INFECTION AND IMMUNITY, Apr. 1994, p. 1489–1493
0019-9567/94/$04.00+0
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Vol. 62, No. 4

Phagocytosis of Medically Important Yeasts by Polymorphonuclear Leukocytes

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Received 5 October 1993/Returned for modification 28 November 1993/Accepted 11 January 1994

Phagocytosis is a critical function of polymorphonuclear leukocytes in the control of mycotic infections. By using a modified fluorescence quenching assay to distinguish between attached and ingested organisms, we determined the percent phagocytosis of several medically important yeasts. The percentages of phagocytosis of serum-opsonized *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, and *Torulopsis glabrata* were all comparable at 37°C. By comparison, there was significantly less phagocytosis of *Cryptococcus neoformans* and *Trichosporon beigelii* isolates ($P < 0.001$). Thus, phagocytosis of *C. albicans* by polymorphonuclear leukocytes is comparable to that of species other than *C. albicans* but is significantly greater than that of the basidiomycetous yeasts *T. beigelii* and *C. neoformans*.

The occurrence of mycotic infections has been rising rapidly with the progressive improvement in our ability to prolong survival of patients with global defects in their host defense mechanisms (31). It has been clearly demonstrated that phagocytes play a critical role in preventing fungal infections (7, 8, 10, 19, 21, 24). Thus, a better understanding of the host phagocyte-fungus interaction is important for continued progress in the successful management of mycoses in immunocompromised patients. We therefore sought to investigate neutrophil-mediated phagocytosis of several medically important fungi by means of a fluorescence quenching assay, which distinguishes between attached and ingested blastoconidia.

Peripheral blood polymorphonuclear leukocytes (PMNLs) were isolated from heparinized venous blood (10 U/ml) collected from healthy human donors. Granulocytes were separated by dextran sedimentation followed by Ficoll-Hypaque (lymphocyte separation medium; Organon Teknika Corp., Durham, N.C.) centrifugation and hypotonic lysis of contaminating erythrocytes (5). The PMNLs (>95% purity, >95% viability) were suspended in Ca^{2+}- and Mg^{2+}-free modified Hanks' balanced salts solution (MHBSS) and stored on ice until use. Prior to incubation with stimuli, the cells were equilibrated in Ca^{2+}- and Mg^{2+}-containing Hanks' balanced salts solution (HBSS).

All organisms used were clinical isolates obtained from patients with fungemia at the Warren Grant Magnuson Clinical Center, the National Institutes of Health. Identification of the isolates was made by using standard microbiological techniques (32). All isolates were stored in skim milk suspensions at −70°C. The organisms were grown in Emmon's modification of Sabouraud glucose broth in a gyratory water bath at 37°C for 18 h, centrifuged, and washed with 0.2 M phosphate-buffered saline (PBS; pH 7.4) prior to use. The concentrations of blastoconidia were adjusted by hemacytometer counts.

The original procedure described by Hed (12) has been modified so that it may be used with cells in suspension and with viable organisms. Labeling with fluorescein isothiocyanate (FITC) was performed in 0.2 M PBS (pH 8.0) containing 10^7 organisms per ml and 250 μM FITC. The labeling mixture was incubated in the dark for 30 min at room temperature (22 to 27°C) on an end-over-end rotator. FITC-labeled organisms were washed three times with PBS (pH 7.4) and resuspended to a concentration of 10^7/ml in HBSS. The viability of labeled organisms was assessed by plating serial 10-fold dilutions of the organisms onto Sabouraud glucose agar and counting CFU after a 24-h incubation at 37°C. The labeling procedure did not result in any loss in viability of any of the organisms tested (data not shown). The milder conditions employed did not compromise labeling efficiency and allowed for the use of viable organisms. Organisms were opsonized by incubation with 25 to 50% pooled human AB serum for 30 min at 37°C. They were washed one time with cold MHBSS and resuspended to a final concentration of 10^7/ml in HBSS. Organisms were maintained on ice until use.

Equal numbers of PMNLs and serum-opsonized FITC-labeled organisms were mixed in a 1-ml final volume and incubated on a rotator for 15 min at 37°C. Immediately after this incubation, the reaction mixtures were placed on ice to inhibit further phagocytosis. If results were not going to be immediately assessed, 1% paraformaldehyde was added to the reaction mixtures prior to placing them on ice.

Equal volumes of the phagocytic suspension and 0.2% trypan blue at pH 5.0 were mixed and examined with an incident-light fluorescence microscope (Carl Zeiss Instruments, Inc., Hanover, Md.) by alternating between fluorescence and bright-field microscopy. The percent phagocytosis was calculated by determining the percent PMNLs containing fluorescent organisms. The phagocytic index was a measure of the average number of yeast cells ingested by PMNLs that successfully phagocytosed organisms.

Statistical significance was determined by using an unpaired Student's *t* test. All comparisons were two sided, and a *P* value of <0.05 was considered significant.

Figure 1 is a composite photograph of the bright-field image and corresponding fluorescent image of *Candida albicans* without (A and B) and with (C and D) 0.2% trypan blue. As demonstrated, the organisms are efficiently labeled under these experimental conditions, and the fluorescence is effectively quenched by the trypan blue. When organisms are ingested (E and F), however, the fluorescence is not quenched,

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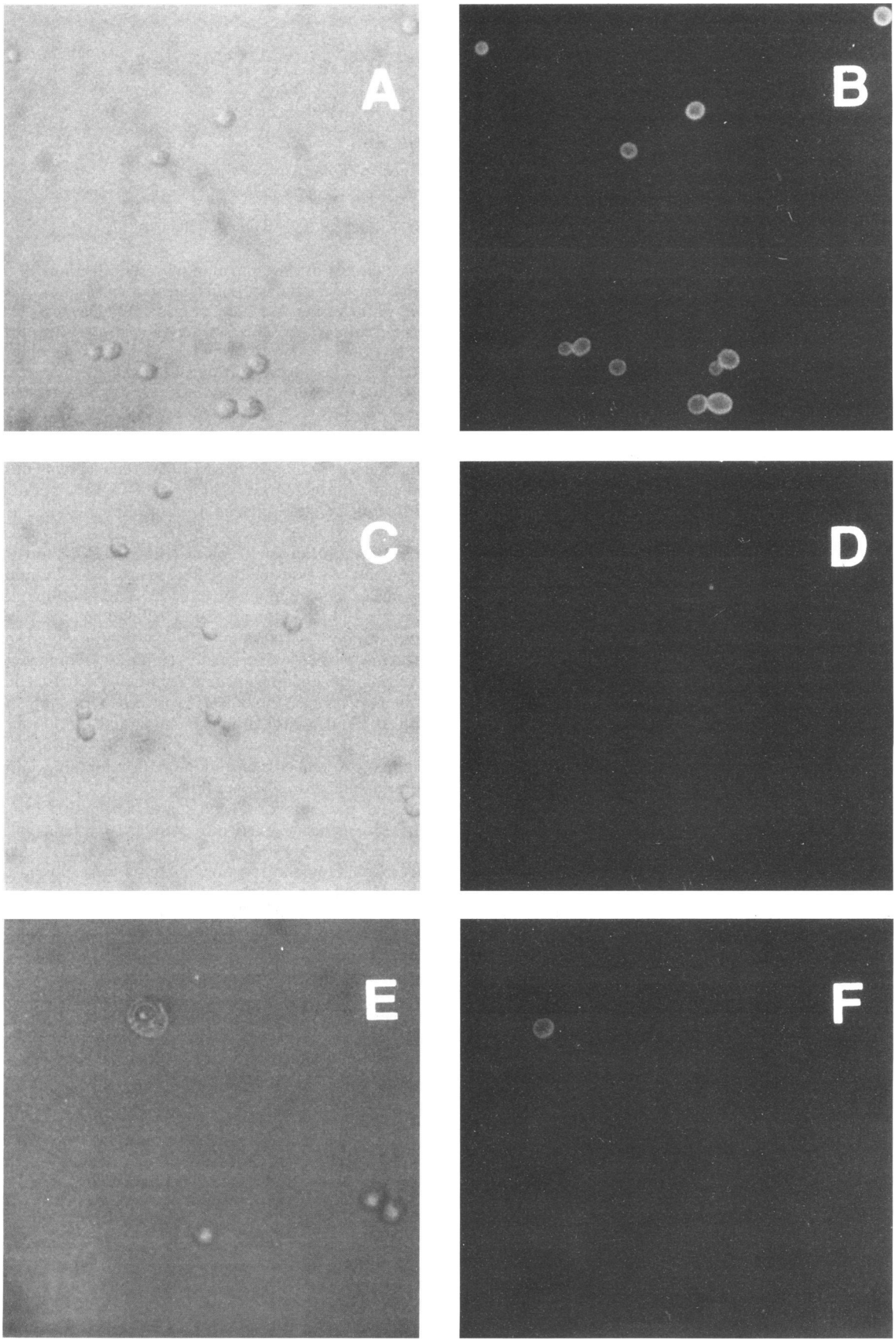
A
B
C
D
E
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FIG. 1. Bright-field (A and C) and corresponding fluorescent (B and D) images of *C. albicans* with (A and B) and without (C and D) 0.2% trypan blue. The bright-field (E) and corresponding fluorescent (F) images of *C. albicans* incubated with PMNLs, demonstrating the quenching of extracellular FITC-labeled organisms and fluorescence of intracellular FITC-labeled *C. albicans*, are also shown.

and thus, intracellular organisms may be effectively differentiated from extracellular organisms.

Figure 2 illustrates the comparative results of phagocytosis of blastoconidia at 37 and 4°C. Five *Candida* strains and five non-*Candida* strains were tested. The results are expressed as means ± standard errors from three to six experiments run in duplicate with PMNLs from different healthy donors. At 37°C, there was 50 to 60% phagocytosis of the two strains of *C. albicans* and *Candida parapsilosis*. There was slightly less but comparable phagocytosis of *Candida tropicalis* and *Torulopsis glabrata* (35 to 45%). The phagocytic index was comparable for all of these organisms, ranging from 1.60 to 2.05 (Table 1). In contrast, there was significantly less phagocytosis of the two strains of *Trichosporon beigelii* tested ($P < 0.001$). However, the phagocytic index for one strain of *T. beigelii* was similar to that of the *Candida* spp., suggesting that there may be subpopulations of PMNLs that are capable of phagocytosing some strains of *T. beigelii*. The resistance of *T. beigelii* to PMNL phagocytosis was similar to that observed with *Cryptococcus neoformans*, which also was poorly phagocytosed.

Phagocytosis is a temperature-dependent process (16). Incubation of phagocytes at 4°C inhibits receptor cycling, effectively inhibiting phagocytosis without altering receptor-mediated attachment. As shown in Fig. 2, at 4°C there was less than 5% phagocytosis of all isolates studied, confirming that organisms were internalized and not simply attached.

The modified fluorescence quenching assay presented in this study utilizes milder labeling conditions than those originally described (12). This allows for the use of viable rather than heat-killed or chemically killed organisms. Surface protein characteristics and natural resistance factors of different organisms may be altered or lost when the organism is dead, and these changes may profoundly affect the ability of phagocytes to attach to and ingest these organisms. Changes in surface hydrophobicity of *C. albicans* have been shown to affect virulence factors such as adherence to host epithelium and avoidance of neutrophil-killing mechanisms (11). Furthermore, conditions which do not alter the viability of the organisms allow them to be used simultaneously to measure other stimulus-induced events in host cells.

A number of phagocytic assays measure cell association without distinguishing between attached and ingested particles. Some methods capable of making this distinction utilize different treatments to destroy or release surface-attached particles. For example, lysostaphin has been used to digest noningested staphylococci (28), and surface-bound erythrocytes can be destroyed by osmotic lysis (1). This type of procedure, however, is very stimulus specific.

In the case of fungi, methylene blue has been used to distinguish between attached and ingested *C. albicans* (20). However, the organisms must be dead, and the assay is not readily applicable to non-*C. albicans* blastoconidia. Another method utilizes fluorescein-labeled conconavalin A for labeling extracellular *C. albicans* on monolayers of PMNLs (25). The fluorescent dye diaethanol, which binds chitin in the cell wall of fungi, provides a more broadly applicable procedure for distinguishing attached from ingested fungal organisms (22). Unfortunately, this dye was not readily available in the United States when these studies began. Other techniques used to differentiate between attached and ingested particles include electron microscopy and immunofluorescence with and without flow cytometry (4, 14, 26, 27).

In fluorescence quenching techniques, targets are prelabeled with a fluorochrome such as FITC. When the excitation energy is transferred from the fluorochrome to a membrane-impermeable quenching dye such as crystal violet or trypan blue, the internalized targets can be discerned from the total number of targets (13). Fluorescein is an inexpensive, readily available fluorochrome, and fluorescence microscopy is not difficult to perform. Nevertheless, some problems in using this technique for determining phagocytosis have been described. Bassoe et

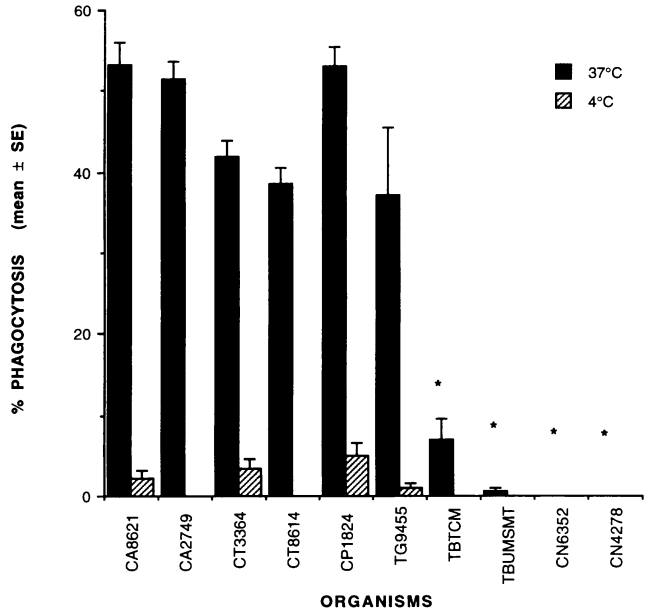

FIG. 2. Percentages of phagocytosis of blastoconidia incubated at 37 or 4°C. Results represent means ± standard errors (SE) of the means of from three to six separate experiments of neutrophils incubated with serum-opsonized *C. albicans* (CA), *C. tropicalis* (CT), *C. parapsilosis* (CP), *T. glabrata* (TG), *T. beigelii* (TB), or *C. neoformans* (CN). An asterisk indicates a *P* of <0.001.

TABLE 1. Phagocytic index[a]

Organism	Phagocytic index
C. albicans 8621	1.81 ± 0.08
C. albicans 2749	1.87 ± 0.07
C. tropicalis 3364	1.60 ± 0.07
C. tropicalis 8614	2.05 ± 0.14
C. parapsilosis 1824	2.00 ± 0.25
T. glabrata 9455	1.79 ± 0.07
T. beigelii TCM	2.03 ± 0.19
T. beigelii UMSMT	1.00 ± 0.02
C. neoformans 4278	0.00 ± 0.00
C. neoformans 6352	0.00 ± 0.00

[a] The phagocytic index equals the average number of yeast cells ingested by PMNLs that successfully phagocytosed organisms ± standard error.

al. (2) demonstrated a loss in fluorescence intensity of fluorescein-labeled targets upon acidification of the surrounding environment when measured by flow cytometry. However, Drevets and Campbell (9) demonstrated that this was not a problem with fluorescence microscopy and suggested that the human eye may not be sufficiently sensitive to detect the decrease in fluorescence intensity measured by flow cytometry. We occasionally detected a slight decrease in fluorescence intensity of fluorescein-labeled *Candida* species, which is most likely due to acidification of the phagosome. However, this decrease did not compromise the counting of internalized organisms and did not approach the magnitude of change in intensity elicited by the quenching agent.

This study revealed differences not previously elucidated in the percent phagocytosis of different yeast isolates. Two strains each of clinical isolates of *C. albicans* and *C. tropicalis* as well as one clinical strain each of *C. parapsilosis* and *T. glabrata* yielded similar susceptibilities to PMNL phagocytosis. While *C. albicans* remains the predominant organism causing fungemia in immunocompromised patients and nosocomial infections in nonimmunocompromised patients (3), the percent phagocytosis of this species was similar to that of species other than *C. albicans*. This correlates with clinical data reported by Komshian et al., who found that in a mixed population of oncological and surgical patients with fungemia caused by *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *T. glabrata*, mortality was not significantly affected by the *Candida* species (17).

These findings contrast with the resistance to PMNL phagocytosis of *T. beigelii* and *C. neoformans*. *T. beigelii* is an uncommon but increasingly recognized cause of disseminated fungal infection in immunocompromised hosts (15, 29). The majority of reported cases of disseminated *Trichosporon* infections have occurred in granulocytopenic or corticosteroid-treated patients. However, host defense mechanisms against the organism are not well understood. Phagocytosis of *T. beigelii* was significantly less than that of *Candida* spp. but was similar to that of *C. neoformans*. It has been shown that the polysaccharide capsule that surrounds *C. neoformans* is responsible for its resistance to phagocytosis (6, 18). *T. beigelii* is phylogenetically related to *C. neoformans* but is not thought to be an encapsulated organism. The mechanism of inhibition of phagocytosis by *T. beigelii* is not well understood. Perhaps the cell wall antigen of *T. beigelii* (23, 30), which is shared with *C. neoformans*, mediates this resistance to phagocytosis. Our results suggest that PMNLs alone may not efficiently control this organism since phagocytosis is significantly less than that which is seen with other opportunistic yeast-like fungi such as *Candida* spp. Further investigations of host defense mechanisms against *T. beigelii* are warranted.

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