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# Influence of Opsonization Conditions on C3 Deposition and Phagocyte Binding of Large- and Small-Capsule *Cryptococcus neoformans* Cells

THOMAS R. KOZEL,[1]* ABDULMONEIM TABUNI,[2] BRYAN J. YOUNG,[1] AND STUART M. LEVITZ[2]

*Department of Microbiology and the Cell and Molecular Biology Program, University of Nevada, Reno, Nevada 89557,[1] and Evans Memorial Department of Clinical Research and Department of Medicine, University Hospital, Boston University Medical Center, Boston, Massachusetts 02118[2]*

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**Previous studies demonstrated that, following opsonization with normal human serum (NHS), phagocytes bind greater numbers of small-capsule *Cryptococcus neoformans* cells than yeast cells with large capsules. The present study tested the hypothesis that suboptimal deposition of opsonic C3 fragments contributes to this disparity. *C. neoformans* was grown under conditions promoting large or small capsules and was incubated at various concentrations in NHS. At low concentrations of yeast cells (125 cells per μl of NHS), the deposition of C3 fragments per unit of capsule volume and the binding of yeast cells to cultured human monocytes were similar for yeast cells having large and small capsules. However, at higher cell concentrations, large-capsule cells exhibited suboptimal coating with C3 fragments and markedly diminished monocyte binding compared with small-capsule cells. Thus, the inverse correlation between capsule size and phagocyte binding can be overcome by conditions promoting optimal C3 deposition.**

The polysaccharide capsule of *Cryptococcus neoformans* has long been viewed as an antiphagocytic surface structure. Numerous studies showed that nonencapsulated cryptococci are readily ingested by phagocytes, such as macrophages and neutrophils, whereas encapsulated cryptococci are relatively resistant to phagocytosis (1, 9, 13, 18). However, there are several reports that encapsulated cryptococci will be ingested to a variable extent if the yeast cells have been opsonized with serum that contains an intact complement system (6, 14, 17). Opsonization of cryptococci by normal human serum (NHS) is most likely due to the activation of C3 via the alternative pathway and the subsequent binding of opsonic fragments of C3 at the capsular surface (6, 10, 11).

Previous studies have found a consistent pattern in which small-capsule isolates opsonized with nonimmune serum attached to phagocytes at a much higher frequency than did opsonized isolates with large capsules (10, 17). A comparison of the binding of C3 fragments to large- and small-capsule strains showed that (i) C3 fragments are found at the surface and throughout the capsules of both large- and small-capsule strains, (ii) the bound C3 is primarily in the form of iC3b on both cell types, and (iii) similar numbers of C3 fragments bind to each cell type (10). These findings raise the following question: what accounts for the relative resistance to phagocytosis of large-capsule cryptococci that are opsonized with NHS? The observation that similar numbers of C3 fragments bound to large- and small-capsule isolates raises the possibility that the density of C3 fragments in and at the surfaces of large-capsule isolates is lower than the density that occurs with small-capsule isolates.

The present study was designed both to identify conditions that lead to optimal deposition of C3 fragments onto an encapsulated strain of *C. neoformans* that was grown under conditions which produce yeast cells with large or small capsules (7, 15) and to compare the binding of optimally and suboptimally opsonized yeast cells to cultured human monocytes. For induction of large capsules, *C. neoformans* 145 (serotype A) was propagated in RPMI 1640 containing 24 mM bicarbonate, pH 7.2, at 37°C in air supplemented with 5% $CO_2$. Small-capsule formation was produced by growth in RPMI 1640 without bicarbonate, pH 6.0, at 37°C in air not supplemented with $CO_2$. Yeast cells were harvested on day 4, heat killed by immersion in a 50°C water bath for 30 min, and stored at 4°C until use. Clumping of *C. neoformans* was not observed under either set of growing conditions. Moreover, all of the cells were present as either single cells or single-budding organisms. Capsular diameters were determined in India ink mounts by use of an ocular micrometer. The diameter of the cell (outer edge to outer edge of the cell wall) and the diameter of the cell plus the capsule (outer edge to outer edge of the capsule) were measured. The capsule thickness was calculated by subtracting the diameter of the cell from the diameter of the cell plus the capsule and dividing the difference by 2. The capsule volume was calculated by assuming that the shape was spherical and subtracting the cell volume from the volume of the cell plus the capsule. Yeast cells grown for induction of large capsules had a mean capsular thickness (± standard deviation) of 5.8 ± 1.0 μm and a mean capsular volume of 3,400 ± 1,300 $\mu m^3$. Yeast cells grown for small-capsule synthesis had a mean capsular thickness of 2.8 ± 1.0 μm and a mean capsular volume of 1,200 ± 790 $\mu m^3$.

Assays to examine the effects of yeast cell numbers on C3 deposition were done in a 1.0-ml reaction mixture consisting of $GVB^{2+}$ (142 mM saline buffered with 5 mM sodium barbital and containing 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% gelatin), 17% NHS, $^{125}$I-labeled C3 added to produce a final specific activity of 50,000 cpm/μg, and yeast cells in numbers corresponding to 4,000, 2,000, 1,000, 500, 250, or 125 cells per

* Corresponding author. Mailing address: Department of Microbiology/320, University of Nevada, Reno, NV 89557-0046. Phone: (702) 784-6161. Fax: (702) 784-1620.

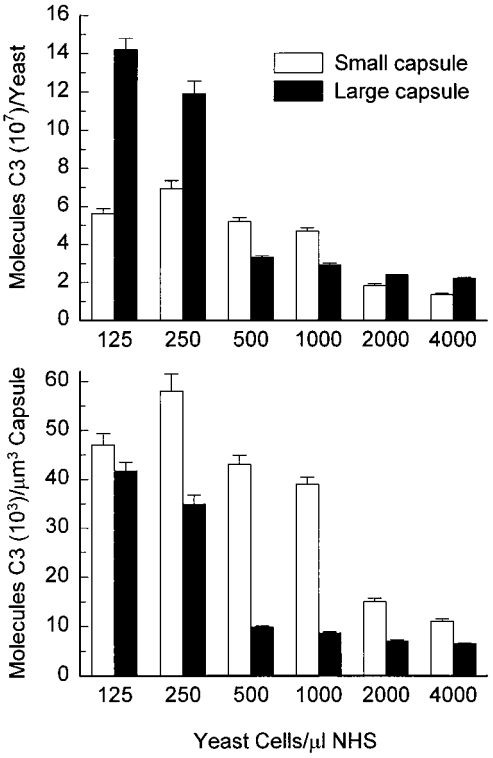


FIG. 1. Effects of the numbers of small- and large-capsule cryptococci per microliter of NHS on the numbers of C3 fragments bound per yeast cell and the number of C3 fragments bound per $\mu m^3$ of capsular volume.

μl of NHS. The mixture was incubated for 30 min at 37°C, and the reaction was stopped by the addition of 0.5 volume of PBS (127 mM saline, pH 7.3, buffered with 0.01 M phosphate) containing 10 mM EDTA. Aliquots (300 μl) from each reaction mixture were distributed in quadruplicate into wells of a MultiScreen-BV 96-well filtration plate (Millipore Corporation, Bedford, Mass.) and washed four times with PBS containing 0.1% sodium dodecyl sulfate. The membranes were removed and the amount of radioactivity was determined. Specific binding was determined by subtracting the radioactivity of samples for which heat-inactivated serum was used from the total binding observed with NHS. The number of molecules of bound C3 was calculated by assuming a specific activity of 50,000 cpm/μg and a molecular weight for bound C3 fragments of 185,000. Data are expressed as the number of C3 molecules bound to an average yeast cell and as the number of C3 molecules bound per cubic micrometer of capsular volume.

Phagocyte binding assays were done with monocytes isolated from human peripheral blood. Blood was collected via venipuncture, anticoagulated with heparin, and centrifuged at 500 × *g* for 15 min; the leukocyte-rich buffy coat was then harvested. Mononuclear cells were collected from the interface following centrifugation of the buffy coat over a gradient of Ficoll-Hypaque and were depleted of T cells and some natural killer cells by rosetting with neuraminidase-treated sheep erythrocytes (12, 15). The monocyte-rich fraction was cultured at 50,000 cells per well in 96-well flat-bottom plates for 6 to 8 days. Each well contained 100 μl of RPMI 1640 plus 10% male human AB serum; the atmosphere was humidified air supplemented with 5% $CO_2$ at 37°C. Following three washes, each well received RPMI 1640 containing 17% NHS and various numbers of *C. neoformans* in a total volume of 150 μl. Wells were incubated at 37°C for 2 h, washed to remove unbound *C. neoformans*, incubated for 30 min in PBS containing 1% formaldehyde and 0.1% Fungiqual A (also known as Uvitex 2B and diaethanol; Specialty Chemicals for Medical Diagnostics, Kandern, Germany), and washed again. Under these conditions, both intracellular and extracellular *C. neoformans* cells stain with diaethanol. By using an inverted microscope equipped with epifluorescence, at least 100 leukocytes per well were scored for the presence of cell-associated (bound and internalized) *C. neoformans*. The results are expressed as a binding index, which represents the mean number of cell-associated *C. neoformans* per 100 leukocytes (14, 15). Statistical analyses of the binding of C3 fragments to cryptococcal cells and of the binding of cryptococci to cultured human monocytes were done by analysis of variance with SigmaStat software (Jandel Scientific, San Rafael, Calif.).

An initial experiment evaluated the extent to which the numbers of large- and small-capsule cryptococci in an incubation mixture influenced the amount of bound C3. Increasing numbers of cryptococci were incubated for 30 min with a constant amount of 17% NHS. The results (Fig. 1) showed that increasing the concentration of large-capsule cryptococci led to a dose-dependent decrease in the amount of C3 bound per cell over the range of 125 to 500 yeast cells per μl of NHS. In contrast, incubation of various numbers of small-capsule cryptococci with 17% NHS showed similar binding of C3 over the range of 125 to 1,000 yeast cells per μl of NHS. Expression of the data as the number of C3 fragments per unit of capsular volume showed that there was no significant difference ($P = 0.13$) in the density of bound C3 on large- and small-capsule isolates when activation and binding were assessed at 125 yeast cells per μl of NHS. However, when the incubation mixture contained 250 or more yeast cells per μl of NHS, small-capsule cells bound significantly ($P < 0.001$) more C3 fragments per unit of volume than did large-capsule isolates.

The impact on opsonization of the number of yeast cells in the reaction mixture was assessed by incorporating various numbers of large- or small-capsule cryptococci into a phagocytosis mixture containing 17% NHS. The results (Fig. 2) showed similar ($P = 0.42$) binding of large- and small-capsule yeast cells if the experiment was done at a cell density of 3,125 yeast cells per well. This cell density corresponds to 125 yeast cells per μl of NHS, a cell density at which similar, maximal numbers of C3 fragments are bound to each yeast type in terms of unit of capsular volume (≈45,000 C3 fragments per $\mu m^3$ [Fig. 1]). If the number of yeast cells in the phagocytosis mixture was increased to 12,500, 50,000, or 200,000 per well, there

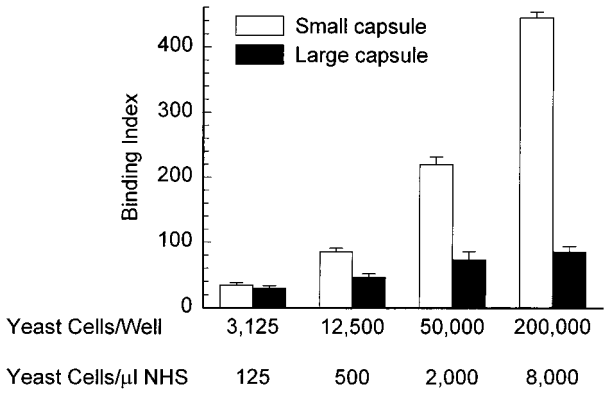


FIG. 2. Effect of capsule size on binding of *C. neoformans* to cultured monocytes. The number of yeast cells per well was varied as indicated. The serum concentration was kept constant at 17%.

was significantly ($P < 0.001$) greater binding of small-capsule than of large-capsule yeast cells. These higher cell densities correspond to the concentrations of yeast cells per μl of NHS which produce significant differences between large- and small-capsule yeast cells in the numbers of C3 fragments bound per unit of capsular volume (Fig. 1). The binding of cryptococci to cultured human monocytes was presumed to be complement dependent because binding was inhibited by more than 90% if the NHS was heated to 56°C for 30 min (data not shown). There was no clumping of the yeast cells at any of the concentrations of yeast cells in either 17% NHS or heat-inactivated serum.

For the past 30 years, the cryptococcal capsule has been described as an antiphagocytic surface structure (1, 2). Results from the present study suggest that the failure to demonstrate phagocytosis of encapsulated cryptococci is accounted for, in part, by the experimental design used for phagocytosis studies. Large-capsule cryptococci may activate and bind numbers of C3 fragments per yeast cell that are equal to or greater than the numbers bound by small-capsule cryptococci (Fig. 1) (19). However, the density of C3 fragments within the capsular space tends to be lower with large-capsule than with small-capsule cryptococci unless the experiment is done under conditions in which the concentration of large-capsule cells is not excessive (Fig. 1). What constitutes "excessive" is quite small: suboptimal deposition of C3 fragments occurred when 250 or more highly encapsulated cells were used per μl of NHS. The specific mechanism that accounts for the suboptimal deposition of C3 fragments at higher concentrations of yeast cells is not known with certainty. Presumably, concentrations greater than 125 cells per μl of NHS produce robust activation of the complement system, leading to depletion of the available complement before controlled amplification of solid-phase C3b can occur within the capsular matrix. An examination of the experimental conditions used for several studies of opsonization and phagocytosis of *C. neoformans* revealed that 2,000 (1), 10,000 (10), 25,000 (14, 17), and 200,000 (5) yeast cells were used per μl of serum. All of these numbers are well above the 125 yeast cells per μl of NHS needed for optimal deposition of C3 fragments into the capsules of yeast cells with very large capsules (Fig. 1).

Data in Fig. 2 also indicate that the number of C3 fragments bound per unit of volume is not the sole variable that influences phagocyte binding. Increasing the ratio of yeast cells per phagocyte produced an increase in the number of small-capsule cells bound to cultured monocytes, even at numbers predicted to deposit less than the optimal 45,000 C3 fragments per $\mu m^3$ of capsule. Increasing the number of large-capsule yeast cells per phagocyte also drove the system toward more binding but not to the extent observed with small-capsule cells. These results suggest that small-capsule cryptococci are less susceptible to the effects of suboptimal opsonization than large-capsule cells, assuming that the difference in the physical size of large- and small-capsule cryptococci is not by itself a major determinant of phagocyte binding.

An alternative method for stimulating phagocytosis of encapsulated cryptococci is the use of cytokines (4, 8). Collins and Bancroft found that tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor stimulate resident murine peritoneal macrophages for highly efficient complement-dependent ingestion of encapsulated cryptococci (4). Of particular importance is the observation that tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor reduce the amount of serum required for effective opsonization of encapsulated cryptococci.

Our results have implications for understanding the pathogenesis of disseminated cryptococcosis. Given limited numbers of cryptococci and adequate production of cytokines, opsonization of encapsulated cryptococci and binding to phagocytes appear to be quite effective. However, this is a balance that is easily disrupted by an excess of yeast cells. Studies of serum complement levels in cryptococcemia found that complement depletion occurs in vivo (16). This may be an important factor that potentiates cryptococcosis in patients with widely disseminated disease, such as patients with AIDS (3).

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