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Activation of the Complement System by *Cryptococcus neoformans* Leads to Binding of iC3b to the Yeast

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The complement system plays a key role in resistance to cryptococcosis. In the present study, we examined several factors that influence the binding of C3 cleavage fragments to *Cryptococcus neoformans*. Binding of C3 was determined by using normal human serum supplemented with ^{125}I-labeled C3. Incubation of encapsulated cryptococci in 20% serum led to the binding of approximately 3.2 × 10^6 molecules of C3 to each cell. The binding of C3 was markedly inhibited by heating the serum at 56°C for 30 min or by chelation of the serum with EDTA. Chelation of the serum with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] reduced binding of C3 by 37%. These results indicated that activation of C3 cleavage fragments and their binding to *C. neoformans* was primarily dependent upon the alternative pathway. Bound C3 could be removed by incubation with 1.0 M hydroxylamine (pH 10) but not by incubation with 3.5 M NaSCN or with phosphate-buffered saline containing 0.1% sodium dodecyl sulfate. These results suggested that C3 fragments were bound to *C. neoformans* by ester bonds. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of C3 fragments eluted from the yeast showed the presence of protein bands consistent with the presence of iC3b. C3b was not detected on the yeast after incubation with serum for time intervals as short as 2.5 min, indicating a rapid conversion of cell-bound C3b to iC3b. These results indicate that iC3b is the ligand which most likely interacts with the phagoctye C3 receptors involved in the phagocytosis of *C. neoformans*.

Components of the complement system play a critical role in resistance to infection by *Cryptococcus neoformans*. Incubation of encapsulated *C. neoformans* with normal human serum leads to deposition of cleavage fragments of C3 onto the yeast (6, 9). Ultrastructural studies showed that the C3 fragments were bound at and immediately beneath the surface of the capsule (15). These C3 fragments appear to be essential for optimal phagocytosis of the yeast because heat-inactivated serum neither deposits C3 fragments at the capsular surface (15) nor supports phagocytosis of the encapsulated yeast by human neutrophils (3, 6, 15). Thus, it is not surprising that guinea pigs treated with cobra venom factor to deplete terminal pathway components (C3 to C9) displayed a markedly diminished capacity to clear cryptococci from extraneural sites (5).

Several studies examined the role of the complement system in the opsonization of *C. neoformans* (3, 6); however, these studies did not address the critical events involving the binding of complement components to the yeast. The importance of C3 cleavage fragments as opsonic ligands (26) led us to examine the factors that influence the binding of C3 cleavage fragments to the yeast. We focused on the activity of serum from normal, presumably nonimmune individuals. The objectives of our study were (i) to determine the role of the alternative pathway in the deposition of C3 fragments onto the yeast, (ii) to examine the mechanism of binding to the yeast, and (iii) to identify the primary fragment(s) of C3 that are bound.

MATERIALS AND METHODS

Yeast strain. A *C. neoformans* serotype D isolate (ATCC 24067; American Type Culture Collection, Rockville, Md.) was used throughout this study. Yeast cells were grown in a yeast extract dialysate medium (12), killed with Formalin (16), and stored at 4°C as a suspension in sterile saline (0.15 M NaCl).

Reagents and buffers. The following buffers were used: VBS (Sodium Veronal [5 mM]-buffered saline [142 mM], pH 7.3), GVB (VBS containing 0.1% gelatin, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$), EDTA-GVB (VBS containing 0.1% gelatin and 10 mM EDTA), and magnesium EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]-GVB (VBS containing 0.1% gelatin, 10 mM $MgCl_2$, and 10 mM EGTA).

Isolation of C3. C3 was isolated from frozen human plasma. The plasma was treated with protease inhibitors and precipitated with polyethylene glycol by using the procedures described by Tack et al. (28, 29). The 5 to 15% polyethylene glycol fraction was depleted of plasminogen by affinity chromatography on a lysine-Sepharose column, and the C3 was isolated by chromatography on DEAE-Sephacel and on Sepharose CL-6B (27). The C3 was sterilized by filtration through a 0.22-μm (pore size) membrane filter (Millipore Corp., Bedford, Mass.) and stored at 4°C at a concentration of 10 mg/ml in 10 mM potassium phosphate-sodium phosphate buffer, pH 7.0, containing 170 mM NaCl and 2 mM EDTA. Analysis of reduced preparations of purified C3 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed two bands characteristic of the 110,000-dalton α chain and the 70,000-dalton β chain of C3 (25). A faint additional band at approximately 41,000 daltons was occasionally observed and was probably due to autolytic cleavage of the C3 during preparation of the sample for SDS-PAGE (26). Gels that were loaded with large amounts of C3 also showed the presence of a faint high-

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molecular-weight contaminant that did not penetrate the running gel. This protein was not found among those eluted from the yeast (Fig. 1) and did not, therefore, influence interpretation of the data. C3 preparations to be labeled by using lactoperoxidase-glucose oxidase beads were adsorbed immediately before use with 0.1 volume of Affi-Gel 501 (Bio-Rad Laboratories, Richmond, Calif.) to absorb any inactive C3 that might be present (23). Inactive C3 was assayed by spectrophotometric titration of the SH content with 5,5'-dithiobis(2-nitrobenzoic acid) as described by Janatova et al. (13). Contamination of C3 preparations by inactive C3 never exceeded 30% before adsorption with Affi-Gel 501.

Radioiodination of C3. C3 was labeled with ^{125}I by using lactoperoxidase-glucose oxidase beads (Enzymobeads; Bio-Rad) according to the directions of the manufacturer. In some experiments, C3 was labeled by using Iodogen (Pierce Chemical Co., Rockford, Ill.) in accordance with the protocol provided by the manufacturer. 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 5×10^5 cpm/μg. Radiolabeled C3 remained functionally stable for at least 7 days, but was used within 4 days.

Serum treatment. 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. Analysis of the ability of heated serum to support alternative or classical pathway activity showed that there was a complete loss of hemolytic activity in serum heated at 56°C. Classical pathway activity was assessed as the ability of serum to support lysis of sheep erythrocytes optimally coated with antibody (14). Antibody to sheep erythrocyte stroma was obtained from Cordis Laboratories, Miami, Fla., and was used at a final dilution of 1/1,000. Alternative pathway activity was determined as the ability of serum to lyse rabbit erythrocytes (25).

C3-binding assay. C3 activation and binding to *C. neoformans* were done in a 2.5-ml reaction mixture containing 5×10^6 cryptococci in 200 μl of GVB, ^{125}I-labeled C3 (10^6 cpm) in 200 μl of GVB, 1,600 μl of buffer (GVB, magnesium EGTA-GVB, or EDTA-GVB), and 500 μl of human serum. 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 six times with phosphate-buffered saline (PBS), and the amount of bound radioactivity was determined with a gamma counter (Packard Auto-Gamma 5650; Packard Instrument Co., Inc., Rockville, Md.). Tubes containing heat-inactivated serum (56°C) in place of normal human serum served as controls for nonspecific binding of C3 to the cells and tubes. The amount of specific binding was determined by subtracting the amount of nonspecific binding from the total for each tube. All of the assays were done in quadruplicate. The data are presented as the average of 3 to 5 individual experiments.

Elution of bound C3. Cryptococci were incubated with normal human serum containing [^{125}I]C3 in the same manner as that described above. The cells were washed five times with PBS and suspended in 2.5 ml of (i) PBS, (ii) 1 M NH_2OH in 0.2 M $NaHCO_3$ (pH 10.0), or (iii) 3.5 M NaSCN (pH 7.0). The yeast cells were incubated for 1 h with the eluting agents and washed two times with PBS; the amount of bound C3 was then determined. The controls for nonspecific binding were cryptococci treated in an identical manner except that heat-inactivated serum (56°C) was used in place of normal serum.

Isolation of eluted C3 and analysis by SDS-PAGE. C3 cleavage fragments were bound to cryptococci by incubation for 30 min at 37°C of a reaction mixture consisting of 5×10^7 cryptococci, ^{125}I-labeled C3 (10^8 cpm), 5 ml of normal or heat-inactivated (56°C) human serum, and GVB to a final reaction volume of 10 ml. The reaction was stopped by placing the tubes on ice and adding EDTA and phenylmethylsulfonyl fluoride to final concentrations of 10 and 1 mM, respectively. The cells with bound C3 were washed six times with PBS, suspended in 2.0 ml of 1.0 M NH_2OH in 0.2 M $NaHCO_3$ (pH 10.0), and incubated for 1 h at 37°C. The cells were removed by centrifugation, and the eluted C3 fragments were precipitated from the supernatant fluid by dialysis at 4°C against methanol containing 1 mM phenylmethylsulfonyl fluoride. The precipitated samples were dissolved in 0.05 M Tris (pH 6.8) and analyzed under reducing conditions by SDS-PAGE with 7% acrylamide gels by the method of Laemmli (18).

RESULTS

Binding of C3 to *C. neoformans*. Previous studies provided qualitative evidence that C3 or a cleavage fragment of C3 binds to the capsule of *C. neoformans* (15). We sought a quantitative estimate of the amount of C3 that binds to the yeast. Cryptococcal cells (5×10^6) were incubated for 30 min with 20% human serum containing ^{125}I-labeled C3 at a specific activity of 2,000 cpm/μg of C3. The cells were washed, and the amount of specifically bound C3 was measured. If the bound fragment was iC3b with a molecular weight of 186,000 (10, 17), approximately 3.2×10^6 molecules of iC3b bound per cell.

An experiment was done to determine the kinetics of deposition of C3 on the yeast. Cryptococcal cells were incubated with 20% serum containing ^{125}I-labeled C3 for time intervals ranging from 2.5 to 80 min. The amount of bound radioactivity was then determined. The results (Table 1) showed that maximum binding did not occur until the cryptococci had been incubated with the serum for 20 to 40 min.

The binding of C3 fragments to the yeast was determined in the presence and absence of magnesium EGTA. EGTA chelates Ca^{2+}, which is in turn necessary for activation of the classical pathway (7, 25). Activation of the alternative pathway occurs in the presence of magnesium EGTA because the alternative pathway requires Mg^{2+} for activation but not Ca^{2+} (7, 25). Control experiments used rabbit erythrocytes as indicators of alternative pathway activity (25) and antibody-coated sheep erythrocytes as indicators of classical pathway activity. These control experiments

TABLE 1. Kinetics of binding of C3 to *C. neoformans*

Incubation time (min)	Amt of bound radioactivity (cpm, mean ± SEM)[a]
2.5	260 ± 30
5	970 ± 70
10	3,900 ± 230
20	10,000 ± 240
40	14,000 ± 350
80	14,000 ± 280

[a] Each value is the average of three individual experiments, each of which had four replications.

showed that there was a complete loss of classical pathway activity but only a 41% loss of alternative pathway activity when serum was chelated with magnesium EGTA. Incubation of cryptococci with serum in the presence of magnesium EGTA resulted in 37% inhibition of binding of ^{125}I-labeled C3 as compared with binding in uninhibited serum. The amount of bound radioactivity (mean ± standard error of the mean) for serum incubated with and without magnesium EGTA was 8,800 ± 960 cpm and 14,000 ± 2,200 cpm, respectively. Each value is the average of three individual experiments, each of which had four replications. Complete inhibition was observed when serum was chelated with EDTA.

Elution of C3 from *C. neoformans*. We examined the manner by which C3 cleavage fragments were bound to the cryptococcal capsule. Specifically, we investigated whether the C3 was bound to the capsule via a hydroxylamine-sensitive bond. Cryptococci were incubated with normal human serum containing ^{125}I-labeled C3. The cells were washed with PBS and incubated for 60 min at 37°C with PBS, PBS–0.1% SDS, 1 M NH_2OH, or 3.5 M NaSCN. The cells were again washed, and the amount of bound radioactivity was determined. The results (Table 2) showed that NaSCN and PBS–0.1% SDS were unable to elute the bound C3. Incubation with hydroxylamine led to the release of 80% of the radiolabeled C3.

Molecular form of bound C3. The previous experiments demonstrated that some form of C3 was bound to the yeast, but they did not identify the molecule as intact C3 or as C3b, iC3b, or some other fragment of C3. This question was investigated by incubating cryptococci for 2.5, 5, 10, or 30 min at 37°C with normal serum containing ^{125}I-labeled C3. The bound C3 was eluted with hydroxylamine, and the eluted C3 was reduced with 2-mercaptoethanol and analyzed by SDS-PAGE. As a control, C3 alone was incubated with hydroxylamine and analyzed by SDS-PAGE. The results (data not shown) showed that the hydroxylamine had no apparent effect on the SDS-PAGE pattern; this is in agreement with an earlier report by Law and Levine (19). Analysis of the C3 fragments released from *C. neoformans* by hydroxylamine (Fig. 1) showed the presence of bands corresponding to molecular weights of approximately 76,000, 69,000, and 41,000. A faint band at approximately 33,000 was found in samples that were incubated with serum for 10 or 30 min. Incubation with heat-inactivated serum produced an amount of bound and released C3 that was below the limits of detection for this method (data not shown).

DISCUSSION

We and others (6, 9, 15) have reported the binding of C3 to the surface of the cryptococcal capsule. The results of the present study provide a quantitative estimate of the amount of C3 that is bound to the yeast. Approximately 3.2×10^6 iC3b molecules bound to each yeast. This is in the same range as the results reported by Pangburn et al. (24), who found that saturation of rabbit erythrocytes occurs between 1.5×10^6 and 3×10^6 C3b or iC3b molecules per erythrocyte and between 3×10^6 and 4×10^6 molecules per zymosan particle. In the case of *C. neoformans*, it is unlikely that all of the C3 molecules are bound at the capsular surface because our previous ultrastructural studies showed C3 binding at multiple layers within the capsular matrix (15). Thus, the apparent number of opsonic C3 fragments at the immediate surface of the yeast is probably less than the number observed for other particles.

TABLE 2. Elution of [^{125}I]C3 from *C. neoformans*

Eluting agent	Amt of bound radioactivity (cpm, mean ± SEM)[a]
PBS	6,500 ± 740
PBS–0.1% SDS	6,200 ± 440
NH_2OH (1 M)	1,200 ± 240
NaSCN (3.5 M)	7,000 ± 620

[a] Each value is the average of three individual experiments, each of which had four replications.

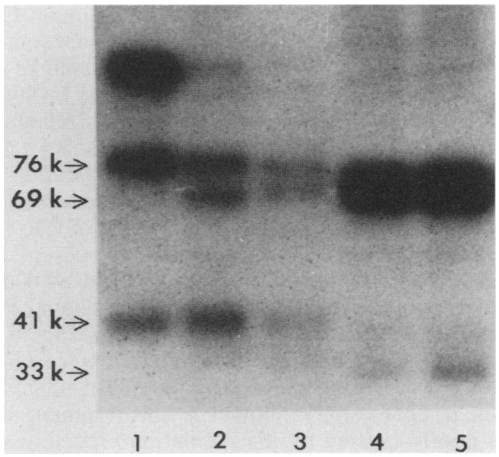

FIG. 1. Autoradiogram of SDS-PAGE of hydroxylamine eluate from cryptococci incubated for various times with human serum containing ^{125}I-labeled C3. Samples were reduced with 2-mercaptoethanol before analysis. Lanes: 1, [^{125}I]C3 alone; 2 to 5, eluates from cryptococci incubated for 2.5, 5, 10, and 30 min, respectively. k, Molecular weight in thousands.

The alternative complement pathway was the primary mechanism for the activation of C3 for binding to the yeast. Alternative pathway activity was indicated by the fact that most binding (63% of the total) occurred in serum chelated with EGTA (7, 25). In addition, maximum binding of iC3b to the yeast required incubation of cryptococci with serum for 20 to 40 min. This rate of deposition is consistent with the relatively slow activation kinetics of the alternative pathway. Indeed, it is slower than the rates observed for the alternative-pathway-mediated deposition of C3b on rabbit erythrocytes, zymosan, and *Escherichia coli* O4 (24).

The binding of C3 to the capsule occurred via a bond that was resistant to detergents and chaotropes but was sensitive to hydroxylamine. Several investigators have suggested that activated C3b reacts with hydroxyl groups on receptive molecules to form an ester bond via an acyl transfer reaction (11, 20, 21). This ester bond is sensitive to hydrolysis with hydroxylamine (19, 20, 21). Activated C3b can also form an amide (hydroxylamine-resistant) bond with receptive molecules (8, 21). The sensitivity of the C3 bound to *C. neoformans* to hydrolysis by hydroxylamine suggests the presence of an ester bond. This differs from the results of a study of antibody-dependent binding of C3b to pneumococci in which C3b was covalently bound to antibody via bonds that were only partially sensitive to incubation with hydroxylamine (1).

Previous studies of C3 binding to *C. neoformans* used fluorescein- or peroxidase-labeled antisera to C3 to determine the presence of C3. This approach did not identify the

molecular form of the capsule-bound C3 as intact C3 or as C3b, iC3b, or some other fragment of C3. Consequently, we used SDS-PAGE and autoradiography to analyze the C3 fragments that were released from the yeast by hydroxylamine. Fragments with molecular weights of 76,000, 69,000, and 41,000 were found after incubation periods of 2.5, 5, 10, and 30 min. The absence of both the 126,000-dalton protein characteristic of the α chain of intact C3 and the 117,000-dalton protein characteristic of the α′ chain of C3b indicate that these proteins were either absent or present in very small amounts. The presence of 76,000- and 69,000-dalton bands suggests the presence of the intact β chain of C3 and a factor I-derived cleavage fragment of C3b, respectively (4). The origin of the 41,000-dalton band is less certain. This band could have been generated by factor I-mediated cleavage of the α′ chain of C3b; however, this fragment of the α′ chain is poorly labeled by the iodination procedures which were used (22). Alternatively, this fragment could be C3d,g generated by cleavage of iC3b. The presence of a 33,000-dalton protein after incubations of 10 and 30 min suggests the eventual decay of a portion of the iC3b to C3d (10, 17, 26). The presence of the 33,000-dalton protein strengthens the argument that the 41,000-dalton protein is C3d,g.

The absence of detectable C3b and the presence of iC3b after incubations as short as 2.5 min indicates a rapid decay of C3b to iC3b at the surface and within the capsule of *C. neoformans*. This parallels the results of a similar study by Pangburn et al. (24) in which the functional activity of the C3b bound to rabbit erythrocytes rapidly decayed after 5 to 10 min at 37°C. In contrast, the C3b bound to pneumococci showed little if any decay to iC3b and could be isolated from the bacteria as C3b (2). A study of C3 fragments bound to *E. coli, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumonia* type 3, and *Haemophilus influenzae* type b showed that only 16 to 28% of the bound C3 was in the form of iC3b (22). These results indicate that the manner of decay of C3b on the surface of a microbial capsule differs from one species of microorganism to another. The presence of large amounts of iC3b bound to *C. neoformans* further suggests that the phagocyte receptor for iC3b (CR3) plays an important role in the phagocytosis of the encapsulated yeast (26).

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