

Activation and Binding of C3 by *Candida albicans*

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Interaction with components of the complement system is an important aspect of the pathogenesis of infection by *Candida albicans*. The key role of C3 as an opsonic ligand and as an element in amplification of complement activation led us to examine several factors that influence the activation and binding of C3 cleavage fragments to the yeast. Activation and binding of C3 were determined by use of normal human serum containing ^{125}I -labeled C3. Incubation of yeast-phase cells in 20% serum led to deposition of 2.5×10^5 to 3.0×10^5 molecules of C3 per yeast cell. Binding of C3 was absent in serum that was heat inactivated for 30 min at 37°C or in serum that was chelated with EDTA. Chelation of serum with EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] reduced binding of C3 fragments by 31%. These results suggest that the alternative complement pathway is the primary mechanism for activation and binding of C3 fragments to *C. albicans*. Bound C3 could be partially removed (50%) by treatment with 1.0 M hydroxylamine. In contrast, 1.0 M hydroxylamine removed 98% of the C3 fragments bound to encapsulated *Cryptococcus neoformans*. These results suggest that ester bonds are the primary mechanism for binding C3 to *C. neoformans*, whereas binding of C3 to *C. albicans* involves both ester and amide bonds. Monoclonal antibodies specific for C3c and an iC3b neoantigen were used to identify the fragment of C3 that was bound to *C. albicans*. The results showed that the primary fragment bound to the yeast was C3b. An examination of the kinetics of activation and binding of C3 fragments showed that activation and binding were very rapid. Near-maximal binding occurred after a 2.5- to 5-min incubation period. In contrast, activation and binding of C3 fragments to *C. neoformans* proceeded at a slower rate, with maximal binding requiring 10 to 20 min. These results indicate that activation and binding of C3 fragments by the yeasts *C. albicans* and *C. neoformans* differ in several important characteristics.

Interaction with components of the complement system is an important aspect of the pathogenesis of *Candida albicans* infection. Whole yeast cells and extracts from yeast cell walls activate the complement cascade (20, 21, 28). The importance of the complement system is demonstrated by the increased susceptibility to candidiasis of animals depleted of alternative pathway activity by treatment with cobra venom factor (7).

Phagocytosis of *C. albicans* yeast cells is critically dependent upon opsonization by components of the complement cascade. Little phagocytosis occurs without opsonization with serum components (25). Moreover, the opsonic activity of serum is markedly reduced by heat inactivation at 56°C (3, 23, 25) or 50°C (16). Serum chelated with 10 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid containing 10 mM MgCl_2 (magnesium EGTA) would support phagocytosis of the yeast, whereas serum chelated with EDTA showed no opsonic activity (8, 16, 25). Taken together, these reports provide strong evidence that the alternative complement pathway is the mechanism for opsonization of *C. albicans* yeast cells by normal serum.

The importance of C3 in phagocytosis of *C. albicans* was demonstrated by Morrison and Cutler (16), who found that serum depleted of C3 would not opsonize *C. albicans* for phagocytosis. Complement component C3 plays a key role in amplification of the complement cascade. In addition, cleavage fragments of C3 can act as opsonic ligands for phagocytosis (24). These properties of C3 led us to examine the factors that influence the binding of C3 cleavage fragments to *C. albicans*. We assessed the ability of serum from normal individuals to deposit C3 fragments on *C. albicans*.

The objectives of our study were (i) to determine the role of the alternative pathway in activation and binding of C3 to *C. albicans*, (ii) to identify the mechanism by which C3 fragments are bound to *C. albicans*, (iii) to identify the fragment(s) of C3 that is bound to *C. albicans*, and (iv) to determine the time course of activation and binding of C3 fragments. The results showed that C3 fragments are activated and bound from normal human serum onto *C. albicans*. However, *C. albicans* differs in several key characteristics from *Cryptococcus neoformans*, another activator of the complement cascade.

MATERIALS AND METHODS

Yeast strains. *C. albicans* 3153A (ATCC 28367) was used throughout the study. *C. neoformans* 217 was used in selected experiments. Strain 217 is a serotype A isolate that has a capsule diameter of 2.7 μm . *C. albicans* cells were grown in the yeast phase in Lee medium (15), pH 6.6, on a rotary shaker at 200 rpm for 48 h at room temperature. The cells were harvested by centrifugation and killed by incubation with 1% formaldehyde in phosphate-buffered saline (PBS) for 6 h at 37°C. The yeast cells were killed with formaldehyde to reduce the possibility that secretion of proteases by the yeast cells might influence complement activation by the yeast surface. Preliminary experiments established that the number of C3 fragments activated from normal serum and deposited onto the yeast cells was similar in yeast cells that were live or killed by treatment with thimerosal, sodium azide, or formaldehyde or heating at 80°C for 30 min. Cryptococci were cultured and killed with formaldehyde as previously described (10). All yeast cells were stored as a sterile suspension in PBS at 4°C.

Reagents and buffers. The following buffers were used: VBS (sodium Veronal [5 mM]-buffered saline [142 mM], pH

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7.3), GVB (VBS containing 0.1% gelatin, 1 mM MgCl₂, and 0.15 mM CaCl₂), EDTA-GVB (VBS containing 0.1% gelatin and 10 mM EDTA), and magnesium EGTA-GVB (VBS containing 0.1% gelatin, 10 mM MgCl₂, and 10 mM EGTA).

Serum and serum proteins. Normal human serum samples were obtained from 10 volunteers after their informed consent was obtained. The sera were pooled, and portions were stored at -70°C. For some studies, the serum was heated at 56°C for 30 min. C3 was isolated from frozen human plasma as previously described (11). Any contaminating immunoglobulin G in the C3 preparation was removed by adsorption with protein A-Sepharose, followed by adsorption on an anti-immunoglobulin G column (26). Analysis of reduced preparations of C3 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis showed the presence of two bands characteristic of the alpha and beta chains of C3 (26) and the absence of any detectable contaminating proteins.

C3 was labeled with ¹²⁵I by the Iodogen procedure (Pierce Chemical Co., Rockford, Ill.) as previously described (5). Labeled C3 was separated from free iodine by gel filtration through Sephadex G-25. Typically, 1.0 mg of C3 was labeled with an efficiency of 80% to a specific activity of 5 × 10⁵ cpm/μg. Radiolabeled C3 remained functionally active for at least 7 days but was used within 4 days.

Antibodies. A monoclonal antibody specific for C3c was purchased from Genzyme Corp., Boston, Mass. A monoclonal antibody specific for iC3b neoantigen (27) was purchased from Cytotech, San Diego, Calif. The monoclonal antibodies were labeled with ¹²⁵I by the Iodogen technique.

Assays for C3 fragments bound to yeast cells. Activation and binding of C3 to yeast cells were assessed as previously described for *C. neoformans* (11). Briefly, binding assays were done in a 2.5-ml reaction volume containing (i) 2 × 10⁷ candidal cells or 5 × 10⁶ cryptococci in 200 μl of GVB, (ii) ¹²⁵I-labeled C3 (10⁶ cpm) in 200 μl of GVB, (iii) 1,600 μl of buffer (GVB, magnesium EGTA-GVB, or EDTA-GVB), and (iv) 500 μl of human serum. Unless otherwise indicated, tubes were incubated for 30 min at 37°C. The tubes were then immediately placed on ice, and EDTA was added to a final concentration of 10 mM. The cells were washed five times with PBS containing 0.1% SDS, and the amount of bound radioactivity was determined. SDS was used to remove C3 fragments that were not covalently bound to the yeast cells (12). Tubes containing heat-inactivated (56°C for 30 min) serum in place of normal human serum served as controls for nonspecific binding of C3 to the cells and tubes. Nonspecific binding was consistently less than 5% of the total specific binding. The amount of specific binding was determined by subtracting the amount of nonspecific binding from the total of each tube. The number of C3 molecules bound to each yeast cell was calculated from the specific activity of the radiolabeled C3, assuming the molecular weight of C3 to be 187,500 (26). All assays were done in quadruplicate.

In some experiments, radiolabeled antibodies to C3c or iC3b neoantigen were used to assess the presence of C3 fragments. Yeast cells were incubated with normal human serum as described above. The yeast cells were washed five times with GVB and incubated for 30 min at room temperature with saturating amounts of ¹²⁵I-labeled anti-C3c or anti-iC3b neoantigen. The yeast cells were washed four times with PBS containing 0.1% SDS, and the amount of bound radioactivity was determined. Data are reported as the number of bound antibody molecules per yeast cell.

Elution of bound C3 fragments. Yeast cells were incubated

TABLE 1. Effect of serum treatment on activation and binding of C3 fragments to *C. albicans*

| Serum treatment | No. of C3 molecules (10 ⁴)/yeast cell ^a |
|-----------------|--|
| None | 27.9 ± 3.7 |
| 56°C | 0.8 ± 0.3 |
| EDTA | 0.8 ± 0.6 |
| Magnesium EGTA | 19.1 ± 2.3 |

^a Each value is the mean ± the standard error of the mean of two individual experiments, each of which had four replications.

with normal human serum containing ¹²⁵I-labeled C3 in the manner described above. The cells were washed five times with PBS-SDS. The amount of bound C3 was determined, and the yeast cells were incubated for 60 min at 37°C with 2.5 ml of (i) PBS-SDS, (ii) 1 M NH₂OH in 0.2 M NaHCO₃ (pH 10.0), or (iii) 3.5 M NaSCN (pH 7.0). The treated yeast cells were washed twice with PBS-SDS, and the amount of remaining C3 was determined. The amount of released C3 was calculated, and the data are reported as the percentage of C3 removed after treatment with NH₂OH or NaSCN relative to the amount of C3 remaining after treatment with PBS-SDS.

RESULTS

Previous studies have shown that effective opsonization of *C. albicans* by heat-labile opsonins was due to activation of the alternative complement pathway. An experiment was done to assess the roles of the classical and alternative pathways in activation and deposition of C3 onto yeast cells. *Candida* cells were incubated with normal human serum, heat-inactivated (56°C for 30 min) human serum, normal human serum chelated with EDTA, or human serum treated with magnesium EGTA. Radiolabeled C3 was added to each serum source as a marker for C3 binding. EGTA chelates Ca²⁺, which is necessary for activation of the classical complement pathway (4, 19). Activation of the alternative pathway occurs in the presence of magnesium EGTA because the alternative pathway requires Mg²⁺ for activation but not Ca²⁺ (4, 19). The results (Table 1) showed complete loss of activation and binding of C3 to the yeast cells in serum that was heat inactivated or chelated with EDTA. Treatment of serum with magnesium EGTA produced 32% inhibition of binding of ¹²⁵I-labeled C3 when compared with binding in uninhibited serum.

Binding of C3 to a substrate can occur via an ester bond or an amide bond (6, 12, 13). Ester bonds are quite sensitive to hydrolysis with hydroxylamine, whereas amide bonds are relatively resistant to hydroxylamine (12-14). Alternatively, the C3 could be bound to antibody, which in turn is bound to the yeast cells. As a consequence, we determined whether C3 bound to *C. albicans* could be eluted with NH₂OH (1 M) or the chaotrope NaSCN (3.5 M). Candidal cells were incubated with normal human serum containing ¹²⁵I-labeled C3. The cells were washed with PBS containing 0.1% SDS and incubated for 1 h at 37°C with each eluting agent. Previous studies have shown that C3 fragments bound to encapsulated *C. neoformans* are eluted by treatment with NH₂OH (11). We conducted a parallel assay in which cryptococcal cells were treated in an identical manner with normal serum containing ¹²⁵I-labeled C3 followed by treatment with the various eluting agents. This was done to ensure that the treatment with NH₂OH was sufficient to remove any C3 that was bound via an ester bond. The results

TABLE 2. Elution of C3 fragments from *C. albicans* and *C. neoformans*

| Treatment ^a | % of C3 removed from ^b : | |
|------------------------|-------------------------------------|----------------------|
| | <i>C. albicans</i> | <i>C. neoformans</i> |
| NH ₂ OH | 50 | 98 |
| NaSCN | 6.4 | 0 |

^a Yeast cells were incubated with human serum containing ¹²⁵I-labeled C3, washed, incubated for 60 min at 37°C with the indicated dissociating agent or PBS-SDS, and washed with PBS-SDS.

^b Data are reported as the percentage of bound ¹²⁵I-labeled C3 removed by treatment of yeast cells with NH₂OH or NaSCN relative to the amount removed by incubation with PBS-SDS. The data shown are the mean of two separate experiments. Each experiment had four replications.

(Table 2), reported as the percentage of bound C3 removed by each treatment, showed that hydroxylamine removed almost 100% of the C3 bound to *C. neoformans* but only 50% of the C3 bound to *C. albicans*. Treatment with the chaotrope NaSCN did not remove appreciable amounts of bound C3 from either *C. albicans* or *C. neoformans*.

The previous experiments demonstrated that some form of C3 was bound to *C. albicans*, but the molecular form of the bound C3 fragment was not identified. Monoclonal antibodies specific for C3c and iC3b neoantigen were used as probes for the molecular form of the bound C3 fragments. Anti-C3c is reactive with C3b and iC3b (17). In contrast, anti-iC3b neoantigen is not reactive with C3b but is reactive with iC3b (27). Candidal yeast cells were incubated with normal human serum for 2.5 to 40 min. The reaction was stopped, and the yeast cells were washed and incubated with saturating amounts of ¹²⁵I-labeled anti-C3c or anti-iC3b neoantigen. The results (Fig. 1) showed rapid activation and binding of C3 fragments to the yeast cells, as shown by the binding of anti-C3c to the serum-treated yeast cells. Very little anti-iC3b neoantigen bound to candidal cells that were incubated with normal human serum, indicating little conversion of bound C3b to iC3b (27). As a positive control for the reactivity of the anti-iC3b neoantigen, cryptococci were incubated with normal human serum, washed, and incubated

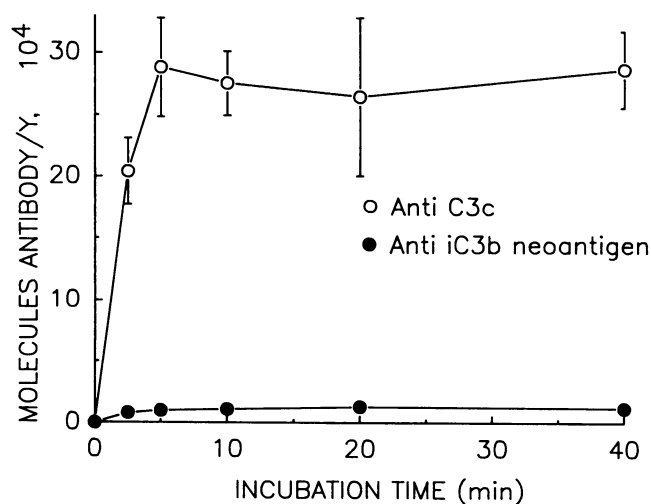


FIG. 1. Binding of monoclonal anti-C3c and anti-iC3b neoantigen to *C. albicans* incubated for various times in 20% normal human serum. Monoclonal anti-C3c binds to both C3b and iC3b. Monoclonal anti-iC3b neoantigen binds to iC3b. The data shown are the mean \pm the standard error of the mean of three separate experiments. Each experiment had four replications.

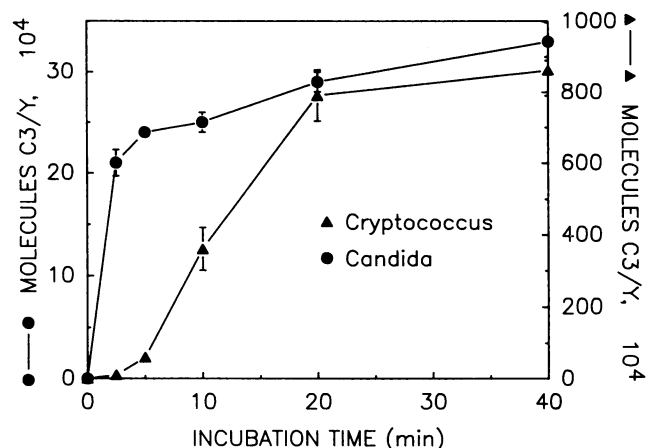


FIG. 2. Kinetics of binding of C3 to *C. albicans* and *C. neoformans*. The data shown are the mean \pm the standard error of the mean of three separate experiments. Each experiment had four replications.

with the radiolabeled anti-iC3b neoantigen, and the bound radioactivity was determined. The results (data not shown) showed appreciable conversion of C3b to iC3b. Previous studies have shown that large amounts of C3b bound to the cryptococcal capsule are converted to iC3b (11).

The rapid activation and binding of C3b to *C. albicans* shown by the anti-C3c antibody probe suggested a much more rapid activation kinetics than has been observed for *C. neoformans* (11). This rapid activation was confirmed by comparing the activation and binding of ¹²⁵I-labeled C3 from normal human serum onto *C. albicans* and *C. neoformans* (Fig. 2). Incubation of *C. albicans* in human serum containing ¹²⁵I-labeled C3 for 2.5 to 40 min showed that 64% of the maximum binding of C3 fragments occurred after a 2.5-min incubation period. In contrast, only 42% of maximum binding of C3 fragments to *C. neoformans* had occurred after a 10-min incubation period.

DISCUSSION

Yeast cell walls have been studied as activators of the complement cascade for many years. Indeed, zymosan is the prototype of the particulate activator of the alternative complement pathway. Our studies demonstrate that marked differences may occur in the manner in which C3 is activated and bound to pathogenic yeasts. Activation and binding of C3 fragments to *C. albicans* and *C. neoformans* are compared in Table 3.

The polysaccharide capsule is the site for activation and binding of C3 fragments to *C. neoformans*. Ultrastructural

TABLE 3. Activation and binding of C3 fragments to *C. albicans* and *C. neoformans*^a

| Organism | Binding site | No. of C3 molecules (10 ⁵)/yeast cell | % Release by NH ₂ OH | C3 fragment(s) | Activation kinetics |
|----------------------|--------------|---|---------------------------------|----------------|---------------------|
| <i>C. albicans</i> | Cell wall | 2-4 | 50 | C3b | Rapid |
| <i>C. neoformans</i> | Capsule | 50-100 | 90-100 | C3b, iC3b | Relatively slow |

^a Activation and binding of C3 fragments to *C. albicans* and *C. neoformans* were inhibited markedly by EDTA and 30 to 40% by magnesium EGTA.

studies have shown that C3 binds predominantly to the periphery of the capsule, with little C3 bound deep within the capsule or at the cell wall (9). *C. albicans* lacks a capsule; consequently, the cell wall is the only structure available for activation and binding of C3. The cryptococcal capsule is a large, three-dimensional matrix which presents a much larger surface area for C3 binding than would be available on the two-dimensional structure presented by the candidal cell wall. This undoubtedly accounts in a large part for the much greater amount of C3 that binds to *C. neoformans*. Results of other quantitative studies of binding of C3 to nonencapsulated yeasts more closely resemble the numbers of C3 molecules binding to *C. albicans* than *C. neoformans*. For example, Newman and Mikus report 7×10^4 to 9×10^4 C3 molecules bound per cell of bakers' yeast (17).

C3 was bound to *C. albicans* by both hydroxylamine-sensitive and hydroxylamine-resistant bonds. In contrast, the C3 bound to *C. neoformans* was completely sensitive to hydrolysis with hydroxylamine. Previous studies have shown that hydroxylamine-sensitive bonds are most likely to be ester bonds between C3 fragments and hydroxyl groups on receptive molecules (6, 12, 13). Hydroxylamine-resistant bonds are most likely due to amide bonds between C3 fragments and receptive molecules (12-14). The absence of amide bonds between C3 and *C. neoformans* is probably due to the absence of appropriate amino groups in cryptococcal capsular polysaccharide (1). Previous ultrastructural studies have shown that C3 binds to the periphery of the capsule, an area that would be relatively free of amino groups. *C. albicans*, in contrast, lacks a capsule, and the cell wall is the most likely binding site for C3. The source of available amino groups is not known for the candidal cell wall; however, the composition of the cell wall of *C. albicans* does include protein (22).

C. albicans and *C. neoformans* exhibit qualitative differences in the C3 fragments that are bound to the yeast cells. These qualitative differences suggest that complement activation is regulated differently on the two yeasts. The absence of iC3b on *C. albicans* suggests that complement activation is not regulated by factors H and I. Presumably, activation proceeds in a relatively unrestricted manner until saturating amounts of C3b have been deposited on the yeast surface. In contrast, the presence of iC3b on *C. neoformans* indicates that factors H and I effect the decay of a portion of C3b as it is formed. The molecular differences between the surfaces of the two yeasts that account for this differential regulation are not known.

The relatively slow activation kinetics by *C. neoformans* may be a reflection of the decay of C3b to iC3b in the cryptococcal capsule. Conversion of C3b to iC3b may inhibit the rate of activation to some extent. *C. albicans*, in contrast, exhibits little or no decay of C3b to iC3b; thus, the rate of the reaction may be faster. Alternatively, the slow reaction kinetics by cryptococci may be due to the three-dimensional nature of the cryptococcal capsule. It is possible that the capsule retards to some extent the diffusion of reactants within the capsule. This would, then, account for both the large number of bound molecules and the slow reaction rate. Pangburn et al. (18) have also noted differences in the time course of C3 activation and binding by rabbit erythrocytes, zymosan particles, and cells of *Escherichia coli*.

Despite differences in the ways in which *C. albicans* and *C. neoformans* interact with the complement cascade, there is little doubt that activation of the complement cascade is an important event in the pathogenesis of infection by both yeasts. The fact that treatment of guinea pigs with cobra

venom renders the animals more susceptible to both yeasts underscores the key role of the complement system in resistance to both yeasts (2, 7).

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