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Generation of Hydrogen Peroxide by *Candida albicans* and Influence on Murine Polymorphonuclear Leukocyte Activity

DAVID L. DANLEY,[1]* ANTHONY E. HILGER,[2] AND CRAIG A. WINKEL[1]

Department of Clinical Investigation, Letterman Army Medical Center, San Francisco, California 94129,[1] and Division of Medical Technology, Medical School, The University of North Carolina, Chapel Hill, North Carolina 27514[2]

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Iodide fixation by murine polymorphonuclear leukocytes (PMN) incubated with viable *Candida albicans* blastoconidia increases directly with yeast cell concentration up to about 3×10^6 cells per ml, but above this concentration bound activity declines dramatically. To understand the basis for this decline, we examined the oxidative metabolism of fungi and stimulated PMN and found some remarkable similarities between these cell types. Both produced $^{14}CO_2$ when incubated with [1-^{14}C]glucose, both reduced cytochrome *c*, and both fixed radiolabeled iodide, although the fungi required exogenous lactoperoxidase. In dose-response experiments, iodination by fungi with lactoperoxidase was identical to that with PMN, i.e., the maximum bound activity occurred in cultures with 10^6 to 3×10^6 blastoconidia per ml. Iodination by fungi with lactoperoxidase was reduced when blastoconidia were incubated at 25°C or in the presence of catalase and the metabolic inhibitors rotenone, antimycin A, and 2-deoxyglucose. Results from assays for oxidation of scopoletin and *o*-dianisidine showed that 10^6 blastoconidia in 1.0 ml of medium released 0.5 to 0.7 nmol of H_2O_2 after 1 h, but 3×10^6 and 10^7 cells released significantly less H_2O_2. These results suggest that iodide fixation by PMN and low numbers of fungal cells may reflect a cooperative effort, with fungi generating some H_2O_2 that reacts with the myeloperoxidase released from the PMN. With high concentrations of blastoconidia, H_2O_2 activity appeared to be specifically inhibited, possibly to protect fungal cells from damage.

Candida albicans and some related species are part of the normal flora of the upper respiratory, alimentary, and urogenital tracts of most humans. However, in a small percentage of the population, *Candida* spp. may colonize epi- or endothelial tissues and produce primary infections, such as thrush or vaginitis, and in immunosuppressed patients these fungi may behave like pathogens and become invasive. Although considerable research has been conducted on the physiology of *Candida* spp. and their mechanisms of pathogenesis, no single factor has been identified that consistently correlates with invasion by this fungus.

In previous studies on the interaction of murine polymorphonuclear leukocytes (PMN) with *C. albicans* cells in vitro, we reported that iodide fixation by PMN is considerably different when stimulation is by viable or killed fungi; i.e., iodination increases when PMN are incubated with increased numbers of killed blastoconidia, but decreases when PMN are incubated with increased numbers of viable blastoconidia (5). Since phagocytic cells may damage and kill fungi through iodination (10, 13), these results suggest that viable blastoconidia may augment their survival by modulating PMN activity. To test this hypothesis, we examined the oxidative metabolism of blastoconidia and stimulated PMN. In this communication, we report the similarities between the oxidative metabolisms of these two cell types. These similarities make it difficult to evaluate the individual aspects of metabolic function when PMN and fungi are incubated together; however, in the iodination assay our data suggested that both cell types may cooperate to fix iodide.

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MATERIALS AND METHODS

Fungal cells. A strain of *C. albicans* originally isolated from a patient (9) was grown as blastoconidia in Sabouraud dextrose broth at either 25 or 37°C and subcultured every 24 to 48 h. The yeast cells used in the following assays were inoculated at 5×10^6 cells per ml into 50 ml of fresh medium and incubated at 37°C for 2 to 3 h unless otherwise noted. The cells were washed twice in Krebs-Ringer phosphate-buffered saline (pH 7.2) with 3.0 mg of glucose per ml (KRPG) and suspended in an appropriate medium for assay.

PMN-enriched spleen cells. Murine PMN were obtained from DBA/2 female mice as previously reported (5). Briefly, mice were injected intraperitoneally with complete Freund adjuvant H37RA (Difco Laboratories, Detroit, Mich.), and 3 to 5 days later a spleen cell suspension was prepared from these animals. Cells were treated with iron carbonyl to remove macrophages and put on a two-step Ficoll-Hypaque gradient. Cells at the first interface from the bottom were recovered and washed twice in KRPG before being used. Cell viability, determined by trypan blue dye exclusion, was greater than 99%, and the PMN concentration ranged from 35 to 45%, with less than 5% contamination by macrophages.

Assays for oxidative metabolism. The oxidative metabolism of PMN and fungi was measured with standard assays. Hexose monophosphate shunt (HMPS) activity in the PMN was determined by measuring $^{14}CO_2$ production by cells incubated with D-[1-^{14}C]glucose (16). Fungal cells or PMN were incubated in 15- by 85-mm culture tubes containing 1.0 ml of KRPG with 1.0 μCi of D-[1-^{14}C]glucose (specific activity, 7.7 mCi/mmol; New England Nuclear Corp., Boston, Mass.). Each tube was sealed with a serum stopper holding a center well, and the cultures were incubated at 37°C for 1 h. Afterwards, 0.2 ml of 2 N H_2SO_4 was added, and $^{14}CO_2$ was collected over 60 min on filter paper saturated with 1.0 N NaOH. The contents of the center well were mixed with 10 ml of Aquasol (New England Nuclear), the vials were refrigerated overnight to reduce chemiluminescence, and the radioactivity was measured in a Packard Tricarb liquid scintillation counter.

Superoxide anion (O_2^-) production was determined by measuring the reduction of cytochrome *c* (1) in the presence and absence of superoxide dismutase (SOD). Fungal cells or PMN were incubated in 15- by 85-mm siliconized glass tubes containing 0.8 ml of KRPG for 15 min at 37°C. After that time, 500 μg of cytochrome *c* (type III; Sigma Chemical Co., St. Louis, Mo.) in 0.1 ml of KRPG and either 0.1 ml of KRPG or 300 U of SOD (Sigma) in 0.1 ml of medium were added to each tube. Cultures were incubated for an additional 45 min at 37°C, and the optical density at 550 nm of each culture supernatant was determined with a Beckman DU8 spectrophotometer. Concentrations of O_2^- were calculated by subtracting the mean absorbance of culture supernatant with SOD from those values without SOD and dividing the difference by the millimolar extinction coefficient for cytochrome *c* (23 mM cm^{-1}).

To detect H_2O_2 release, we employed three assays: iodide fixation (11), scopoletin oxidation (14), and *o*-dianisidine oxidation (15). In assays of iodide fixation, cells were incubated in siliconized 15- by 85-mm glass tubes with 1.0 ml of KRPG, 2.0 U of lactoperoxidase (LP) (Sigma), and 1.0 μCi of Na^{125}I (specific activity, 13 to 17 mCi/μg; Amersham Corp., Arlington Heights, Ill.). Control tubes lacked cells or LP. The cultures were incubated at 37°C for 1 h, and reactions were terminated by adding 0.2 ml of 0.01 M sodium thiosulfate and 3.0 ml of 10% trichloroacetic acid. Precipitates were sedimentated by centrifugation and washed three times with 3 ml of 10% trichloroacetic acid. Radioactivity was measured in a Packard autogamma counter. In assays of scopoletin oxidation, cells were incubated in 15- by 85-mm culture tubes with 1.0 ml of KRPG, 2.0 U of LP, and 2.0 μg of scopoletin (Sigma). Control tubes lacked LP. Cultures were incubated at 37°C for 1 h and centrifuged, and 0.1 ml of supernatant was added to 1.9 ml of water with 10^{-3} M cyanide. Fluorescence was measured with an FOCI A-4 fluorometer with a 7-39 primary filter and a 3-72 secondary filter. To determine the amount of H_2O_2 produced, we compared these results with a standard curve obtained with predetermined concentrations of H_2O_2 in lieu of viable cells. In assays of *o*-dianisidine oxidation, cells were incubated in 15- by 85-mm culture tubes with 1.0 ml of KRPG for 1 h at 37°C. The tubes were centrifuged, and 0.8 ml of supernatant was recovered and mixed with 1.0 U of LP and 100 μg of *o*-diansidine in 0.2 ml of KRPG. The optical density at 460 nm was measured spectrophotometrically, and the amount of H_2O_2 in each culture was determined from a standard curve.

RESULTS

Measurement of HMPS activity. During an oxidative metabolic burst, PMN metabolize glucose through the HMPS, which can be assessed by measuring their production by $^{14}CO_2$ from [1-^{14}C]glucose. For instance, in our assays PMN released (mean ± standard error of the mean) 1,109 ± 122 cpm when incubated alone, but in the presence of 100 μg of zymosan they released 9,670 ± 953 cpm. The $^{14}CO_2$ released by PMN incubated with viable blastoconidia increased from 23,198 ± 293 to 104,097 ± 7,607 cpm as the number of fungi increased from 10^5 to 10^7 cells (Fig. 1). However, when comparable numbers of viable blastoconidia were incubated alone, $^{14}CO_2$ release increased from 31,453 ± 1,288 to 156,908 ± 14,477 cpm. Because blastoconidia alone released 2 to 10 times more $^{14}CO_2$ than did PMN activated with zymosan, we could not determine the contribution made by each cell type when they were incubated together. Moreover, $^{14}CO_2$ release was lower in cultures containing both PMN and fungi than in cultures containing fungi alone.

O_2^- release by PMN and blastoconidia. As an alternate means of detecting PMN activation by blastoconidia, we measured O_2^- release by quantifying the reduction of cytochrome *c* in the presence and absence of SOD. Spleen cells enriched for PMN released 0.2 nmol of O_2^- when incubated alone and 4.7 ± 0.4 nmol of O_2^- when activated with 100 μg of zymosan; these values were in close agreement with earlier observations (5). PMN stimulated with 3×10^5 viable blastoconidia generated 1.7 nmol of O_2^-, but only 0.9 nmol when stimulated with 10^7 blastoconidia. However, blastoconidia alone had significant activity in the assay, since 3×10^5 yeast cells released 1.1 nmol and 3×10^6 and 10^7 cells released about 1.7 nmol of O_2^- (Fig. 2). Once again, the oxidative metabolism of the fungus obscured determinations of PMN metabolism; in fact, these data suggest that little

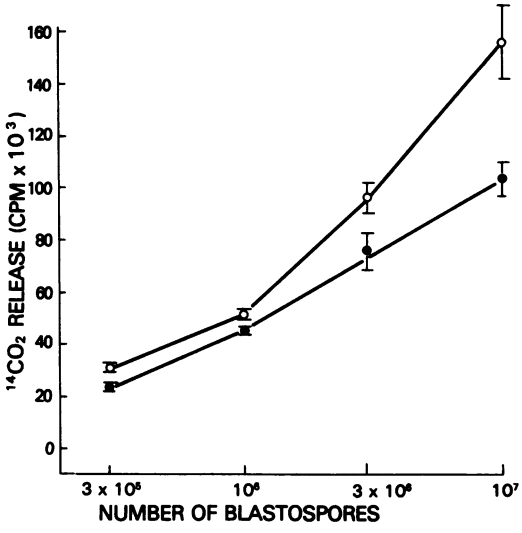

FIG. 1. Release of $^{14}CO_2$ by increasing numbers of *C. albicans* blastoconidia (○) or blastoconidia with 2 × 10^6 PMN-enriched spleen cells (●) incubated with 1.0 μCi of [1-^{14}C]glucose for 1 h at 37°C. Each point represents the geometric mean of six values from duplicate experiments. Bars represent the standard error of the mean.

difference exists in the amounts of O_2^- generated whether blastoconidia are incubated alone or with PMN.

Iodination by viable blastoconidia. The fact that viable blastoconidia released O_2^- gave us reason to hypothesize that they would also release H_2O_2 and fix iodide in the presence of a suitable peroxidase. ^{125}I fixation by PMN incubated with various numbers of fungi and by fungi incubated with LP were compared (Fig. 3). Iodination by PMN (mean ± standard error of the mean) increased from 14,329 ± 1,805 cpm with 3 × 10^5 blastoconidia to 46,728 ± 6,352 cpm with 3 × 10^6 yeast cells, but declined dramatically to 8,408 ± 1,491 cpm with 10^7 fungi. Yeast cells incubated with LP had a similar pattern of iodination, i.e., 3 × 10^5 cells fixed 43,021 ± 7,485 cpm, and 10^6 and 3 × 10^6 cells fixed about 60,000 cpm, but 10^7 cells fixed only 20,662 ± 3,495 cpm.

To determine whether iodination in the presence of LP was the result of H_2O_2 release by blastoconidia, we measured the cell-bound activity when yeast cells were incubated under various conditions (Table 1). Iodination did not occur in cultures incubated at 25°C or in those lacking glucose or LP. The addition of catalase at 3,000 or 10,000 U/ml inhibited iodination in a dose-dependent fashion, and the metabolic inhibitors antimycin A and rotenone also inhibited iodination. Antimycin A and rotenone, however, did not inhibit iodination of blastoconidia when exogenous H_2O_2 was added to the cultures. We also examined the effects of hexose on iodination, since we had previously reported that mannose inhibits iodination by PMN incubated with viable blastoconidia (5). Adding 3.0 mg of glucose or mannose to KRPG decreased iodination by blastoconidia 30 to 40%, whereas 2-deoxyglucose at a similar concentration reduced iodination by 78%.

In the studies described above, blastoconidia were recovered from cultures grown at 25°C and incubated at 37°C for 2 h. To determine whether H_2O_2 release occurred with blastoconidia at any age, we measured iodide fixation by cells grown in Sabouraud broth for various times at 37°C. Because fungi transferred to 37°C tended to produce filaments, iodination was quantified as counts per minute per microgram of fungal cell protein, and serial threefold dilutions of cells were assayed to compensate for the inhibition we observed in assay tubes with more than 3 × 10^6 cells per ml. Blastoconidia removed from overnight cultures at 25°C and put in KRPG at 37°C failed to show iodination. However, after a 2-h incubation at 37°C in fresh medium, cells at low concentrations (less than 1 μg of protein per ml) fixed 55,000 to 60,000 cpm/μg of protein, and at higher concentrations (greater than 1 μg of protein per ml) iodination declined in a dose-dependent fashion. A similar pattern of iodination by cells incubated in fresh medium for 4 h was observed. However, peak iodination was only 20,000 cpm/μg, and after a 6-h incubation at 37°C the cells failed to fix iodide regardless of the cell concentration (Fig. 4).

Because we had transferred the cells after overnight culture at 25°C to fresh medium at 37°C, we hypothesized that metabolic activity in

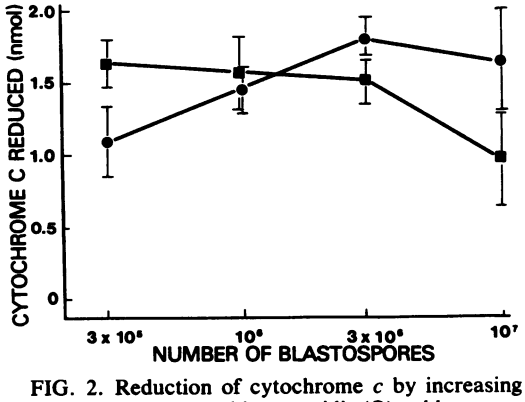

FIG. 2. Reduction of cytochrome *c* by increasing numbers of *C. albicans* blastoconidia (●) or blastoconidia with 2 × 10^6 PMN-enriched spleen cells (■) incubated for 1 h at 37°C. Each point represents the geometric mean of nine values from triplicate experiments. Bars represent the standard error of the mean.

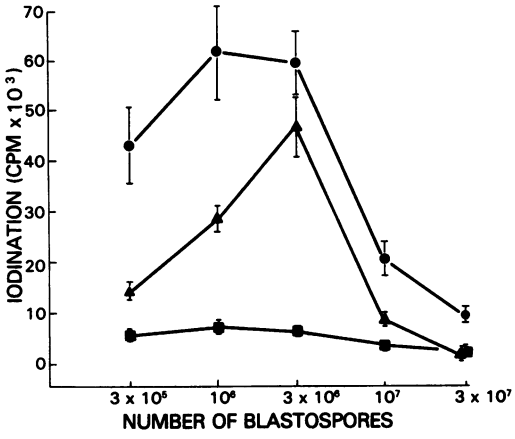

FIG. 3. Iodide fixation by increasing numbers of *C. albicans* blastoconidia incubated alone (■), with 2 × 10^6 PMN-enriched spleen cells (▲), or with 2.0 U of LP (●). Each point represents the geometric mean of 12 values from quadruplicate experiments. Bars represent the standard error of the mean.

the fungi might have increased dramatically, accounting for the transient release of H_2O_2. Therefore, we cultured blastoconidia at 37°C, transferred them to fresh medium at 37°C, and measured iodination at various times after the transfer. The pattern of iodination observed in these blastoconidia was identical to that observed in cells from cultures grown at 25°C, i.e., they failed to fix iodide; after 2 h, cultures with 1 μg of fungal cell protein fixed 50,000 to 60,000 cpm/μg, and older cells fixed progressively less iodide (data not shown). These findings indicate that H_2O_2 release did not occur throughout the cell cycle but appeared to be limited to early log-phase growth.

Examination of H_2O_2 release by other assays. Although iodide fixation is related directly to H_2O_2 concentration, this assay is not suitable for quantitating H_2O_2 release because the number of sites for iodide fixation is not constant. To eliminate this variable and to quantitate H_2O_2 release, we used two assays that measure H_2O_2 concentration through oxidation of the exogenous substrates scopoletin and *o*-dianisidine. Data from the scopoletin assay showed that 3 × 10^5 and 10^6 blastoconidia released 0.5 nmol of H_2O_2 after 1 h at 37°C. However, we were not able to use this assay to measure H_2O_2 in cultures with more than 10^6 blastoconidia since there was a significant reduction in fluorescence in cultures lacking LP (9). When *o*-dianisidine was used in lieu of scopoletin, we found that it was not oxidized by supernatant from tubes containing 3 × 10^5 to 10^7 blastoconidia unless LP was present. The greatest amount of oxidation (optical density at 460 nm, 0.0069 ± 0.0003) was observed with supernatant from tubes containing 10^6 blastoconidia, and this activity was reduced 90% when fungi were incubated with 100 U of catalase. Using a standard curve, we determined the amount of H_2O_2 in cultures with increased numbers of blastoconidia. Supernatants from cultures with 3 × 10^5 and 10^6 fungal cells contained (mean ± standard error of the mean) 0.51 ± 0.03 and 0.67 ± 0.03 nmol of H_2O_2, respectively (Fig. 5). However, the optical density at 460 nm was significantly lower for the media in which 3 × 10^6 and 10^7 cells were incubated, and we calculated that only 0.29 ± 0.2 and 0.13 ± 0.03 nmol of H_2O_2, respectively, was present. Results from this assay and the scopoletin assay agreed that 3 × 10^5 and 10^6 blastoconidia produced 0.5 to 0.7 nmol of H_2O_2 after 1 h at 37°C. Moreover, in cultures with greater numbers of yeast cells, results from the *o*-dianisidine assay showed that less H_2O_2 is available for reaction.

DISCUSSION

In studying the interaction between phagocytic cells and *C. albicans*, we and others (5, 6) have reported changes in the oxidative metabolism of PMN that are consistent with activation of the oxidative metabolic burst. However, in these studies there was little or no consideration given to the contribution made by the fungi. The results of studies reported in this communication indicate that *C. albicans* cell metabolism is significant and definitely contributes to the results observed in a number of assays.

Initially, we measured HMPS activity in PMN and showed that *C. albicans* cells generated $^{14}CO_2$ from [1-^{14}C]glucose as do PMN activated with zymosan. On a per-cell basis, blastoconidia generated more activity than PMN, since 3 × 10^5 blastoconidia released (mean ± standard error of the mean) 31,453 ± 1,288 cpm from [1-

TABLE 1. Effect of various treatments on iodination by *C. albicans* blastoconidia in vitro

Treatment	% Reduction in iodination from control[a] (mean ± SEM)[b]
Cells incubated (25°C)	87 ± 7
LP deleted	98 ± 0
Glucose deleted	86 ± 1
Catalase added (3,000 U)	59 ± 3
Catalase added (10,000 U)	89 ± 1
Antimycin A added (18 μM)	79 ± 3
Rotenone added (10 μM)	83 ± 2
2-Deoxyglucose added (3 mg)	78 ± 5
Glucose added (3 mg)	43 ± 6

[a] In treated controls, 10^6 blastoconidia were incubated in 1.0 ml of KRPG with 2.0 U of LP and 1.0 μCi of Na^{125}I for 1 h at 37°C.

[b] Data represent at least six values from duplicate experiments.

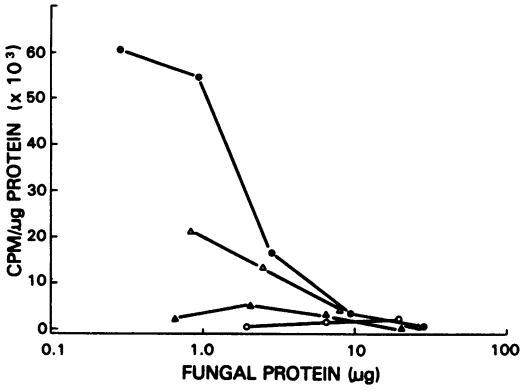

FIG. 4. Iodide fixation by increasing numbers of *C. albicans* cells transferred from overnight cultures at 25°C and incubated in Sabouraud broth at 37°C for 0 (○), 2 (●), 4 (△), or 6 h (▲) before being used in the iodination assay. Each point represents the geometric mean of six values from duplicate experiments.

^{14}C]glucose, whereas 2×10^6 activated spleen cells containing about 10^6 PMN released only 9,670 ± 953 cpm. When PMN were incubated with various numbers of yeast cells, the $^{14}CO_2$ release was about 30% less than in cultures with fungi alone, suggesting that PMN may inhibit blastospore metabolism. However, the extent of this inhibition could not be determined from our test results.

Chattaway et al. (3) reported significant HMPS activity in blastoconidia grown at 30°C, and noted that the HMPS is a major source of NADPH that could be used by protein disulfide reductase to promote budding. Our data suggest that this metabolic pathway may be very active in blastoconidia grown at 37°C, and the reducing power generated by HMPS activity also may be spent in generating O_2^-. The amount of O_2^- released by yeast cells alone was comparable to that released by PMN plus blastoconidia, so this assay was not suitable for measuring PMN activation by viable fungi. However, these results suggest that H_2O_2 was also being generated extracellularly by fungi, since O_2^- spontaneously dismutates to H_2O_2. Using an iodination assay to measure H_2O_2 release, we found that blastoconidia incubated with $Na^{125}I$ and LP fixed iodide in a manner similar to PMN incubated with viable blastoconidia, i.e., bound radioactivity was highest in cultures with 10^6 to 3×10^6 yeast cells and declined dramatically in cultures with 10^7 cells.

That iodination did not occur in the absence of LP and was significantly reduced by catalase indicate that iodide fixation was mediated by the H_2O_2 released by the blastoconidia. Generation of H_2O_2 within the mitochondria of baker's yeast (*Saccharomyces cerevisiae*) has been reported by Boveris (2). He postulated that O_2^- generated from ubisemiquinone is dismutated to H_2O_2 by mitochondrial SOD in the intermembrane space. He noted, however, that H_2O_2 is probably neutralized by cytochrome *c* peroxidase, since H_2O_2 diffusion from the mitochondria to the yeast cytosol could result in cell injury or death. Our results showed that H_2O_2 or O_2^- was generated in the mitochondria of the blastoconidia, since iodide fixation by blastoconidia was inhibited by antimycin A and rotenone, compounds that block the mitochondrial respiratory chain. However, it is not clear why these reactive oxygen species did not cause cell injury. We have observed that the mitochondria in yeast cells are localized next to the cell membrane (unpublished results), which would reduce the intracellular distance through which these potentially cytotoxic oxygen species migrate.

Eremina and Lozinov (7) used an *o*-dianisidine assay to measure H_2O_2 in filtrates from cultures of *C. albicans*. They reported that the blastoconidia released H_2O_2 throughout log-phase growth and that resting cells inoculated into Tris-phosphate buffer produced a maximum concentration of H_2O_2 of 20 nmol/ml after 30 min. These results differed from our findings that H_2O_2 release by blastoconidia was greatly reduced in the absence of glucose and that 10^6 cells released 0.5 to 0.7 nmol of H_2O_2 after 1.0 h at 37°C. Of interest, however, is our observation that H_2O_2 was released by cells during early log-phase growth, a time during which many blastoconidia are forming germ tubes. Evans et al. (8)

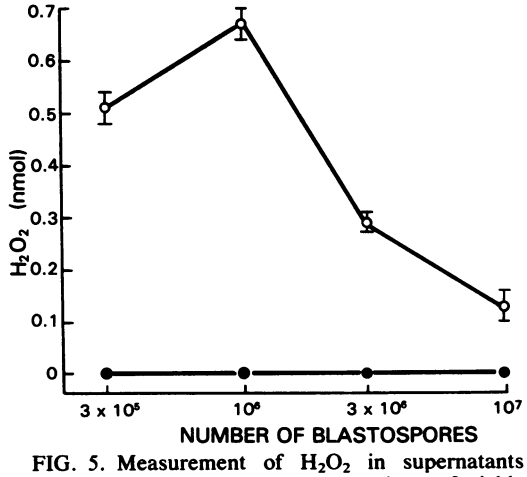

FIG. 5. Measurement of H_2O_2 in supernatants from cultures containing various numbers of viable blastoconidia as determined by the oxidation of *o*-dianisidine in the presence (○) or absence (●) of 1.0 U of LP. Each point represents the geometric mean of six values from duplicate experiments. Bars represent the standard error of the mean.

reported that blastoconidia transferred to fresh Sabouraud medium at 37°C initiated filamentation and that filamentation peaked within 1.5 to 2.5 h. After this time, reproduction occurred by lateral budding, and the blastoconidia formed did not produce germ tubes. There appears to be a correlation between filamentation and maximal H_2O_2 release per microgram of cell protein; however, we do not know whether H_2O_2 release occurs in all cells or exclusively in cells forming germ tubes.

The process of filamentation has been associated with the pathogenicity of *C. albicans* since the physical extrusion of a germ tube permits the extension of the fungus into tissue and hinders its containment by phagocytic cells. The release of reactive oxygen species by *C. albicans* cells also could serve to augment invasion, since O_2^- and H_2O_2 are toxic to mammalian cells as well as to microorganisms (12). However, the release of H_2O_2 by fungi also could enhance their killing by phagocytic cells. We observed that PMN incubated with 10^6 viable blastoconidia fixed four times more radioiodide than did PMN incubated with comparable numbers of killed fungi (5). This difference could indicate that PMN are stimulated more by viable organisms than by killed organisms (6). However, we postulate that viable blastoconidia release H_2O_2, which reacts with PMN myeloperoxidase to fix halide. We did not determine whether iodination by blastoconidia affects cell function, but it may be significant that iodination is suppressed when the fungal cell concentration is greater than 3×10^6 blastoconidia per ml, whereas glucose metabolism and O_2^- release by the fungi are not. We suggest that blastoconidia may tolerate low concentrations of H_2O_2 but will inhibit levels that would be detrimental to cell function. If and how these functions are related to invasion by *C. albicans* remains to be determined; but we believe that reexamination of the oxidative metabolism in both fungi and phagocytes may lead to a better understanding of *Candida* pathogenicity.

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