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# Killing of *Aspergillus fumigatus* Spores and *Candida albicans* Yeast Phase by the Iron-Hydrogen Peroxide-Iodide Cytotoxic System: Comparison with the Myeloperoxidase-Hydrogen Peroxide-Halide System

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A new fungicidal system composed of ferrous ion, $H_2O_2$, and iodide is described and compared with the myeloperoxidase-hydrogen peroxide-halide system. Both systems had similar activity against *Aspergillus fumigatus* spores and the *Candida albicans* yeast phase, but only the ferrous ion-hydrogen peroxide-iodide system was inhibited by hydroxyl radical scavengers.

The combination of myeloperoxidase (MPO), hydrogen peroxide ($H_2O_2$), and a halide appears to play an important role in the antimicrobial activity of neutrophils and monocytes (2, 10). Previous studies in which cell-free systems were used have demonstrated the antifungal activity of the MPO-hydrogen peroxide-halide system (3, 4, 18). Evidence exists, however, that other antimicrobial systems are operative in phagocytes. First, patients with hereditary MPO deficiency are often asymptomatic, and in vitro studies on their leukocytes show delayed but ultimately effective microbicidal activity (10, 15, 16). Second, mature macrophages which lack MPO undergo a respiratory burst during phagocytosis and are capable of killing ingested organisms (8, 17). Recently, Klebanoff (12) described an antimicrobial system composed of ferrous ion ($Fe^{2+}$), $H_2O_2$, and iodide. In this paper, we compare the fungicidal properties of MPO-hydrogen peroxide-iodide (MPO system) with that of $Fe^{2+}$-hydrogen peroxide-iodide ($Fe^{2+}$ system) against *Aspergillus fumigatus* spores and the *Candida albicans* yeast phase.

*A. fumigatus* spores were harvested from a previously described isolate as in prior studies (3). Killing of spores was determined by inhibition of germination performed in a final volume of 100 μl of 20 mM sodium acetate buffer (pH 5.5) in flat-bottomed microwells (Corning Glass Works, Corning, N.Y.) containing $1.0 \times 10^3$ spores. MPO was prepared from canine pus neutrophils by the method of Agner (1) through the end of the sixth step and assayed by the *o*-dianisidine method. One unit of MPO uses 1 μmol of substrate per min at 25°C (13). MPO was used at a concentration of 10 mU/ml. $Fe^{2+}$ was added as 10 μM ferrous sulfate. Microwell plates were centrifuged at 1,000 × *g* for 3 min and then placed on a shaker at 37°C and incubated for 1 h. Plates were subsequently centrifuged at 4°C, 1,000 × *g* for 5 min, the supernatant was gently aspirated, and 200 μl of Sabouraud broth containing 100 U of penicillin and 100 μg of streptomycin per ml was added. The centrifugation and washing steps were repeated twice, and the plates were incubated at 22°C for 20 h. Percent germination was then determined by counting the number of germinated spores among the first 100 organisms per well from randomly selected fields with an inverted microscope. Wells with 0% germination were incubated for an additional 48 h and in all cases remained sterile. Heat-inactivated (100°C, 15 min) MPO and $Fe^{3+}$ (10 μM ferric chloride) were used as controls. Neither inhibited germination when substituted for MPO or $Fe^{2+}$.

$Fe^{2+}$ could be substituted for MPO in the presence of $H_2O_2$ and iodide to inhibit germination of *A. fumigatus* spores (Table 1). Minimum concentrations of 10 μM $H_2O_2$ and 100 μM iodide were required in both systems. $H_2O_2$ generation by glucose oxidase with glucose could replace reagent $H_2O_2$ (data not shown). However, when iodide was replaced by chloride or bromide, no inhibition of germination was seen. When 1 μM $Fe^{2+}$ was used in place of 10 μM $Fe^{2+}$, germination was no longer inhibited.

The $Fe^{2+}$ system, but not the MPO system, was inhibited by the hydroxyl radical scavengers mannitol and ethanol and the iron chelator EDTA (Table 2). In contrast, the MPO system was inhibited by concentrations of azide known to inhibit MPO. Catalase inhibited both systems, confirming the requirement for $H_2O_2$. When the pH of the 20 mM acetate buffer was varied, both systems completely inhibited germination at pH 4.5 through 6.0, pHs which are thought to be attainable in phagocytic vacuoles (7, 19). At pH 6.5, inhibition of germination was reduced by 30% with the $Fe^{2+}$ system and 76% with the MPO system, and neither system inhibited germination at pH 7.0. $H_2O_2$ and iodide without MPO or $Fe^{2+}$ failed to inhibit germination in the pH range and concentrations tested.

The ability of both the $Fe^{2+}$ and MPO systems to iodinate *A. fumigatus* conidia was demonstrated with an iodination assay as described by Klebanoff and Clark (14) but modified for use in a cell-free system. In a final volume of 1 ml of 20 mM sodium acetate buffer (pH 5.5) $2 \times 10^6$ spores, 100 nCi of [$^{125}$I]Na, 10 μM NaI, 100 μM $H_2O_2$, and either 50 mU of MPO or 10 μM $Fe^{2+}$ were added to borosilicate culture tubes (12 by 75 mm; Fisher Scientific Co., Pittsburgh, Pa.) and incubated at 37°C with shaking for 1 h. The tubes were centrifuged at 4°C, 1,000 × *g* for 5 min, the supernatant was discarded, and the spore-containing pellet was washed four times with 4 ml of isotonic saline and counted in a gamma counter. Micromoles of iodide bound to spores was calculated as follows: 10 × [(cpm of spores − cpm with spores omitted)/cpm of total $^{125}$I added], where 10 equals micro-

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TABLE 1. Effect of varying concentrations of $H_2O_2$ and iodide on germination of *A. fumigatus* spores in the presence of MPO or $Fe^{2+}$

| $H_2O_2$ concn | Iodide concn (μM) | Germination (%)[a] | |
|---|---|---|---|
| | | MPO | $Fe^{2+}$ |
| 0 | 0 | 99 | 99 |
| 1 mM | 100 | 0[b] | 0[b] |
| 1 mM | 10 | 99 | 94 |
| 100 μM | 100 | 0[b] | 0[b] |
| 100 μM | 10 | 99 | 96 |
| 10 μM | 100 | 0[b] | 0[b] |
| 10 μM | 10 | 100 | 99 |
| 1 μM | 100 | 99 | 99 |

[a] Results are the means of two to six experiments, each performed in duplicate.
[b] $P < 0.001$ by Student's $t$ test compared with controls containing no $H_2O_2$ or iodide.

moles of iodide in the reaction mixture (cpm, counts per minute). Both the MPO and $Fe^{2+}$ systems iodinated spores (Table 3), whereas no appreciable iodination occurred in the absence of $H_2O_2$. EDTA and mannitol significantly inhibited only the $Fe^{2+}$ system (by 99.8 and 93.9%, respectively), whereas azide significantly inhibited only the MPO system (by 95.3%).

The fungicidal properties of the $Fe^{2+}$ system were demonstrated and compared with those of the MPO system with dilutions and pour plates made from tubes of reagents in a 1-ml volume with $10^5$ *C. albicans* yeast phase organisms (Table 4). At this density, no significant clumping of yeast cells was observed. With both MPO and $Fe^{2+}$, compared with *A. fumigatus* spores, *C. albicans* yeast phase organisms were sensitive to concentrations of iodide 10- to 100-fold lower (complete killing at 10 μM, partial killing at 1 μM) and concentrations of $H_2O_2$ 10-fold lower (partial killing at 1 μM). When 100 μM chloride was substituted for iodide, no significant killing of *C. albicans* was seen in either the MPO or the $Fe^{2+}$ system with 100 μM $H_2O_2$ (data not shown).

The results presented here establish $Fe^{2+}$ as a component of a potent fungicidal system comparable in activity to the MPO system against spores of *A. fumigatus* and *C. albicans* blastospores. Both systems were also active against *Rhizopus oryzae* spores (data not shown). Klebanoff (11, 12) proposed that $Fe^{2+}$ reacts with $H_2O_2$ to generate hydroxyl radical, which then reacts with iodide to form the toxic product(s) of the $Fe^{2+}$ system. Our observations of inhibition of killing by the hydroxyl radical scavengers mannitol and ethanol are consistent with this interpretation. Since cells such as macrophages which lack MPO can kill *A. fumigatus* spores (20), MPO-independent fungicidal mechanisms must be operative. Since iodination reactions do not normally occur in macrophages (21), our data suggesting that the sporicidal activity of the $Fe^{2+}$ system is accompanied by iodination argues somewhat against, but does not preclude, a role for the $Fe^{2+}$ system in the microbicidal defenses of that cell type. First of all, iodination need not be required for killing of organisms, and the two processes might be separable and dependent upon different concentrations of reagents. It has been suggested, moreover, that macrophages might utilize exogenous peroxidase from other sources present within mixed cell populations composing inflammatory exudates in vivo (5, 6). By analogy, trapping mechanisms might exist which enable macrophages to utilize the $Fe^{2+}$ system. Although the iodide concentration necessary for fungicidal activity of the MPO and $Fe^{2+}$ systems far exceeds those present in serum (less than 1 μg/100 ml), leukocytes have efficient iodide-concentrating mechanisms and may contain sufficient intracellular iodide for the system to be operative (9). Finally, the combined sporicidal activity of the $Fe^{2+}$ system in vitro could potentially constitute a useful method for control or elimination of these difficult-to-eradicate fungal forms.

TABLE 3. Comparison of the iodinating activities of the MPO and $Fe^{2+}$ systems

| Changes to system[a] | Iodination (μM $^{125}I$ fixed by system)[b] | |
|---|---|---|
| | MPO | $Fe^{2+}$ |
| None | 16.55 | 4.27[c] |
| $H_2O_2$ omitted | 0.85[d] | 0.09[e] |
| 10 μM EDTA | 19.29 | 0.01[e] |
| 100 mM mannitol | 14.40 | 0.26[e] |
| 100 μM azide | 0.78[d] | 2.56 |

[a] Standard system contains either MPO or $Fe^{2+}$ with 100 μM $H_2O_2$ and 10 μM iodide.
[b] Results are the means of two experiments in which $2 \times 10^6$ spores were used per tube.
[c] $P < 0.005$ by Student's $t$ test compared with the standard MPO system.
[d] $P < 0.001$ by Student's $t$ test compared with the standard MPO system.
[e] $P < 0.01$ by Student's $t$ test compared with the standard $Fe^{2+}$ system.

TABLE 2. Effect of inhibitors on germination of *A. fumigatus* spores in the presence of the MPO or the $Fe^{2+}$ system

| Inhibitor (concn) | Germination (%)[a] | |
|---|---|---|
| | MPO system | $Fe^{2+}$ system |
| None | 0 | 0 |
| Mannitol (100 mM) | 0 | 99[b] |
| Ethanol (100 mM) | 1 | 18[b] |
| Azide (100 μM) | 94[b] | 0 |
| EDTA (10 μM) | 0 | 100[b] |
| Catalase (1,000U/ml) | 97[b] | 96[b] |

[a] Results are the means of two experiments in a system containing 10 mU of MPO per ml or 10 μM ferrous sulfate, 20 μM $H_2O_2$, and 100 μM sodium iodide.
[b] $P < 0.001$ compared with no inhibitors (none) by Student's $t$ test.

TABLE 4. Effect of varying concentrations of $H_2O_2$ and iodide on killing of *C. albicans* in the presence of MPO or $Fe^{2+}$

| $H_2O_2$ concn | Iodide concn | Killing (%)[a] | |
|---|---|---|---|
| | | MPO | $Fe^{2+}$ |
| 10 μM | 10 μM | 100.0[b] | 100.0[b] |
| 10 μM | 1 μM | 17.8[c] | 60.3[b] |
| 10 μM | 100 pM | 2.7 | 6.8 |
| 1 μM | 10 μM | 97.3[b] | 65.8[d] |
| 100 pM | 10 μM | 0.0 | 2.7 |

[a] Results were calculated from the control system without $H_2O_2$ or iodide and are the means of five experiments with 10 mU of MPO per ml or 10 μm $Fe^{2+}$ ferrous sulfate in 20 mM sodium acetate buffer (pH 5.5).
[b] $P < 0.001$ by Student's $t$ test compared with the control system.
[c] $P < 0.05$ by Student's $t$ test compared with the control system.
[d] $P < 0.01$ by Student's $t$ test compared with the control system.

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