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Rapid Killing of Monocytes In Vitro by *Candida albicans* Yeast Cells

DAVID L. DANLEY* AND JOSEPHINE POLAKOFF

Department of Clinical Investigation, Letterman Army Medical Center, Presidio of San Francisco, California 94129-6700

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To study the interaction between *Candida albicans* blastoconidia and human phagocytes, we incubated peripheral leukocytes with fungi for 1 h at 37°C and stained the cells with fluorescent vital stains ethidium bromide (EB) and fluorescein diacetate. Fungi that had been phagocytosed showed little staining; however, some leukocytes containing blastoconidia exhibited nuclear staining with EB, even though their cell membranes showed no signs of penetration by fungi. The number of EB-positive leukocytes was related to viability of the yeast cells and the temperature at which they were maintained before use. Because efforts to quantitate EB-positive leukocytes microscopically were frustrated by cell aggregation, we labeled the leukocytes with ^{51}Cr and measured isotope release. We determined that leukocytes incubated with viable fungi released significantly more isotope than cells incubated alone or with killed blastoconidia. Furthermore, ^{51}Cr release correlated directly with concentration of fungi in the assay, time of incubation, and temperature at which fungi were maintained before use. Using a number of isolates of *C. albicans* and several other species of *Candida*, we found that all exhibited cytotoxic activity against leukocytes, but the level of activity varied among organisms. Finally, we depleted or enriched peripheral leukocytes for specific cell populations and determined that only monocytes released more ^{51}Cr after incubation with viable blastoconidia. Blastoconidia can lyse phagocytic cells through germination and penetration of cell membranes within 1 to 2 h, but the cytotoxic phenomenon we describe occurs within 15 to 30 min after yeast cells have been phagocytosed. Therefore, this capacity may represent a more immediate response by blastoconidia against phagocytosis and killing by monocytes.

Polymorphonuclear leukocytes (PMN), monocytes, and macrophages play a central role in immunity to *Candida albicans* and related species. Results of clinical studies have shown that the incidence of candidiasis increases in patients with defective neutrophil function (11, 13) or defective cellular immunity (8, 26, 27). In patients with neutropenia, candidiasis may range in severity from colonization of mucocutaneous surfaces to systemic dissemination; while in patients with defective cellular immunity, the disease is more often limited to cutaneous and mucocutaneous surfaces. The fungicidal capabilities of phagocytic immunocytes has been elucidated through in vitro studies. In particular, Lehrer and co-workers demonstrated that blastoconidia are susceptible to both oxidative and nonoxidative microbicidal mechanisms of PMN and monocytes (12, 14–17).

Blastoconidia of *C. albicans* are not without some means of resistance to phagocyte activity. Louria et al. (19) and Stanley and Hurley (24) showed that PMN and macrophages are vulnerable to penetration by blastoconidia that germinate within the phagosome. Moreover, Diamond and co-workers (4, 5) found that PMN could damage *C. albicans* pseudohyphae in vitro, but they did not measure fungal cell killing. Therefore, the ability of blastoconidia to germinate may serve to lyse phagocytes that engulf the fungi, and in the hyphal form, the fungus may be more resistant to killing because it is more difficult to contain.

We have reported that blastoconidia grown at 37°C for 2 h release significant quantities of hydrogen peroxide (3). While studying the effects of H_2O_2 on mammalian and fungal cells, we observed that some leukocytes, which had phagocytosed blastoconidia, exhibited nuclear staining with ethidium bromide (EB), suggesting that there was damage to the cell membrane in the absence of germination by the yeasts. To determine if phagocytes were being lysed, they were labeled with ^{51}Cr, and cell disruption was measured by increased isotope release. Using this assay, we also found that monocytes are exquisitely sensitive to membrane disruption after viable blastoconidia are phagocytosed. We did not determine whether this finding has any significance to the pathogenicity of *C. albicans*; however, it may function, like germination, to prevent containment and killing of fungal cells by this population of leukocytes.

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MATERIALS AND METHODS

Fungal cells. For initial studies we used an isolate of *C. albicans* that was recovered from a patient and maintained in our laboratory for 6 years. Additional isolates were provided by David Stevens (Santa Clara Medical Center, Santa Clara, Calif.). All strains were grown as blastoconidia in Sabouraud dextrose broth at 25°C and subcultured every 24 or 48 h. Prior to use in cytotoxicity assays, blastoconidia were inoculated at 5×10^6 cells per ml into 50 ml of fresh medium and incubated at 37°C for 2 h unless otherwise noted. Cells were washed twice in Krebs-Ringer phosphate-buffered saline (pH 7.2) with 3.0 mg of glucose per ml (KRPG). Killed fungi were prepared by heating cells for 15 to 20 min in a boiling water bath and washing them three times in KRPG before use.

Isolation of target leukocyte populations. Blood was obtained by venipuncture from the forearms of healthy volunteers and collected in evacuated tubes containing either EDTA or heparin. Consent was obtained from participants under guidelines established by the Human Use Committee

* Corresponding author.

at Letterman Army Medical Center. Initially, leukocytes were isolated by dextran sedimentation. Three milliliters of 6.0% dextran (molecular weight, 70,000) was added to 10 ml of blood, the blood was allowed to sediment at 1 × *g* for 1 h, and leukocytes were recovered from the plasma. For experiments in which mononuclear cells and PMN were used, blood was diluted with an equal volume of phosphate-buffered saline and layered on a gradient with two densities of Ficoll-Hypaque (purchased as Histopaque 1077 and 1119 from Sigma Chemical Co., St. Louis, Mo.) according to the instructions of the manufacturer. Gradients were centrifuged at 700 × *g* for 30 min at 25°C, and the mononuclear cells were recovered at the interface of the plasma and the Ficoll-Hypaque, whereas the PMN were recovered at the interface of the two Ficoll-Hypaque layers. Cells were washed twice in KRPG and examined for viability and morphology before use. Cell viability by trypan blue dye exclusion was greater than 98%, and both cell populations exhibited less than 4% contamination by the other.

The mononuclear cell preparation was processed further to isolate monocytes and lymphocytes. Phagocytic cells were depleted from the mononuclear fraction by the procedure of Mishell and Mishell (20). Briefly, 2×10^7 to 3×10^7 mononuclear cells were loaded on a 16- by 160-mm Sephadex G-25 column, which was equilibrated with KRPG plus 10% fetal calf serum. After 5 min, cells were eluted with 5 ml of medium, washed, and examined for morphology. By staining for α-naphthyl acetate esterase (Sigma), we determined that the number of esterase-positive cells was reduced from about 20% of the mononuclear cell fraction to less than 3% by adherence to the Sephadex column. Cell viability by trypan blue dye exclusion was greater than 98%. Monocytes and lymphocytes were also isolated by the technique of Ulmer and Flad (25). Mononuclear cells were suspended in Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) that had been diluted to a density of 1.080 with Hanks balanced salt solution. Cells were layered under a discontinuous gradient containing five dilutions of Percoll (densities of 1.070, 1.068, 1.066, 1.064, and 1.062). Gradients were centrifuged at 390 × *g* for 30 min, and monocytes were recovered near the top, between density layers 1.062 and 1.064. Lymphocytes were distributed in more diffuse bands beneath the monocytes. Monocyte preparations, stained for α-naphthyl acetate esterase, contained 90 to 97% esterase-positive cells, whereas lymphocyte fractions contained only 1 to 2% positive cells. Cell viability by trypan blue dye exclusion was greater than 99%.

Evaluation of yeast cell-mediated cytotoxicity. To visualize adverse effects of blastoconidia on leukocytes, we used the procedure of Calich et al. (1). In this assay, 10^7 blastoconidia and 2×10^6 leukocytes were incubated for 1 h at 37°C in 0.5 ml of KRPG with 20% pooled human serum. One drop of the cell suspension was placed on a microscope slide and mixed with one drop of dye mixture containing 0.005% fluorescein diacetate and 0.01% EB. Cells were examined with a Leitz microscope (E. Leitz, Inc., Rockleigh, N.J.) configured for illumination of fluorescein by epifluorescence and phase contrast to visualize phagocytic cells containing blastoconidia. Rather than enumerating uptake of EB by yeast cells, we determined the percentage of EB-positive leukocytes by examining 100 leukocytes containing one or more yeast cells and calculating the percentage that had EB-positive nuclei. Three determinations were made for each experiment.

To measure yeast cell-mediated cytotoxicity, leukocytes were labeled with ^{51}Cr as follows. A total 2×10^7 to 5×10^7 cells were incubated for 30 min at 37°C in 0.5 ml of KRPG with 10% fetal calf serum and 100 μCi of ^{51}Cr (as sodium chromate; specific activity, 200 to 900 Ci/g; New England Nuclear Corp., Boston, Mass.). Leukocytes were washed three times in KRPG before use. To measure cytotoxicity, we prepared cultures in 12- by 75-mm glass tubes that contained 2×10^6 leukocytes, various numbers of blastoconidia, and 0.5 ml of KRPG with 20% pooled human serum. Cultures were incubated at 37°C; after 1 h they were centrifuged at 800 × *g*, and 0.25 ml of supernatant was recovered

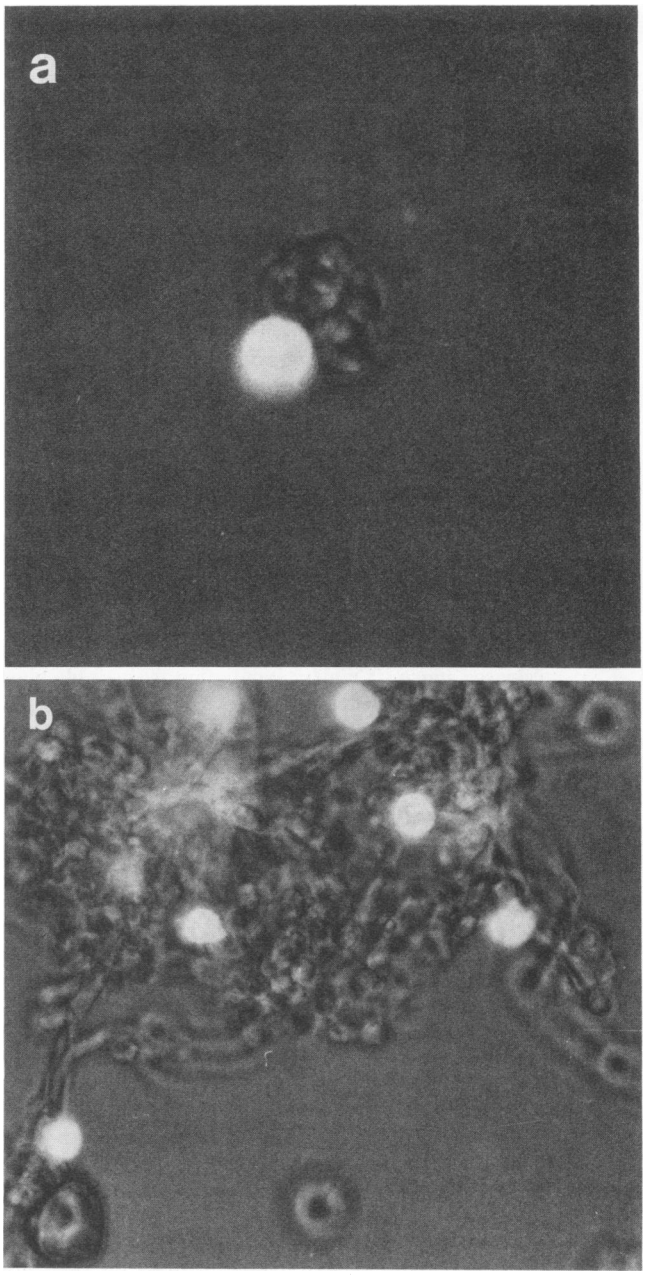

FIG. 1. Photographs taken through a light microscope configured for epifluorescence and phase contrast to illuminate phagocytosis of blastoconidia by leukocytes and nuclear staining with EB. (a) Phagocytic leukocyte contains numerous blastoconidia and has an EB-positive nucleus (×1,000). (b) Aggregation of fungi and leukocytes, with EB-positive nuclei of the latter being interspersed (×800).

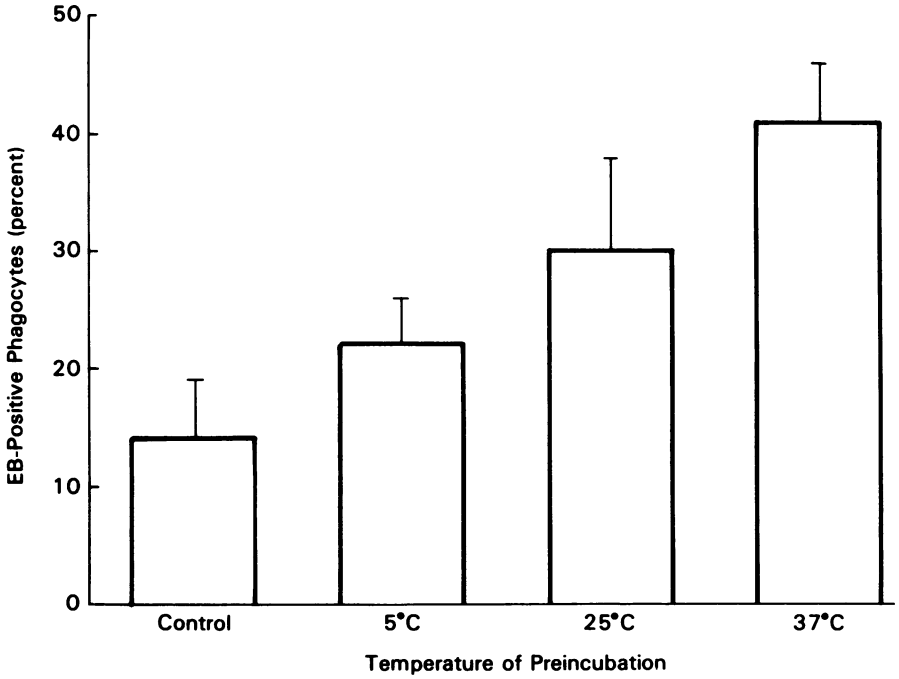

FIG. 2. EB staining of leukocytes that phagocytosed blastoconidia in vitro. For controls, leukocytes were incubated for 1 h at 37°C with killed blastoconidia, whereas in the other groups, leukocytes were incubated with viable yeast cells preincubated at 5, 25, or 37°C. Each value represents the arithmetic mean (± standard error of the mean) of average uptake obtained from triplicate observations in five experiments.

for counting in an autogamma scintillation counter. Total releasable counts were obtained by adding 2×10^6 cells to 0.5 ml of distilled water and subjecting the cells to three freeze-thaw cycles. After centrifugation, 0.25 ml of the supernatant was recovered for counting. Percent release was calculated by dividing the activity released by cells alone or with fungi (less background) by the total releasable counts and multiplying by 100. Control cultures with leukocytes alone or with killed blastoconidia were included in all experiments, and all cultures were run in triplicate.

Statistical analysis. Differences between groups were tested by the Student-Newman-Keuls multiple comparison test.

RESULTS

Evaluation of yeast cell-mediated cytotoxicity. Our initial aim was to study the effect that H_2O_2, produced by blastoconidia, has on the fungicidal capabilities of PMN. Blastoconidia were incubated in fresh Sabouraud medium for 2 h at 37°C to stimulate the production of H_2O_2, after which time they were incubated with leukocytes for 1 h at 37°C. We evaluated fungal cell viability by staining cells with EB and fluorscein diacetate and examining the preparations by fluorescence microscopy. We observed blastoconidia within phagocytes, but the majority of the fungi exhibited no fluorescence, and we could not determine their status.

During these studies, we noted that some of the leukocytes that had phagocytosed blastoconidia had EB-positive nuclei. Leukocytes with stained nuclei had good cellular integrity, and some of the cells stained positive with fluorescein diacetate. There was no evidence to suggest that nuclear staining was related to penetration of the leukocyte by germinating fungi (Fig. 1A); however, many positive nuclei were observed in aggregates with fungi (Fig. 1B), and rupture of leukocyte membranes by hyphae could not be ruled out. Phagocytosis of blastoconidia appeared to be a prerequisite of nuclear staining, since we rarely observed lymphocytes with EB-positive nuclei.

To determine what significance fungal cell viability and metabolism had for EB staining of leukocytes, we incubated leukocytes with heat-killed yeast cells or viable blastoconidia preincubated at 5°C overnight, at 25°C for 2 h, or at 37°C for 2 h. Significantly more leukocytes ($P < 0.05$) had EB-positive nuclei when incubated with viable blastoconidia, grown at 37°C, than when incubated with killed fungi (Fig. 2). Results with fungi grown at 5 or 25°C suggest that staining of phagocytes with EB is related not only to the viability of the fungi but also to the metabolic activity of the fungi.

The rapid uptake of EB in low concentration indicates that the cells have membrane damage (7) that could lead to cell lysis. We did observe a decline in the number of phagocytes containing blastoconidia after 2 h of incubation. However, this decline was accompanied by aggregation of luekocytes and fungi, which prevented us from quantitating leukocyte damage.

As an alternative, we labeled leukocytes with ^{51}Cr and measured isotope release. Results from this assay (Fig. 3) correlate well with our previous observations. Significantly more isotope was released ($P < 0.05$) from leukocytes incubated with 5×10^6 blastoconidia, grown at 37°C, than from cells incubated alone or cells with fungi grown at 5°C. Blastoconidia grown at 25°C caused more ^{51}Cr release than did yeasts maintained at 5°C but less than those grown at 37°C, indicating that leukocyte membrane disruption is dependent on yeast cell metabolism. Release of isotope also depended on the number of blastoconidia in culture with the leukocytes. Our data indicated that 5×10^6 yeast cells caused optimum damage, so this number was used in all subsequent assays.

The length of time that fungi and leukocytes were incubated together also affected the amount of ^{51}Cr released (Fig.

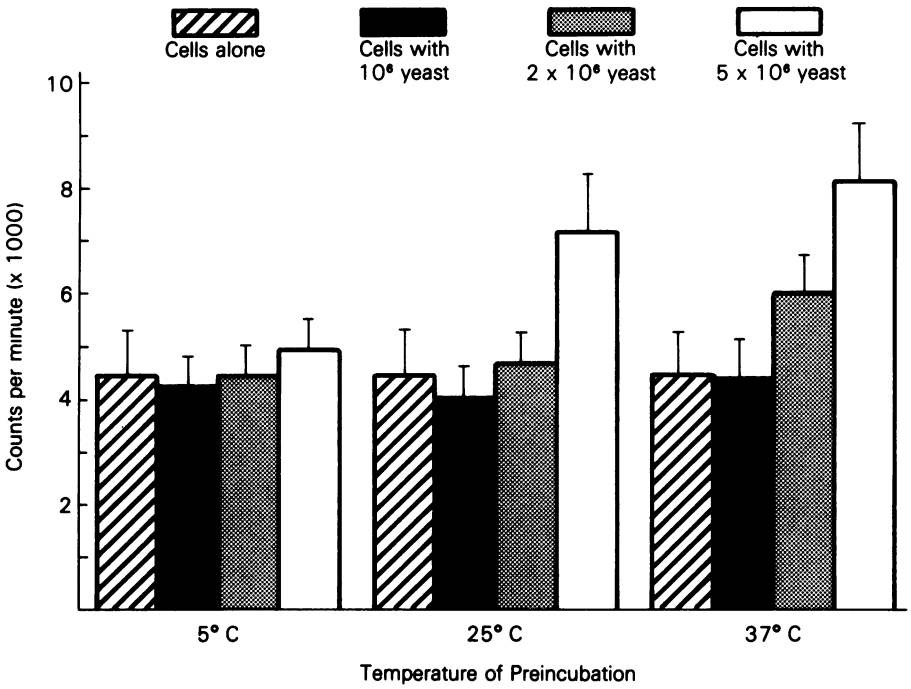

FIG. 3. ^{51}Cr release from 2×10^6 radiolabeled leukocytes incubated at 37°C for 1 h either alone or with various concentrations of blastoconidia that were grown at 5, 25, or 37°C. Each value represents the arithmetic mean (± standard error of the mean) of average isotope release in triplicate cultures from five experiments.

4). At 15 min there was no significant difference in the amount of isotope released by leukocytes alone or by cells incubated with blastoconidia. At 30 and 60 min, however, the amount of isotope released was significantly greater ($P < 0.05$) in cultures with leukocytes and viable blastoconidia, showing that cytotoxicity did not occur immediately but progressed substantially after 15 min.

To determine whether other isolates of *Candida* exhibit cytotoxicity against leukocytes, we tested six isolates of *C. albicans* which had been used by other investigators (18) to study cell metabolism and virulence, as well as isolates of *C. krusei* and *C. stellatoidea*. Results of this survey (Table 1) indicate that all isolates exhibited cytotoxicity against leukocytes; however, isolates 140/1 and H12 showed signifi-

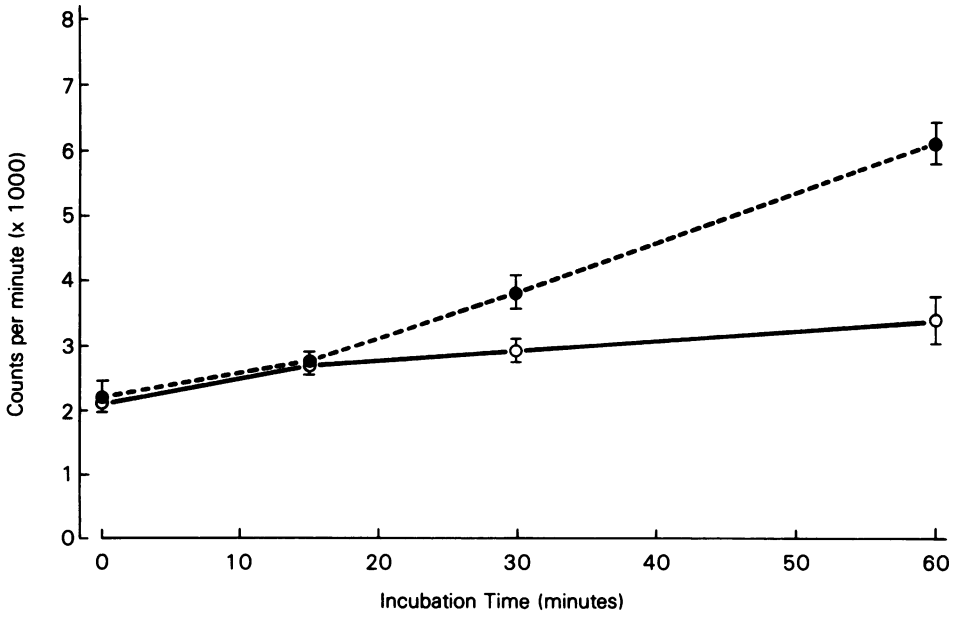

FIG. 4. ^{51}Cr release from 2×10^6 radiolabeled leukocytes incubated at 37°C for various times alone (○) or with 5×10^6 viable blastoconidia (●). Each point represents the arithmetic mean (± standard error of the mean) of average isotope release in triplicate cultures from three experiments.

TABLE 1. Percentage of isotope released by ^{51}Cr-labeled leukocytes incubated in vitro with isolates of *C. albicans* and other *Candida* species[a]

Leukocytes incubated with the following isolates[b]:	% ^{51}Cr released[c]
Alone	20 ± 3
C. albicans	29 ± 4
C. albicans Sh8	32 ± 4
C. albicans 610	28 ± 5
C. albicans 140/1	40 ± 5[d]
C. albicans 603	34 ± 7
C. albicans Sh27	28 ± 3
C. albicans H12	41 ± 4[d]
C. krusei	26 ± 3
C. stellatoidea	26 ± 2

[a] Arithmetic mean (± standard error of the mean) of average isotope release from five experiments with *Candida* isolates in triplicate cultures.

[b] Leukocytes (2×10^6) were incubated for 1 h at 37°C with 5×10^6 blastoconidia preincubated at 37°C for 2 h in fresh Sabouraud broth.

[c] Calculated by dividing counts released (less background) in the culture supernatant by the total releasable counts (less background) obtained by repeated freezing and thawing of 2×10^6 leukocytes.

[d] Significantly greater values ($P < 0.05$), as determined by Student-Newman-Keuls multiple comparison test.

cantly more ($P < 0.05$) cytotoxicity than the other isolates. Both 140/1 and H12 are reported to be highly virulent in mice (18), but other virulent isolates (610 and 603) showed cytotoxicity comparable to that of isolates with low virulence (Sh8 and Sh27). Isolate 140/1 showed little germination, whereas H12 germinated extensively when incubated with leukocytes. For subsequent studies, isolate 140/1 was used to reduce the effect of yeast cell germination on leukocyte lysis.

Identification of the target leukocyte population. Using fluorescence microscopy, we determined that phagocytic leukocytes are uniquely susceptible to yeast cell-mediated cytotoxicity. As an initial experiment to define the sensitive cell type(s) in the population, we separated peripheral leukocytes into PMN and mononuclear cell populations, labeled each population with ^{51}Cr, and incubated them with viable or killed blastoconidia (Fig. 5). No change in the amount of isotope released was observed when PMN were incubated alone, with killed yeast cells, or with viable blastoconidia, indicating that this population is not disrupted by viable fungi. However, mononuclear cells incubated with viable blastoconidia released about twice as much isotope as cells incubated alone or with killed fungi.

The mononuclear cell population was depleted of adherent cells by passing the cells through a Sephadex column. This procedure removed about 75% of the esterase-positive cells, and the percentage of ^{51}Cr released from mononuclear cells incubated with viable yeast cells was reduced from 20% to 10%, a level comparable with values from controls. To separate monocytes and lymphocytes within the mononuclear cell fraction, we used a discontinuous Percoll gradient. Monocytes isolated by this procedure were killed by viable blastoconidia, whereas lymphocytes were not (Fig. 6). In the three experiments done for this study, the amount of isotope released from monocytes incubated with viable blastoconidia ranged from 40 to 90% of the total amount releasable, which was three times the amount released in the presence of killed yeast cells. These results substantiate our microscopic observations and show that monocytes are uniquely sensitive to damage by viable blastoconidia of *C. albicans*.

DISCUSSION

The capacity of *C. albicans* to damage mammalian cells has been studied largely in terms of their ability to germinate

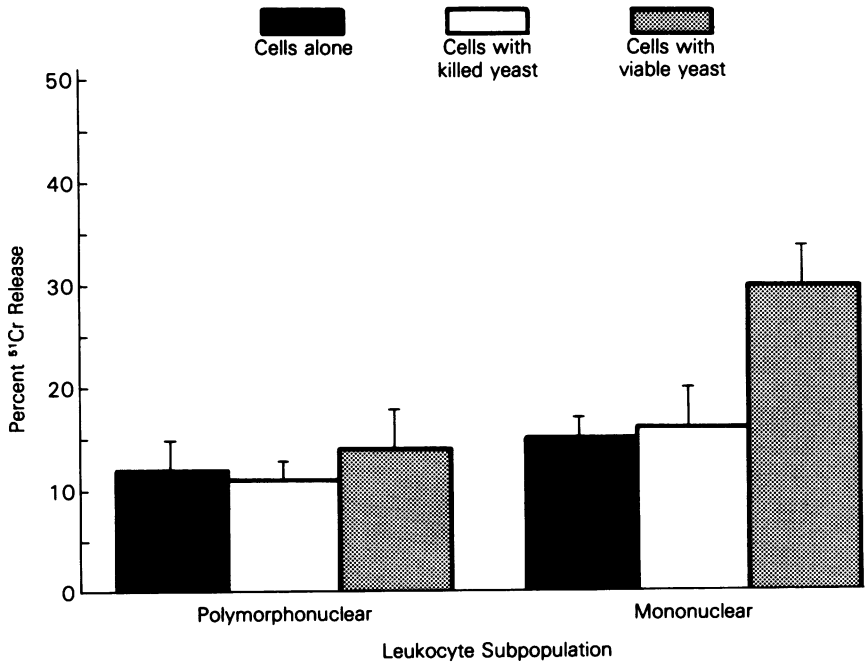

FIG. 5. ^{51}Cr release as a percentage of maximum isotope released from 2×10^6 PMN or mononuclear leukocytes incubated for 1 h at 37°C alone, with 5×10^6 killed blastoconidia, or with 5×10^6 viable blastoconidia. Each value represents the arithmetic mean (± standard error of the mean) of average isotope release in triplicate cultures from five experiments.

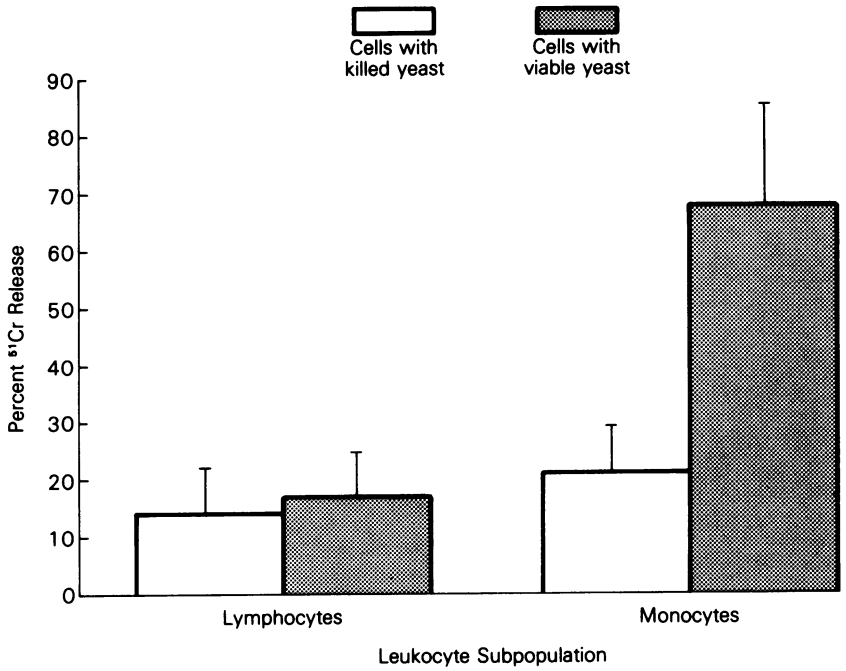

FIG. 6. ^{51}Cr release as a percentage of maximum isotope release from 2×10^6 lymphocytes or monocytes incubated at 37°C for 1 h with 5×10^6 killed or viable blastoconidia. Each value represents the arithmetic mean (± standard error of the mean) of average isotope release in triplicate cultures from three experiments.

and rupture cell membranes. The first investigators to use in vitro culture for studying interactions between blastoconidia and phagocytes recognized that macrophages and PMN that engulf blastoconidia are in jeopardy of lysis if the fungi germinate (19, 24). These observations bolstered the concept that the dimorphic nature of *C. albicans* is responsible for its pathogenicity, although, as pointed out by Odds (21): ". . . it would be incorrect to suggest that mycelial and blastospore forms of *C. albicans* are respectively pathogenic and nonpathogenic, or invasive and non-invasive."

In our assays, germinating blastoconidia probably contributed to monocyte lysis; however, results of studies with fluorescence microscopy provide consistent evidence that most monocytes exhibit changes in membrane permeability after phagocytosing blastoconidia but before fungal cell germination. Diamond and Haudenschild (4) suggested that monocytes are uniquely susceptible to disruption by *C. albicans*. Using electron microscopy to study monocytes and hyphal forms in vitro, they observed monocytes, attached to hyphae, with swollen and disrupted membranes and hyphae with surface-adherent vesicles that they postulated came from destroyed monocytes. They noted that PMN examined under the same conditions do not exhibit these anomalies. More recently, Klotz et al. (9), using an in vitro procedure, reported that blastoconidia can transverse vascular epithelium in the absence of germ tube formation. Their electron micrographs show that yeast cells destroy endothelial cells and penetrate tissue without mechanical extension. None of these investigators explored the means by which blastoconidia cause cell disruption; however, Klotz et al. (9) postulated that enzyme secretion by yeast cells is responsible, and *Candida* species have been reported to release enzymes that degrade protein and lipid (21).

At present, we do not know how blastoconidia cause monocyte lysis. Results of our studies show that cytotoxicity is dependent on yeast cell viability and the temperature at which the fungi are grown. In other studies we have found that cytotoxicity declined when blastoconidia were treated with amphotericin B, a drug that inhibits fungal metabolism in low concentrations (10). Therefore, we believe that cytotoxicity is related to the metabolic activity of the blastoconidia, but we do not know whether cytotoxicity is mediated by a product of protein synthesis, such as a catabolic enzyme, or a product of oxidative metabolism, such as H_2O_2.

In addition, it is not clear why monocytes are uniquely susceptible to yeast cell-mediated cytotoxicity. Diamond and Haudenschild (4) noted that monocytes must be in contact with *Candida* to exhibit swelling and membrane disruption; therefore, adherence of monocytes to fungi may be a requirement for cytotoxicity. However, PMN, which avidly phagocytose blastoconidia, are resistant to this cytotoxic phenomenon, suggesting that monocytes are deficient in their ability to cope with this fungus or its products. Cutler and Thompson (2) reported that murine PMN kill blastoconidia in vitro, whereas resident or elicited macrophages do not. Sasada and Johnston (22) observed that activated murine macrophages kill blastoconidia two to three times more efficiently than resident macrophages. These observations raise the possibility that monocytes are more vulnerable to cytotoxicity; hence, they are less candidicidal than PMN. However, in an activated state monocytes may behave more like PMN, exhibiting less vulnerability and more fungicidal activity. Studies addressing this possibility are currently in progress.

In considering the differences in susceptibility of monocytes and PMN to yeast cell-mediated cytotoxicity, we were struck by the correlation of our observations with clinical findings that show that neutropenic patients are more susceptible to invasive forms of candidiasis than are patients who have a monocyte dysfunction. In a review on immunity to *C. albicans*, Domer and Carrow (6) concluded that PMN

appear to be critical to the nonspecific defense against this fungus; however, the role of monocytes and macrophages is less clear. At present, we do not know if the capacity of blastoconidia to lyse monocytes in vitro reflects a pathogenic mechanism that is significant in vivo, particularly in neutropenic patients. Results of our tests of various isolates of *Candida* show that there is no correlation between yeast cell-mediated cytotoxicity and virulence in mice. Yet, the ability to lyse cells biochemically produces results identical to those of a germinating hypha, i.e., penetration of host tissue or release from within a host phagocyte. Recently, Sobel et al. (23) reported on the role of germ tube formation in the pathogenesis of candidal vaginitis, and they concluded that the ability to produce hyphae ". . . appeared to be an important but nonessential virulence factor in the pathogenesis of candidal vaginitis." The fact that there may be a redundancy in the mechanisms by which *C. albicans* destroys cells and invades host tissue would thwart our efforts to identify a unique physiological factor that correlates with virulence.

The current model for immunity to *C. albicans* and related species is ambiguous because studies have failed to show that one form of immunity (innate versus specific, humoral versus cellular) is solely responsible for protection against invasion. Immunity to *Candida* species is more complex than immunity to other fungi (6), and this undoubtedly reflects an evolutionary history that permits this fungus to coexist within a variety of mammalian hosts.

The capacity of blastoconidia to lyse monocytes in vitro is subtle and may have no bearing on the relationship of these two cell types in vivo. However, better understanding of immunity to *C. albicans* may result from studying such subtle interactions and determining how, as a whole, they affect susceptibility or resistance to infection.

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