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Oxidative Killing of *Aspergillus fumigatus* Proceeds by Parallel Myeloperoxidase-Dependent and -Independent Pathways

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The relative importance of several oxygen intermediates in fungicidal action against opsonized *Apergillus fumigatus* conidia was investigated with monocytes from normal volunteers and patients with either chronic granulomatous disease or myeloperoxidase (MPO) deficiency. Results from experiments in which catalase, taurine, mannitol, or glucose-glucose oxidase were added to these phagocytes indicated that the MPO-hydrogen peroxide-halide system and an MPO-independent oxidative system exerted comparable conidiacidal activity. These findings offer a plausible explanation for the susceptibility of patients with chronic granulomatous disease to invasive *Aspergillus* infections; their phagocytes fail to generate hydrogen peroxide, a substrate necessary for both systems. Patients with MPO deficiency are not known to be predisposed to invasive aspergillosis, suggesting that an MPO-independent oxidative system may provide an alternative mechanism for the oxidative killing of *Aspergillus* spp.

Aspergillus fumigatus can cause severe infections in immunocompromised hosts. Leukopenia and chronic granulomatous disease (CGD) represent risks for acquisition of these infections, suggesting that polymorphonuclear neutrophils and monocytes are important in host defense against *Aspergillus* spp. (1, 18). *A. fumigatus*, like most organisms that cause serious infections in patients with CGD, produces catalase (15). The defective production of oxidative metabolites by monocytes and neutrophils of CGD patients could lead to increased susceptibility to aspergillosis. Conversely, it seems plausible that hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (·OH), or hypochlorous acid (HOCl) might be important metabolites for the oxidative killing of *Aspergillus* conidia by normal phagocytes.

Diamond and co-workers presented electron microscopic evidence that normal polymorphonuclear neutrophils damage *A. fumigatus* hyphae and that only a limited inhibition of this effect could be produced by adding catalase to degrade H_2O_2 (3). Mannitol, which scavenges ·OH, does not alter the extent of damage to hyphal forms by these effector cells. Monocytes from normal subjects damage hyphae by using myeloperoxidase (MPO), whereas monocytes from patients with CGD or a patient with MPO deficiency damage hyphae via the action of cationic proteins (2).

We further investigated the susceptibility of CGD patients to invasive *Aspergillus* infections by focusing our research on host defense against conidia rather than against hyphae. Conidia are the inhaled particles which initiate infection and can be used in quantitative fungicidal assays. Killing of opsonized resting conidia by human neutrophils has been difficult to demonstrate. For this reason, we chose to study human monocytes, which we found to ingest and kill resting conidia provided they were opsonized in normal human serum.

H_2O_2, HOCl, and ·OH have all been shown to kill *A. fumigatus* conidia in cell-free systems (6–8), but the relative contributions made by these agents in a system containing human effector cells have not previously been defined. We report here a series of experiments with probes of oxidative killing which demonstrate that H_2O_2 plays a central role in defense against *A. fumigatus* conidia by human fresh peripheral blood mononuclear cells. Our results indicate that the MPO-H_2O_2-halide system and an MPO-independent pathway, probably the ferrous ion-H_2O_2-halide system, each contribute significantly to the oxidative killing of this fungal target particle. The chief toxic metabolites of these two reactions are hypochlorous acid and the hydroxyl radical, respectively. These results offer a plausible explanation for the susceptibility of patients with CGD to invasive aspergillosis and suggest that redundancy is incorporated into the oxidative component of host defense against this infection.

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MATERIALS AND METHODS

Preparation of *Aspergillus* inoculum. Sabouraud agar (SA) was prepared with 1% neopeptone (Difco Laboratories, Detroit, Mich.) and 1% glucose. A clinical isolate of *A. fumigatus* was grown on SA for 72 h at 30°C. Suspensions of single conidia were prepared by washing the mycelial mat with Hanks balanced salt solution (HBSS), filtering the harvested conidia through cheesecloth, and vortexing vigorously. Viability, as judged by quantitative cultures on SA, averaged greater than 95%.

Leukocyte separation. Mononuclear cells were separated from whole heparinized blood (20 U/ml) by Ficoll-Hypaque density centrifugation, which yielded a mean of 22% monocytes (nonspecific esterase-positive cells) and 75% lymphocytes, with 3% basophils and eosinophils. In most experiments, monocytes were further purified by using continuous Percoll gradients (4) (colloidal suspension of silica coated with polyvinylpyrrolidone; Pharmacia Fine Chemicals, Piscataway, N.J.), which resulted in a mean of 67% monocytes, less than 5% basophils, and the remainder lymphocytes. The viability of monocytes remained greater than 95%, as judged by trypan blue exclusion.

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TABLE 1. Reagents used to alter level of killing of *A. fumigatus* conidia by human monocytes

Reagent[a] (concn)	Effect (reference)
Catalase (6,000 U/ml)	Degrades H_2O_2 (10)
Glucose-glucose oxidase (10 mM–50 mU/ml)	Generates H_2O_2
Mannitol (40 mM)	Scavenges · OH
Sodium benzoate (20 mM)	Scavenges · OH
Taurine (10 mM)	Scavenges HOCl
Superoxide dismutase (0.1 mg/ml)	Scavenges O_2^-

[a] All reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Catalase (EC 1.11.1.6): 1 U decomposes 1.0 μmol of H_2O_2 per min. Superoxide dismutase (EC 1.15.1.1).

Opsonization of *A. fumigatus*. Conidia were incubated in 50% fresh normal type AB human serum in HBSS at 37°C for 30 min. They were then washed twice in HBSS at 4°C and suspended at a density of 5×10^7/ml for immediate use in phagocytic assays.

Phagocytic assay. Monocytes (10^6) were mixed with 10^7 *A. fumigatus* organisms as determined by hemacytometer count (effector/target ratio, 1:10) in a final volume of 0.5 ml of HBSS in plastic tubes with or without added reagents (see below). These were tumbled at 8 rpm for 2 h at 37°C. After 15, 30, 60, and 120 min, cytocentrifuge slides were prepared with nonspecific esterase stain and toluidine blue counterstain. Cell association indices (mean number of intracellular and attached organisms per esterase-positive monocyte) were calculated; 200 consecutive monocytes were counted. Most of the cell-associated conidia appeared to have been ingested and were not simply attached to monocytes. Monocytes were lysed within the incubation tubes with 0.5% sodium deoxycholate and vigorous vortexing; serial 10-fold dilutions were performed in phosphate-buffered saline (pH 7.5) with 0.05% Tween 20 (Fisher Scientific Co., Pittsburgh, Pa.). Microscopic examination of lysed-cell suspensions revealed that conidia were dispersed as single cells, and no debris from mononuclear cells could be seen. Inspection of the inner surfaces of the plastic incubation tubes with an inverted microscope after the vortexing step revealed no adherent conidia or mononuclear cells. Portions (0.2 ml) were plated in triplicate on SA to determine the percentage of conidia killed. The observed killing was limited almost entirely to cell-associated conidia, as shown by the fact that the addition of a phagocytosis inhibitor, cytochalasin B (10 μg/ml), reduced killing to ≤8% of the conidia added to the tube. This number was derived from four experiments with mononuclear cells from either Ficoll-Hypaque or Percoll density gradients. Fungicidal activity was therefore expressed according to the following formula: % cell-associated organisms killed = [(% total inoculum killed/% inoculum associated with monocytes)] × 100. Fungal colonies which grew on the 10-cm petri dishes were counted after a 24-h incubation at 37°C; the mean number of colonies per plate was 132. As a control, duplicate tubes without mononuclear cells were included in several representative experiments.

In most of the experiments, mononuclear cells from Percoll gradients were used. For the experiments in which cells from Ficoll-Hypaque density gradients were used, adjustment of the leukocyte concentration was necessary to maintain the monocyte/conidium ratio of 1:10, resulting in a level of conidiacidal activity that was similar to that observed with monocytes from Percoll gradients. Lymphocyte contamination did not influence the results. These data were therefore combined with data obtained with cells from Percoll gradients to calculate the mean ± the standard error of the mean (SEM).

Patients. Mononuclear cells were obtained from five normal volunteers, one patient with MPO deficiency, and eight patients with CGD. Two male CGD patients had X-linked disease, and two others had a possible autosomal recessive pattern of inheritance (11). One female had autosomal recessive CGD, one had suspected autosomal dominant disease, and the pattern of inheritance in two other females was unclear.

Reagents added to phagocytic assay. To learn the relative importance of several oxygen intermediates for oxidative killing of *A. fumigatus* conidia, we added the reagents listed in Table 1 to the phagocytic assays. In three experiments, glucose-glucose oxidase was added to opsonized conidia without mononuclear phagocytes to assess the cell-free fungicidal activity of H_2O_2. Generation of H_2O_2 by this enzymatic system was documented in two experiments by measuring fluorescence quenching of scopoletin (7-hydroxy-6-methoxycoumarin) (13, 14). These control tubes contained only HBSS with a final concentration of 10 mM glucose–glucose oxidase (50 mU/ml) without serum, conidia, or effector cells. The mean rate of H_2O_2 generation over a 5-min interval was 8.4 nmol/ml per min.

RESULTS

Killing of *A. fumigatus* conidia by three donor groups. After the 2-h incubation, monocytes from normal volunteers killed 61.1 ± 3.8% (mean ± SEM; 18 experiments) of cell-associated fungi (Fig. 1). Most (88%) of this fungicidal activity occurred within the first 30 min of incubation. Killing of conidia by monocytes from the MPO-deficient patient was comparable to that by control cells at 2 h, but the kinetics were slowed. Only 12.1% killing occurred within 30

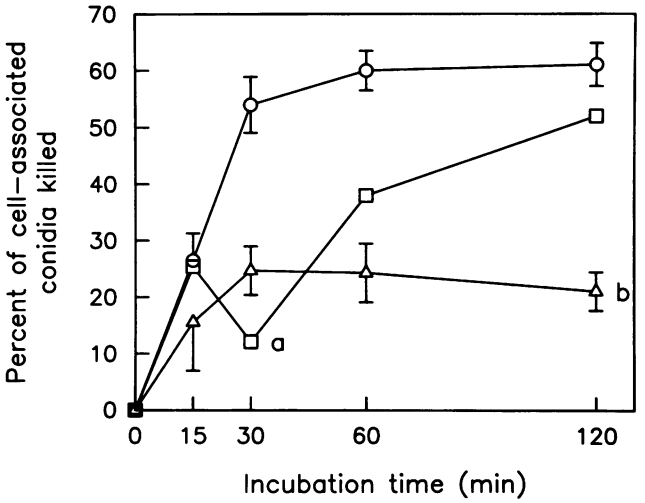

FIG. 1. Kinetics of fungicidal activity against *A. fumigatus* conidia by monocytes from three different patient populations. Symbols: ○, normal volunteers (mean ± SEM; 18 experiments); □, MPO-deficient patient (points represent the mean of two experiments performed in duplicate); △, CGD patients (mean ± SEM; 11 experiments). *P* (versus controls) is <0.001 at 30 min (a) and <0.001 at 120 min (b). Statistical significance was determined by the Student's *t* test for summary data.

TABLE 2. Effect of scavengers on level of killing by normal monocytes

Reagent	% Cell-associated conidia killed (mean ± SEM [no. of expts]) at:			
	15 min	30 min	1 h	2 h
None	26.5 ± 4.8 (7)	54.0 ± 4.9 (15)	60.0 ± 3.5 (14)	61.1 ± 3.8 (18)
Catalase	22.9 ± 5.7 (4)	21.2 ± 3.8 (7)	16.7 ± 3.1 (7)	18.7 ± 2.1 (9)[a]
Taurine	20.2 ± 8.2 (4)	33.5 ± 4.6 (5)[b]	60.2 ± 9.3 (5)	52.1 ± 4.1 (6)
Mannitol	10.5 ± 6.1 (4)	38.0 ± 6.8 (6)	48.9 ± 2.3 (6)	58.5 ± 4.1 (7)
Taurine-mannitol	15.9 ± 6.6 (3)	17.0 ± 6.4 (4)	17.1 ± 4.3 (4)	17.9 ± 4.4 (4)[a]
Superoxide dismutase	25.6 ± 7.8 (3)	62.2 ± 13.0 (4)	70.0 ± 13.0 (4)	71.3 ± 13.0 (5)

[a] P [versus no reagents] < 0.001.
[b] P [versus no reagents] < 0.05.

min (P [versus controls] < 0.001). Monocytes from CGD patients exhibited abnormally low fungicidal activity, with 21.1 ± 3.4% killing at 2 h (n = 11 experiments; P [versus controls] < 0.001; Student's t test with summary data [16]). As a control, tubes not containing mononuclear cells were included in several representative experiments. Greater than 95% of the original inoculum could be recovered from these tubes.

Effect of added reagents on killing of *A. fumigatus*. The addition of fresh catalase reduced killing of conidia by normal monocytes to 18.7 ± 2.1% at 2 h (n = 9 experiments; P [versus no additives] < 0.001) (Table 2). Catalase which had been boiled for 30 min had no effect on normal killing. The addition of taurine to the phagocytic assay to scavenge HOCl delayed killing by normal monocytes (P [versus no additives] at 30 min < 0.05), but the results at 2 h were not significantly lower than the results observed when no reagents were added. A similar effect was noted in the presence of mannitol, a scavenger of ·OH, though this did not reach statistical significance. The mean fungicidal endpoint at 2 h for two experiments with another ·OH scavenger, sodium benzoate, was normal at 52.7%. The simultaneous addition of both taurine and mannitol decreased killing to 17.9 ± 4.4% (n = 4 experiments). Fungicidal activity was unaffected by superoxide dismutase.

Although mannitol caused only a modest slowing of conidiacidal activity by normal monocytes, this scavenger of ·OH significantly inhibited killing by monocytes from a patient with MPO deficiency even at 2 h. The percentage of conidia killed at the final incubation time was only 27.9% (P [versus no additives] < 0.001) (Fig. 2).

The addition of the H_2O_2-generating combination of glucose-glucose oxidase increased the killing of conidia by monocytes from patients with CGD to a level which was midway between the results observed with CGD monocytes without additives and the results with normal monocytes, i.e., 46.5 ± 6.9% (n = 9 experiments; P [versus no additives] at 120 min < 0.005) (Fig. 3). No killing was detected in three experiments in which the H_2O_2-generating combination of glucose-glucose oxidase was added to opsonized conidia without mononuclear cells.

Lack of effect of added reagents on monocyte viability or phagocytosis. Trypan blue exclusion by mononuclear cells averaged greater than 90% after the 2-h incubation period and was not affected by any of the reagents. The mean cell association index at 2 h was 5.78 when cells from normal volunteers were used; cell association indices with cells from patients with CGD and MPO deficiency were 6.28 and 7.60, respectively. In all experiments, the extent of association of conidia with monocytes was over 95% complete within 30 min, and the cell association index was unchanged by any of the added reagents at 15, 30, 60, and 120 min.

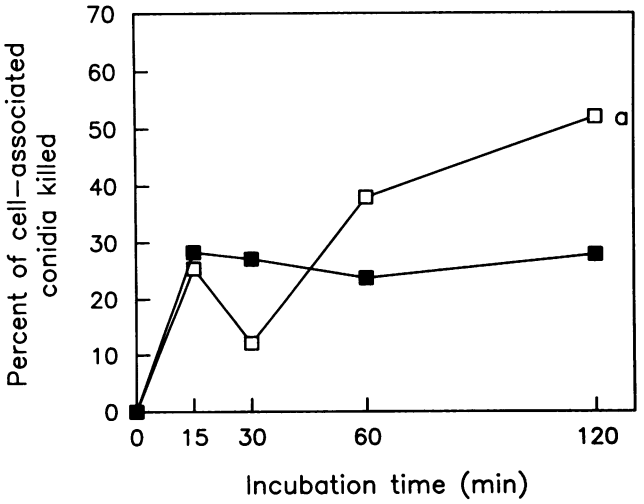

FIG. 2. Inhibition of *A. fumigatus* killing by mannitol with monocytes from an MPO-deficient patient. Symbols: □, MPO-deficient patient; ■, MPO-deficient patient plus mannitol. Each experiment was performed in duplicate on two separate occasions. (a) P was <0.001 at 120 min, as calculated by one-way analysis of variance (16).

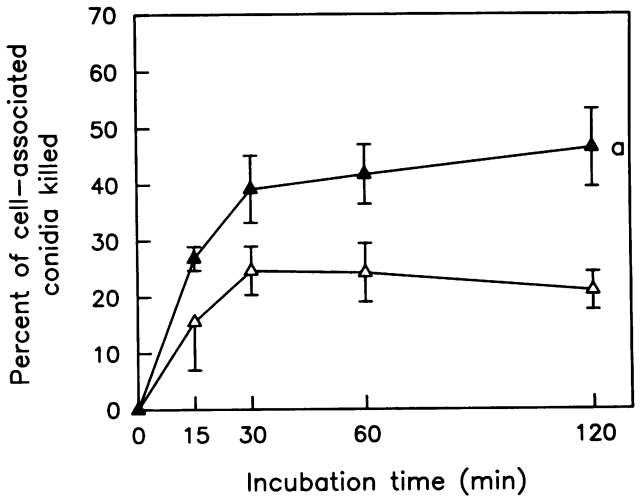

FIG. 3. Improvement of fungicidal activity of CGD monocytes by the inclusion of an H_2O_2-generating system. Symbols: △, CGD patients; ▲, CGD patients plus glucose-glucose oxidase (mean ± SEM; nine experiments). (a) P (versus no additives) was <0.005 at 120 min.

DISCUSSION

Human monocytes were found to kill *A. fumigatus* conidia by several mechanisms. CGD cells, which elaborate no hydrogen peroxide, exhibited modest but significant killing. MPO-deficient cells generate H_2O_2 but killed conidia slower than normal cells do. Hydrogen peroxide was therefore not the only factor necessary for optimal killing but was nevertheless found to play a central role. Impaired killing by normal cells in the presence of added catalase supported such a role for H_2O_2. Furthermore, extracellular generation of H_2O_2 by the glucose-glucose oxidase combination improved killing of *A. fumigatus* by cells from patients with CGD.

Other researchers have shown that catalase enters phagocytes only in small quantities during phagocytosis (9). Therefore, the inhibitory effect of catalase in our experiments was probably due to the depletion of intracellular H_2O_2 by degradation of the extracellular pool with which it was in equilibrium. It is also probable that *Aspergillus* catalase contributed to the depletion of H_2O_2 within the phagolysosome.

The observed improvement in killing by monocytes from patients with CGD after the addition of glucose-glucose oxidase confirmed earlier work which showed that the function of CGD granulocytes could be partially corrected with the addition of extracellular H_2O_2 (R. K. Root, Clin. Res. **22**:452, 1974). Extracellular H_2O_2 recently has been shown to diffuse into azurophil (primary) granules of human neutrophils (12).

It has been demonstrated in cell-free systems that H_2O_2 used alone must be present in concentrations of between 1 and 100 mM to kill *A. fumigatus* conidia (8). However, only micromolar concentrations were required for fungicidal activity when either MPO-halide or Fe^{2+}-halide was present at pH levels of 4.5 to 6.0 (7). These concentrations of H_2O_2 were closer to those achieved by our H_2O_2-generating system. From our studies with catalase and glucose-glucose oxidase, we postulated that H_2O_2 could play a central role in oxidative defense against *Aspergillus* conidia by serving as a substrate for one or both of these reactions. It seems unlikely that concentrations of H_2O_2 generated within the cell acting alone would be adequate to kill *Aspergillus* spp. This supposition is supported by the fact that together mannitol and taurine, which are ·OH and HOCl scavengers that do not decrease the H_2O_2 concentration, markedly impaired the cellular killing of *A. fumigatus*. Similarly, our H_2O_2-generating system was not fungicidal when acting alone, but enhanced killing by CGD cells.

The delayed killing observed with monocytes from an MPO-deficient patient or with taurine added to normal monocytes suggested that the MPO-H_2O_2-halide system contributes to the oxidative killing of *A. fumigatus* conidia. However, in both cases the percentage of conidia killed at 2 h was the same as that by control cells. This indicated that MPO was probably less important in killing this fungal target than it is against *Candida albicans*, which is killed poorly if at all in the absence of an intact MPO-H_2O_2-halide system (5, 17).

The addition of mannitol also slowed the killing of conidia, suggesting a role for ·OH in fungicidal activity. However, this metabolite evidently made only a partial contribution, as indicated by a normal level of killing at 2 h. When monocytes from a patient with MPO deficiency were used, the inhibition of killing by the addition of mannitol was more pronounced. This is consistent with the hypothesis that both the MPO-H_2O_2-halide system and an MPO-independent oxidative system might contribute significantly to fungicidal activity against *Aspergillus* conidia. Simultaneous scavenging of HOCl and ·OH in normal monocytes with a combination of taurine and mannitol resulted in significant impairment of killing at 2 h.

In summary, fungicidal action against *A. fumigatus* conidia remained intact even when only one of the two pathways was operational. However, *A. fumigatus* conidia were no longer effectively killed when neither system functioned because of (i) the degradation of H_2O_2 by catalase, (ii) the innate inability of effector cells to generate H_2O_2, (iii) the addition of mannitol to cells from a patient with MPO deficiency, or (iv) the simultaneous scavenging of HOCl and ·OH from normal monocytes by taurine and mannitol.

There appear to exist two comparably effective oxidative pathways for conidiacidal activity. This observation is clinically significant because it offers a plausible explanation for both the susceptibility of CGD patients to invasive aspergillosis and the resistance of MPO-deficient patients to this fungal infection.

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