrin, failed to alter damage to hyphae by normal neutrophils (2).

The value of oxidative metabolism of leukocytes in host defenses against *Candida* has been highlighted by the apparent increased frequency of *Candida* infections in patients with chronic granulomatous disease and hereditary myeloperoxidase deficiency (3, 4). Although oxidative intermediates appeared to be of critical importance in damage to *Candida* hyphae in vitro, neither the results with chronic granulomatous disease neutrophils nor the data obtained with inhibitors indicated which oxidative intermediates or pathways might be directly responsible for hyphal damage (1, 2). For example, in addition to inhibition of myeloperoxidase, sodium azide and sodium cyanide may inhibit cell respiration (5) generally, whereas sodium azide in high concentrations acts as a quencher of singlet oxygen (6). Therefore, effects on whole cells may not reflect specific effects of inhibitors on an isolated system. Oxidative mechanisms present in whole neutrophils may be simulated in vitro using cell-free components of known systems to generate microbicidal intermediates. For example, effects of the myeloperoxidase system can be tested by using the purified enzyme with a halide and either reagent hydrogen peroxide or peroxide-generating systems (7). Hydrogen peroxide may be produced by the reactions of glucose oxidase with glucose, and of xanthine oxidase with either hypoxanthine or acetaldehyde (6–8). The latter reactions generate superoxide radical, which may react to form hydrogen peroxide or may, itself, be microbicidal for some organisms. Other oxidative products may also be produced, such as hydroxyl radical or singlet oxygen (6, 7). Singlet oxygen, a possible product of intact neutrophils, may be produced in vitro as the major oxidative product of photooxidation of the dye, rose bengal (6).

In these studies, we have found that neither whole neutrophils from additional patients with chronic granulomatous disease, nor neutrophils deficient in myeloperoxidase, could damage *Candida* hyphae. In vitro cell-free systems for generation of oxidative intermediates appeared to damage hyphae if myeloperoxidase, halide, and a source of hydrogen peroxide were present, or if singlet oxygen and, perhaps, hydroxyl radical were produced.

METHODS

Fungi. An isolate of *Candida albicans* was originally obtained from a patient with systemic candidiasis. Blastospores were germinated to form hyphae and pseudohyphae in Eagle's minimal essential medium which had been supplemented with nonessential amino acids (Grand Island Biological Co., Grand Island, N. Y.). *Candida* were incubated at 37°C until ≥95% of cells had formed germ tubes ≥30 μg long. Before incubation with neutrophils or in vitro microbicidal systems, hyphae and pseudohyphae were washed in Hanks' balanced salt solution.

Neutrophils. Patients without history of serious systemic infections, with typical chronic granulomatous disease (three sex-linked, one autosomal) and myeloperoxidase deficiency were identified in the laboratories of Dr. Harvey Cohen (Children's Hospital, Boston, Mass.) and Dr. Richard K. Root (Yale-New Haven Hospital, New Haven, Conn.). Blood from these patients was kindly supplied for our studies. Blood from normal volunteers was used for simultaneous controls. Neutrophils were obtained by first separating them from mononuclear cells on a Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.)-Hypaque (sodium and meglumine diatrizoates, Winthrop Laboratories, New York) gradient, followed by sedimentation with 3% dextran (average mol wt, 250,000, Pharmacia Fine Chemicals, Piscataway, N. J.) as described by Böyum (9). Contaminating erythrocytes were lysed and cells were washed as in previous studies (1).

Assessment of damage to fungi by effects on uptake of [^{14}C]cytosine by hyphae. Tubes contained 5×10^5 *Candida* and either 5×10^6 neutrophils or compounds for generating oxidative intermediates. Neutrophils were suspended in Hanks' balanced salt solution. The reaction mixture for cell-free systems was suspended in chloride-free 0.1 M phosphate buffer (sodium salts), pH 7.4, with no added serum or albumin in the reaction mixture. Assays for damage to hyphae were performed as previously described (1). In short, hyphae were rotated in triplicate tubes with neutrophils or soluble generating systems for 1 h at 37°C. After incubation, in the tubes which contained neutrophils, 5×10^6 neutrophils were added to each matching control tube, and neutrophils in all tubes were lysed with deoxycholate. Remaining *Candida* were washed twice in distilled water and once in yeast nitrogen base broth (supplemented with 1% dextrose and 0.15% asparagine). To each tube was then added 0.25 μCi of [^{14}C]cytosine (Amersham Searle Corp., Arlington Heights, Ill.; Research Products International, Elk Grove Village Corp., Ill.) dissolved in supplemented yeast nitrogen base broth. After incubation at 30°C for 1 h, hyphae were harvested onto filters with an automated multiple sample harvester (Otto Hiller Co., Madison, Wis.). Filters were air-dried and incorporation of ^{14}C was determined by liquid scintillation counting. Damage to *Candida* was expressed as

[1] *Abbreviations used in this paper*: DABCO, 1,4-diazobicyclo[2.2.2]octane; DMSO, dimethyl sulfoxide; MPO, myeloperoxidase; XO, xanthine oxidase.

the percent reduction in uptake of [^{14}C]cytosine by hyphae: (mean cpm in control tubes) − (mean cpm in experimental tubes)/(mean cpm in control tubes) × 100.

Ingredients of in vitro systems for generation of microbicidal activity. Myeloperoxidase was prepared from canine pus neutrophils by the method of Agner through the end of this sixth step (10) and was assayed by the *o*-dianisidine method (11). One unit of myeloperoxidase uses 1 μmol of substrate per minute at 25°C (12). Glucose oxidase (type V, 200 U/mg) was obtained from Sigma Chemical Co. (St. Louis, Mo.), as were hypoxanthine and xanthine oxidase (bovine buttermilk, suspended in 2.3 M ammonium sulfate, 10 mM sodium phosphate buffer, pH 7.8, and containing 1 mM EDTA and 1 mM sodium salicylate). Catalase (bovine liver, 6.1 mg/ml, 50,000 U/mg) was obtained from Worthington Biochemical Corp. (Freehold, N. J.) and dialyzed against water before use. Superoxide dismutase (bovine erythrocyte, lyophilized powder, 12,300 U/mg) obtained from Miles Research Products (Elkhart, Ind.) was dissolved in water at a concentration of 5 mg/ml and stored at −20°C until used. For some experiments, myeloperoxidase, xanthine oxidase, and catalase were heated at 100°C for 15 min, and superoxide dismutase was autoclaved at 121°C for 30 min before use. Acetaldehyde (Fisher Chemical Manufacturing, Fairlawn, N. J.) was distilled and stored in aliquots at −20°C. Rose bengal was also obtained from Fisher Chemical Manufacturing. Inhibitors used included sodium azide, sodium cyanide, histidine, tryptophan, dimethyl sulfoxide (DMSO) (Sigma Chemicals, St. Louis, Mo.) and DABCO (supplied as triethylenediamine by Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.).

Electron microscopy. After 60 min of exposure to in vitro microbicidal systems or whole neutrophils, *Candida* hyphae were fixed in suspension by addition of phosphate-buffered glutaraldehyde-formaldehyde (final concentrations of 1 and 4%, respectively). The cells were spun at 450 *g* for 10 min and resuspended in the next solution for each of the following preparative steps: 1% aqueous osmium tetroxide for 60 min, 0.1% aqueous uranyl acetate for 30 min, graded alcohols, propylene oxide, and Epon 812 (Fisher, Boston, Mass.). Toluidine blue–stained sections, 1 μM thick, were cut in both a plane perpendicular and a plane parallel to the axis of centrifugal force to examine the distribution of cells within the pellet. Ultra-thin sections through the center of the pellet (including all cells from top to bottom) were cut with diamond knives, stained with lead citrate and uranyl acetate, and examined with a Philips EM300 (Philips, Amsterdam, The Netherlands) at 80KV.

Statistical methods. Means and standard errors of means were compared using two sample *t* tests (2-tailed, unpaired).

RESULTS

Damage to hyphae by intact neutrophils with deficient oxidative metabolism. To confirm and extend previous studies on the importance of oxidative pathways in damage to hyphae (2), neutrophils from an additional three patients with chronic granulomatous disease were obtained. Neutrophils from only one (typical sex-linked) of the total of four patients with chronic granulomatous disease caused minimal damage to the hyphae, whereas neutrophils from the other three subjects (two sex-linked, one autosomal) were totally inactive (Table I). Similarly, neutrophils from two patients with myeloperoxidase deficiency tested for the first time were unable to damage *Candida* hyphae. Neutrophils from normal volunteer subjects tested simultaneously (Table I) damaged hyphae within the range established for normal subjects in previous experiments (1).

TABLE I

Effect of Defects in Oxidative Metabolism on Ability of Intact Neutrophils to Damage Candida Hyphae

Source of neutrophils	Number of subjects	Damage to hyphae
		%
Chronic granulomatous disease	4	5.6±5.6*
Normal control subjects‡	4	62.2±4.2§
Myeloperoxidase deficiency	2	0.0
Normal control subjects‡	2	69.3§

* Mean±SEM damage to fungi determined by reduction of uptake of [^{14}C]cytosine by *Candida* hyphae after 1-h incubation of 5×10^6 neutrophils with 5×10^5 hyphae.
‡ Neutrophils from normal subjects tested simultaneously with neutrophils from patients with deficient cell function.
§ ($P < 0.05$) by two sample *t* tests.

Generation of microbicidal oxidative intermediates in in vitro cell-free systems. The experiments described above, in which whole neutrophils were used, established the importance of oxidative metabolism in damaging *Candida* hyphae by neutrophils. However, oxidative metabolism by neutrophils generates several intermediate products that are potentially microbicidal (6–8) but that may or may not damage *Candida* hyphae. To make this distinction, we used in vitro cell-free systems for generating these intermediate products. A wide range of concentrations was tested for each reagent used in these cell-free systems. After preliminary experiments, concentrations of reagents were selected that did not damage *Candida* hyphae by themselves. We then repeated the experiments using concentrations that caused maximum hyphal damage along with other ingredients of the cell-free systems.

As shown in Table II, the myeloperoxidase system was effective in damaging hyphae. Damage to the hyphae was greatest where iodide and chloride were present as cofactors. Although iodide alone was relatively efficient, chloride used without iodide was associated with less damage to the fungi. Without myeloperoxidase, higher concentrations of iodide alone (1 mM and above) damaged hyphae, and were not tested in the system. Active myeloperoxidase was essential, inasmuch as there was no damage to hyphae when heated myeloperoxidase was used or when myeloperoxidase was omitted from incubations. Moreover, damage to the fungi was inhibited by known inhibitors of myeloperoxidase, sodium azide and sodium cyanide (5, 13). Though hydrogen peroxide in the concentration used (0.1 mM) did not damage hyphae by itself,

TABLE II

Damage to Hyphae by the Myeloperoxidase (MPO) System and its Reversal by Inhibitors

Components of system*										
MPO 4 mU	Heated MPO	NaI 0.1 mM	NaCl 0.1 M	H_2O_2 0.1 mM	NaN_3 0.1 mM	NaCN 1.0 mM	Catalase 2,100 U	Heated catalase	Damage to hyphae	Inhibition of damage
									%	%
+	–	+	+	+	–	–	–	–	91.2±2.5(11)‡	—
+	–	+	+	–	–	–	–	–	0.0 (3)§	—
–	–	+	+	+	–	–	–	–	0.0 (3)§	—
–	(+)	+	+	+	–	–	–	–	3.0 (2)§	96.7‖
+	–	+	+	+	(+)	–	–	–	40.1±9.9(3)§	56.0
+	–	+	+	+	–	(+)	–	–	55.9±6.4(3)§	38.7
+	–	+	+	+	–	–	(+)	–	15.7±8.8(5)§	82.8
+	–	+	+	+	–	–	–	(+)	91.5±4.8(4)	0.0
+	–	+	–	+	–	–	–	–	81.7±9.1(8)	—
+	–	+	–	+	(+)	–	–	–	5.6±2.1(3)*	93.2
+	–	+	–	+	–	(+)	–	–	40.8±6.1(3)§	50.0
+	–	+	–	+	–	–	(+)	–	3.7±2.1(3)§	95.5
+	–	+	–	+	–	–	–	(+)	91.2 (2)	0.0
+	–	–	+	+	–	–	–	–	40.4 (2)	—
+	–	–	+	+	(+)	–	–	–	0.0 (2)§	100.0
+	–	–	+	+	–	(+)	–	–	0.0 (2)§	100.0
+	–	–	+	+	–	–	(+)	–	0.0 (2)§	100.0
+	–	–	+	+	–	–	–	(+)	52.7 (2)	0.0

* *Candida* hyphae (5×10^5) were tumbled for 1 h at 37°C with components of the MPO system (denoted by + if present) and its inhibitors [denoted by (+) if present].

‡ Mean±SEM (number of experiments) determining damage to hyphae by reduction of uptake of [^{14}C]cytosine by *Candida*.

§ ($P < 0.05$) by two sample *t* tests.

‖ Calculated from damage to hyphae with active myeloperoxidase and with inactive myeloperoxidase or with inhibitors.

its presence was required as part of the myeloperoxidase system; active but not heated catalase reduced damage to fungi by 80.8–100%, depending on which halide cofactors were used. Higher concentrations of hydrogen peroxide damaged hyphae in the absence of myeloperoxidase and halide. With myeloperoxidase, halide, and lower concentrations of hydrogen peroxide (0.01 mM), damage to hyphae was reduced by approximately half. There was no hyphal damage with 0.001 mM hydrogen peroxide. Without halide, myeloperoxidase with 0.01–0.1 mM hydrogen peroxide did not damage hyphae.

Hydrogen peroxide could also be supplied by peroxide generating systems. As shown in Table III, glucose oxidase with added glucose (8) could be substituted for direct addition of hydrogen peroxide in the myeloperoxidase system with halide present. Azide, cyanide, and catalase again were inhibitory. Without myeloperoxidase and halide, however, glucose oxidase and glucose failed to damage *Candida* significantly.

Xanthine oxidase, together with hypoxanthine (6, 7), caused minimal, if any, damage to hyphae. The little damage that occurred appeared to be eliminated by the addition of active, but not autoclaved, superoxide dismutase (Table IV). However, addition of myeloperoxidase and iodide to the xanthine oxidase and hypoxanthine resulted in marked fungal damage. Active enzymes were required, because heated myeloperoxidase or xanthine oxidase did not damage the hyphae. Sodium azide and sodium cyanide inhibited damage, thus supporting the role played by myeloperoxidase in this system (13). Catalase completely blocked the antifungal effects of this system, again emphasizing the importance of hydrogen peroxide in the reaction. Hypoxanthine alone, in concentrations ≥1.0 mM, appeared to damage hyphae directly. In the presence of myeloperoxidase and iodide, hypoxanthine was active at concentrations as low as 10 μM (15.5% damage, $P < 0.05$).

Acetaldehyde could be substituted for hypoxanthine (6, 7) in the xanthine oxidase–myeloperoxidase–halide system (Table V). Iodide and chloride, together or separately, were effective in this system. The presence of a halide was required, because myeloperoxidase with acetaldehyde and xanthine oxidase caused no damage to hyphae. Hyphal damage was completely blocked by cyanide and was partially inhibited by azide and catalase. Superoxide dismutase failed to inhibit the activity of the myeloperoxidase–xanthine oxidase–acetaldehyde–halide system. If anything, this enzyme slightly enhanced activity ($P = 0.07$ with iodide and

TABLE III

Glucose Oxidase–mediated Damage to Candida Hyphae

Components of system*								Damage to hyphae	Inhibition of damage
Glucose oxidase 1.4 mU	Glucose 1.0 mM	MPO 4 mU	NaI 0.1 mM	NaCl 0.1 M	NaN_3 0.1 mM	NaCN 1 mM	Catalase 2,100 U		
								%	%
+	+	+	+	+	–	–	–	97.2±1.5(5)‡	—
+	+	–	–	–	–	–	–	1.8±1.8(6)§	—
+	+	+	+	+	(+)	–	–	0.0 (2)§	100.0
+	+	+	+	+	–	(+)	–	65.7 (2)§	33.8
+	+	+	+	+	–	–	(+)‖	30.1 (2)§	71.7
+	+	+	+	–	–	–	–	92.2±0.2(3)	—
+	+	+	–	+	–	–	–	50.1 (2)	—

* *Candida* (5×10^5) tumbled for 1 h with indicated compounds.

‡ Mean±SEM (number of experiments) damage to hyphae (by reduction of uptake of [^{14}C]cytosine).

§ ($P < 0.05$) by two sample t tests.

‖ Control medium containing heated catalase did not inhibit damage to *Candida*.

chloride, $P < 0.05$ with iodide alone as the halide cofactor). This suggests that superoxide anion serves primarily as an intermediate in hydrogen peroxide formation under these conditions.

Rose bengal reacts with light and oxygen to form singlet oxygen as its principal oxidative product (6). Rose bengal caused appreciable damage to hyphae only when both light and oxygen were present (Table VI). Damage was completely or nearly completely reversed by the addition of any of three putative quenchers of singlet oxygen, histidine (14), tryptophan (15), or DABCO (16). These agents also inhibited the myeloperoxidase system, both with direct addition of hydrogen peroxide and with generation of hydrogen peroxide from xanthine oxidase and acetaldehyde (Table VII). Under the conditions stated, histidine was the most potent inhibitor in the presence of reagent hydrogen peroxide, whereas tryptophan and DABCO were more effective with the xanthine oxidase–acetaldehyde system. It should be noted that DABCO could not be used in concentrations > 0.1 mM because at those levels it caused nonspecific damage to hyphae. These same myeloperoxidase-mediated reactions were also inhibited by DMSO (Table VIII).

TABLE IV

Damage to Hyphae by the Xanthine Oxidase (XO)–Hypoxanthine System

Components of system*												Damage to hyphae	Inhibition of damage
XO 4 mU	Heated XO	Hx‡ 0.1 mM	MPO 4 mU	Heated MPO	NaI 0.1 mM	NaN_3 0.1 mM	NaCN 1.0 mM	Catalase 2,100 U	Heated catalase	SOD‡ 1 μg/ml	Heated SOD		
												%	%
+	–	+	–	–	–	–	–	–	–	–	–	10.8±4.3(6)‖	—
+	–	+	–	–	–	–	–	–	–	(+)	–	0.0 (2)¶	100.0
+	–	+	–	–	–	–	–	–	–	–	(+)	11.6 (2)	0.0
+	–	+	+	–	+	–	–	–	–	–	–	67.8±3.9(3)	—
+	–	+	–	(+)	–	–	–	–	–	–	–	0.0 (2)¶	100.0
–	(+)	+	+	–	+	–	–	–	–	–	–	1.4 (2)¶	97.9
+	–	+	+	–	+	(+)	–	–	–	–	–	34.9 (2)¶	51.5
+	–	+	+	–	+	–	(+)	–	–	–	–	22.2 (2)¶	67.3
+	–	+	+	–	+	–	–	(+)	–	–	–	0.0 (2)¶	100.0
+	–	+	+	–	+	–	–	–	(+)	–	–	57.6 (2)	15.0
+	–	+	+	–	+	–	–	–	–	(+)	–	70.6 (2)	0.0

* Hyphae (5×10^5) tumbled with indicated compounds for 1 h.

‡ Hypoxanthine.

§ Superoxide dismutase.

‖ Mean±SEM (number of experiments) damage to fungi by reduction of uptake of [^{14}C]cytosine.

¶ ($P < 0.05$) by two sample t tests.

TABLE V

Damage to Fungi by the XO–Acetaldehyde System

Components of system*										
Acetaldehyde 0.1 mM	XO 4 mU	MPO 4 mU	NaI 0.1 mM	NaCl 0.1 M	SOD 1 μg/ml	Catalase 2,100 U	NaCN 1 mM	NaN_3 1.1 mM	Damage to hyphae	Inhibition of damage
									%	%
+	+	–	–	–	–	–	–	–	14.6±1.9(4)‡	—
+	+	+	+	+	–	–	–	–	37.8±2.0(4)	—
+	+	+	+	+	–	(+)	–	–	17.3±1.6(3)§	54.2‖
+	+	+	+	+	–	–	(+)	–	0.0±0.0(3)§	100.0
+	+	+	+	+	–	–	–	(+)	19.6±1.8(3)§	48.1
+	+	+	+	+	(+)	–	–	–	56.1±3.8(3)	—
+	+	+	+	–	–	–	–	–	24.7±2.6(4)	—
+	+	+	+	–	(+)	–	–	–	42.2±2.7(3)§	—
+	+	+	–	+	–	–	–	–	32.9±1.1(4)	—
+	+	+	–	+	(+)	–	–	–	48.8±1.9(3)§	—

* Triplicate tubes, each containing 5×10^5 hyphae, were tumbled for 1 h at 37°C.

‡ Mean±SEM (number of experiments) quantitated by reduction of uptake of [^{14}C]cytosine induced in hyphae by incubation systems.

§ ($P < 0.05$) by two sample t tests.

‖ Heated catalase did not inhibit damage to the fungi.

Electron microscopy. The morphologic changes seen in myeloperoxidase-treated hyphae were subtle and less pronounced than the ones seen in our previous study with intact neutrophils (1). However, Fig. 1 demonstrates that myeloperoxidase can increase the electron density of the cell wall and cause prominent internal structures to appear which cannot be identified as usual organelles.

DISCUSSION

Our previous studies established that neutrophils could damage and probably kill *Candida albicans* hyphae (1, 2). We now have reaffirmed and extended our observations that oxidative mechanisms of neutrophils were necessary for this damage to hyphae. When neutrophils from four patients with chronic granulomatous disease were tested, cells from only one of the patients could damage hyphae at all, and these cells performed at well below the level of neutrophils from normal volunteers. In chronic granulomatous disease, neutrophils ingest (or attach to) organisms normally, but fail to increase oxygen uptake and consumption and are defective in production of superoxide, hydrogen peroxide, and other products of oxidative metabolism

TABLE VI

Damage to Hyphae by Rose Bengal and its Inhibition by Scavengers of Singlet Oxygen

Components of system*									
			Histidine						
1 μM Rose bengal	Light exposure	Oxygen present	10 mM	1.0 mM	0.1 mM	Tryptophan 1.0 mM	DABCO 0.1 mM	Damage to hyphae	Inhibition of damage
								%	%
+	+	+	–	–	–	–	–	56.9±4.4 (8)‡	—
+	+	–	–	–	–	–	–	19.2±11.0(8)§	—
+	–	+	–	–	–	–	–	15.1±9.1 (8)§	—
+	+	+	(+)	–	–	–	–	0.0 (2)§	100.0
+	+	+	–	(+)	–	–	–	21.6 (2)§	68.4
+	+	+	–	–	(+)	–	–	0.0 (2)§	100.0
+	+	+	–	–	–	(+)	–	0.0 (2)§	100.0
+	+	+	–	–	–	–	(+)	11.9 (2)§	82.6

* Indicated components tumbled for 1 h with 1×10^6 hyphae.

‡ Mean±SEM (number of experiments). Damage assessed by reduction in [^{14}C]cytosine uptake by hyphae.

§ ($P < 0.05$) by two sample t tests.

TABLE VII

Effects of Singlet Oxygen Quenchers on Damage to Candida Hyphae by the MPO System

Additions to MPO and I^-*			Inhibitor added			
H_2O_2 0.1 mM	Cl^- 0.1 M	4 mU XO + CH_3CHO 0.1 mM	Histidine 10 mM	Tryptophan 10 mM	DABCO 0.1 mM	Inhibition of damage to hyphae‡
						%
+	–	–	(+)	–	–	100.0±0.0§
+	–	–	–	(+)	–	20.2±1.1§
+	–	–	–	–	(+)	11.6±3.8
+	+	–	(+)	–	–	73.1±3.4§
+	+	–	–	(+)	–	31.9±2.4§
+	+	–	–	–	(+)	3.4±2.2
–	–	+	(+)	–	–	10.0±3.1
–	–	+	–	(+)	–	88.2±4.1§
–	–	+	–	–	(+)	100.0±0.0§

* Each tube contained hyphae with 4 mU of MPO and 0.1 mM NaI. To some tubes, one or more of the following were added: NaCl and a source of hydrogen peroxide, i.e., either H_2O_2 or a combination of xanthine oxidase and acetaldehyde (CH_3CHO), with or without a singlet oxygen quencher. Tubes were incubated on a rotator at 37°C for 1 h.

‡ Mean±SEM results from three experiments on effects of inhibition on uptake of [^{14}C]cytosine by hyphae in the presence and absence of singlet oxygen quenchers.

§ ($P < 0.05$) by two sample t tests.

(17). Lack of these oxidative intermediates in phagocytic cells apparently leads to a marked inability to kill a wide range of microorganisms (18). Many of the pathways yielding potentially microbicidal oxidative products are present or even enhanced in myeloperoxidase-deficient neutrophils (17, 19), but absence of this enzyme results in impaired microbicidal activity (3, 5, 17, 19, 20). In our studies, neutrophils from two patients with myeloperoxidase deficiency failed to damage *Candida* hyphae at all. Therefore, it appeared that damaging or killing hyphae by neutrophils not only required intact oxidative metabolism but that the reaction

TABLE VIII

Effects of a Scavenger of Hydroxyl Radical on Damage to Hyphae by the MPO System

Additions to MPO (4 mU) and NaI (0.1 mM)*					
H_2O_2 0.1 mM	4 mU XO + 0.1 M CH_3CHO	NaCl 0.1 M	DMSO 14 mM	DMSO 1.4 mM	Inhibition of damage to hyphae
					%‡
+	–	–	(+)	–	37.1±2.9§
+	–	–	–	(+)	17.7±1.0§
+	–	+	(+)	–	83.2±3.4§
+	–	+	–	(+)	34.0±1.3§
–	+	+	(+)	–	48.8±2.7§
–	+	+	–	(+)	16.0±0.9§

* Each tube contained hyphae with 4 mU MPO and 0.1 m NaI. To some tubes, one or more of the following were added: NaCl and a source of hydrogen peroxide, i.e., either H_2O_2 or a combination of xanthine oxidase (XO) and acetaldehyde (CH_3CHO), with or without DMSO. Tubes were incubated on a rotator at 37°C for 1 h. DMSO alone did not significantly alter cytosine uptake by hyphae.

‡ Mean±SEM results from three experiments calculated from uptake of [^{14}C]-cytosine by hyphae in the presence and absence of DMSO.

§ ($P < 0.05$) by two sample t tests.

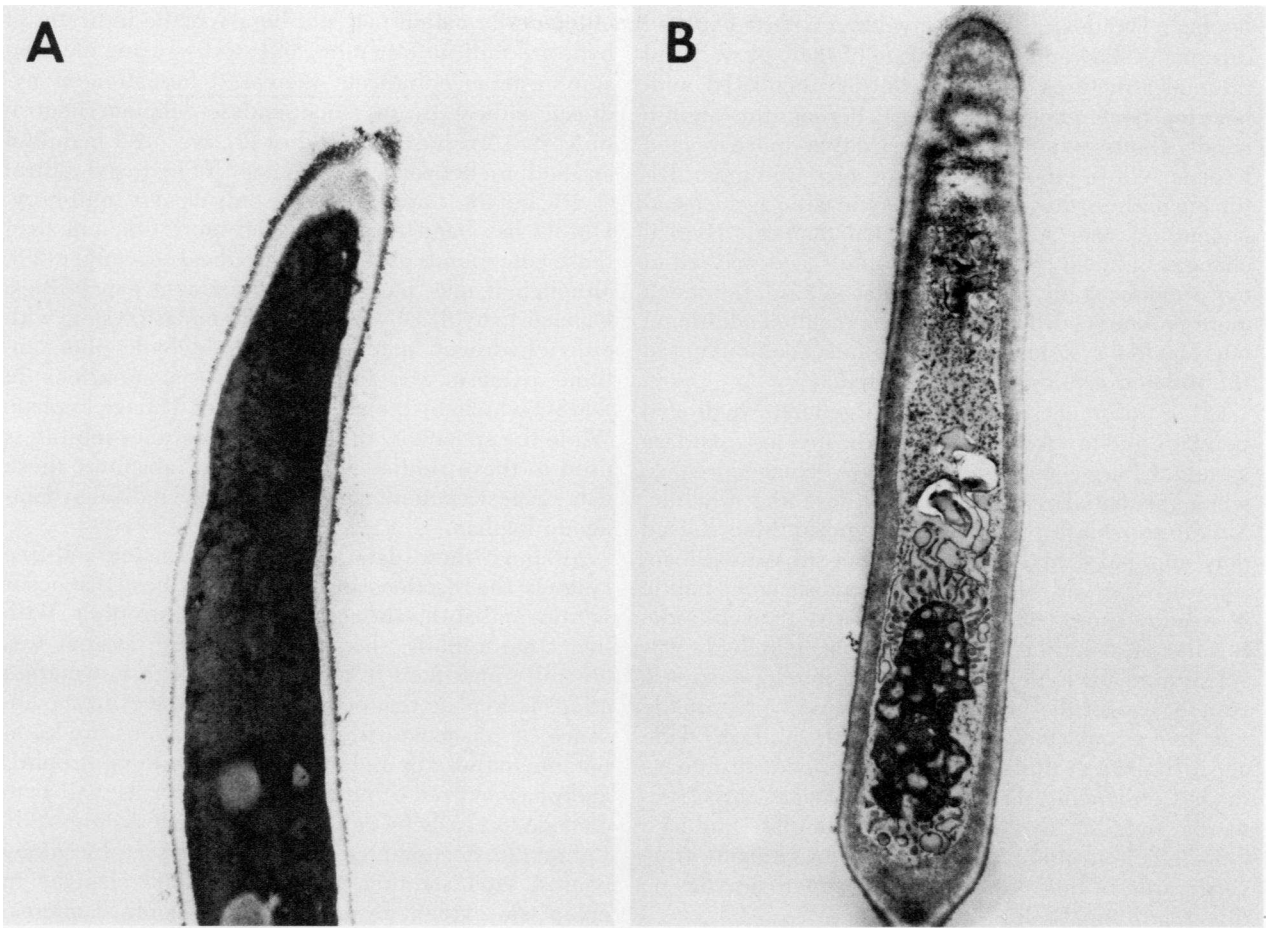

FIGURE 1 Transmission electron micrographs demonstrating the effects of the myeloperoxidase system on *Candida* hyphae. ×10,260. (A) Control: incubation in heat-inactivated myeloperoxidase in presence of H_2O_2 and NaI for 1 h. The cell wall is thick and electron translucent except for the outermost layer. The internal structures appear relatively dark and indistinct. (B) Experimental: incubation with active myeloperoxidase in presence of H_2O_2 and NaI for 1 h. The cell wall shows increased diffuse electron density. Although outlines of many internal structures appear distinct, organelles cannot be identified.

of myeloperoxidase with hydrogen peroxide and a halide was also of primary importance. The myeloperoxidase system may exert microbicidal activity through halogenation reactions or by the formation of potent oxidants, possibly including halogens, hypohalous acids, chloramines, aldehydes, and singlet oxygen (17, 18). Therefore, even myeloperoxidase-mediated damage to *Candida* hyphae might be attributable to a number of different substances or reactions.

To determine which of these potentially microbicidal mechanisms could damage *Candida* hyphae, we exposed the hyphae to purified reagents in cell-free systems. Such systems have been used by others to document cytotoxic effects on a variety of target cells, including leukocytes (8), platelets (21), tumor cells (22), and bacteria (7). Using *Candida* hyphae as target cells, we found that the myeloperoxidase system with both chloride and iodide induced cell damage very efficiently. There was less damage to hyphae when chloride or iodide was omitted from incubations. Active myeloperoxidase was required, inasmuch as there was no hyphal damage when the enzyme was inactivated by heating or was omitted from incubations. Furthermore, sodium cyanide and sodium azide inhibited damage to hyphae when used in concentrations that primarily affected myeloperoxidase activity (5). However, this effect is not wholly specific inasmuch as both substances may affect cell respiration, and sodium azide in high concentrations is also a singlet oxygen scavenger (6, 7). The importance of hydrogen peroxide in antihyphal activity was emphasized by the almost complete inhibition of damage induced by catalase.

The myeloperoxidase system induced subtle but clearly visible changes in the ultrastructure of the

hyphae. Paradoxically, the myeloperoxidase-exposed organisms showed a better image of the cell wall and internal structures than the controls incubated with heat-inactivated myeloperoxidase. Perhaps this was the result of better penetration of the fixatives into damaged hyphae. While internal structures appeared more distinct in outline, they were not recognizable as the usual organelles, also suggesting hyphal damage. Hyphal changes induced by intact neutrophils as described in our previous study (1) were similar in kind, but much more pronounced. This may be the result of additional microbicidal systems acting in the intact cell but not in the isolated myeloperoxidase system.

Other substances were used to generate hydrogen peroxide and to substitute for it in the myeloperoxidase system. Glucose and glucose oxidase damaged hyphae when incubated with myeloperoxidase and a halide. Xanthine oxidase with 0.1 mM hypoxanthine caused only minimal damage to hyphae, but substantial damage was observed when myeloperoxidase and a halide were added, iodide being more effective than chloride. Results were similar when 0.1 mM acetaldehyde was substituted for hypoxanthine in the myeloperoxidase system, except that iodide and chloride were equally effective as cofactors. Cyanide (1 mM) inhibited damage to hyphae by this system, but superoxide dismutase did not, enhancing, if anything, the damage. This observation suggests that superoxide dismutase induced more efficient production of hydrogen peroxide from superoxide in this system, but that superoxide has no direct antifungal role.

Given the central importance of the myeloperoxidase system in damage to hyphae, it would be useful to identify potential products of this reaction that have antifungal activity. One such substance, singlet oxygen (23), is produced as the major product of the photooxidation of the dye, rose bengal (6). Hyphae were damaged by this reaction and damage was almost completely inhibited by three scavengers of singlet oxygen: DABCO, histidine, and tryptophan. Singlet oxygen scavengers also partially inhibited the myeloperoxidase system, both when hydrogen peroxide was directly included in incubations and when supplied by the reaction of acetaldehyde with xanthine oxidase. Though these data suggest antifungal activity of singlet oxygen in these systems, the lack of absolute specificity of the quenching agents (24, 25) precludes a definite statement regarding singlet oxygen involvement.

Hydroxyl radical has been proposed as another potentially microbicidal substance produced during oxygen consumption by neutrophils (26–28). It has been suggested that hydroxyl radical may be produced as a by-product of the myeloperoxidase system (13). However, this suggestion is based on use of ethylene formation as an index of hydroxyl radical formation by neutrophils and by the myeloperoxidase system. However, ethylene formation may not be a specific indicator of hydroxyl radical formation, and studies using electron spin resonance indicate increased formation of hydroxyl radical by myeloperoxidase-deficient neutrophils (28). In previous studies (2), we used mannitol and sodium benzoate as scavengers of hydroxyl radical (7, 26), but these appeared to be only weakly inhibitory. DMSO has been reported to be more efficient than these compounds as a hydroxyl radical scavenger (29), although it may have other antioxidant capabilities. Damage to hyphae by the myeloperoxidase system with either hydrogen peroxide or acetaldehyde plus xanthine oxidase was inhibited by concentrations of DMSO which, by themselves, did not damage hyphae. While the specificity of this as well as other inhibitors used in these studies is by no means absolute, these data suggest a potential role for hydroxyl radical in damage to hyphae.

Although these data were obtained using cell-free systems, the reactions are analogous to those that occur within, and at the surfaces of, intact neutrophils. With intact neutrophils, the myeloperoxidase system was critically important in mediating damage to *Candida albicans* hyphae that occurs at cell surfaces in the absence of phagocytosis. These observations are comparable to those by Lehrer and Cline with neutrophils and phagocytized *Candida* yeasts (3, 20), as well as to damage to yeasts by cell-free peroxidase systems (30). Lehrer (30) documented candidacidal effects by colony counts, vital staining, and morphologic changes in cytoplasm evident by light or phase-contrast microscopy. Killing occurred only when hydrogen peroxide and halide were combined with purified peroxidase or with neutrophil granules, and each component alone did not damage the yeasts. We have now extended these observations using cell-free systems capable of forming superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen to damage the tissue-invasive forms of *Candida*. Cell-free enzyme systems, themselves, may have a potential role in antifungal therapy. In one study, peroxidase and xanthine oxidase coupled to specific antibodies to *Candida albicans* improved survival in experimental murine candidiasis (31). Hydrogen peroxide (in the presence of myeloperoxidase and a halide), and perhaps singlet oxygen, may also prove to mediate damage to hyphae by intact neutrophils inasmuch as they are products of oxidative metabolism or of the myeloperoxidase system (13, 17, 18).

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