INFECTION AND IMMUNITY, Mar. 1986, p. 731–735
0019-9567/86/030731-05$02.00/0

Vol. 51, No. 3

Inhibition of Neutrophil Killing of *Candida albicans* Pseudohyphae by Substances Which Quench Hypochlorous Acid and Chloramines

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Using a microtiter plate killing assay, we investigated the in vitro killing of *Candida albicans* by human neutrophils and by hypochlorous acid/hypochlorite ion ($HOCl/OCl^-$) or chloramine solutions to evaluate the inhibition of this process by quenchers of these oxidants. Methionine, tryptophan, and alanine were able to effectively inhibit neutrophil killing of candida pseudohyphae. These substances were capable of quenching the oxidant activity of NaOCl, monochloramine (NH_2Cl), and to a lesser extent, taurine chloramine. NaOCl and NH_2Cl were able to kill *C. albicans* in the absence of inhibitors in concentrations of less than 5 μM, whereas greater than 100 μM taurine chloramine was required for killing. Methionine and tryptophan were capable of markedly inhibiting killing by all three oxidants, whereas alanine affected only killing by NaOCl. The oxidant activity of NaOCl was more readily quenched by opsonized or unopsonized *Candida* yeast than was the oxidant activity of either NH_2Cl or taurine chloramine. These results suggest that some substances which quench the oxidizing activity of the products of the neutrophil myeloperoxidase system can inhibit the killing of *C. albicans* by these cells.

Killing of microorganisms by human neutrophils involves several events which begin with attachment of the neutrophil to the microbial cell. The attachment process appears to activate the neutrophil, resulting in initiation of the respiratory burst and degranulation (2, 9). For *Candida albicans*, attachment is followed by phagocytosis of yeast-phase organisms and spreading over the fungal surface for pseudohyphae (6). Spreading of the neutrophils over the surface of the organism appears to be essential for the neutrophil to be capable of inflicting damage to fungal cells (4, 5).

The neutrophil oxygen-dependent killing mechanisms are thought to play a primary role in neutrophil killing of candida pseudohyphae under aerobic conditions (6). When neutrophil membranes are first stimulated, there is an increase in oxygen consumption. This oxygen undergoes a one-electron reduction to superoxide, which then spontaneously dismutates to hydrogen peroxide (H_2O_2) (10). Hydrogen peroxide itself has some bactericidal activity, but its potency is greatly augmented in the presence of myeloperoxidase and a halide ion (9). Myeloperoxidase can convert H_2O_2 and chloride ion to a strong oxidizing compound which can diffuse away from the enzyme as free hypochlorous acid, in contrast to the oxidizing compounds of other peroxidases (8). Since the pKa of HOCl is 7.53, it exists as a mixture of the unionized acid and hypochlorite ion ($HOCl/OCl^-$) at a physiologic pH. There is a significant amount of evidence to support the role of $HOCl/OCl^-$ as a major toxic product produced by neutrophils (1, 12, 13, 16–19). Interaction of $HOCl/OCl^-$ with certain substances present in the organisms, neutrophils, or surrounding milieu can result in the production of other compounds such as chloramines which may also be toxic for microorganisms (9, 16). Stimulated human neutrophils have recently been demonstrated to release into the surrounding medium two types of chloramines, monochloramine (NH_2Cl) (7) and taurine chloramine (15). These two species could be responsible for killing of microorganisms or for damage to surrounding tissues.

In the present study, we investigated killing of *C. albicans* by human neutrophils or the oxidants produced by their myeloperoxidase system in a microtiter plate killing assay and the inhibition of these processes by several substances. We also attempted to correlate the effect of inhibitory substances on candida killing with their ability to quench the oxidants or to react with the organisms themselves. The results of these assays indicate that substances which quench the oxidizing activity of the products of the neutrophil myeloperoxidase system can inhibit killing of *C. albicans* by these cells.

MATERIALS AND METHODS

Organisms. Yeast-phase organisms from a clinical isolate of *C. albicans* were obtained for the experiments after overnight growth in Sabouraud broth. The yeast cells were washed twice in saline, counted in a counting chamber, and suspended at the appropriate concentrations.

Neutrophils. Human neutrophils were isolated from peripheral blood of healthy volunteers by Hypaque-Ficoll density centrifugation as previously described (14). Contaminating erythrocytes were lysed by hypotonic shock; the resulting preparations consisted of 98 to 99% neutrophils.

Chemicals. Taurine, methionine, tryptophan, alanine, ammonium chloride (NH_4Cl), mannitol, superoxide dismutase, cytochrome *c*, horseradish peroxidase, and catalase were obtained from the Sigma Chemical Co., St. Louis, Mo.; sodium hypochlorite (NaOCl) was obtained from the Aldrich Chemical Co., Milwaukee, Wis.; and *o*-phenylenediamine (opd) was from Eastman Kodak Co., Rochester, N.Y. Catalase was dialyzed against saline before use. NH_2Cl was prepared by incubating NaOCl with a 10-fold excess of NH_4Cl; taurine chloramine was prepared by incubating NaOCl with a 10-fold excess of taurine. Conversion to the chloramine was evaluated under these conditions by UV spectroscopy; in both cases, the NaOCl peak at 292 nm

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disappeared, to be replaced by the NH_2Cl peak at 242 nm or the taurine chloramine peak at 252 nm. Molarity of the H_2O_2 and NaOCl stock solutions was confirmed by using absorbance at 230 nm (extinction coefficient of 81 M^{-1} cm^{-1}) and at 292 nm at pH 12.0 (extinction coefficient 350 M^{-1} cm^{-1}), respectively.

Quenching assays. Quenching of NaOCl, NH_2Cl, taurine chloramine, and H_2O_2 was evaluated by using the oxidation of opd to determine the remaining oxidant concentration. Standard curves were first prepared by using half-log dilutions of the oxidants. Solutions containing 500 μM concentrations of the oxidants were placed in 12-by-75-mm plastic tubes, to which were added the inhibitors. After 5 min at room temperature, 0.1 ml of the opd solution (10 mg/ml final concentration for the NaOCl, NH_2Cl, or taurine chloramine assays and 10 mg of opd per ml plus 10^{-5} mg of horseradish peroxidase per ml for the H_2O_2 assay [total volume 1 ml]) was added. After the addition of opd, the solutions were incubated at room temperature for an additional 40 min and read at 420 nm. Candida cells were prepared for use as an inhibitor by being washed in saline and by opsonization, which was carried out by placing 10^9 candida cells in 1 ml of 95% human plasma with 5% rabbit anti-candida antiserum (obtained from rabbits immunized with candida cells in Freund complete adjuvant), followed by washing in phosphate-buffered saline (PBS).

The data were expressed as the reduction of the concentration (micromolar) of NaOCl, NH_2Cl, taurine chloramine, or H_2O_2 after incubation with the inhibitor. Inhibitor concentrations evaluated were those used in the neutrophil killing experiments and in some cases $\log_{10}$ dilutions of those. In some experiments, the inhibitors were added after the oxidants to evaluate the possibility that the inhibitor reacted with oxidized opd (such a result was not found).

Microtiter plate killing assays. The killing assay used was a modification of that of Schaffner et al. (11). Costar flat-bottom tissue culture plates (Costar, Cambridge, Mass.) were used with either human neutrophils or oxidant solutions to kill candida cells or pseudohyphae. For experiments involving pseudohyphae, 10 cells were incubated with 0.05 ml of fetal calf serum at 37°C for 2 to 4 h until greater than 95% of the cells had converted to the pseudohyphal phase, as evaluated microscopically in parallel samples carried out in larger volumes. In vitro killing by oxidants was studied with washed candida cells (instead of pseudohyphae) to avoid exposing the organisms to potential inhibitors during the germination process. After incubation, the plates were centrifuged at 800 × *g* for 15 min, and the supernatant was removed with a 23-gauge needle. For the NaOCl killing experiments, the wells were washed two times with saline.

For the neutrophil killing assay, 10^5 neutrophils in sterile PBS (containing 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 5 mM glucose at pH 7.4) and 10% human plasma were added to wells containing 10 organisms. Preliminary experiments demonstrated this ratio to be optimal; sterilization of the wells occurred less frequently with lower neutrophil to candida organism ratios. The inhibitors were added to appropriate wells before addition of neutrophils or NaOCl. Final assay volume was 0.1 ml. The plates were then centrifuged (150 × *g* for 5 min) and placed in a CO_2 incubator overnight. After incubation, the plates were again centrifuged (800 × *g* for 15 min), the supernatant was removed, and Sabouraud broth was added. Plates were read at 3 days, and killing of *C. albicans* was expressed as the percentage of wells sterilized.

In the oxidant killing assay, solutions of NaOCl, NH_2Cl, or taurine chloramine were diluted in sterile PBS (pH 7.0) to final concentrations of 1 to 1,000 μM. After the addition of the inhibitors to the appropriate wells, the oxidant solutions were added to wells containing 10 yeast cells with or without inhibitor solutions. The final assay volume was 0.1 ml. The plates were incubated for 1 h at room temperature and centrifuged (800 × *g* for 15 min). The supernatant was removed, Sabouraud broth (0.1 ml) was added, and the plates incubated at 37°C for 3 days. Killing of *C. albicans* was expressed as the percentage of wells sterilized. The effects of the inhibitors were expressed as the increase in the concentration of the oxidant required for sterilization of duplicate wells.

TABLE 1. Effect of potential inhibitors on neutrophil killing of candida pseudohyphae[a]

Inhibitor	% Reduction in no. of wells sterilized (mean ± SE)	Statistical significance[b]
Methionine (10^{-2} M)	100 ± 0	$P < 0.001$
Tryptophan (10^{-2} M)	91.7 ± 8.5	$P < 0.01$
Alanine (10^{-2} M)	63.8 ± 21.4	$P < 0.05$
Mannitol (10^{-2} M)	2.1 ± 2.1	NS[c]
Superoxide dismutase (20 μg/ml)	13.9 ± 7.5	NS
Catalase (500 μg/ml)	20.1 ± 11.9	NS

[a] For percentage of wells sterilized, the control value (killing by 10^5 neutrophils [mean ± standard error]) was 72.9 ± 5.1.

[b] Statistical significance as determined by using the one-sample *t*-test. Data represent results (mean ± standard error) from 3 to 9 experiments per point with 12 to 24 wells used in each determination for each condition.

[c] NS, Not significant.

Superoxide assay. Superoxide production was measured by using a modification of the method of Babior et al. (3) in which 10^6 neutrophils were added to 12-by-75 mm plastic tubes containing a concentration of 100 μM cytochrome *c* in PBS with a final volume of 1 ml. Inhibitor solutions were added after the cytochrome *c*. The cells were stimulated with phorbol myristate acetate (100 ng/ml). Duplicate samples were prepared, and 20 μg of superoxide dismutase per ml was added to one. The tubes were incubated at 37°C for 5 min and centrifuged, and the supernatants were placed on ice and read at 550 nM. The results were converted to nanomoles of cytochrome *c* reduced, using the extinction coefficient of 2.1×10^4 M^{-1} cm^{-1}. The production of superoxide was determined by subtracting the value in the sample containing superoxide dismutase from that without superoxide dismutase.

Statistics. The results were evaluated statistically by using the single sample *t*-test. Significance was taken as $P < 0.05$. The experiments were repeated at least three times.

RESULTS

Neutrophil killing. Table 1 shows the results of the potential inhibitors on neutrophil killing of candida pseudohyphae. Most experiments were done with pseudohyphae (the invasive phase of *C. albicans*), although studies with *C. albicans* yeast revealed similar results (data not shown). In wells containing 10 pseudohyphae and 10^5 neutrophils, the mean percent sterilization of wells was 72.9 ± 5.1. Complete or almost complete inhibition of killing occurred in the presence of tryptophan and methionine; alanine caused less inhibition (63.8%), but this value was statistically significant. Superoxide dismutase and catalase produced minor degrees

TABLE 2. Effect of potential inhibitors on production of superoxide by stimulated neutrophils

Inhibitor (10^{-2} M)	nmol of cytochrome *c* reduced by 10^6 phorbol myristate acetate-stimulated neutrophils	Statistical significance[a]
None	46.4 ± 5.9	–
Methionine	44.8 ± 3.1	NS[b]
Tryptophan	30.9 ± 6.7	$P < 0.05$
Alanine	47.8 ± 6.1	NS
Mannitol	47.8 ± 5.2	NS

[a] Statistical significance by the one-sample *t*-test as compared to control. (Data represent mean ± standard error of values from three experiments.)
[b] NS, Not significant.

of inhibition (13.9 and 20.1%, respectively), and there was almost no inhibition with mannitol.

Superoxide production. Table 2 shows the effects of the potential inhibitor solutions on the production of superoxide by phorbol myristate acetate-stimulated neutrophils. Tryptophan showed partial suppression of this neutrophil function, whereas the other inhibitors had no effect.

Quenching assays. Figure 1 gives standard curves for the quantitation of H_2O_2, HOCl/OCl^- (as NaOCl), NH_2Cl, and taurine chloramine by the oxidation of opd. The sensitivity of this method was the highest for H_2O_2 and lowest for taurine chloramine. Table 3 shows the quenching of NaOCl, NH_2Cl, taurine chloramine, and H_2O_2 by the potential inhibitors, using the oxidation of opd method. Oxidation of iodide or nitrobenzoic acid could not be used to assay HOCl-OCl^- in this system since some reducing substances have the potential to convert the oxidized species back to the reduced form. Oxidized opd (the colored product) was not affected by the substances used as inhibitors. As can be seen in the first two columns, methionine, tryptophan, and alanine effectively quenched the oxidative capacity of 500 μM NaOCl or NH_2Cl. Taurine chloramine and H_2O_2 were quenched to a lesser extent by the three substances which were capable of inhibiting killing of *C. albicans* by neutrophils, except that tryptophan did react with H_2O_2. Mannitol did not significantly quench any of the oxidants under these conditions.

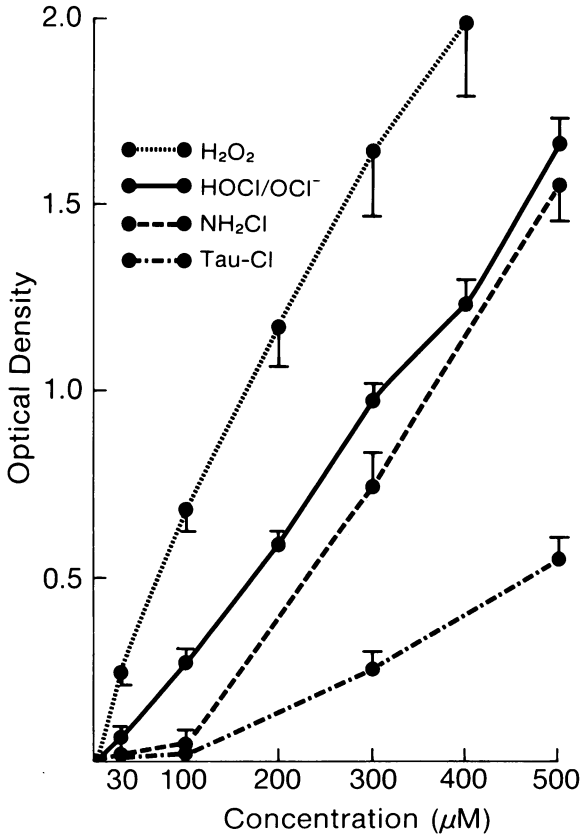

FIG. 1. Standard curves for the detection of hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl/OCl^-), monochloramine (NH_2Cl), and taurine chloramine (Tau-Cl) by the oxidation of opd method. The data represent the means ± 1 standard error of results of 8 experiments for H_2O_2, 10 for HOCl/OCl^-, 11 for NH_2Cl, and 10 for Tau-Cl.

TABLE 3. Quenching of oxidants by potential inhibitors of candida killing

Inhibitor	Oxidant quenched (μM)[a]			
	NaOCl	NH_2Cl	Taurine chloramine	H_2O_2
Methionine				
10^{-2} M	493 ± 0	452 ± 26	383 ± 23	39 ± 20
10^{-3} M	496 ± 0	418 ± 36	455 ± 31	0 ± 0
Tryptophan				
10^{-2} M	491 ± 2	354 ± 2	150 ± 17	466 ± 18
10^{-3} M	465 ± 9	152 ± 9	7 ± 7	328 ± 63
Alanine				
10^{-2} M	443 ± 11	394 ± 15	137 ± 23	1 ± 1
10^{-3} M	444 ± 6	193 ± 4	23 ± 15	0 ± 0
Mannitol				
10^{-2} M	32 ± 19	0 ± 0	0 ± 0	0 ± 0

[a] Amounts quenched represents reduction in concentration (μM) of a 500 μM solution of the oxidant after incubation with the inhibitors. (Data represent mean ± standard error of values from three experiments.)

Oxidant killing. The concentration of the oxidants required to sterilize duplicate wells containing 10 organisms each in the presence of the same inhibitors is shown in Table 4. Complete sterilization of wells required a mean of 2 μM NaOCl, 3 μM NH_2Cl, and 143 μM taurine chloramine. Marked increases in the concentration of all three oxidants required for sterilization were seen in the presence of methionine and tryptophan. Alanine produced an inhibitory effect with NaOCl, but not with NH_2Cl or taurine chloramine. Mannitol did not appear to be an effective inhibitor of killing by these oxidants.

TABLE 4. Effect of potential inhibitors on killing of *C. albicans* yeast by oxidants

Inhibitor (10^{-2} M)	μM oxidant required for killing[a]		
	NaOCl	NH_2Cl	Taurine chloramine
None	2 ± 0	3 ± 0	143 ± 81
Methionine	1,000 ± 0	533 ± 233	1,000 ± 0
Tryptophan	1,000 ± 0	1,000 ± 0	1,000 ± 0
Alanine	766 ± 2	23 ± 7	137 ± 1
Mannitol	17 ± 7	10 ± 0	700 ± 300

[a] Concentration of oxidant required for sterilization of microtiter plate wells containing 10 *C. albicans* cells in the presence of the inhibitors. (Data represent mean ± standard error of three determinations.)

TABLE 5. Quenching of oxidants by *C. albicans*

Candida preparation[a]	Oxidant quenched (μM)[b]			
	NaOCl	NH_2Cl	Taurine chloramine	H_2O_2
Unopsonized yeast	402 ± 23.7	85 ± 17.6	33 ± 18.6	134 ± 30.8
Opsonized yeast	369 ± 49.3	65 ± 18.9	32 ± 22.4	124 ± 39.0

[a] *C. albicans* cells were used at a concentration of 0.1% by volume.
[b] Amount quenched represents reduction in concentration (μM) of a 500 μM solution of the oxidant after incubation with the yeast cells. (Data represent mean ± standard error of values from three experiments.)

Quenching by *C. albicans*. The ability of *C. albicans* to quench the oxidants was used as a measure of the ability of the organism to interact with these species. This interaction was greatest with NaOCl and much less with NH_2Cl and taurine chloramine (Table 5). Opsonization of the organisms did not affect the quantity of the oxidants quenched.

DISCUSSION

The data from these studies indicate that some substances which react with products of the myeloperoxidase system of the neutrophil can inhibit killing of *C. albicans* pseudohyphae by these cells. Myeloperoxidase has the ability to oxidize halogens to hypohalous acids; since the major halide ion available to the cell is chloride, the resulting product is $HOCl/OCl^-$. This oxidant can react with taurine contained inside the neutrophil to produce taurine chloramine (15) or with NH_4^+ produced by the cell to produce NH_2Cl (7). NaOCl and NH_2Cl appear to be more potent in killing *C. albicans* than is taurine chloramine. Methionine, tryptophan, and alanine can all inhibit neutrophil killing of candida pseudohyphae, and all are capable of effectively quenching both NaOCl and NH_2Cl. Whereas tryptophan suppresses to some degree superoxide production by stimulated neutrophils, methionine and alanine do not. Tryptophan is also capable of reacting with H_2O_2. The pattern in inhibition produced by these three substances might suggest that $HOCl/OCl^-$ itself is the target in intact cells because this species is more readily quenched by these inhibitors and also reacts to a greater degree with the organisms themselves than does NH_2Cl. However, there are enough differences between the quenching assays and the neutrophil killing assay (see below) to make such a distinction difficult.

Killing of *C. albicans* by neutrophils involves internalization of the organism, whereas killing of pseudohyphae involves attachment of the neutrophil to the surface of the organism and damage produced extracellularly (5, 6). Candida pseudohyphae were used in the neutrophil killing assays to allow better access of the potential inhibitor to the site of attack on the surface of the organism. Recent studies on the adherence of macrophages to glass surfaces coated with phagocytosis-promoting ligands have shown that proteins over 50,000 daltons are excluded from the site of attachment (20). Smaller molecules, such as the inhibitors used in these studies, may have been able to enter the area of neutrophil attachment to the organisms. Catalase, on the other hand, is a high-molecular-weight substance that might be expected to inhibit myeloperoxidase-mediated killing by neutrophils in this system; we suspect that it was excluded from the area of attack at the fungal surface in these studies. In the in vitro killing assays, we used ungerminated *C. albicans* to study killing of the organisms in the absence of potentially inhibitory substances (e.g., serum, glycine) used to germinate the cells. In addition, the medium for the neutrophil killing assays contained 10% human serum to promote attachment of the cells to the organisms. Despite these differences, the activity of the three inhibitors in the quenching assays paralleled fairly closely their effects in preventing neutrophil killing of the candida pseudohyphae, suggesting that the oxidants, particularly $HOCl/OCl^-$, produced by the myeloperoxidase system of the neutrophil are involved in killing of this organism.

ACKNOWLEDGMENTS

This work was supported by the Veterans Administration and the Kroc Foundation.
We thank Sue Schneider for excellent editorial assistance.

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