Candidacidal mechanisms of peritoneal macrophages activated with lymphokines or γ -interferon

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Summary. The mechanisms by which resident peritoneal macrophages, activated *in vitro* by lymphokines (LK) or recombinant γ -interferon (IFN), kill *Candida parapsilosis* or *C. albicans* were studied. Resident non-activated peritoneal macrophages killed *C. parapsilosis* (55.5%SD6.8%), but not *C. albicans*. This killing was completely inhibited by superoxide dismutase (SOD), partially by dimethyl sulphoxide (DMSO), but not by catalase or azide. Killing correlated with a brisk lucigenin-dependent chemiluminescence (CL) response by macrophages interacting with *C. parapsilosis*. No enhanced luminol-dependent CL response was observed in this system. This suggests that *C. parapsilosis* is killed by resident macrophages *via* a mechanism dependent on the presence of superoxide anion. By contrast, killing of *C. parapsilosis* by activated macrophages (49.0%SD5.9%) was not inhibited by SOD or DMSO, suggesting the induction of a non-oxidative candidacidal mechanism. *C. albicans* was killed only by macrophages activated with IFN (52.0%SD3.7%) or LK (55.7%SD2.8%). Inhibition of killing by SOD was greater in IFN- than in LK-activated macrophages. Conversely, killing by LK-, but not IFN-, activated macrophages was significantly inhibited by catalase, DMSO or azide. The killing by LK-activated macrophages, and its inhibition by scavengers, correlated with the luminol-dependent CL response. The non-killing resident macrophages interacting with *C. albicans* made lucigenin-dependent CL responses similar to those of activated macrophages. The mechanisms enabling killing of *C. albicans* induced by activation appear to be different for LK and IFN, and appear to depend upon the myeloperoxidase systems and superoxide respectively.

Introduction

Unlike normal polymorphonuclear neutrophils (PMN) and monocytes which kill *Candida albicans* (Leijh *et al.*, 1977), alveolar (Biggar and Sturgess, 1976) and peritoneal (Peterson *et al.*, 1985) macrophages are deficient in myeloperoxidase (MPO) and lack significant ability to kill *C. albicans* (Lehrer *et al.*, 1980; Peterson *et al.*, 1985). Furthermore, *C. albicans* resists killing by PMN and monocytes that are genetically deficient in myeloperoxidase (Lehrer and Cline, 1969). Nevertheless, such MPO-deficient leucocytes readily kill other *Candida* species, e.g., *C. parapsilosis*, *C. krusei*, and *C. pseudotropicalis*, and bacteria (Lehrer, 1972). Although the MPO-independent mechanism is slower, it is efficient (Lehrer, 1972; Lehrer *et al.*, 1975).

Peritoneal macrophages from mice infected with

bacillus Calmette-Guerin (BCG) (Sasada and Johnston, 1980) or resident peritoneal macrophages treated with lymphokines *in vitro* (Kagaya and Fukazawa, 1981) have been reported to kill *C. albicans*. Recently we have reported that recombinant γ -interferon (IFN) activates resident peritoneal macrophages *in vitro* for significant killing (44%) of *C. albicans* (Brummer *et al.*, 1985). Although killing of *C. albicans* by activated macrophages is likely to be important in host defence against *C. albicans*, the mechanisms by which activated macrophages kill *C. albicans* is unclear.

The purpose of the present study was to elucidate candidacidal mechanisms of resident and activated peritoneal macrophages by use of new reagents and techniques. With the use of lucigenin, that reacts primarily with superoxide anion (Minkenberg and Ferber, 1984; Muller-Peddinghaus, 1984) and luminol, oxygen radicals generated by interaction of activated or resident macrophages with viable

candida were measured by chemiluminescence. Concomitantly, the ability of oxygen radical scavengers, or enzyme inhibitors, to decrease the killing of candida by macrophages was assessed.

Materials and methods

Animals

Male BALB/cByJIMR mice, 8–12 weeks of age, were obtained from the breeding colony of the Institute for Medical Research, San Jose, CA, for use in these experiments.

Reagents and media

Tissue-culture medium RPMI-1640 with L-glutamine, Eagle's Minimum Essential Medium (MEM), heat-inactivated fetal bovine serum (FBS), and penicillin-streptomycin, 10 000 U/ml–10 000 µg/ml, respectively, (P/S) (Gibco, Grand Island, NY) were used and complete tissue culture medium (CTCM) comprised 89 ml of RPMI-1640, 10 ml of FBS, and 1 ml of P/S. Concanavalin A (Con A) and α -methyl-D-mannoside, grade III (aMM) (Sigma Chemical Co., St Louis, MO), preservative-free heparin (American Scientific Products, Sunnyvale, CA), 96-well half-area (A/2) tissue-culture plates (Costar Co., Cambridge, MA) and murine γ -interferon produced by recombinant DNA technology, and hyperimmune rabbit antibody (0.155 mg/ml) to murine γ -IFN (Genentech Corp., South San Francisco, CA) as described by Celada *et al.* (1984) and Svedersky *et al.* (1984) were employed. Luminol (5-amino-2,3-dihydro-1, 4-phthaliazinedione) and lucigenin (bis-*N*-methylairidinium nitrate) (Sigma) were dissolved in dimethyl sulphoxide (DMSO; Eastman Kodak Co., Rochester, NY) to form stock solutions of 10 mg/ml. Superoxide dimutase (SOD; 3000 U/mg of protein) and catalase (25 000 U/mg of protein) (Sigma) were dialysed against phosphate-buffered saline (PBS) before use. Stock 0.1 M sodium azide (Fisher Scientific Co., Fair Lawn, NJ) was prepared in saline. Horseradish peroxidase (HPO; Sigma) was dialysed and used as previously described (Sugar *et al.*, 1983).

Fungi

C. parapsilosis and *C. albicans* ATCC 56882 were grown in yeast nitrogen broth (Difco) at 35°C for 3–4 days. *Blastomyces dermatitidis* ATCC 26199, an isolate shown to be virulent in mice, was grown and used as previously described (Brummer *et al.*, 1985; Brummer and Stevens, 1984). Fungi were washed twice in 4 ml of saline, pelleted cells were suspended in CTCM, and units (single or multicellular) counted with a haemocytometer. Viable cfu were determined by plating 1 ml of appropriate dilutions in quadruplicate on blood-agar plates. Killed *C. albicans* was prepared by heating for 1 h at 60°C.

Peritoneal macrophages

Peritoneal cells (PC) were collected from the abdominal cavity of each mouse by repeated lavage with a total of 10 ml of MEM containing heparin 10 U/ml. PC from mice were pelleted by centrifugation (200 g, 10 min) and pooled. The number of cells/ml was determined with a haemocytometer. PC were dispensed (0.1 ml of 5×10^6 PC/ml of CTCM) into the wells of an A/2 Costar plate and the plate was incubated at 37°C in CO₂ 5% in air for 2 h, then the non-adherent cells were removed by aspiration and each well was washed once with CTCM. When the number of non-adherent cells was subtracted from the number of incubated PC, the average number of adherent PC/well was 2.5×10^5 . Adherent cells were previously shown to be 90% positive for non-specific esterase (Brummer *et al.*, 1983).

Treatment and challenge of macrophages

Macrophage monolayers were incubated overnight (37°C, CO₂ 5% in air) with 0.1 ml of CTCM or CTCM containing IFN 200 U/ml or Con A-stimulated spleen-cell culture supernates (obtained as described in the next paragraph) containing 50 mM aMM. After incubation, culture supernates were aspirated and monolayers were challenged with 0.1 ml of *C. albicans* or *C. parapsilosis* suspensions (10 000 fungal units/ml of CTCM) plus 0.01 ml of fresh mouse serum with or without SOD, catalase, DMSO, or sodium azide. Co-cultures were incubated at 37°C in CO₂ 5% in air for 2 h. Cultures were harvested by aspiration and repeated washing of culture wells with distilled water to a final volume of 10 ml. Microscopic examination of washed wells indicated that there was complete removal of macrophages. To determine the number of cfu/culture, 1 ml of the 10 ml of distilled water from the wells was plated on a blood-agar plate and colonies were counted after incubation for 2 days at 35°C.

Spleen-cell supernates

Spleen cells were obtained from normal mice and cultured as previously described (Brummer and Stevens, 1984). Supernates were generated by incubation of 10^7 spleen cell/ml of CTCM with or without Con A 5 µg/ml in 20-well tissue-culture plates (Falcon, Oxnard, CA), 2 ml/well at 37°C in CO₂ 5% in air for 1 day. Supernates were harvested from pooled cultures by centrifugation (400 g, 10 min) and filtration through 0.45-µm filters (Millipore Corp., Bedford, MA). Portions of supernates were stored at –70°C until used.

Chemiluminescence

To detect the products of oxidative metabolism subsequent to challenge of macrophages with fungal units in fresh serum, the luminol method of Allen and Loose (1976) and the lucigenin method of Minkenberg and Ferber (1984) were used. Briefly, 0.5 ml of PC (5×10^6

PC/ml of CTCM) were dispensed into each glass micro-sample vial (15 × 45 mm) and incubated at 37°C for 2 h in CO₂ 5% in air. Non-adherent cells were removed by aspiration and the monolayers were washed once with CTCM. Monolayers were treated overnight with 0.5 ml of CTCM, Con A supernates containing 50 mM aMM, or IFN (200 U/ml CTCM). After treatment and removal of the supernates, monolayers were challenged with 0.1 ml of luminol or lucigenin (1 in 250 or 1 in 100 dilutions of stock 10 mg/ml in DMSO, respectively), 0.05 ml of fresh mouse serum, 0.15 ml of PBS, and 0.1 ml of fungal suspension (10 × 10⁶ cells/ml of PBS) and assayed at room temperature. When catalase or SOD was added to the mixtures, corresponding volumes of PBS were omitted. Photon emission was measured in a scintillation counter (Mark II, Nuclear Chicago) at room temperature with the windows set on "manual" and levels at L-infinity. The manual setting permitted rapid counting of samples, and the cpm was calculated from the counting time (≤ 0.2 min) registered by the scintillation counter. Chemiluminescence values in the absence of fungi were less than the time 0 values in the presence of fungi shown in the figures, in all cases.

Phagocytosis

PC (5 × 10⁶/ml of CTCM) were dispensed, 0.25 ml/chamber of Lab-Tek tissue-culture slides (Miles Scientific, Naperville, IL). After incubation for 2 h at 37°C, non-adherent cells were removed by aspiration and the monolayers were washed once with CTCM. Monolayers were treated overnight with 0.25 ml of CTCM, IFN (200 U/ml of CTCM) or Con A supernates containing 50 mM aMM at 37°C in CO₂ 5% in air. After treatment, supernates were aspirated and monolayers challenged with 0.2 ml of *C. albicans* (5 × 10⁶ cells/ml of CTCM) plus 10% fresh mouse serum. Challenged monolayers were incubated for 2 h at 37°C in CO₂ 5% in air. After incubation, challenged monolayers were washed three times with saline, then dried, fixed, and stained with Diff-Quik (American Scientific Products, McGraw Park, IL).

Statistics

The statistical significance of the differences between the means was determined by Student's *t* test.

Results

Susceptibility of *C. parapsilosis* and *C. albicans* to the H₂O₂-HPO-halide system

The sensitivity of *C. parapsilosis* and *C. albicans* to the toxic products of a cell-free H₂O₂-HPO-halide system was tested as previously described (Sugar *et al.*, 1983) with *B. dermatitidis* being used in comparison. All fungi were sensitive to 5 × 10⁻⁶ M

Table I. Killing of *C. albicans*, *C. parapsilosis* and *B. dermatitidis* in the H₂O₂-HPO-halide system*

Fungus species	Percentage killing at H ₂ O ₂ concentration (M)			
	5 × 10 ⁻⁵	5 × 10 ⁻⁶	5 × 10 ⁻⁷	5 × 10 ⁻⁸
<i>C. parapsilosis</i>	100	100	2	0
<i>C. albicans</i>	100	100	23	0
<i>B. dermatitidis</i>	100	100	0	0

*KI, 5 × 10⁻⁴ M; HPO as peroxidase 14 U/ml.

H₂O₂ interacting with HPO 14 U/ml and KI 5 × 10⁻⁴ M (table I). In a second experiment similar killing results were obtained. Individually the components—5 × 10⁻⁴ M H₂O₂, 5 × 10⁻⁴ M KI or HPO 14 U/ml—were not toxic. Killing was completely inhibited by catalase 200 U/ml, but not by SOD 450 U/ml. These results indicate that the resistance of *C. albicans* to killing by resident macrophages in comparison with *C. parapsilosis*, is not due to resistance to the products of the H₂O₂-HPO-halide system.

Phagocytosis

Differences in phagocytosis of *C. albicans* by macrophage monolayers treated overnight with CTCM, IFN, or Con A supernates were assessed by the slide Lab-Tek tissue-culture chamber method. Macrophages in CTCM ingested *C. albicans* as readily as those treated with IFN or Con A supernates and the inability of resident macrophages to kill *C. albicans* is not explained by lack of phagocytic ingestion.

Effect of oxygen-radical scavengers on killing of *C. parapsilosis* by macrophages

The killing of *C. parapsilosis* in 2 h by resident macrophages (55.5%SD6.8%) was extremely sensitive to inhibition (100%) by SOD 450 U/ml and, to a lesser extent, by DMSO (33% inhibition) (table II). Killing was not inhibited by heated SOD 450 U/ml, catalase 20 000 U/ml or 1 mM sodium azide. Killing increased with incubation time, e.g., at 3 h it increased to 75.0%SD2.8% (n = 2). These findings indicate that resident macrophages killed *C. parapsilosis*, not by a H₂O₂-MPO-halide system, but by a mechanism requiring superoxide anion, and to some extent the generation of hydroxyl radicals.

Resident macrophages treated with Con A supernates or IFN did not exhibit enhanced killing

(49.0%SD5.9% and 57.6SD7.2%) compared to resident macrophages (55.5%SD6.8%) (table II). The primary effect of these treatments of macrophages was resistance to inhibition of killing by SOD 450 U/ml or 300 mM DMSO (table II). Azide (1 mM) did not significantly inhibit killing of *C. parapsilosis* by macrophages treated with Con A supernate (66% vs 52% in the presence of azide) or by IFN 200 U/ml (77% vs. 69% in the presence of azide). Since killing was not inhibited by catalase, or significantly by azide, the mechanism by which treated macrophages killed *C. parapsilosis* was relatively independent of a need for generating oxygen radicals.

Effect of oxygen-radical scavengers on killing of C. albicans by activated macrophages

Although resident peritoneal macrophages killed *C. parapsilosis* efficiently, they were not able to kill *C. albicans*. However, as previously reported,

macrophages treated overnight with γ -IFN or supernates from Con A-stimulated spleen-cell cultures killed *C. albicans* (Brummer *et al.*, 1985). We confirmed those results in the present study (table III). Killing of *C. albicans* by IFN treated macrophages was inhibited 76% by SOD 450 U/ml but not by catalase or DMSO (table III). Moreover, 1 mM azide did not significantly inhibit killing (50% vs. 47% in the presence of azide).

By contrast, killing by macrophages activated with Con A supernates was inhibited significantly by catalase, DMSO, and SOD 4500 U/ml (table III). These agents resulted in an inhibition of killing by 24%, 42%, and 46%, respectively. Furthermore, 1 mM azide significantly inhibited killing (54% vs. 13% in the presence of azide; 77% inhibition). These results clearly implicate involvement of the H_2O_2 -MPO-halide system in the killing of *C. albicans* by macrophages activated with Con A supernates. These findings show that IFN-activated macrophages killed *C. albicans* as efficiently as Con

Table II. Effect of oxygen-radical scavengers on the killing of *C. parapsilosis* by resident and activated peritoneal macrophages

Macrophage* treatment	Percentage (mean $\pm$ SD) reduction of inoculum cfu in 2 h (number of experiments)				
	SOD (U/ml)			Catalase (20 000 U/ml)	DMSO (300 mM)
	CTCM	4500	450		
CTCM	55.5 $\pm$ 6.8(6)	ND	†0.0 $\pm$ 0.0(3)	65.0 $\pm$ 5.6(2)	†37.3 $\pm$ 4.9(3)
Con A sup	49.0 $\pm$ 5.9(4)	43.3 $\pm$ 16.1(3)	48.2 $\pm$ 8.0(4)	67.0 $\pm$ 2.8(2)	64.5 $\pm$ 9.1(2)
γ -IFN	57.6 $\pm$ 7.2(3)	†31.6 $\pm$ 11.0(3)	56.3 $\pm$ 5.7(3)	69.0 $\pm$ 2.8(2)	53.5 $\pm$ 4.9(2)

*Resident peritoneal macrophages incubated overnight with complete tissue culture medium (CTCM), Con A-stimulated spleen cell culture supernates (Con A sup) or recombinant γ -IFN 200 U/ml.

†Significantly different ($p < 0.05$) from CTCM control.

ND = not done.

Table III. Effect of oxygen-radical scavengers on the killing of *C. albicans* by lymphokine or γ -IFN-activated macrophages

Macrophage* Treatment	Percentage (mean $\pm$ SD) reduction of inoculum cfu in 2 h (number of experiments)				
	SOD (U/ml)			Catalase (20 000 U/ml)	DMSO (300 mM)
	CTCM	4000	450		
γ -IFN	52.0 $\pm$ 3.7(5)	†2.3 $\pm$ 2.5(3)	†12.3 $\pm$ 8.0(3)	65.6 $\pm$ 4.6(3)	46.3 $\pm$ 4.9(3)
Con A sup	55.7 $\pm$ 2.8(4)	†30.0 $\pm$ 13.7(3)	45.3 $\pm$ 9.0(3)	†42.5 $\pm$ 5.8(4)	†32.2 $\pm$ 9.2(4)

*Resident peritoneal macrophages incubated overnight with complete tissue culture medium (CTCM; see table II); Con A-stimulated spleen cell culture supernates (Con A sup); or recombinant γ -IFN 200 U/ml.

†Significantly different ($p < 0.05$) from CTCM control.

Fig. 1. Generation of lucigenin-enhanced CL by the interaction of macrophages and *C. parapsilosis*. The CL produced by interaction of macrophages, treated with CTCM (□), Con A supernates (■) or IFN 200 U/ml (●), and *C. parapsilosis* are plotted against time.

A supernate-activated macrophages but by a novel and different mechanism, e.g., one that required only the generation of superoxide anion.

Lucigenin-enhanced chemiluminescence responses generated by interaction of macrophages and C. parapsilosis

The greatest superoxide anion-dependent lucigenin CL responses were made by the interaction of resident macrophages and *C. parapsilosis* (fig. 1). The response reached near maximum by 30 min and was maintained for at least 120 min. Interaction of Con A supernate- or IFN-activated macrophages with *C. parapsilosis* produced a similar CL profile but with one half log₁₀ and one log₁₀ fewer cpm, respectively (fig. 1). Similar results were obtained in a second experiment in which the resident macrophages gave a greater lucigenin CL response to *C. parapsilosis* ($10^{3.95}$ cpm) than did activated (Con A supernate) macrophages ($10^{3.57}$ cpm). Since resident and activated macrophages killed *C. parapsilosis* to the same extent, the magnitude of lucigenin CL responses and superoxide anion production *per se* did not parallel killing. However, the brisk lucigenin CL response made by resident macrophages interacting with *C. parapsilosis* was probably related to crucial candidacidal mechanisms, as evidenced by inhibition of that mechanism with SOD.

Fig. 2. Generation of luminol-enhanced CL by the interaction of macrophages and *C. parapsilosis*. The CL produced by the interaction of macrophages treated with CTCM (□), IFN 200 U/ml (●) or Con A supernates (■) and *C. parapsilosis* is given with time.

Luminol-enhanced chemiluminescence responses generated by interaction of macrophages and C. parapsilosis

Resident macrophages plus *C. parapsilosis* gave a peak luminol-enhanced CL response 143% that of macrophages that had not interacted with *C. parapsilosis*. However, Con A supernate-treated macrophages exhibited an early five-fold greater CL response to *C. parapsilosis* than resident macrophages (fig. 2), and this was sustained for at least 120 min. IFN-treated macrophages plus *C. parapsilosis* produced a two-fold greater CL response than resident macrophages after incubation for 15 min. These results suggest that activated macrophages interacting with *C. parapsilosis* generated oxygen radicals detectable by luminol-enhanced CL. However, the levels of these products did not play a critical role in the mechanisms of killing because killing was not inhibited by catalase or DMSO, and because activated macrophages did not kill *C. parapsilosis* better than resident macrophages.

Lucigenin-enhanced chemiluminescence generated by interaction of macrophages and C. albicans

When resident or activated macrophages were challenged with viable *C. albicans*, similar levels of lucigenin-enhanced CL were recorded (fig. 3). Killed *C. albicans* cells were more effective stimulators than viable cells and elicited a 10-fold greater CL response (fig. 3). In other experiments, killed *C. albicans* elicited greater peak CL responses in

Fig. 3. Lucigenin-enhanced CL generated by interaction of macrophages and *C. albicans*. The CL produced by interaction of macrophages treated with CTCM (□), Con A supernates (■) or IFN 200 U/ml (●) and viable *C. albicans* is given with that of macrophages treated with CTCM (x-x) or Con A supernates (⊗-⊗) interacting with killed *C. albicans*.

resident macrophages than viable cells did, e.g., $10^{5.24}$ cpm vs. $10^{4.28}$ cpm, and viable cells stimulated resident and Con A supernate-activated macrophages to a similar extent, e.g., $10^{3.79}$ cpm vs. $10^{3.86}$ cpm, respectively. Since only activated macrophages killed *C. albicans*, generation of a lucigenin-enhanced CL response by macrophages did not correlate with their capacity to kill *C. albicans*. Lucigenin-enhanced CL responses were superoxide anion-dependent because the response was completely inhibited by SOD 450 U/ml, but not by catalase 20 000 U/ml (data not shown).

Luminol-enhanced CL generated by interaction of *C. albicans* with macrophages

Macrophages activated by treatment with supernates from Con A-stimulated spleen cells generated a luminol-enhanced CL response with viable or killed *C. albicans* (fig. 4). These CL responses were 13–15-fold greater than that of resident macrophages with *C. albicans* (fig. 4). In a second experiment, CL responses were 8.75-fold greater with Con A supernate-activated macrophages interacting with *C. albicans* than with resident macrophages and *C. albicans*. The enhanced CL responses paralleled the ability of Con A supernate-treated macrophages to kill *C. albicans*. The CL response was inhibited by 1 mM azide and catalase

Fig. 4. Luminol-enhanced CL generated by the interaction of macrophages with *C. albicans*. The CL produced by the interaction of macrophages treated with CTCM (□), Con A supernates (■) or IFN 200 U/ml (●) and viable *C. albicans* is given with the responses of macrophages treated with CTCM (⊗-⊗) and Con A supernates (x-x) interacting with killed *C. albicans*.

20 000 U/ml, indicating its dependence on the H_2O_2 -MPO-halide system (data not shown). These results also correlated with significant inhibition of killing by 1 mM azide, catalase 20 000 U/ml and 300 mM DMSO (table III).

Macrophages treated with IFN, when interacting with *C. albicans*, also produced a luminol-enhanced CL response but at a three-fold lower level (fig. 4). Similar results were obtained in a second experiment. This low CL response correlated with failure of catalase, azide, or DMSO to inhibit killing of *C. albicans* by IFN-activated macrophages, and provided additional evidence for a killing mechanism for *C. albicans* independent of the H_2O_2 -MPO-halide system.

Discussion

In this study we have shown that resident macrophages can kill *C. parapsilosis*, and that this killing activity is susceptible to SOD and DMSO and resistant to catalase and azide. Macrophages treated with Con A supernates or IFN did not show increased killing of *C. parapsilosis*. The killing by Con A supernate-treated macrophages was resistant to SOD, catalase, DMSO, and azide, whereas killing by macrophages treated with IFN was partially resistant to SOD and resistant to the other three inhibitors. Chemiluminescence after contact with this fungus, assayed with lucigenin, showed that resident macrophages had greater activity than treated macrophages, even though killing was the

same. Resident macrophages did not demonstrate chemiluminescence with luminol; treated macrophages had enhanced chemiluminescence with this method, but killing was the same.

In contrast, only treated macrophages became activated to kill *C. albicans*. The killing induced by Con A supernates was susceptible to azide, catalase, DMSO, and SOD. The killing induced by IFN was susceptible to SOD but resistant to the other inhibitors. Chemiluminescence after contact with *C. albicans* assayed with lucigenin was similar in resident and activated macrophages, though only activated macrophages killed the fungus. Macrophage chemiluminescence assayed with luminol was enhanced by Con A supernates; this enhancement was neutralised by the four inhibitors, which correlated with their inhibition of killing of *C. albicans* by these cells. Luminol-assayed chemiluminescence after IFN treatment showed a smaller increase; the resistance of this enhancement to inhibitors correlated with their lack of effect on killing of *C. albicans* by IFN-treated cells.

We confirmed that *C. albicans* is sensitive to products of the H_2O_2 -MPO-halide system first reported by Klebanoff (1968), and showed that resistance of *C. albicans* to killing by macrophages, compared to *C. parapsilosis*, is not due to greater resistance to these toxic products. Although Lehrer (1972) and Sasada and Johnston (1980) have reported that *C. parapsilosis* is more resistant to killing by H_2O_2 than *C. albicans*, the concentrations required for killing candida (10^{-3} and 10^{-2} M) are not physiologically realistic. On the other hand, resistance of *C. albicans* to killing in a MPO-deficient system has been linked to marked insensitivity of *C. albicans* to killing by lysosomal cationic proteins (50 $\mu\text{g}/\text{ml}$) compared to *C. parapsilosis* (Lehrer *et al.*, 1975).

Phagocytosis of *C. albicans* by non-activated macrophages did not differ from that by activated macrophages. These observations agree with those of Sasada and Johnston (1980), who did not find a correlation between the degree of phagocytosis and ability to kill *C. albicans* or *C. parapsilosis*. Resident macrophages phagocytose candida as efficiently as activated macrophages, i.e., >90% of macrophages ingest candida.

The data presented agree with earlier reports by Lehrer (1972) that *C. parapsilosis* can be killed by microbicidal mechanisms of macrophages that do not require the MPO system. We confirm that 1–2 mM sodium azide does not inhibit killing and, in our hands, catalase also does not inhibit killing. Furthermore, we showed that the killing mechanism is superoxide anion-dependent and that killing

is mediated partially by generation of hydroxyl radicals. In this respect, our results agree with those of Sasada and Johnston (1980), who reported that killing was inhibited best by SOD and to a lesser extent by benzoate, another hydroxyl radical scavenger.

On the other hand, the mechanism for the killing of *C. parapsilosis* by in-vitro activated macrophages was different; it was insensitive to inhibition by SOD and DMSO. Thus, the mechanism appears to be independent of any need to generate superoxide anions or hydroxyl radicals. We speculate that this mechanism may involve enhancement of the candidacidal effects of lysosomal cationic proteins described by Patterson-Delafield *et al.* (1980). Our results with in-vitro activated macrophages differ very much from those reported by Sasada and Johnston (1980) for macrophages elicited by LPS in normal mice or by antigen in BCG-infected mice, e.g., they reported that killing by elicited macrophages was inhibited by SOD to the same extent as killing by resident macrophages. Elicited macrophages may contain recently migrated MPO-containing monocytes and also resident macrophages that have ingested MPO from PMN (Heifets *et al.*, 1980; Chapes and Haskill, 1983; Peterson *et al.*, 1985); consequently, it is not possible to compare these data with our results with resident macrophages activated *in vitro*.

Treatment of resident macrophages with IFN enables them to kill *C. albicans* by a mechanism that requires generation of superoxide anion but does not require the H_2O_2 -MPO-halide system. The induced killing mechanism does not depend upon enhanced levels of superoxide production, because superoxide anion production by resident and activated macrophages in response to *C. albicans* were similar, as measured by lucigenin-enhanced CL. Our results suggest that IFN-activated macrophages are able to kill *C. albicans* by a presently undefined mechanism that uses normally available superoxide anion levels which *per se* are not toxic. Furthermore, the killing mechanism does not appear to involve the generation of hydroxyl radicals by the Haber-Weiss reaction ($\text{O}_2^- + \text{H}_2\text{O}_2$ to give $\text{OH}\cdot + \text{OH}^- + \text{O}_2$) because killing is not inhibited by either catalase or DMSO.

Supernates from Con A-stimulated spleen-cell cultures activate resident macrophages to kill *C. albicans* by mechanisms involving the H_2O_2 -MPO-halide system, as evidenced by inhibition of killing with azide, catalase, and DMSO. A luminol-enhanced CL response to *C. albicans* also indicates the presence of such a system (DeChatelet *et al.*,

1982; Dahlgren and Stendahl, 1983). These results indicate that these supernates contained factors (lymphokines) that induced enough MPO in the treated macrophages to support these reactions. The supernates contained natural γ -IFN (Brummer *et al.*, 1985), which is a glycosylated dimer of 28 Kda (Havell and Spitalny, 1984) and is the major activating factor present (Brummer *et al.*, 1985). However, the action of other factors present in supernates, working alone or synergistically with natural γ -IFN, cannot be ruled out. Apparently recombinant γ -IFN, which is nonglycosylated (15 Kda), alone does not produce this level of effects. When the concentration of IFN was increased above 200 U/ml, killing was not increased (Brummer *et al.*, 1985) nor were the luminol-enhanced CL responses.

Our findings that Con A supernates and recombinant γ -IFN caused killing of *C. albicans* by different mechanisms in resident peritoneal macrophages are in contrast with those reported by Murray *et al.* (1983) in the human monocyte-derived macrophage vs. *Leishmania donovani* or *Toxoplasma gondii* systems, e.g. killing of *T. gondii* by macrophages treated for 3 days with Con A supernates or recombinant γ -IFN was inhibited by SOD, catalase, and mannitol, suggesting that either treatment induced the MPO system (Murray *et al.*, 1985). Further study with our system is needed to determine if a 3 day treatment of resident macrophages with murine γ -IFN would also induce such levels of MPO. On the other hand, evidence for the induction of a MPO-independent killing mechanism in macrophages from patients with chronic granulomatous disease by IFN was obtained (Murray *et al.*, 1985). Although this mechanism was only 50% as effective as the MPO-dependent system, it was inducible by recombinant γ -IFN. More recently

it has been reported that recombinant γ -IFN induces a MPO-independent mechanism in macrophages that inhibits intracellular replication of *Chlamydia psittaci* (Rothermel *et al.*, 1986).

Using viable organisms as stimuli for macrophages, we did not find enhanced superoxide anion production by activated macrophages in comparison with non-activated macrophages, when assessed by lucigenin CL. Killed *C. albicans* produced a 10-fold greater CL response than viable *C. albicans* and similar enhanced responses were induced by activated and non-activated macrophages. Others have used particulate material (opsonised zymosan) or soluble compounds such as phorbol myristate acetate as stimulants to demonstrate enhanced capacity of activated macrophages to generate superoxide anion or H_2O_2 (Murray *et al.*, 1985). Although these stimuli distinguish activated from non-activated macrophages, extrapolation from enhanced superoxide anion and H_2O_2 production to their role as a microbicidal mechanism may be unwise as evidenced by our results with *C. albicans* presented here.

Candidacidal mechanisms of peritoneal macrophages activated *in vitro* by Con A supernates reported here resemble those recently described for human monocytes by Sasada *et al.* (1987). Killing of *C. albicans* (35SD5%) by monocytes was inhibited by SOD, catalase or thiourea and was associated with MPO-positive cells. Monocytes cultured *in vitro* for 14 days differentiated into macrophages, became MPO negative, and failed to kill *C. albicans*. In this respect monocyte-derived macrophages were similar to resident murine peritoneal macrophages.

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