Enhanced oxidative mechanisms in immunologically activated versus elicited polymorphonuclear neutrophils: correlations with fungicidal activity

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Summary. Peritoneal polymorphonuclear neutrophils (PMN) from mice were tested for their ability to kill the yeast form of *Blastomyces dermatitidis* (Bd) *in vitro* and for their fungicidal mechanisms. PMN elicited from immune mice by the intraperitoneal injection of non-viable Bd (referred to as immunologically activated PMN or ActPMN) showed significantly enhanced fungicidal activity in comparison with PMN elicited with thioglycollate medium (ThioPMN) [means = 44.7% (SD 12.8%) and 16.4% (SD 9.2%) killed; $n = 14$; $p < 0.001$]. Production of superoxide anion (O_2^-) by ActPMN after stimulation with phorbol myristate acetate was enhanced in comparison with production by ThioPMN. Superoxide dismutase, which removes O_2^- , inhibited ActPMN killing by 75% ($p < 0.001$) when added to cultures immediately before challenge with Bd (optimal concentration: 6000 U/ml). Sodium azide, which inhibits myeloperoxidase and scavenges singlet oxygen (1O_2), and catalase, which breaks down hydrogen peroxide (H_2O_2), inhibited ActPMN killing by 64% ($p < 0.001$) and 52% ($p < 0.001$), with optimal concentrations of 1 mM and 10 000 U/ml, respectively. Two agents that both scavenge 1O_2 and antagonise hypochlorous acid ($HOCl^-$), histidine and tryptophan, were also powerful inhibitors of ActPMN killing. Quenchers of hydroxyl radical ($\cdot OH$), dimethylsulfoxide and sodium benzoate, had less effect, and required higher concentrations. These data suggest that the enhanced killing of Bd by ActPMN involves one or more oxidative mechanisms, and that there is a prominent role for O_2^- , either directly or as a precursor of other active oxygen species, a probable role for H_2O_2 , and possible roles for 1O_2 , $HOCl^-$, and $\cdot OH$.

Introduction

Upon interaction with micro-organisms, polymorphonuclear neutrophils (PMN) display enhanced oxidative metabolism including increased consumption of oxygen, increased hexose monophosphate shunt activity, and the production of chemically reactive metabolites of oxygen such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Klebanoff and Clark, 1978). H_2O_2 and O_2^- can react to produce additional metabolites (Badwey and Karnovsky, 1980) including hydroxyl radical ($\cdot OH$) and singlet oxygen (1O_2). H_2O_2 in the presence of halide and myeloperoxidase (MPO), an enzyme present in PMN, can also generate halogenated oxidative anions such as hypochlorous

acid ($HOCl^-$) (Klebanoff and Clark, 1978). The reactive oxygen species generated by PMN mediate much of their microbicidal activity (Johnston *et al.*, 1975; Klebanoff and Clark, 1978) although oxygen independent killing mechanisms may also be involved (Lehrer *et al.*, 1975; Klebanoff and Clark, 1978; Spitznagel and Shafer, 1985).

It was previously reported that immunologically activated PMN (ActPMN), induced in *Blastomyces dermatitidis*-immune mice by intraperitoneal injection of *B. dermatitidis* antigen, had enhanced fungicidal activity *in vitro* in comparison with PMN elicited by thioglycollate medium (ThioPMN), and it was suggested they had an increased oxidative burst in chemiluminescence studies (Brummer *et al.*, 1984, 1985). In a more specific investigation of the oxygen metabolites involved in the killing of *B. dermatitidis* by PMN, the production of O_2^- by

ActPMN or ThioPMN after stimulation with phorbol myristate acetate (PMA) has been measured and the effect of scavengers of active oxygen species on the PMN killing of *B. dermatitidis* yeasts *in vitro* has been examined; the results are reported below.

Materials and methods

Animals

Pathogen-free BALB/cByJIMR male mice (Institute for Medical Research, San Jose, CA) 8–12 weeks old were used.

Fungi

Inocula of *B. dermatitidis*, strain ATCC 26199, were prepared for in-vitro challenge from cultures grown for 48–72 h on blood agar (BA) incubated at 35°C. Fungal cells were washed and appropriate dilutions cultured in triplicate on BA to determine the number of cfu/ml. *B. dermatitidis* cells frozen and thawed 12 times in an acetone-dry ice bath or stored at –70°C for more than 2 years were used as non-viable fungal antigen.

Reagents and media

These were: tissue culture medium (TCM)—RPMI-1640 medium supplemented with heat-inactivated fetal bovine serum 10% v/v and (ml) penicillin 100U plus streptomycin 100µg (Gibco Laboratories, Grand Island, NY); Hanks's Balanced Salt Solution without phenol red (HBSS) (Gibco); preservative-free ammonium heparin (American Scientific Products, Sunnyvale, CA); metrizamide (2-(3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose) (Sigma Chemical Company, St Louis, MO), a 35.5% isotonic solution in distilled water. Metrizamide gradients were made by diluting the 35.5% solution in Dulbecco's phosphate-buffered saline (Gibco). Ferricytochrome c (type VI, from horse heart), phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (from bovine erythrocytes) (SOD), sodium azide (AZ), and dimethylsulphoxide (DMSO) were from Sigma. Catalase (from bovine liver) (Sigma) was dialysed against 0.05 M potassium phosphate buffer, pH 7.0, for 24 h before use. Catalase activity was assayed by the method of Beers and Sizer (1952) in which the disappearance of H₂O₂ was followed spectrophotometrically at 240 nm. Heat-inactivated catalase and SOD were prepared by autoclaving the enzymes for 15 min at 121°C. Triton X-100 (New England Nuclear, Boston, MA), tryptophan (Matheson, Coleman and Bell, Norwood, OH), histidine (Eastman Kodak, Rochester, NY), and sodium benzoate (Mallinkrodt, St Louis, MO) were used as stated.

Immunisation of mice

Mice given 20 000 cfu of *B. dermatitidis* subcutaneously at two dorsal sites are referred to as immune mice after resolution of the infection over a 4-week period (Morozumi *et al.*, 1982).

Polymorphonuclear neutrophils (PMN)

Peritoneal exudate cells (PEC) enriched for PMN were elicited by injecting 1 ml of thioglycollate broth (Clinical Standards Laboratories, Carson, CA) intraperitoneally into mice 24 h before cell harvest. Immunologically activated PMN were obtained by injecting 500 µg (wet weight) of non-viable *B. dermatitidis* cells in 0.5 ml of isotonic saline intraperitoneally into immune mice 24 h before collection. PEC obtained after either stimulus were collected by repeated lavage of the peritoneal cavity with heparinised Eagle's Minimal Essential Medium (MEM) (Gibco), suspending the cells in 0.2% saline to lyse erythrocytes, reconstituting to isotonicity with 1.6% saline, washing once in isotonic saline and counting in a haemocytometer. These PEC were purified (Brunner and Stevens, 1983) by layering 1 ml of $(3-4) \times 10^7$ cells on a metrizamide gradient (1.5 ml of 14.5% over 1.5 ml of 15.5% metrizamide) and centrifuging at 500 *g* for 20 min. Pelleted cells were washed once, resuspended in TCM and counted in a haemocytometer. The purified ThioPMN preparation was 83.6% (SD 5.6%) PMN, and the purified preparation of ActPMN was 79.9% (SD 6.6%) PMN (*n*=8).

Determination of O₂⁻ production

PMN were suspended in HBSS, dispensed into 24-well flat-bottom microtest plates—1.0 ml/well; $(3-6) \times 10^6$ cells/ml (Corning Glass Works, Corning, NY)—and incubated for 1–2 h at 37°C in CO₂ 5%, air 95%. Non-adherent cells were removed and adherent cells were washed once with isotonic saline before the assay mixture was added to each well. The adherent cells, for ThioPMN and ActPMN, were both 92% PMN. The assay mixture (2 ml total volume/well) consisted of HBSS plus ferricytochrome c 40–80 µg/ml, PMA 2–4 µg/ml and fresh normal mouse serum 5–10%. SOD in 0.2 ml of HBSS (6000 U/ml, final concentration) was substituted for 0.2 ml of HBSS in selected wells to determine the SOD-inhibitable reduction of ferricytochrome c. After incubation for 1 h at 37°C in CO₂ 5%, air 95%, the fluid was removed from the wells and placed in test tubes on ice. The test tubes were then centrifuged at 1000 *g* for 10 min at 4°C and the absorbance at 550 nm of the supernates measured in a Beckman Acta III (Beckman Inc., Fullerton, CA) double beam spectrophotometer. The number of nmol of ferricytochrome c reduced was determined with an extinction coefficient of 2.1×10^4 M⁻¹ cm⁻¹. Fluid from wells containing PMN and all assay mixture components except PMA was assessed for background reduction of ferricytochrome c and the values obtained were subtracted from those of test samples.

Adherent cells were washed twice with HBSS and disrupted by exposure to 0.5 ml of 0.2% Triton X-100 detergent. Each well was then scraped with a pasteur pipette. The protein content of cell digests was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Co-cultures of PMN and *B. dermatitidis*

PMN were suspended in TCM and dispensed (0.1 ml of 5×10^6 /ml) into 96-well flat-bottom microtest plates (Corning Glass Works), and incubated for 1 h at 37°C in CO₂ 5%, air 95%. *B. dermatitidis* (0.1 ml of 1×10^4 cfu/ml) was added plus fresh normal mouse serum 0.02 ml/well. To selected wells, various potential inhibitors were added immediately before the addition of *B. dermatitidis*.

Co-cultures were incubated for 2 or 3 h at 37°C in CO₂ 5%, air 95% and harvested with distilled water (Brummer and Stevens, 1984). Dilutions were made in isotonic saline and the number of cfu/well was determined by culture on BA. The percentage reduction of inoculum cfu was determined with the formula:

$$[1 - (\text{co-culture cfu/inoculum cfu})] \times 100$$

Statistical analyses

Student's *t* test was used to determine the statistical significance between groups.

Results

Killing of *B. dermatitidis* by activated versus elicited PMN

It was previously reported that immunologically activated PMN (ActPMN) had an enhanced capacity to kill *B. dermatitidis* yeast cells *in vitro* in comparison with thioglycollate elicited PMN (ThioPMN) (Brummer *et al.*, 1985). In those studies, thioglycollate was injected intraperitoneally 4 h before peritoneal exudate cell collection. To follow more closely the elicitation schedule used for ActPMN (Brummer *et al.*, 1985), where whole, killed *B. dermatitidis* were injected intraperitoneally 24 h before PEC collection, thioglycollate was injected 24 h before PEC harvest for elicitation of ThioPMN in these experiments. This 24-h ThioPMN population isolated after centrifugation on a single metrizamide gradient was studied extensively for the first time. Whereas PMN elicited by thioglycollate injection 4 h before failed to kill *B. dermatitidis* yeasts (0–8% reduction of inoculum cfu) (Brummer *et al.*, 1984, 1985), these 24-h ThioPMN killed a small (16.4 SD 9.2%, mean and SD) but significant ($p < 0.001$) part of the fungal

inoculum (figure). During the co-culture period, the cfu in the absence of PMN did not significantly decline (1.4 SD 9.0%), and thus killing by these PMN was also significant ($p < 0.001$) if the post-co-culture period cfu are used for comparison. ActPMN demonstrated a significantly enhanced ability to kill *B. dermatitidis* *in vitro* (44.7 SD 12.8%) compared with 24-h ThioPMN ($p < 0.001$) (figure), as well as compared with 4-h ThioPMN as previously reported (Brummer *et al.*, 1984, 1985).

Production of O₂⁻ by activated versus elicited PMN

It was previously reported that ActPMN produced a 10-fold greater chemiluminescent burst 15 min after stimulation with *B. dermatitidis* cells than did ThioPMN (Brummer *et al.*, 1985). Luminol enhanced chemiluminescence by phagocytic cells has been attributed to the production of active oxygen species that depend upon O₂⁻ for their formation; therefore, the production of O₂⁻ by ActPMN or ThioPMN was examined to determine whether O₂⁻ production correlated with fungicidal activity.

After stimulation with PMA, ActPMN produced more than twice the quantity of O₂⁻ produced by ThioPMN ($p < 0.01$) (table I). No differences in the numbers of cells adhering to culture plates or in the quantity of adherent cell protein were observed between ThioPMN and ActPMN cultures; 24 and 25% of ThioPMN and ActPMN preparations, respectively, were nonadherent. The two preparations resulted in 0.022 SD 0.0007 and 0.021 SD 0.0007 mg adherent cell protein/10⁶ cells, respectively.

Table I. Production of O₂⁻ by ActPMN and ThioPMN after stimulation with phorbol myristate acetate

PMN type	O ₂ ⁻ released*	
	nmoles O ₂ ⁻ /10 ⁶ PMN/h	nmoles O ₂ ⁻ /mg cell protein/h
ThioPMN	1.20 SD 0.48 (6)	54.6 SD 21.8 (6)
ActPMN	3.10 SD 1.28 (6)	149.5 SD 63.5 (6)

* O₂⁻ production was measured in a ferricytochrome c reduction assay. Mean number of SOD (6000 U/ml)-inhibitable nmoles of O₂⁻ produced/10⁶ PMN or mg cell protein and the SD from the mean for the number of experiments given in parentheses. Values shown for ThioPMN were significantly different from those for ActPMN ($p < 0.01$).

Effect of SOD on killing of B. dermatitidis by activated versus elicited PMN

Exogenous SOD, a scavenger of O_2^- , inhibited ActPMN killing by as much as 74.9% when added to cultures immediately before challenge with *B. dermatitidis* (figure). The optimum SOD concentration tested was 6000 U/ml, with significant inhibition of killing at 3000– $\geq$ 9000 U/ml (maximum concentration tested, 66 000 U/ml) (figure). The *p* values for the comparisons between SOD treated and untreated ActPMN were $p < 0.005$, $p < 0.001$ and $p < 0.01$ for 3000, 6000, and ≥ 9000 U/ml, respectively. Enzyme concentrations ≤ 1000 U/ml or heated SOD did not significantly inhibit the ActPMN fungicidal capacity (figure). The addition of catalase (20 000 U/ml) in conjunction with SOD (6000 U/ml) did not inhibit fungal killing any more than SOD alone (figure). SOD had no direct effect on *B. dermatitidis* viability and did not inhibit fungal killing by Thio PMN at the concentrations tested (≤ 1000 , 3000, 6000, or ≥ 9000 U/ml in 4–6 experiments at each of these concentrations).

Effect of catalase on killing of B. dermatitidis by activated versus elicited PMN

Catalase, an enzyme that catalyses the breakdown of H_2O_2 to H_2O and O_2 (Beers and Sizer,

1952), inhibited the ActPMN-mediated killing of *B. dermatitidis* by 51.9% ($p < 0.001$) when added to cultures at an optimum concentration of 10 000 U/ml (table II). Catalase concentrations above or below the optimum were not significantly protective, although inhibition of killing was suggested (table II). Heated catalase (20 000 U/ml) did not inhibit fungal killing by ActPMN (table II) or ThioPMN, and catalase at the concentrations tested— ≤ 500 , 2000–5000, 10 000 or ≥ 20 000 (maximum concentration tested, 42 000) U/ml in 3 to 9 experiments at each of these concentrations—did not inhibit killing of *B. dermatitidis* by ThioPMN (data not shown).

Effect of other potential inhibitors of oxidative microbicidal mechanisms on killing of B. dermatitidis by ActPMN and ThioPMN

AZ added to co-cultures at a concentration of 1 or 10 mM significantly inhibited ActPMN killing of *B. dermatitidis* ($p < 0.001$) (table II). AZ at ≤ 10 mM had no direct effect on *B. dermatitidis* viability and did not significantly reduce killing of fungi by ThioPMN. A higher concentration of AZ (100 mM) was directly toxic to *B. dermatitidis* (18.0 SD 6.4% killing in 2 h, $n = 2$) in the absence of PMN.

Scavenging of hydroxyl radical ($\cdot OH$) (Brummer

Figure. Effect of superoxide dismutase (SOD) concentration on reduction of inoculum cfu by PMN. SOD had no direct effect on *B. dermatitidis* viability and did not inhibit killing of *B. dermatitidis* by ThioPMN at the concentrations tested (≤ 1000 – ≥ 9000 U/ml in 4–6 experiments). The mean percentage reduction of inoculum cfu and SD for the number of experiments in parentheses is given.

Table II. Effect of potential inhibitors of oxidative microbicidal mechanisms on reduction of inoculum cfu by PMN*

PMN type	Inhibitor added ^a	Percentage reduction of inoculum cfu	Number of experiments	Percentage inhibition of killing [†]	p value [‡]
No PMN	—	1.4	14	...	...
ActPMN	None	44.7	14	...	...
	Catalase 2000–5000 U/ml	31.0	9	30.6	n.s.
	10,000 U/ml	21.5	6	51.9	<0.001
	≥20,000 U/ml	29.5	3	34.0	n.s.
	Heated catalase [§]	48.2	5	0	...
	Azide 0.1 mM	36.9	5	17.4	n.s.
	1.0 mM	16.0	5	64.2	<0.001
	10.0 mM	16.2	3	63.8	<0.001
	DMSO 300 mM	29.0	5	35.1	n.s.
	Benzoate 50.0 mM	26.9	2	39.8	n.s.
	Tryptophan 1.0 mM	25.8	2	42.3	<0.001
	10.0 mM	0	2	100	<0.001
	Histidine 10.0 mM	33.6	2	24.8	<0.01
ThioPMN	None	16.4	14	...	...
	Benzoate 50.0 mM	8.9	2	45.7	<0.05
	Histidine 1.0 mM	0.3	2	98.2	<0.001
	10.0 mM	4.4	2	73.2	<0.02

* For brevity of presentation, only those experiments where differences in reduction of inoculum cfu in cultures with potential inhibitors compared with the same PMN type containing no inhibitors was at least 7.5% are shown. Not shown therefore are experiments (number of experiments in parentheses) with ActPMN and <500 U catalase (three), DMSO 3 mM (five) and 30 mM (three), tryptophan 0.1 mM (two), histidine 0.1 mM (two) and 1.0 mM (two), and benzoate 10.0 mM (two) and 20 mM (two). Similarly, not shown are experiments with ThioPMN and catalase (see text); azide 0.1 mM (four), 1.0 mM (five) and 10.0 mM (three); DMSO 3 mM (four), 30 mM (three), and 300 mM (five); tryptophan 0.1 mM (two), 1.0 mM (two), and 10.0 mM (two); histidine 1.0 mM (two); and benzoate 10.0 mM (two) and 20.0 mM (two). Results in none of these experiments were statistically significant.

† Percent inhibition of killing by inhibitor for each PMN type compared to samples containing no inhibitor.

‡ Student's *t* test, comparison between PMN killing with and without inhibitor for each PMN type; n.s. = *p* > 0.05.

§ Catalase (20 000 U/ml) was autoclaved at 121°C for 15 min before addition to PMN cultures.

^a Final concentration of inhibitor in co-culture medium.

et al., 1985) with 300 mM DMSO reduced ActPMN fungicidal activity by a mean of 35% (table II), but this reduction was not significant because of variability in the DMSO effects in repeated experiments resulting in a large SD. Lower concentrations of DMSO did not significantly reduce killing by ActPMN, nor were ThioPMN affected by any of the concentrations tested. DMSO had no direct effect on *B. dermatitidis* viability at the concentrations suggested to be inhibitory for ActPMN killing in table II, but a higher concentration (2800 mM) of DMSO reduced inoculum cfu in the absence of PMN (95.1% in 2 h).

Another ·OH scavenger, sodium benzoate (Sasada and Johnston, 1980), inhibited ActPMN killing, but again only at the highest concentration tested, and not significantly, because of variability between experiments (table II). At this concentration, ThioPMN killing was significantly reduced. We also studied the effects of two agents which both quench ¹O₂ and antagonise HOCl[·], histidine and tryptophan, at previously studied concentra-

tions (Hodgson and Fridovich, 1974; Singh and Valdez, 1978). The latter was a potent inhibitor of ActPMN killing, whereas the former inhibited ActPMN and was also the most potent inhibitor of ThioPMN killing (table II).

Discussion

It has been suggested that in luminol-dependent chemiluminescence studies (Brummer *et al.*, 1984, 1985) an enhanced in-vitro fungicidal activity of ActPMN versus ThioPMN correlates with an increased oxidative burst by ActPMN upon stimulation with *B. dermatitidis* cells. We report here that ActPMN produce greater quantities of O₂^{·-} in response to PMA than do ThioPMN. Furthermore, killing of Bd by ActPMN was inhibited by scavengers of active oxygen species. Exogenous superoxide dismutase, catalase, sodium azide, tryptophan and histidine and, to a lesser extent, DMSO and benzoate, inhibited killing when added to cultures immediately before fungal challenge.

These data, in conjunction with the earlier reports (Brummer *et al.*, 1984, 1985), indicate that the killing of *B. dermatitidis* by ActPMN involves one or more oxidative mechanisms, and suggest a prominent role for O_2^- , a probable role for H_2O_2 , and possible roles for 1O_2 , $HOCl^-$ and $\cdot OH$ in fungal killing by ActPMN. Because O_2^- and H_2O_2 are responsible for the formation of $\cdot OH$ and 1O_2 , scavengers that interfere with the activity of O_2^- or H_2O_2 or both may also interfere with the production of $\cdot OH$ and 1O_2 , as well as other products of H_2O_2 . Therefore, the direct contribution of O_2^- and H_2O_2 to fungal killing may be less profound than indicated by the results with scavengers of these compounds, as these scavengers may also indirectly remove more distal oxidative compounds that contribute to fungal killing.

The action of SOD to produce H_2O_2 from O_2^- would be expected to suppress microbicidal reactions dependent upon O_2^- and enhance those dependent upon H_2O_2 . Because SOD markedly suppresses fungicidal activity, H_2O_2 alone is probably a less potent killing agent than O_2^- or its by-products. H_2O_2 may serve as the precursor of 1O_2 or $\cdot OH$ upon interaction with O_2^- (Badwey and Karnovsky, 1980). The addition of SOD to cultures may have resulted in the removal of O_2^- before it could react with H_2O_2 to form toxic oxygen metabolites further along the oxidative pathway, thereby inhibiting ActPMN fungicidal activity.

Although catalase significantly reduced fungal killing by ActPMN, the addition of catalase in conjunction with SOD did not reduce killing further. Residual killing activity by ActPMN after SOD treatment was, therefore, not due to H_2O_2 formation catalysed by SOD, because H_2O_2 would have been destroyed by the addition of catalase and killing would have been further inhibited.

The reduction of ActPMN killing activity by sodium azide may have been partly due to its removal of 1O_2 (Hasty *et al.*, 1972). Sodium azide has also been shown to inactivate MPO, the enzyme mediating the MPO- H_2O_2 -halide killing system of PMN (Klebanoff and Clark, 1978). This system generates $HOCl^-$, which is microbicidal. Thus, azide may have also acted to eliminate oxidative killing mechanisms dependent upon H_2O_2 .

The activity of tryptophan and histidine, which also have potential activity against both 1O_2 and $HOCl^-$, in inhibiting ActPMN killing again emphasises the possible role of one or both of these potentially cidal oxidative by-products in fungal killing by ActPMN.

Hydroxyl radical ($\cdot OH$) is not the most likely mediator of ActPMN killing activity based on data

obtained with DMSO and benzoate as $\cdot OH$ scavengers. These agents inhibited ActPMN fungicidal activity by 35% and 40%, respectively, which, while suggestive, was not significantly inhibitory, and required the highest concentrations studied. However, previous work (Brummer *et al.*, 1985) has demonstrated reduced chemiluminescence in the presence of 300 mM DMSO, suggesting a possible role for $\cdot OH$ in ActPMN fungicidal mechanisms. Other scavengers of $\cdot OH$ might allow a further assessment of the contribution of $\cdot OH$ to fungal killing.

The absence of inhibition of the small amount of ThioPMN-mediated killing of fungi by almost all the oxidative scavengers studied indicates that ThioPMN may use primarily non-oxidative mechanisms in their limited capacity for fungal killing. Non-oxidative microbicidal mechanisms using lysosomes or granule-associated cationic proteins are known to exist in rabbit, human and guinea pig PMN (Lehrer *et al.*, 1975; Spitznagel and Shafer, 1985). Whether or not non-oxidative pathways are involved in ThioPMN killing of *B. dermatitidis* is worth investigation. Residual killing by ActPMN, after SOD treatment, may reflect the alternative non-oxidative fungicidal mechanisms present in these cells. No direct effects on the viability of *B. dermatitidis* were observed at the optimal concentrations of scavengers used to inhibit PMN killing in this study, and no effects on chemotaxis or the attachment of PMN to *B. dermatitidis* were found in the presence of the scavengers employed (unpublished observations).

These data indicate that the ActPMN killing of *B. dermatitidis* involves one or more active oxygen species but does not rule out a contribution by non-oxidative mechanisms as well. ThioPMN killing of *B. dermatitidis*, although limited, is presumably mediated by predominantly non-oxidative mechanisms, because killing is generally not inhibited by scavengers of oxidative metabolites. Based on the enhanced production of O_2^- by ActPMN and the efficacy of inhibition of killing by oxygen metabolite scavengers, the agents responsible for ActPMN fungicidal activity appear to be primarily O_2^- or mechanisms dependent on O_2^- production, with a probable role for H_2O_2 , and possible involvement by 1O_2 , $HOCl^-$ and $\cdot OH$ as well.

This study was supported by a contract (#N00014-83-K-0018) with the Office of Naval Research, US Navy. The results were presented in part to the 22nd National Meeting of the Reticuloendothelial Society, Ithaca, NY, 1985. We thank Chiyo Inouye for assistance with the manuscript.

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