Susceptibility of *Blastomyces dermatitidis* Strains to Products of Oxidative Metabolism

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Three strains of *Blastomyces dermatitidis* which differ in their virulence for mice were exposed in their yeast form to various components of the peroxidase-hydrogen peroxide-halide system. Susceptibility to H_2O_2 alone correlated with virulence, with the most virulent strain (ATCC 26199) least susceptible (50% lethal dose, >50 mM) and an avirulent strain (ATCC 26197) most susceptible (50% lethal dose <3.3 mM). A strain of intermediate virulence (ATCC 26198) was of intermediate susceptibility (50% lethal dose, 11.5 mM). The addition of a nontoxic concentration of KI (5×10^{-4} M) did not increase H_2O_2 toxicity. However, the addition of either myeloperoxidase or horseradish peroxidase and KI markedly decreased the amount of H_2O_2 required to kill the organisms, with 100 ± 0% of all strains killed at 5×10^{-5} M H_2O_2 and 97 ± 4, 100 ± 0, and 94 ± 8% of ATCC 26199, ATCC 26198, and ATCC 26197 killed, respectively, at 5×10^{-6} M H_2O_2. Kinetic studies with H_2O_2 alone revealed a delayed onset of killing, but virtually 100% of organisms were killed by 120 min of exposure in all strains. By comparison, the peroxidase-hydrogen peroxide-halide system was 100% lethal for all strains at 1 min. The relatively high concentrations of H_2O_2 required to kill the yeast phase of *B. dermatitidis* suggest that H_2O_2 alone does not account for host resistance to the organism. However, the rapidly lethal effect of the peroxidase-hydrogen peroxide-halide system at physiologically relevant concentrations suggests that this may be one mechanism of host defense to *B. dermatitidis*.

Products of oxidative metabolism are microbicidal and presumably constitute an important microbicidal system in phagocytic cells (11). In particular, the peroxidase-hydrogen peroxide-halide (PPH) system is effective in the killing of bacteria (10, 16), fungi (7, 9), protozoa (13, 14), and tumor cells (17). With the fungi, attention has been paid to *Candida* species, which are opportunistic pathogens (7, 12). However, there are scant data regarding the interaction of this system with the dimorphic fungi responsible for the endemic mycoses, which are principally diseases of normal hosts (9). Susceptibility to oxidative systems has been reported with *Histoplasma capsulatum* (9).

In previous studies, we reported that peritoneal macrophages activated by *Mycobacterium bovis* BCG or concanavalin A, in contrast to resident macrophages, killed the yeast phase of *Blastomyces dermatitidis* during a 4-h cocultivation (4). We therefore studied whether the interaction of this organism with products of the PPH system could be a possible mechanism for killing by activated macrophages. We used three strains of *B. dermatitidis* which vary in their

virulence, as studied in a murine model (1, 2). The results of the present study, the first to report the interaction of products of oxidative metabolism with *B. dermatitidis*, show that the three strains exhibit differences in susceptibility to H_2O_2 and to products of the PPH system.

MATERIALS AND METHODS

Fungi. Three strains of *B. dermatitidis* were maintained on Sabouraud dextrose agar slants at 4°C. They were subcultured, with no more than 15 transfers, into 3 ml of a synthetic medium (8). The organisms were subcultured in all experiments during the log phase of growth, 2 to 3 days before an experiment. The three strains used had previously been defined in terms of virulence for mice: ATCC 26199 (V, virulent), ATCC 26197 (GA-1, avirulent), and ATCC 26198 (KL-1, intermediate in virulence) (1, 2).

Chemicals. Hydrogen peroxide was obtained as a reagent-grade 30% solution (Matheson, Coleman and Bell Manufacturing, Norwood, Ohio). Human myeloperoxidase (MPO; 2,655 U/mg) was the generous gift of Robert I. Lehrer, University of California Medical Center, Los Angeles. Horseradish peroxidase type VI (HPO; 250 to 330 U/mg) and catalase (bovine liver; 2,250 U/mg) were obtained from Sigma Chemical Co.,

TABLE 1. Percent killing of *B. dermatitidis* by high concentrations of H_2O_2

Strain	Mean % killing ± SD at H_2O_2 concn (M) of:		
	5×10^{-2a}	5×10^{-3b}	5×10^{-4}
V	45 ± 6 (4)^c	10 ± 13 (5)	0 (5)
KL-1	97 ± 3 (3)	24 ± 12 (3)	0 (3)
GA-1	96 ± 4 (3)	61 ± 17 (4)	0 (4)

^{a} Killing of V versus KL-1 or GA-1, $P < 0.001$.
^{b} Killing of KL-1 versus GA-1, $P < 0.02$; killing of V versus GA-1, $P < 0.005$.
^{c} Number of experiments.

St. Louis, Mo. Before use, the catalase was dialyzed against 0.2 M sodium phosphate buffer, pH 6.5, for 24 h. Catalase activity was assayed by the *O*-dianisidine (Sigma) method, using standard techniques (6). Potassium iodide (reagent grade; J. T. Baker Chemical Co., Phillipsburg, N.J.) was prepared as a 1 M stock solution and stored at 4°C for no longer than 2 weeks. Sodium phosphate buffer (0.2 M, pH 6.5) was utilized in all experiments.

In vitro system. The following protocol was followed in all experiments, in the order of the addition of the components: (i) buffer; (ii) KI (final concentration, 5 × 10^{-4} M); (iii) catalase, 400 U in 0.1 ml; (iv) H_2O_2 in 0.1 ml (the molar concentrations indicated in the text are the final concentrations in the reaction mixture); (v) *B. dermatitidis*, 10,000 CFU in 0.1 ml; (vi) HPO or MPO, 28 U in 0.2 ml. The total volume of the reaction mixture (2.0 ml) was contained in plastic tubes (17 by 100 mm; no. 2051; Falcon Plastics, Oxnard, Calif.). Tubes were incubated at 37°C on a rotary shaker at 250 rpm for 1 h. Incubation was extended to 2 h for the kinetic experiments.

Statistics. Percent killing was calculated as {[(CFU at $t = 0$) − (CFU in experimental group)]/[CFU at $t = 0$]} × 100, where $t = 0$ was the time at which the reaction mixture was initially combined. Negative values reflect an increase in CFU. The Student t test was used where indicated.

RESULTS

H_2O_2 susceptibility of three strains of *B. dermatitidis*. V was significantly less susceptible to 5 × 10^{-2} M H_2O_2 than was KL-1 or GA-1 ($P < 0.01$) (Table 1). At 5 × 10^{-3} M H_2O_2, killing of GA-1 was significantly greater than killing of KL-1 ($P < 0.02$) and V ($P < 0.005$). No killing was observed at 5 × 10^{-4} M H_2O_2 for any of the strains. The 50% lethal dose for V, KL-1, and GA-1 was >0.05, 0.01, and 0.003 M H_2O_2, respectively. Thus, in vivo virulence for mice correlated with degree of resistance to H_2O_2.

Effect of addition of KI or PO to H_2O_2. Viability of the strains in buffer alone was good, with no significant decrease noted after the 1-h incubation period: V, 4 ± 3% standard deviation; KL-1, −4 ± 7%; GA-1, 2 ± 9%. For all three

strains, KI (5 × 10^{-4} M) and HPO (14 U/ml) alone or in combination had no effect on viability (Table 2). The results with MPO were identical to those with HPO; therefore, the data were combined. In addition, H_2O_2 plus KI or HPO was no more toxic than H_2O_2 alone (data not shown).

There were no major differences in susceptibility of the three strains to H_2O_2 at any concentration tested when KI and peroxidase (PO) were present (Table 3). The steep dose-response curve of the lethal effect is readily apparent, with virtually all or none of the organism killed within a 10-fold range. However, close inspection of this critical interval reveals a similarity of H_2O_2 concentrations required to kill all three strains (Fig. 1).

Effect of adding catalase. The specificity of the killing of *B. dermatitidis* by the PPH system was demonstrated by the complete abrogation of killing with the addition of catalase (20 U/ml) to the reaction mixture (Table 4). Heated catalase had no effect on H_2O_2-mediated killing of any of the three strains (Table 4).

Evidence of lack of endogenous H_2O_2 production by *B. dermatitidis*. Incubation of each of the three strains with KI and HPO would be expected to result in killing in the presence of H_2O_2 (Table 3). However, viability of the organisms was unaffected during inoculation with these components (Table 2), suggesting that the yeast form of *B. dermatitidis* does not produce appreciable quantities of H_2O_2 under these conditions or that enzymes (such as catalase) present in the organism have degraded any H_2O_2 that is produced.

Kinetics of killing by H_2O_2 and the PPH system. The data from this set of experiments are presented in Fig. 2. Although no killing of any of the strains was observed at 1 min with H_2O_2 (5 × 10^{-2} M for KL-1 and GA-1; 10^{-1} M for V), a significant degree of killing of KL-1 (49 ± 7%) was observed at 30 min ($P < 0.05$ for KL-1 versus GA-1 or V). Killing of KL-1 increased to 95 ± 0% by 60 min, and the other two strains also showed a decrease in viability at this time

TABLE 2. Effect of components of the PPH system^a on growth of *B. dermatitidis*

Strain	Mean % growth ± SD		
	KI	HPO or MPO	KI + HPO or KI + MPO
V	6 ± 3 (5)^b	5 ± 6 (6)	0 ± 8 (9)
KL-1	−7 ± 2 (4)	−3 ± 9 (3)	−2 ± 3 (3)
GA-1	−2 ± 11 (4)	−5 ± 11 (3)	−10 ± 8 (3)

^{a} KI, 5 × 10^{-4} M; HPO or MPO, 14 U/ml.
^{b} Number of experiments.

TABLE 3. Percent killing of *B. dermatitidis* by the PPH system[a]

Strain	Mean % killing ± SD at H_2O_2 concn (M) of:					
	5×10^{-3}	5×10^{-4}	5×10^{-5}	5×10^{-6}	5×10^{-7}	5×10^{-8}
V	98 ± 3 (2)[b]	100 ± 0 (4)	100 ± 0 (4)	97 ± 4 (14)	0 (3)	0 (7)
KL-1	93 ± 3 (3)	100 ± 0 (4)	100 ± 0.6 (3)	100 ± 0.4 (5)	0 (2)	0 (4)
GA-1	100 ± 0.7 (2)	100 ± 0 (2)	100 ± 0 (6)	94 ± 8 (8)	0 (8)	0 (5)

[a] KI, 5×10^{-4} M; HPO as source of PO, 14 U/ml.
[b] Number of experiments.

($P < 0.01$ for KL-1 versus V; $P < 0.005$ for KL-1 versus GA-1).

It is interesting that V was affected least by H_2O_2 (46 ± 14% at 60 min), even though H_2O_2 was present at twice the concentration as compared with that present with KL-1 and GA-1. By 120 min, however, virtually all of the organisms of all three strains were nonviable (V, 99 ± 1%; KL-1, 100 ± 0%; and GA-1, 100 ± 0%).

In marked contrast was the rapid onset of action of the PPH system against the three strains. At 1 min, the earliest point measured, 100% killing was observed (H_2O_2, 5×10^{-5} M; KI, 5×10^{-4} M; and HPO, 14 U/ml).

DISCUSSION

This is the first report of the susceptibility of *B. dermatitidis* to the products of oxidative metabolism. The results suggest that in the presence of PO and halide, the concentration of

H_2O_2 required to kill this organism is markedly decreased. However, in contrast to the correlation of virulence with susceptibility to H_2O_2 alone (Table 1), there was no correlation of virulence with susceptibility to the entire PPH system (Table 3). The decrease in the amount of H_2O_2, when combined with halide and PO, required for lethality is consistent with prior reports of the synergistic effects of these components (11).

The only other dimorphic fungus to be similarly studied has been *H. capsulatum* (9). In that study and similar to the results reported here with *B. dermatitidis*, Howard found that blastospores of the fungus are susceptible to high concentrations of H_2O_2 ($>10^{-4}$ M). This lethal effect was increased by the addition of halide, an observation not noted in the present study with *B. dermatitidis*. An even more substantial lethal effect was noted when MPO was added to the H_2O_2-halide system. KI was a much more effec-
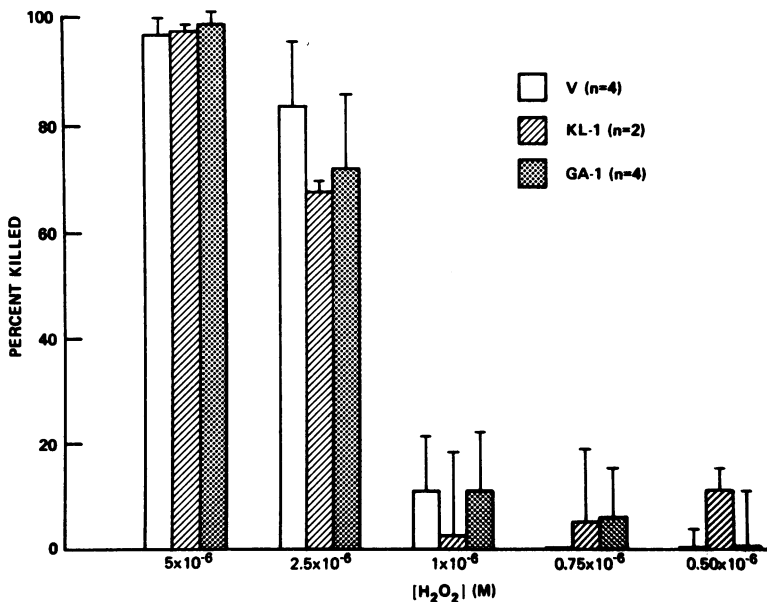

FIG. 1. Percent killing of *B. dermatitidis* by H_2O_2 in the presence of HPO (14 U/ml) and KI (5×10^{-4} M). Bars represent standard deviation. n, Number of experiments.

TABLE 4. Effect of catalase on killing by HPO-KI-H_2O_2 system[a]

Strain	Without catalase	With catalase	With heated[b] catalase
V	98 ± 4 $(9)^c$	0 (4)	94 (1)
KL-1	100 ± 0.6 (3)	0 (3)	100 (2)
GA-1	100 ± 0 (4)	0 (2)	100 (2)

[a] Data are expressed as percentage of *B. dermatitidis* killed ± standard deviation. H_2O_2 at 5×10^{-6} M for V and 5×10^{-5} M for KL-1 or GA-1; catalase at 20 U/ml.

[b] Catalase was autoclaved at 121°C for 30 min.

[c] Number of experiments.

tive component, in combination with H_2O_2 and HPO, than KCl in damaging *H. capsulatum*. Preliminary experiments show a similar increased lethal effect of KI over KCl, in combination with H_2O_2 and HPO, on the virulent strain of *B. dermatitidis*. A 0.1 M concentration of KCl alone was not toxic to the virulent strain (100% viable) as opposed to 100% lethality with 0.1 M KI. One problem in the interpretation of Howard's study, however, is the apparent decrease in viability of the fungus in the buffer over the 3-h incubation period. We have found that in the occasional experiment in which viability in buffer is <90% of the initial inoculum (such experiments were not included in this report), the apparent toxicity of the various reagents is markedly enhanced, suggesting that other parameters, e.g., culture age, number of subcultures, and time the inoculum spent at room
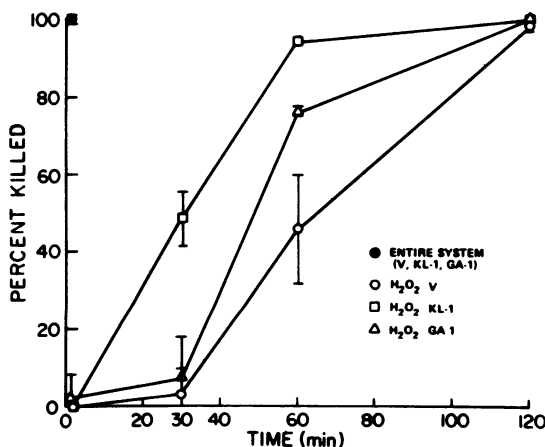

FIG. 2. Kinetics of killing of three strains of *B. dermatitidis* by H_2O_2. Concentrations of H_2O_2 were 5×10^{-2} M for KL-1 and GA-1 and 10^{-1} M, for V. At 30 min, the results were significant for KL-1 versus GA-1 or V, $P < 0.05$. At 60 min, V versus GA-1, $P < 0.05$; V versus KL-1, $P < 0.01$; GA-1 versus KL-1, $P < 0.005$.

temperature rather than at 37°C, may affect fungal survival under study conditions such as these.

In the kinetic experiments, differences between the effects of H_2O_2 alone and in combination with KI and PO were evident. Killing of all three strains in the presence of H_2O_2 alone was time dependent and was only complete after 120 min of incubation. Killing of the virulent strain occurred most slowly, but in the continued presence of H_2O_2, killing was completed. However, the concentrations of H_2O_2 used in these kinetic experiments are above the physiological range (15), and their applicability to the in vivo situation is uncertain.

It is of interest that strains of *B. dermatitidis* which vary in virulence can also be differentiated in their ability to grow in prolonged cocultures with murine peritoneal macrophages (3). In this situation, the virulent strain escapes from the inhibition of its multiplication due to the coculturing with macrophages.

The observation that by 1 min of incubation all three strains of *B. dermatitidis* are killed by H_2O_2 in a physiological concentration (15) in the presence of KI and PO may be relevant to the in vivo situation. We have previously demonstrated that murine peritoneal macrophages activated by BCG or concanavalin A can kill the virulent strain of *B. dermatitidis* (4). Preliminary results also suggest that this killing can be abrogated by the presence of catalase (unpublished data). If PO is involved in this killing, its source is unclear, since mature peritoneal macrophages are devoid of peroxidative activity (5). Heifets et al. (8) have indicated that exogenous PO, such as that from granulocytes, could provide the peritoneal macrophage with this activity and thus could facilitate killing of the fungus.

The lack of differences in susceptibility to the PPH system of the three strains of *B. dermatitidis* of different virulence for mice suggests that pathogen factors or other host factors, unrelated to products of oxidative metabolism as studied in our system, are important virulence factors.

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