CLINICAL MICROBIOLOGY REVIEWS, Jan. 1996, p. 34–46
0893-8512/96/$04.00+0

Vol. 9, No. 1

Activation of the Complement System by Pathogenic Fungi

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INTRODUCTION

Studies of complement activation by fungi have their origins in a report in 1900 by von Dungern that incubation of yeast cells with serum leads to inactivation of the heat-sensitive components in serum that are normally destructive to bacteria and other cells (104). This observation was confirmed in 1902 by Ehrlich and Sachs (25). Since that time, zymosan, a cell wall product of *Saccharomyces cerevisiae*, has received extensive study as a prototype activator of the complement system (28, 85).

The complement system is a group of proteins which can be activated in a cascade fashion to provide a humoral defense against microorganisms. Initiation of the cascade occurs via the classical or alternative pathway (Fig. 1). Despite different activation mechanisms, both pathways converge at a single point, the third component of complement.

Early steps in activation of the classical pathway involve four proteins: C1q,r,s, C2, C3, and C4. The classical pathway is typically initiated when the C1q subcomponent of C1 recognizes and binds to the Fc region of an immunoglobulin G (IgG) or IgM antibody that is bound to an appropriate antigen. An alternative initiator that may be of particular importance for activation of the classical pathway by fungi is mannan-binding protein (MBP). MBP was first identified by its ability to bind to yeast mannan (51). Serum MBP is a lectin that is found in a wide range of concentrations in sera from healthy adults (68). Serum MBP has a structure similar to that of C1q. Binding of serum MBP to carbohydrate structures, including yeast mannan, leads to antibody-C1q-independent activation of the classical pathway (68, 79). Initiation of the classical pathway can also occur through direct binding of C1q to target particles (10); however, this initiation mechanism is probably uncommon. Regardless of the manner of initiation, the activating event sets into motion a cascade of reactions leading to formation of a C3 convertase, with subsequent cleavage of C3 to C3b. The reader is referred to several reviews for a more detailed discussion of the molecular events involved in initiation and regulation of the classical complement pathway (14, 49, 66). Two features of classical pathway initiation have proven useful for identification of the means by which a putative activator triggers the complement system. First, antibody-initiated activation of the classical pathway will not occur if serum lacks specific antibody, is agammaglobulinemic, or has been absorbed to remove specific antibody. Second, the stability of the C1q,r,s complex is dependent on the presence of Ca^{2+}. The classical pathway is blocked by addition of a calcium chelator such as EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. As described below, absorption of serum to remove specific antibody or chelation with EGTA is an effective means to block the classical pathway while leaving the alternative pathway intact.

The alternative pathway consists of six proteins: C3, factor B, factor D, factor H, factor I, and properdin (Fig. 2). Initiation of the alternative pathway does not require antibody. As a consequence, the alternative pathway is an important natural defense in the nonimmune host. Initiation begins with the spontaneous conversion of C3 in serum to C3(H_2O), a form of C3 with C3b-like properties. C3(H_2O) forms a complex with Mg^{2+} and factor B which is susceptible to the enzymatic action

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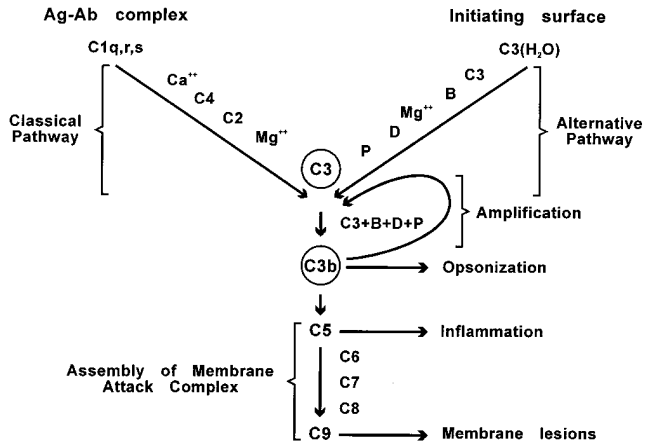

FIG. 1. Classical and alternative pathways for initiation of the complement cascade.

of factor D, leading to the formation of a fluid-phase C3 convertase [C3(H_2O),Bb]. This fluid-phase C3 convertase cleaves C3 from serum to produce metastable C3b, which binds randomly from the fluid phase onto particles. Subsequent interaction of this randomly deposited C3b with proteins of the alternative pathway is determined by the nature of the particle surface.

Particulate activators of the alternative pathway provide a surface that favors binding of factor B in a step that requires Mg^{2+} (45). C3b bound to factor B cannot interact with the inactivating enzyme factor I. Rather, factor B bound to C3b is cleaved by factor D to produce C3b,Bb, a solid-phase C3 convertase which generates new C3b, leading to amplification of C3b deposition on the activator surface. In contrast, the surface of nonactivating particles favors binding of factor H (rather than factor B) to solid-phase C3b (45). Factor H acts as a cofactor for factor I in cleavage of C3b, yielding the inactive fragment iC3b, which cannot participate in further amplification.

Initiation of the complement system may have biological consequences. Cleavage of several proteins during activation of the complement cascade yields soluble fragments that contribute to inflammation. C4a, C3a, and C5a bind to cellular receptors, inducing release of mediators that produce changes in vascular permeability. C5a is the most potent in this regard. C5a is also chemotactic, inducing the directed migration of leukocytes.

Binding of C3 fragments to cellular targets opsonizes the target cells for efficient phagocytosis by cells with receptors for C3 fragments. This process begins with C3, which contains an internal thioester bond that is activated when C3 is cleaved to produce C3b (Fig. 3). This activated thioester has an extremely short life in the newly produced C3b (termed metastable C3b). The activated thioester can form an ester or amide bond with a receptive surface. As noted above, C3b bound to cell surfaces is susceptible to the concerted action of factors H and I. Factor I is a serine protease that cleaves C3b to yield iC3b. Finally, iC3b is susceptible to further protease degradation to produce C3d,g, which remains covalently linked to the activator. C3b, iC3b, and C3d,g are ligands for several complement receptors found on mammalian cells. Interaction between the particle-bound opsonic fragments of C3 and complement receptors on phagocytic cells helps to eliminate the coated target cell. Complement receptor type 1, termed CR1 or CD35, has C3b and C4b as its primary ligands. Complement receptor type 2, termed CR2 or CD21, has C3d,g as its primary ligand. Complement receptor type 3, termed CR3 or CD11b/18, has iC3b as its primary ligand. Finally, complement receptor type 4, termed CR4 or CD11c/18, has iC3b as its primary ligand.

Complement activation can lead to lysis and death of the target cell. This occurs when the terminal pathway components of the complement cascade (C5 to C9) associate into a multimeric membrane attack complex on the target membrane. The membrane attack complex forms transmembrane channels that produce target cell lysis. Direct killing via formation of a membrane attack complex has not been convincingly demonstrated with pathogenic fungi. Presumably, the fungal cell wall blocks formation of an effective attack complex.

CRYPTOCOCCUS NEOFORMANS

Encapsulated cryptococci are powerful activators of the complement system. Goren and Warren found that incubation of encapsulated cryptococci in normal serum leads to binding of a protein, then believed to be C3, to the cryptococcal capsule (34). The first definitive evaluation of the interaction of encapsulated cryptococci with complement proteins was reported by Diamond et al., who found that opsonization of the yeast required an intact complement system (22). Laxalt and Kozel further reported that incubation of *C. neoformans* with normal human serum leads to generation of cleavage fragments of factor B and other cleavage products that are chemotactic for neutrophils (63).

Incubation of encapsulated isolates of *C. neoformans* in normal serum leads to the activation and binding of 10^7 to 10^8 C3 fragments to a typical yeast cell (56, 109). This is considerably more C3 per cell than is found after incubation of other fungi with normal serum. For example, approximately 4×10^6 C3 fragments bind to an average zymosan particle (58), 2×10^6 C3 fragments bind to conidia of *Aspergillus fumigatus* (57), and 2×10^6 C3 molecules bind to a nonencapsulated cryptococcal cell (58).

Initiation and Regulation of the Complement Cascade by Encapsulated and Nonencapsulated *C. neoformans*

Incubation of encapsulated cryptococci in normal human serum leads to activation of the complement system solely via

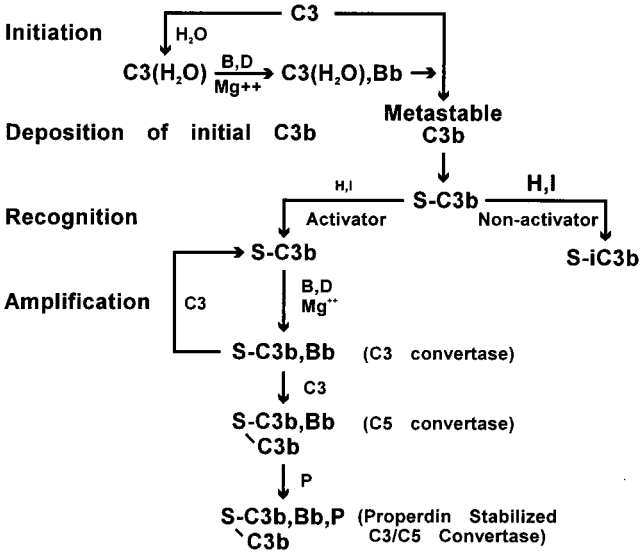

FIG. 2. Schematic representation of molecular events in alternative pathway activation. Adapted from reference 82 with permission of the publisher.

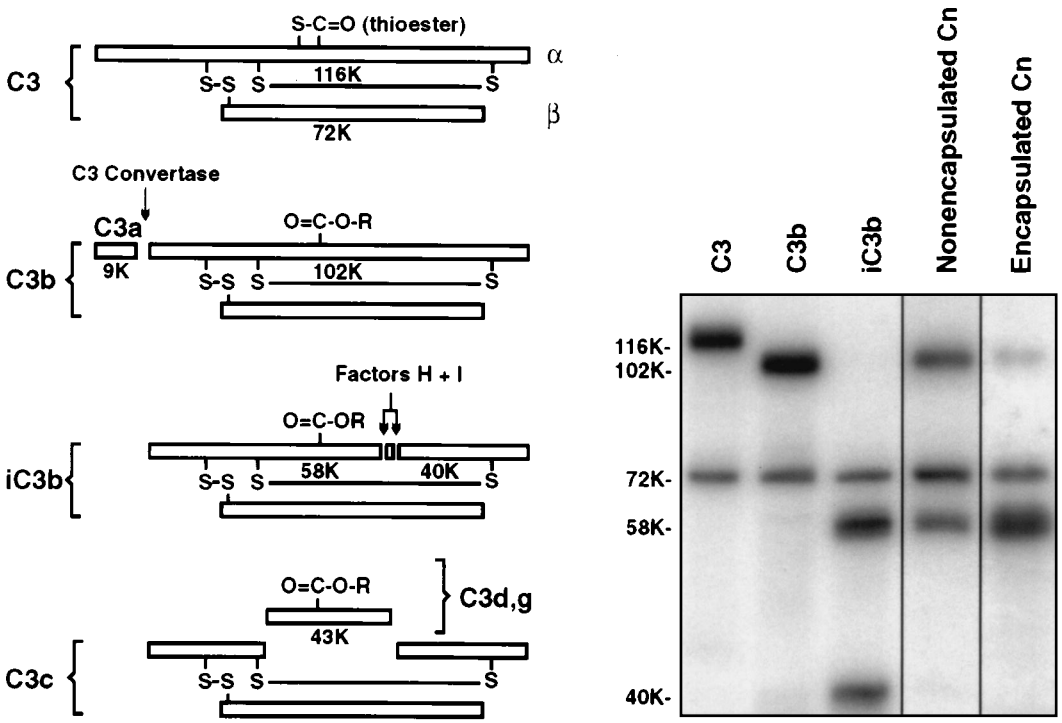

FIG. 3. Schematic representation of the C3 molecule and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of C3, C3b, iC3b, and C3 fragments eluted from encapsulated and nonencapsulated *C. neoformans*. Native C3 has a thioester bond on the alpha chain. Cleavage of C3 by C3 convertase releases a small C3a fragment from the alpha chain and activates the thioester to produce metastable C3b, which can form an ester bond with the yeast surface. C3b is cleaved by the concerted action of factors H and I to produce iC3b. These molecular forms are illustrated in the SDS-PAGE autoradiogram. Analysis of C3 fragments eluted from encapsulated and nonencapsulated *C. neoformans* incubated for 8 min with normal human serum shows that most C3 on encapsulated *C. neoformans* is in the form of iC3b, whereas the C3 fragments on nonencapsulated *C. neoformans* are a mixture of C3b and iC3b.

the alternative pathway. Evidence for an absence of classical pathway initiation comes from two lines of study. First, incubation of encapsulated cells in an alternative pathway reconstituted from purified factors B, D, H, I, and C3 and properdin leads to activation and binding of C3 fragments to the capsule in a manner that is qualitatively and quantitatively indistinguishable from the pattern of activation and binding that occurs with normal serum (59). Second, the kinetics for activation and binding of C3 to encapsulated *C. neoformans* is characterized by a lag of 4 to 6 min before readily detectable amounts of C3 accumulate on the yeast cells (Fig. 4). Once past the lag, accumulation is extraordinarily rapid. The phase of rapid accumulation of C3 fragments on the yeast cells is followed by an abrupt termination of amplification after incubation for approximately 15 min. The kinetics for activation and binding of C3 to encapsulated cryptococci are unaltered if

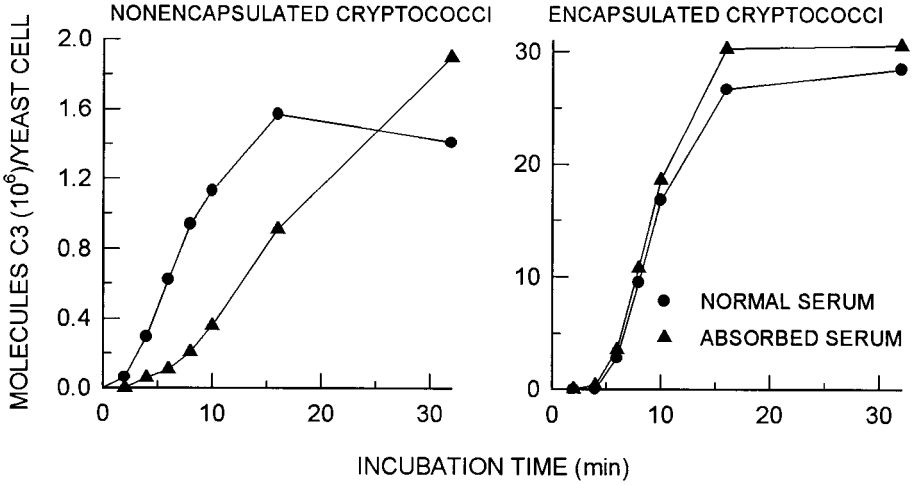

FIG. 4. Kinetics for activation and binding of C3 fragments from normal human serum onto encapsulated and nonencapsulated *C. neoformans*. Yeast cells were incubated for various times with normal human serum (●) or serum that had been absorbed with the homologous yeast to remove antibody (▲). Adapted from reference 108 with permission of the publisher.

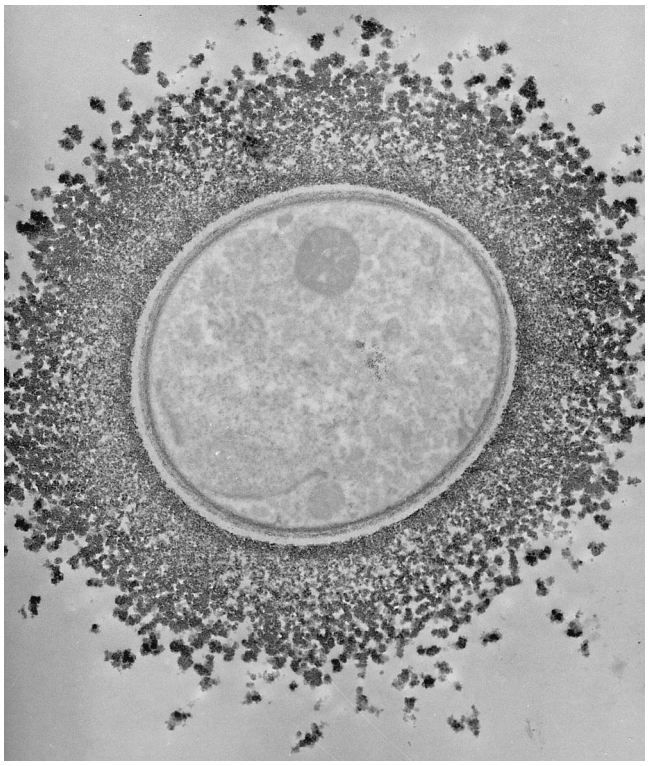

FIG. 5. Ultrastructural location of C3 fragments bound to encapsulated *C. neoformans*. Yeast cells were incubated with normal human serum, and the site of C3 binding was identified by immunoperoxidase staining. C3 deposition is seen as dense staining throughout the capsule, particularly at the capsular surface. Negative controls, using heat-inactivated serum, show a complete absence of staining within the capsule (not shown; see reference 54).

incubation is done in the presence of Mg-EGTA (58). As noted previously, EGTA chelates the Ca^{2+} that is required for classical pathway initiation.

The pattern for activation and binding of C3 to nonencapsulated cryptococci differs quantitatively and qualitatively from the pattern observed with encapsulated *C. neoformans*. First, many more C3 molecules bind to encapsulated cryptococci than to nonencapsulated cells. Second, C3 binds to the cell wall of nonencapsulated cells rather than to the predominant capsular location in the case of encapsulated cells. An electron micrograph illustrating the site for binding of C3 fragments to encapsulated cryptococci is shown in Fig. 5. Third, activation of the complement system by nonencapsulated cryptococci can involve either the classical or the alternative pathway. Incubation of nonencapsulated yeast cells in normal human serum leads to an immediate accumulation of C3 fragments on the yeast cells (58). Blockade of the classical pathway by treatment of serum with Mg-EGTA or by removal of antibodies through absorption of the serum with whole cells introduces a lag into the activation and binding kinetics which is very similar to that observed with encapsulated cryptococci (Fig. 4).

An examination by immunofluorescence microscopy of the cellular sites for early C3 deposition provided an explanation for differences in activation kinetics by encapsulated and nonencapsulated cryptococci. Deposition of C3 into the cryptococcal capsule is focal in nature (58). Incubation of encapsulated yeast cells in normal human serum for 2 to 4 min produces minute sites of C3 deposition (Fig. 6). As the incubation progresses, the early foci expand to form larger patches of C3 deposition, and new minute foci appear at different sites. This pattern of C3 binding suggests a nucleation event in which the formation of sites for alternative pathway initiation is a relatively infrequent event, and the sites spread via alternative pathway amplification to fill the capsule. This pattern is also consistent with the model of alternative pathway initiation described by Pangburn and Müller-Eberhard (illustrated in Fig. 2) which predicts a slow, random formation of solid-phase C3b which would be the nidus for a solid-phase C3 convertase (82). Asynchronous focal initiation is also consistent with the observed kinetics for activation and binding of C3 to encapsulated cryptococci (Fig. 4). The 4- to 6-min lag before appreciable amounts of C3 are bound to the capsule likely reflects the time needed for formation of sufficient focal sites to support vigorous amplification.

Incubation of nonencapsulated *C. neoformans* in normal serum leads to simultaneous and synchronous initiation and rapid accumulation of C3 fragments at sites distributed over the entire cell surface (Fig. 6). This rapid, synchronous initiation is due to the action of the classical pathway. If the classical pathway is disrupted by treatment of serum with Mg-EGTA or by removal of antibody through absorption with nonencapsulated cryptococci, an initiation pattern that is asynchronous and focal in nature occurs, a pattern resembling that observed with encapsulated cryptococci.

Initiation of the classical pathway by nonencapsulated cryptococci is most likely due to the action of a ubiquitous anti-cell wall IgG antibody that is found in sera from all donors tested to date (52). The IgG antibodies are of the IgG2 subclass. The antibodies are absorbed by isolated yeast glucan or zymosan particles, suggesting that the antibodies are glucan specific. This interpretation is consistent with the report by James et al. that the cryptococcal cell wall is composed predominantly of glucan (50). The anticryptococcal cell wall IgG is not removed by absorption with blastoconidia of *Candida albicans* or cells of *S. cerevisiae*. Both *C. albicans* and *S. cerevisiae* have dense accumulations of mannan at the yeast surface (4, 44, 86) which probably block access of the antiglucan antibody to glucan found at deeper sites within the cell wall. This explanation is supported by the ability of zymosan to completely remove the anti-cell wall antibodies in normal sera that are responsible for initiation of the classical pathway by nonencapsulated cryptococci (108). Zymosan consists of "ghosts" of *S. cerevisiae* in which the glucan found in the interior of the cell wall is likely to be available to the antibody during absorption procedures (23). Moreover, zymosan contains considerably less mannan than is found in intact *S. cerevisiae* yeast cells or isolated cell walls (23, 28).

An area that has received little attention is the possible role that high levels of anticapsular IgG or IgM might play in initiation of the classical pathway by encapsulated cryptococci. Although the levels of anticapsular antibody found in normal human serum are not sufficient to alter the kinetics for initiation of the complement system (48), it is possible that passively administered antibody (73, 74) or high levels of antibody raised by active immunization (19) might produce a pattern of activation and binding of C3 fragments that is qualitatively or quantitatively different from the patterns observed with normal serum. The potential importance of antibody-mediated initiation of the complement system is suggested in results from a study by Griffin (36). Encapsulated cryptococci were preincubated with nonimmune mouse serum or nonimmune mouse serum plus rabbit anticapsular IgM. Yeast cells opsonized by IgM plus nonimmune mouse serum were ingested by cytokine-activated murine macrophages in much higher numbers than cryptococci opsonized by incubation with nonimmune mouse serum alone. Quantitative or qualitative alterations in activa-

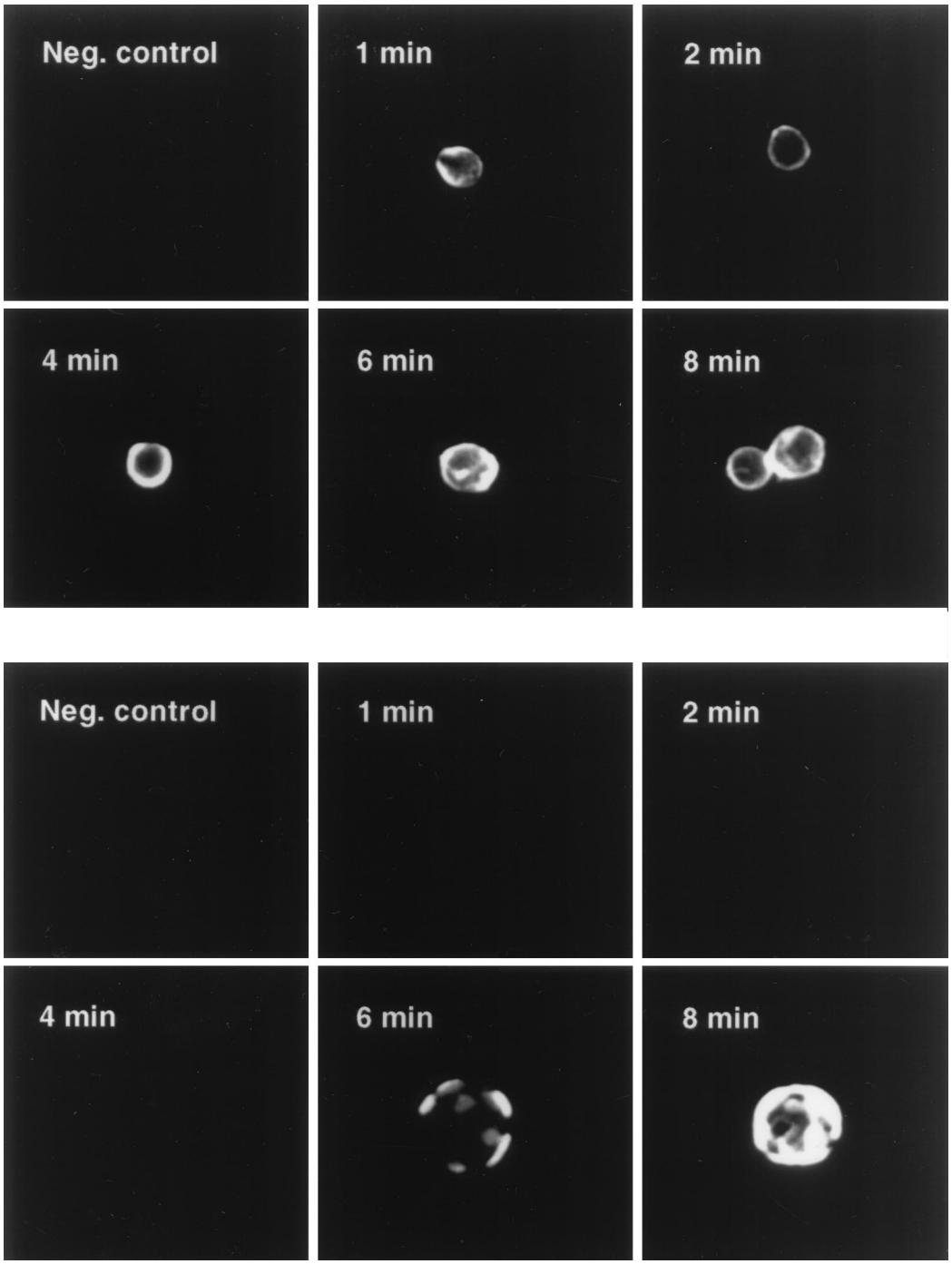

FIG. 6. Localization by immunofluorescence of cellular sites for early deposition of C3 fragments on nonencapsulated (top two rows) and encapsulated (bottom two rows) *C. neoformans*. Yeast cells were incubated with heat-inactivated serum (Neg. control) or incubated for 1, 2, 4, 6, or 8 min with normal human serum and stained with fluorescein-labeled antiserum specific for human C3. Adapted from reference 58 with permission of the publisher.

tion and binding of opsonic C3 fragments to the cryptococcal capsule may contribute to the protective effect observed when mice are passively immunized with anticapsular monoclonal antibodies of the IgM class (73, 74).

Characteristics of C3 Fragments Bound to the Cryptococcal Capsule

C3 fragments are bound to encapsulated cryptococci in a manner that is resistant to elution with detergents or chaotropes. The bound C3 is removed completely by treatment with hydroxylamine (55, 57). Studies of a variety of model compounds and particles have found that activated C3b reacts with hydroxyl groups on receptive molecules to form an ester bond. This ester bond is sensitive to hydrolysis with hydroxylamine (47, 60–62). Activated C3b can also form an amide bond with receptive molecules; the amide bond is resistant to hydrolysis with hydroxylamine. The complete release of C3 fragments from encapsulated cryptococci by treatment with hydroxyl-

amine suggests that metastable C3b binds via an ester bond to the abundant hydroxyl groups found in the cryptococcal capsule. In contrast, a significant percentage of the C3 fragments bound to nonencapsulated cryptococci are resistant to release by treatment with hydroxylamine (55, 84). This suggests the presence of an amide bond. These results obtained with nonencapsulated cryptococci are similar to results from studies of C3 fragments bound to zymosan which found that 20 to 25% of the bound C3 was relatively resistant to elution with hydroxylamine (60).

Once metastable C3b binds to the yeast surface, it can be converted to iC3b when the plasma serine protease factor I cleaves the α′ chain of C3b into three fragments (Fig. 3). Both fragments remain bound to the C3 β chain via disulfide bonds (81). Cleavage of C3b by factor I requires that the complement regulatory protein factor H be bound to the C3b (83). Thus, formation of iC3b is dependent on interaction between particle-bound C3b and factor H. Analysis of C3 fragments eluted from encapsulated *C. neoformans* showed that the vast majority (>95%) are in the form of iC3b (Fig. 3) (55, 84, 105). Indeed, the percentage of bound C3 occurring as iC3b approaches 100% after 8 min of incubation in normal serum (84). In contrast, the percentage of bound C3 occurring as iC3b on nonencapsulated cryptococci does not exceed 70%, even after 60 min of incubation in normal human serum. Rapid conversion of C3b to iC3b within the capsular milieu may explain the abrupt termination in amplification that occurs when encapsulated cryptococci are incubated for approximately 15 min with normal serum (Fig. 4). In contrast, nonencapsulated cryptococci continue to accumulate C3 fragments over incubation periods of 60 min or longer. Continued amplification such as that observed with nonencapsulated cryptococci requires the presence of C3b, which is an essential component of the alternative pathway C3 convertase (Fig. 2).

Rapid conversion of C3b to iC3b suggests that an extremely efficient mechanism operates for conversion of C3b to iC3b on encapsulated cryptococci relative to the conversion that occurs on nonencapsulated cryptococci or zymosan (84). A kinetic analysis of cleavage of C3b to iC3b on encapsulated and nonencapsulated cryptococci suggests that there is a variation in the efficiency with which factor H, factor I, or both influence conversion of C3b to iC3b on these two cell types (84). Dose-response curves of the requirements for both factor H and factor I are quite steep when cleavage of C3b to iC3b is assessed on encapsulated cryptococci. This indicates that the C3b molecules on encapsulated cryptococci are uniform in their interactions with these two proteins. In contrast, dose-response curves for factors H and I in conversion of C3b to iC3b on nonencapsulated cryptococci have shallow slopes. This indicates that C3b molecules bound to nonencapsulated cryptococci are more heterogeneous in their requirements for factors H and I. Indeed, it appears that approximately 30 to 40% of the C3b on nonencapsulated cryptococci is highly resistant to degradation to iC3b. Heterogeneity in the ability of factors H and I to degrade C3b to iC3b is similar to results from a study by Horstmann et al. which found considerable heterogeneity in the affinity of factor H for C3b bound to zymosan (45).

A possible explanation for the uniformity with which factors H and I interact with C3b in the cryptococcal capsule is the homogeneous nature of the capsule itself. This uniform structure, coupled with the three-dimensional matrix of the capsule, may provide an identical binding environment for all C3b molecules. In contrast, the complexity of the cryptococcal cell wall and the two-dimensional nature of the surface of the cell wall may restrict access or provide a variable environment for interaction of factors H and I with C3b.

Molecular Basis for Activation and Regulation of the Complement System by the Cryptococcal Capsule

The cryptococcal capsule presents a surface that activates and regulates the complement system in a manner that is qualitatively and quantitatively different from activation by nonencapsulated cryptococci or zymosan. Indeed, activation and regulation of the alternative pathway appear to be markedly different from those of any particle studied to date. Activation of the alternative pathway represents the end result of several steps in a cascade (Fig. 2). It is likely that one or more molecular features of the capsule influence several of these steps. The cumulative result of these activities is the characteristic pattern by which encapsulated cryptococci activate and regulate the alternative pathway.

Identification of the critical parameters of the capsular structure that influence complement activation requires an understanding of the molecular structure of the capsule. The major constituent of the capsule is a polysaccharide termed glucuronoxylomannan (GXM) (7). Studies by Cherniak and coworkers and reports from other laboratories have greatly clarified the structure of GXM. GXM consists of a (1→3)-linked linear α-D-mannopyran with single β-D-xylopyranosyl, β-D-glucuronopyranosyl, and 6-*O*-acetyl substituents. The polysaccharide occurs as four primary serotypes which differ in the degrees of substitution with xylopyranosyl and *O*-acetyl groups.

The first characteristic of the cryptococcal capsule that influences complement activation is capsular size (109). Large-capsule isolates bind more C3 than small-capsule isolates. However, the increase in C3 binding is not directly proportional to the increase in capsule size. Nevertheless, the large three-dimensional matrix provided by the cryptococcal capsule undoubtedly provides abundant binding sites for C3. This large binding capacity is probably a major factor accounting for the extraordinary amounts of C3 that bind to encapsulated cells.

Capsular serotype influences the efficiency with which C3 binds to the yeast cells as well as the rate at which C3 accumulates on encapsulated cryptococci. A study by Sahu et al. of the binding efficiency of metastable C3b to encapsulated cryptococci of all four serotypes showed a stepwise decrease in binding efficiency that parallels the serotype-dependent decrease in density of xylopyranosyl residues, e.g., C > B > A > D (93). In contrast, evaluations of the rate of accumulation of C3 on encapsulated cryptococci after incubation in normal human serum showed a greater rate of accumulation on cells of serotypes A and D than serotypes B and C (105, 109). These seemingly disparate results illustrate the multifactorial nature of alternative pathway activation by the cryptococcal capsule. Studies of binding efficiency by Sahu et al. rigorously examined a single component of the process, while the rate of amplification reflects the end result of several steps of alternative pathway activation. Thus, serotype-dependent variations in capsular structure may have multiple and independent effects on initiation, amplification, and regulation of the alternative pathway.

O acetylation is another feature of the capsular polysaccharide that impacts activation and/or binding of C3 to encapsulated cryptococci. GXM is O acetylated at position 6 on the mannose backbone (19). Studies by Washburn et al. (105) and Young and Kozel (109) found that de-O-acetylation of encapsulated cryptococci led to a significant increase in the amount of C3 binding to a cryptococcal cell. One explanation for this effect is the possibility that *O*-acetyl groups sterically block potential binding sites on the GXM for metastable C3b.

Biological Consequences of Complement Activation by *C. neoformans*

The complement system is essential for opsonization of encapsulated *C. neoformans* by normal serum. Cryptococci that are incubated with normal serum are engulfed by phagocytes in greater numbers than cryptococci that are not treated with serum (16, 22, 70). The opsonic potential of serum is lost if the serum is inactivated by heating at 50 or 56°C or by treatment with EDTA but not by treatment with EGTA (16, 22). Taken together, these studies of opsonic requirements for phagocytosis of *C. neoformans* indicate the importance of the alternative pathway in opsonization, a result consistent with studies of activation of the complement system by the yeast.

Activation of phagocytes is an important component in ingestion of complement-coated cryptococci. Griffin found that treatment of murine macrophages with a lymphokine derived from murine thymocytes greatly enhanced ingestion of C3-coated cryptococci (36). More recently, Collins and Bancroft found that phagocytosis of serum-opsonized cryptococci was markedly enhanced by treatment of macrophages with tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor, two cytokines that stimulate complement-dependent phagocytosis by murine macrophages (13).

The importance of phagocyte complement receptors in phagocytosis is illustrated by the report from Collins and Bancroft that phagocytosis of serum-opsonized cryptococci is completely blocked by treatment of murine macrophages with monoclonal antibodies specific for CR3, a complement receptor with specificity for iC3b (13). Similarly, Levitz and Tabuni demonstrated that monoclonal antibodies specific for CR1, CR3, or CR4 profoundly inhibited binding of serum-opsonized encapsulated cryptococci to cultured human macrophages (65). The rapid conversion of C3b to iC3b on encapsulated cryptococci suggests that iC3b may be a more important opsonic ligand than C3b; however, it appears that multiple complement receptors are involved in binding of serum-opsonized cryptococci to phagocytes.

Opsonization is not the sole function of the complement system in host resistance to cryptococcosis. The rapid course of cryptococcosis in C5-deficient mice (see below) suggests an important role for C5. C5a, a cleavage fragment of C5, is a powerful mediator of inflammation. Biological activities of C5a that likely contribute to the inflammatory response include induction of increased vascular permeability, chemotaxis, augmented adherence of neutrophils, and degranulation. Indirect evidence for generation of C5a was provided by Laxalt and Kozel, who reported that incubation of encapsulated cryptococci in normal serum leads to release of soluble cleavage fragments that are chemotactic for neutrophils (63). Fragments of C5 were identified as the essential chemotactic factor when Diamond and Erickson found that the chemotactic activity of *C. neoformans*-activated serum is blocked by treatment with antibodies specific for C5 but not by antibodies specific for C3 (20).

Complement Activation In Vivo

Studies of both human cryptococcosis and animal models of cryptococcal disease showed that complement activation occurs in vivo. Macher et al. found a profound depletion of complement activity in sera obtained from patients with cryptococcal sepsis (69). Evidence for complement depletion in patient serum included loss of hemolytic complement activity, loss of the ability to opsonize encapsulated cryptococci for phagocytosis by human neutrophils, depressed levels of circulating C3, the presence of factor B cleavage products, and a decreased ability to deposit C3 fragments on the surface of encapsulated cryptococci. Complement activation was attributed to the alternative pathway because levels of C4 were normal except for a single patient who had circulating antibodies directed against the cryptococcal capsule. Continuous cryptococcal fungemia appears to be necessary for complement depletion because sera obtained from patients with negative fungal blood cultures had normal opsonic activity, normal levels of hemolytic complement activity, and normal levels of circulating C3.

Complement depletion is also observed in experimental models of cryptococcosis, including disseminated cryptococcosis in guinea pigs and mice (29, 69). Intracardiac injection of whole, heat-killed cryptococci led to depletion of hemolytic serum complement activity, indicating that complement activation is due to the yeast cell itself rather than being a secondary consequence of cryptococcosis.

Histopathologic analysis of tissues from patients and animals with experimental cryptococcosis demonstrated deposition of C3 fragments on encapsulated cryptococci. Chiang et al. examined cryptococci found in lesions of patients with cutaneous cryptococcosis and found C3 fragments but not C1q on the surface of the yeast cells, suggesting involvement of the alternative pathway (8). Truelsen et al. examined tissues from mice infected with *C. neoformans* by immunofluorescence to determine the extent of deposition of C3 fragments on encapsulated cryptococci (101). The relative percentages of cryptococci in each tissue having readily visible C3 were greatest for liver and lung, with the kidney having the next highest percentage and the spleen having few C3-coated cryptococci. Binding of C3 fragments to cryptococci in brain tissue was essentially absent. The last result is consistent with studies by Diamond et al., who found little or no C3 detectable on the surface of cryptococci obtained from spinal fluid specimens of four patients with cryptococcal meningitis (22). The absence of deposition of C3 onto cryptococci in brain tissue may provide an additional explanation for the niche that the central nervous system provides for *C. neoformans.*

Role of Complement in Cryptococcosis

Studies of experimental cryptococcosis in animals with congenital or induced deficiencies of the complement system have provided insight into the importance and function of the complement system in resistance to cryptococcosis. One such model is the C4-deficient guinea pig. In these animals, a complete absence of serum C4 is transmitted as a simple autosomal recessive trait (27). C4-deficient guinea pigs lack a classical complement pathway but have an intact alternative pathway; thus, this animal model allows an evaluation of the importance of the classical pathway in the pathogenesis of fungal infections. The second model of complement deficiency is the C5-deficient mouse. Sera from several strains of mice, such as DBA/2, A/J, and B10.D2/Sn "old," were noted to lack hemolytic complement activity (9, 92). Subsequent analysis by Nilsson and Müller-Eberhard found that mice with the inherited complement deficiency lacked C5 (78). The third model of complement deficiency is the mouse or guinea pig that has been treated with cobra venom factor (CVF) from *Naja naja* or *Naja haje*. CVF is a component of cobra venom that shares structural and functional properties with C3 (75, 102, 103). CVF forms an Mg^{2+}-dependent complex with factor B which is activated by factor D to produce a CVF-dependent C3 convertase. The CVF-dependent convertase is much more stable than the normal alternative pathway C3 convertase. As a consequence, treatment of serum or animals with CVF leads to

depletion of C3 by the uncontrolled action of the CVF-dependent convertase. It should be noted that several studies of the effects of CVF on fungal pathogenesis have been done with CVF from *N. naja*, which also has C5-depleting activity (102).

C4-deficient guinea pigs exhibited the same susceptibility to lethal cryptococcal infection as that observed in animals with normal levels of C4 (21). These results indicate that classical pathway initiation is not needed for normal levels of resistance to cryptococcosis. This result is consistent with in vitro studies which found that the classical pathway is not involved in activation and binding of C3 to encapsulated cryptococci. A definitive role for the complement system in resistance to cryptococcosis was demonstrated when treatment of guinea pigs or mice with CVF was found to markedly increase susceptibility to cryptococcosis (21, 35). Experimental animals treated with CVF and subsequently challenged with viable cryptococci died earlier than normal animals. Cryptococcosis in the CVF-treated guinea pig is characterized by increased cryptococcemia.

Cryptococcosis in C5-deficient mice differs in several respects from cryptococcosis in mice with normal C5 levels (67, 90, 91). C5-deficient mice die much more rapidly after intravenous challenge than do C5-sufficient mice. The primary effect of the C5 deficiency is expressed in the lung, where C5-deficient mice develop an acute, fatal cryptococcal pneumonia. C5-deficient mice had as many as 1,000 times more cryptococci in lung tissue than did C5-sufficient mice. Analysis of tissues by light and electron microscopy found that neutrophils accumulated in pulmonary vessels and engulfed the yeast cells 30 min after intravenous inoculation into C5-sufficient mice (67). In contrast, neutrophils failed to accumulate in pulmonary vessels and there was no phagocytosis of encapsulated yeast cells in C5-deficient mice. Deaths in this model have been attributed to a massive pulmonary edema that is most likely due to reduced pulmonary infiltration by neutrophils (90).

CANDIDA ALBICANS

Initiation and Regulation of the Complement Cascade by *C. albicans*

Early studies of the interaction of *C. albicans* with the complement system used generation of chemotactic cleavage fragments, opsonization, and activation of C3 and factor B to generate electrophoretically distinguishable cleavage fragments as indicators of complement activation (72, 88, 100). All such studies demonstrated the essential role of the alternative pathway in complement activation by *C. albicans*. Activation was abolished by heating serum at 50 or 56°C, indicating a requirement for a heat-labile serum factor in the production of chemoattractants. Factor B is particularly sensitive to heat and is inactivated by heating at 50°C, indicating the importance of the alternative pathway. Conversely, activation of the complement system by *C. albicans* occurred in serum chelated with EGTA, in C2-deficient serum, and in C4-deficient serum, indicating that activation can take place in the absence of the classical pathway.

Analysis of the kinetics for activation and binding of C3 fragments to *C. albicans* blastoconidia found very rapid deposition of C3 onto the yeast cells. This early deposition is consistent with a role for classical pathway initiation. The earlier studies which implicated the alternative pathway as the primary mechanism for complement activation assessed activation after incubation of blastoconidia with serum for 30 min. This time interval might have overlooked any contribution of the classical pathway to early activation kinetics. Thus, the possibility exists that initiation via the classical pathway is the normal course of events; however, in the absence of classical pathway initiation, the alternative pathway can be invoked, leading to a similar end result. This pattern would be analogous to the pattern observed with nonencapsulated cryptococci (Fig. 4).

There are at least two candidates for a putative classical pathway initiator. First, IgG antibody that is reactive with *C. albicans* mannan is very common in the general population (64). Second, mannan is a significant component of the *C. albicans* surface (44, 86), raising the possibility that MBP might play a role in initiation of the complement cascade. A central role for mannan in initiation of the complement system is supported by the observation of Denning and Davies that incubation of human serum with a cell wall mannan preparation led to generation of chemotactic complement fragments (18).

Characteristics of C3 Fragments Bound to *C. albicans*

Approximately 50% of the C3 bound to *C. albicans* is resistant to elution with hydroxylamine (53). This suggests that a significant number of the C3 fragments are bound via amide linkages. Proteins found at the candidal surface, perhaps as constituents of the mannoprotein, are possible candidates for the acceptor for binding via amide linkages. Analysis of the molecular form of the C3 fragments bound to *C. albicans* found that most of the fragments were in the form of C3b. These results suggest that phagocyte receptors with high affinity for C3b will be more important in phagocytosis than receptors with high affinity for iC3b. The large percentage of C3 in the form of C3b suggests that C3b bound to *C. albicans* is much more resistant to the action of factors H and I than C3b bound to encapsulated *C. neoformans*. The molecular basis for resistance to degradation from C3b to iC3b within the milieu of the candidal surface is not known.

Candidal Receptors for C3 Fragments

C. albicans hyphae and pseudohyphae form rosettes with sheep erythrocytes coated with C3d or iC3b (24, 41). These results indicate that candidal cells have receptors for cleavage fragments of C3. Although expression of complement receptors appears to be greatest on hyphae (24, 41), there is evidence for iC3b receptor activity on blastoconidia as well (33). The similarity in function between the candidal complement receptors and mammalian CR2 and CR3 led to studies of the structural similarity between mammalian and candidal complement receptors. Fluorescence microscopy was used to show that the anti-CR3 monoclonal antibodies OKM-1, anti-Mac-1, and anti-Mo1 bind to *C. albicans* (24, 33). Binding of anti-CR2 antibodies to *C. albicans* was not readily demonstrable by immunofluorescence, but high concentrations of an anti-CR2 monoclonal antibody and an anti-CR2 polyclonal antibody blocked rosetting with C3d-coated erythrocytes (24).

Expression of candidal complement receptors is influenced by growth conditions. For example, expression of the iC3b-binding protein is temperature sensitive, with reduced expression by cells grown at 37 or 38.5°C (26). In another example, iC3b binding activity is stimulated by growth in medium containing 20 mM glucose (46).

Data indicating a role for complement receptors in virulence is largely correlative. An examination of the species of *Candida* that bind iC3b- and C3d-coated erythrocytes found that only *C. albicans* and the closely related *Candida stellatoidea* exhibited complement receptor activity. Thus, the presence of complement receptors on the most pathogenic of the *Candida* species suggests a role in pathogenesis (24, 41). This association is not

absolute because several species that lack complement receptor activity are also capable of producing disease. Further evidence for a role in virulence was provided when an avirulent mutant of *C. albicans* which exhibited reduced binding of iC3b-coated sheep erythrocytes was identified (80). A role as a candidal adhesin is one mechanism by which complement receptors could contribute to the pathogenesis of candidiasis. Gustafson et al. found that yeast cells grown under conditions that stimulate expression of iC3b binding activity showed greater adherence to human umbilical vein endothelium than cells grown under conditions that suppress receptor expression (37). Moreover, treatment of *C. albicans* with anti-Mo1, an antibody that is reactive with mammalian CR3, blocks adhesion. Finally, pretreatment of *C. albicans* with purified iC3b inhibits glucose-enhanced adhesion to human umbilical vein endothelium. An additional contribution of *C. albicans* complement receptors to virulence was suggested by Moors et al., who found that complement receptors may play a role in iron acquisition by *C. albicans* (71). Expression of iC3b binding activity by *C. albicans* enabled the organism to bind complement-coated erythrocytes and provided a means to obtain heme-derived iron for growth.

Role of Complement in Candidiasis

Immunofluorescence studies found deposits of C3 in biopsies of lesional skin from animals with experimental cutaneous candidiasis and patients with chronic mucocutaneous candidiasis, demonstrating that *C. albicans* activates the complement system in vivo (94, 95).

Studies with experimental models of *C. albicans* infection found that the complement system plays an important role in resistance to disseminated candidiasis. For example, guinea pigs treated with CVF have a higher rate of mortality than untreated animals in response to *C. albicans* infection (32). Quantitative organ cultures done 12 h after infection showed significantly greater numbers of *C. albicans* in kidneys of CVF-treated animals, but there was no difference in the numbers of *C. albicans* in livers from CVF-treated and untreated guinea pigs. The observed effects of CVF treatment are unlikely to be due to a simple decrease in phagocytic killing, because such an effect should produce comparable changes in numbers of *C. albicans* in all tissues. The authors concluded that serum complement plays a major organ-specific role in host defense against septicemia due to *C. albicans* beyond its effect on clearance of the organism from the circulation. Guinea pigs with a genetic deficiency in C4 production are as resistant to disseminated candidiasis as C4-sufficient animals, indicating that classical pathway initiation is not necessary for complement-dependent resistance to disseminated candidiasis.

As is the case with *C. neoformans*, mice with congenital deficiencies in C5 production show decreased resistance to disseminated candidiasis (39, 94). The precise mechanism for increased susceptibility of C5-deficient mice to disseminated candidiasis is not known. C5-deficient serum has opsonic activity that is not appreciably different from the opsonic activity of C5-sufficient serum (72). The most likely explanation is an inability of C5-deficient mice to mount an adequate inflammatory response. The importance of neutrophils in resistance to disseminated candidiasis is well known. A failure in generation of C5a would likely improve the ability of *C. albicans* to survive host tissue defenses.

The complement system is also important in resistance to cutaneous candidiasis. Ray and Wuepper found that intraepidermal injection of *C. albicans* into complement-sufficient mice led to hyphal proliferation that was confined largely to the epidermis. In contrast, C5-deficient mice or mice treated with CVF developed no inflammatory response and permitted *C. albicans* to proliferate into the subcutaneous fat (89).

ASPERGILLUS SPP.

Conidia of *A. fumigatus* exhibit many of the complement-activating parameters found with other fungi, including generation of cleavage fragments of C3 (1), depletion of serum hemolytic complement activity (1), and binding of C3 to the conidial surface (57, 96, 97). There are several unusual aspects of the interaction of *Aspergillus* spp. with the complement system. Resting conidia, swollen conidia, and hyphae differ in the mode of their initiation of the complement cascade (57). Initiation is slowest with resting conidia, a kinetic pattern that is reminiscent of the slow activation of the alternative pathway characteristic of encapsulated *C. neoformans*. This interpretation is consistent with the observation that complement activation by resting conidia is mediated by the alternative pathway, with increasing dependence on classical pathway initiation as the fungal particles mature into swollen conidia and then hyphae. These differences in initiation of the complement system likely reflect differences in the surface of each morphologic form. The surface of resting conidia consists of a smooth, waxy, hydrophobic layer (12, 43). This outer layer may be poorly immunogenic or may be encountered rarely in the environment, leading to an absence of antibody in normal serum. This outer layer is likely to be altered as the resting conidia mature, exposing a new surface for which antibody may be relatively common in normal serum. Alternatively, the classical pathway initiation seen with swollen conidia and hyphae may be due to the action of serum MBP. This is an area that would benefit from more detailed study.

Washburn et al. reported that *A. fumigatus* produces a soluble extracellular inhibitor of the complement system (106, 107). Approximately five times more of the inhibitor was required to produce inhibition of classical pathway-initiated hemolytic activity than was required to produce a comparable reduction in activation and binding of C3 to encapsulated *C. neoformans*. These data suggest that the *Aspergillus* complement inhibitor is a selective inhibitor of the alternative pathway. The specific site of action of the inhibitor has not been reported. *Aspergillus* complement inhibitor is nondialyzable, is hydrophobic, contains little polysaccharide or protein, and contains lipids. The role of the inhibitory factor in the pathogenesis of aspergillosis is not known.

The specific role of complement in the pathogenesis of aspergillosis has not received detailed study. Likely effector functions include opsonization for efficient binding by phagocytes and production of an inflammatory response through generation of C5a. A preliminary study of pulmonary and disseminated aspergillosis in DBA/2N mice found that lower infecting doses are required to produce lethal infections in C5-deficient mice (40). It is notable that a detailed evaluation of the activation and binding of C3 to 29 isolates of nine *Aspergillus* species found that conidia of the highly pathogenic species *A. fumigatus* and *A. flavus* bound fewer C3 molecules per unit of surface area than did conidia of less pathogenic species (42).

PARACOCCIDIOIDES BRASILIENSIS

In vitro evidence for activation of the complement system by cells of *P. brasiliensis* includes opsonization for enhanced phagocytosis by macrophages (5), cleavage of serum C3 to yield electrophoretically identifiable fragments (5, 76), generation of mediators chemotactic for neutrophils (5), and depo-

sition of C3 fragments on the yeast surface (76). Examination of sera from patients with various forms of paracoccidioidomycosis for signs of systemic complement activation found cleavage fragments of C4 and factor B, suggesting activation of the classical and alternative pathways (17, 77). Conversely, clinical improvement following treatment was accompanied by a decline in the presence of activation products of both C4 and factor B (17).

The importance of the complement system in the control of experimental paracoccidioidomycosis is less clear than is the case with *C. neoformans* or *C. albicans*. Calich et al. examined the inflammatory response to subcutaneous inoculation of the yeast into normal mice, C5-deficient mice, and mice pretreated with CVF (5). Histological studies of the lesions found a massive influx of neutrophils that was not appreciably diminished in C5-deficient mice or mice depleted of C3 by treatment with CVF. Similar results were found in an experimental model which evaluated the acute inflammatory infiltrate in mice receiving an intraperitoneal inoculation of *P. brasiliensis* (6). Normal mice, C5-deficient mice, and mice treated with CVF showed comparable inflammatory responses. In studies of lethal disseminated paracoccidioidomycosis, Burger et al. found that the 50% lethal dose of *P. brasiliensis* for C5-sufficient B10.D2/nSn mice did not differ appreciably from that for C5-deficient B10.D2/oSn mice (2). The studies of murine paracoccidioidomycosis utilized an intraperitoneal challenge route, whereas studies done with *C. neoformans* or *C. albicans* used intravenous inoculation. Thus, the observed differences in these models may reflect requirements for complement activation in clearance via the two challenge routes rather than fundamental differences in the role of the complement system in the control of the various mycoses.

TRICHOPHYTON SPP.

Trichophyton species are the most widely studied dermatophytes with regard to complement activation. Incubation of *Trichophyton* mycelia with normal serum leads to depletion of hemolytic complement activity, opsonization, and generation of chemotactic cleavage fragments (15, 98, 99). Thus, there is abundant evidence that *Trichophyton rubrum* and *T. mentagrophytes* mycelia are effective activators of the complement system. The function of the complement system in dermatophytic infections is more controversial. Immunofluorescence studies of experimental dermatophytosis have largely failed to demonstrate the presence of complement fragments on fungal elements in the stratum corneum (38, 99). Moreover, accumulation of neutrophils at the site of dermatophyte infection is highly variable, depending in part on the specific infecting dermatophyte and the site of infection. Dahl and Carpenter (15) have argued that complement-dependent accumulation of neutrophils contributes to the control of dermatophyte infection by (i) stimulating epidermal proliferation, with a consequent shedding of fungi via increased desquamation, and (ii) limiting infection to the stratum corneum through an intense inflammatory response whenever fungal hyphae invade tissue. Studies by Tagami et al. suggest that dermatophytes release chemotaxigenesis factors into the epidermis, leading to complement activation (99). Extracts prepared from lesional epidermis of guinea pigs with experimental *T. mentagrophytes* dermatophytosis exhibited chemotactic activity. Moreover, there was good correlation between the severity of the inflammatory changes noted in the experimental dermatophytosis and the in vitro chemotactic activity of the lesional epidermal extracts.

OTHER FUNGI

One of the most dramatic examples of the importance of the complement system in innate resistance to fungal infections is found in a study of infection by *S. cerevisiae* in C5-deficient DBA/2N mice (3). Commonly considered a nonpathogen, *S. cerevisiae* has been reported as a cause of sepsis in patients with predisposing conditions such as immunosuppression and broad-spectrum antibiotic therapy. Studies in an animal model with immunocompetent CD-1 mice found that isolates varied widely in ability to survive in tissues such as spleen, liver, kidney, lungs, and, particularly, brain (11). With the capacity to proliferate or persist in the brain as a criterion for virulence, clinical isolates of *S. cerevisiae* were found to be more virulent than nonclinical isolates. However, in no instance did either clinical or nonclinical isolates produce lethal infections. Similar studies with C5-deficient DBA/2N mice found that a clinical isolate of *S. cerevisiae* produced a lethal infection in 90% of mice infected intravenously with 10^7 CFU. Studies done with congenic B10.D2/oSn (C5-deficient) and B10.D2/nSn (C5-sufficient) mice demonstrated that the host defect contributing to increased susceptibility to lethal infection was C5 production. Various clinical isolates of *S. cerevisiae* differed widely in ability to produce infection-related deaths. Moreover, the rank order of virulence as shown by infection-related deaths in C5-deficient mice differed from the rank order of virulence as shown by the ability to proliferate and/or survive in the brain in C5-sufficient CD-1 mice. This suggests that virulence is multifactorial, with the importance of each virulence factor being dependent on the animal model and/or the outcome used to measure virulence.

Activation of the alternative pathway by *Histoplasma capsulatum* was reported by Ratnoff et al. (87). *H. capsulatum* yeast cells were shown to consume C3 in C4-deficient guinea pig serum. The extent to which the complement system contributes to either host resistance or the pathogenesis of histoplasmosis is not known.

Galgiani et al. examined complement activation by mycelium- or spherule-phase derivatives of *Coccidioides immitis* (30, 31). Evidence for complement activation in vitro included depression of hemolytic C4 and generation of products of activation of C3, C4, and factor B. Incubation of human serum with extracts from the mycelial or spherule phase generated cleavage fragments chemotactic for human polymorphonuclear leukocytes. Studies of patients with self-limited or disseminated coccidioidomycosis found low levels of total hemolytic complement in sera from 9 of 23 patients, indicating that complement activation occurs in vivo.

FUTURE CONCERNS

In vivo studies have provided convincing evidence that the complement system is an important component in innate resistance to fungal infections. Less clear are the specific means by which complement activation mediates this resistance. Studies to date indicate that opsonization and induction of an inflammatory response are the primary effector systems. However, the relative importance of each effector system may vary between fungal pathogens. The complement system is also likely to exhibit organ-specific effects. That is, activation of the complement system may be important in control of fungal proliferation in some tissues but less important with other tissues.

Although there have been few detailed comparative studies, it is becoming increasingly apparent that the molecular mechanisms for initiation and regulation of the complement system

differ from one fungus to another. These differences likely reflect variation in the composition and molecular architecture of the cell wall surface. Activation of the complement system by the *C. neoformans* capsule offers particular promise for contributing to our understanding of the molecular basis for initiation and regulation. Previous studies of complement activation using model particles such as zymosan have been hampered by the complexity of the activating surface. Uniformity in the structure of the cryptococcal capsule may enable an examination of the contribution of individual constituents of an activating surface to specific steps in the complement cascade. As more is learned about the surface of other fungi, a similar strategy will allow evaluation of paradigms developed in model systems.

In conclusion, studies of complement activation by pathogenic fungi provide valuable insights into an essential aspect of fungal pathogenesis. Results of such studies also have general importance in understanding how components of the complement system interact with pathogenic microorganisms.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI 14209, AI 31696, and AI 37194 and by a grant from the Foundation for Research.

REFERENCES

1. **Budzko, D. B., and R. Negroni.** 1975. Hemolytic, cytotoxic and complement inactivating properties of extracts of different species of aspergillus. Mycopathologia **57:**23–26.
2. **Burger, E., L. M. Singer-Vermes, and V. L. G. Calich.** 1985. The role of C5 in experimental murine paracoccidioidomycosis. J. Infect. Dis. **152:**425.
3. **Byron, J. K., K. V. Clemons, J. H. McCusker, R. W. Davis, and D. A. Stevens.** 1995. Pathogenicity of *Saccharomyces cerevisiae* in complement factor five-deficient mice. Infect. Immun. **63:**478–485.
4. **Cabib, E., B. Bowers, A. Sburlati, and S. J. Silverman.** 1988. Fungal cell wall synthesis: the construction of a biological structure. Microbiol. Sci. **5:** 370–375.
5. **Calich, V. L. G., T. L. Kipnis, M. Mariano, C. F. Neto, and W. Dias Da Silva.** 1979. The activation of the complement system by *Paracoccidioides brasiliensis in vitro*: its opsonic effect and possible significance for an *in vivo* model of infection. Clin. Immunol. Immunopathol. **12:**20–30.
6. **Calich, V. L. G., C. A. C. Vaz, and E. Burger.** 1985. PMN chemotactic factor produced by glass-adherent cells in the acute inflammation caused by *Paracoccidioides brasiliensis*. Br. J. Exp. Pathol. **66:**57–65.
7. **Cherniak, R., E. Reiss, M. E. Slodki, R. D. Plattner, and S. O. Blumer.** 1980. Structure and antigenic activity of the capsular polysaccharide of *Cryptococcus neoformans*. Mol. Immunol. **17:**1025–1032.
8. **Chiang, Y., M. Chuan, C. Chang, H. Yeh, and Y. Lu.** 1985. Cutaneous cryptococcosis: a case with C3 deposition on capsules. J. Dermatol. **12:** 79–85.
9. **Cinader, B., S. Dubiski, and A. C. Wardlaw.** 1964. Distribution, inheritance, and properties of an antigen, MUB1, and its relation to hemolytic complement. J. Exp. Med. **120:**897–924.
10. **Clas, F., and M. Loos.** 1982. Requirement for an additional serum factor essential for the antibody-independent activation of the classical complement sequence by gram-negative bacteria. Infect. Immun. **37:**935–939.
11. **Clemons, K. V., J. H. McCusker, R. W. Davis, and D. A. Stevens.** 1994. Comparative pathogenesis of clinical and nonclinical isolates of *Saccharomyces cerevisiae*. J. Infect. Dis. **169:**859–867.
12. **Cole, G. T.** 1981. Architecture and chemistry of the cell walls of higher fungi, p. 227–231. *In* D. Schlessinger (ed.), Microbiology—1981. American Society for Microbiology, Washington, D.C.
13. **Collins, H. L., and G. J. Bancroft.** 1992. Cytokine enhancement of complement-dependent phagocytosis by macrophages: synergy of tumor necrosis factor-α and granulocyte-macrophage colony-stimulating factor for phagocytosis of *Cryptococcus neoformans*. Eur. J. Immunol. **22:**1447–1454.
14. **Cooper, N. R.** 1985. The classical complement pathway. Activation and regulation of the first complement component. Adv. Immunol. **37:**151–216.
15. **Dahl, M. V., and R. Carpenter.** 1986. Polymorphonuclear leukocytes, complement, and *Trichophyton rubrum*. J. Invest. Dermatol. **86:**138–141.
16. **Davies, S. F., D. P. Clifford, J. R. Hoidal, and J. E. Repine.** 1982. Opsonic requirements for the uptake of *Cryptococcus neoformans* by human polymorphonuclear leukocytes and monocytes. J. Infect. Dis. **145:**870–874.
17. **de Messias, I. T., and D. Mohren.** 1994. Classical and alternative complement pathway activation in paracoccidioidomycosis. J. Invest. Allergol. Clin. Immunol. **4:**91–95.
18. **Denning, T. J. V., and R. R. Davies.** 1973. *Candida albicans* and the chemotaxis of polymorphonuclear neutrophils. Sabouraudia **11:**210–221.
19. **Devi, S. J. N., R. Schneerson, W. Egan, T. J. Ulrich, D. Bryla, J. B. Robbins, and J. E. Bennett.** 1991. *Cryptococcus neoformans* serotype A glucuronoxylomannan-protein conjugate vaccines: synthesis, characterization, and immunogenicity. Infect. Immun. **59:**3700–3707.
20. **Diamond, R. D., and N. F. Erickson III.** 1982. Chemotaxis of human neutrophils and monocytes induced by *Cryptococcus neoformans*. Infect. Immun. **38:**380–382.
21. **Diamond, R. D., J. E. May, M. Kane, M. M. Frank, and J. E. Bennett.** 1973. The role of late complement components and the alternate complement pathway in experimental cryptococcosis. Proc. Soc. Exp. Biol. Med. **144:** 312–315.
22. **Diamond, R. D., J. E. May, M. A. Kane, M. M. Frank, and J. E. Bennett.** 1974. The role of the classical and alternative complement pathways in host defenses against *Cryptococcus neoformans* infection. J. Immunol. **112:**2260–2270.
23. **DiCarlo, F. J., and J. V. Fiore.** 1958. On the composition of zymosan. Science **127:**756–757.
24. **Edwards, J. E., Jr., T. A. Gaither, J. J. O'Shea, D. Rotrosen, T. J. Lawley, S. A. Wright, M. M. Frank, and I. Green.** 1986. Expression of specific binding sites on Candida with functional and antigenic characteristics of human complement receptors. J. Immunol. **137:**3577–3583.
25. **Ehrlich, P., and H. Sachs.** 1902. Ueber die Vielheit der Complemente des Serums. Berl. Klin. Wochschr. **39:**297–299.
26. **Eigentler, A., T. F. Schulz, C. Larcher, E.-M. Breitwieser, B. L. Myones, A. L. Petzer, and M. P. Dierich.** 1989. C3bi-binding protein on *Candida albicans*: temperature-dependent expression and relationship to human complement receptor type 3. Infect. Immun. **57:**616–622.
27. **Ellman, L., I. Green, and M. Frank.** 1970. Genetically controlled total deficiency of the fourth component of complement in the guinea pig. Science **170:**74–75.
28. **Fizpatrick, F. W., and F. J. DiCarlo.** 1964. Zymosan. Ann. N. Y. Acad. Sci. **118:**235–261.
29. **Gadebusch, H. H.** 1961. Natural host resistance to infection with *Cryptococcus neoformans*. I. The effect of the properdin system on the experimental disease. J. Infect. Dis. **109:**147–153.
30. **Galgiani, J. N., R. A. Isenberg, and D. A. Stevens.** 1978. Chemotaxigenic activity of extracts from the mycelial and spherule phases of *Coccidioides immitis* for human polymorphonuclear leukocytes. Infect. Immun. **21:**862–865.
31. **Galgiani, J. N., P. Yam, L. D. Petz, P. L. Williams, and D. A. Stevens.** 1980. Complement activation by *Coccidioides immitis*: in vitro and clinical studies. Infect. Immun. **28:**944–949.
32. **Gelfand, J. A., D. L. Hurley, A. S. Fauci, and M. M. Frank.** 1978. Role of complement in host defense against experimental disseminated candidiasis. J. Infect. Dis. **138:**9–16.
33. **Gilmore, B. J., E. M. Retsinas, J. S. Lorenz, and M. K. Hostetter.** 1988. An iC3b receptor on *Candida albicans*: structure, function, and correlates for pathogenicity. J. Infect. Dis. **157:**38–46.
34. **Goren, M. B., and J. Warren.** 1968. Immunofluorescence studies of reactions at the cryptococcal capsule. J. Infect. Dis. **118:**215–229.
35. **Graybill, J. R., and J. Ahrens.** 1981. Immunization and complement interaction in host defence against murine cryptococcosis. RES J. Reticuloendothel. Soc. **30:**347–357.
36. **Griffin, F. M., Jr.** 1981. Roles of macrophage Fc and C3b receptors in phagocytosis of immunologically coated *Cryptococcus neoformans*. Proc. Natl. Acad. Sci. USA **78:**3853–3857.
37. **Gustafson, K. S., G. M. Vercellotti, C. M. Bendel, and M. K. Hostetter.** 1991. Molecular mimicry in *Candida albicans*: role of an integrin analogue in adhesion of the yeast to human endothelium. J. Clin. Invest. **87:**1896–1902.
38. **Hay, R. J., and E. Nageeb Saeed.** 1981. The immunofluorescence staining of fungi in chronic dermatophyte infections. Clin. Exp. Dermatol. **6:**155–158.
39. **Hector, R. F., J. E. Domer, and E. W. Carrow.** 1982. Immune responses to *Candida albicans* in genetically distinct mice. Infect. Immun. **38:**1020–1028.
40. **Hector, R. F., E. Yee, and M. S. Collins.** 1990. Use of DBA/2N mice in models of systemic candidiasis and pulmonary and systemic aspergillosis. Infect. Immun. **58:**1476–1478.
41. **Heidenreich, F., and M. P. Dierich.** 1985. *Candida albicans* and *Candida stellatoidea*, in contrast to other *Candida* species, bind iC3b and C3d but not C3b. Infect. Immun. **50:**598–600.
42. **Henwick, S., S. V. Hetherington, and C. C. Patrick.** 1993. Complement binding to aspergillus conidia correlates with pathogenicity. J. Lab. Clin. Med. **122:**27–35.
43. **Hess, W. M., and D. L. Stocks.** 1969. Surface characteristics of *Aspergillus* conidia. Mycologia **61:**560–571.
44. **Horisberger, M., and M. Vonlanthen.** 1977. Localization of mannan and chitin on thin sections of budding yeast cells with gold markers. Arch. Microbiol. **115:**1–7.

45. **Horstmann, R. D., M. K. Pangburn, and H. J. Müller-Eberhard.** 1985. Species specificity of recognition by the alternative pathway of complement. J. Immunol. **134:**1101–1104.
46. **Hostetter, M. K., J. S. Lorenz, L. Preus, and K. E. Kendrick.** 1990. The iC3b receptor on *Candida albicans*: subcellular localization and modulation of receptor expression by glucose. J. Infect. Dis. **161:**761–768.
47. **Hostetter, M. K., M. L. Thomas, F. S. Rosen, and B. F. Tack.** 1982. Binding of C3b proceeds by a transesterification reaction at the thiolester site. Nature (London) **298:**72–75.
48. **Houpt, D., G. S. T. Pfrommer, B. Young, T. Larson, and T. R. Kozel.** 1994. Characteristics of antibodies in normal human serum that are reactive with the *Cryptococcus neoformans* glucuronoxylomannan. Infect. Immun. **62:** 2857–2864.
49. **Hughes-Jones, N. C.** 1986. The classical pathway, p. 21–44. *In* G. D. Ross (ed.), Immunobiology of the complement system: an introduction for research and clinical medicine. Academic Press, Inc., Orlando, Fla.
50. **James, P. G., R. Cherniak, R. G. Jones, C. A. Stortz, and E. Reiss.** 1990. Cell-wall glucans of *Cryptococcus neoformans* CAP 67. Carbohydr. Res. **198:** 23–38.
51. **Kawasaki, T., R. Etoh, and I. Yamashina.** 1978. Isolation and characterization of a mannan-binding protein from rabbit liver. Biochem. Biophys. Res. Commun. **81:**1018.
52. **Keller, R. G., G. S. T. Pfrommer, and T. R. Kozel.** 1994. Occurences, specificities, and functions of ubiquitous antibodies in human serum that are reactive with the *Cryptococcus neoformans* cell wall. Infect. Immun. **62:** 215–220.
53. **Kozel, T. R., R. R. Brown, and G. S. T. Pfrommer.** 1987. Activation and binding of C3 by *Candida albicans*. Infect. Immun. **55:**1890–1894.
54. **Kozel, T. R., B. Highison, and C. H. Stratton.** 1984. Localization on encapsulated *Cryptococcus neoformans* of serum components opsonic for phagocytosis by macrophages and neutrophils. Infect. Immun. **43:**574–579.
55. **Kozel, T. R., and G. S. T. Pfrommer.** 1986. Activation of the complement system by *Cryptococcus neoformans* leads to binding of iC3b to the yeast. Infect. Immun. **52:**1–5.
56. **Kozel, T. R., G. S. T. Pfrommer, A. S. Guerlain, B. A. Highison, and G. J. Highison.** 1988. Strain variation in phagocytosis of *Cryptococcus neoformans*: dissociation of susceptibility to phagocytosis from activation and binding of opsonic fragments of C3. Infect. Immun. **56:**2794–2800.
57. **Kozel, T. R., M. A. Wilson, T. P. Farrell, and S. M. Levitz.** 1989. Activation of C3 and binding to *Aspergillus fumigatus* conidia and hyphae. Infect. Immun. **57:**3412–3417.
58. **Kozel, T. R., M. A. Wilson, and J. W. Murphy.** 1991. Early events in initiation of alternative complement pathway activation by the capsule of *Cryptococcus neoformans*. Infect. Immun. **59:**3101–3110.
59. **Kozel, T. R., M. A. Wilson, G. S. T. Pfrommer, and A. M. Schlageter.** 1989. Activation and binding of opsonic fragments of C3 on encapsulated *Cryptococcus neoformans* by using an alternative complement pathway reconstituted from six isolated proteins. Infect. Immun. **57:**1922–1927.
60. **Law, S. K., and R. P. Levine.** 1977. Interaction between the third complement protein and cell surface macromolecules. Proc. Natl. Acad. Sci. USA **74:**2701–2705.
61. **Law, S. K., N. A. Lichtenberg, and R. P. Levine.** 1979. Evidence for an ester linkage between the labile binding site of C3b and receptive surfaces. J. Immunol. **123:**1388–1394.
62. **Law, S. K. A., T. M. Minich, and R. P. Levine.** 1981. Binding reaction between the third human complement protein and small molecules. Biochemistry **20:**7457–7463.
63. **Laxalt, K. A., and T. R. Kozel.** 1979. Chemotaxigenesis and activation of the alternative complement pathway by encapsulated and nonencapsulated *Cryptococcus neoformans*. Infect. Immun. **26:**435–440.
64. **Lehmann, P. F., and E. Reiss.** 1980. Comparison by ELISA of serum anti-*Candida albicans* mannan IgG levels of a normal population and in diseased patients. Mycopathologia **70:**89–93.
65. **Levitz, S. M., and A. Tabuni.** 1991. Binding of *Cryptococcus neoformans* by human cultured macrophages: requirements for multiple complement receptors and actin. J. Clin. Invest. **87:**528–535.
66. **Liszewski, M. K., and J. P. Atkinson.** 1993. The complement system, p. 917–939. *In* W. E. Paul (ed.), Fundamental immunology. Raven Press, Ltd., New York.
67. **Lovchik, J. A., and M. F. Lipscomb.** 1993. Role of C5 and neutrophils in the pulmonary intravascular clearance of circulating *Cryptococcus neoformans*. J. Respir. Cell Mol. Biol. **9:**617–627.
68. **Lu, J., S. Thiel, H. Wiedmann, R. Timpl, and K. B. M. Reid.** 1990. Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme $C1r_2C1s_2$ complex, of the classical pathway of complement, without involvement of C1q. J. Immunol. **144:**2287–2294.
69. **Macher, A. M., J. E. Bennett, J. E. Gadek, and M. M. Frank.** 1978. Complement depletion in cryptococcal sepsis. J. Immunol. **120:**1686–1690.
70. **Mitchell, T. G., and L. Friedman.** 1972. In vitro phagocytosis and intracellular fate of variously encapsulated strains of *Cryptococcus neoformans*. Infect. Immun. **5:**491–498.
71. **Moors, M. A., T. L. Stull, K. J. Blank, H. R. Buckley, and D. M. Mosser.** 1992. A role for complement receptor-like molecules in iron acquisition by *Candida albicans*. J. Exp. Med. **175:**1643–1651.
72. **Morrison, R. P., and J. E. Cutler.** 1981. In vitro studies of the interaction of murine phagocytic cells with *Candida albicans*. RES J. Reticuloendothel. Soc. **29:**23–34.
73. **Mukherjee, J., G. Nussbaum, M. D. Scharff, and A. Casadevall.** 1995. Protective and non-protective monoclonal antibodies to *Cryptococcus neoformans* originating from one B-cell. J. Exp. Med. **181:**405–409.
74. **Mukherjee, J., M. D. Scharff, and A. Casadevall.** 1992. Protective murine monoclonal antibodies to *Cryptococcus neoformans.* Infect. Immun. **60:**4534–4541.
75. **Müller-Eberhard, H. J., and K.-E. Fjellstrom.** 1971. Isolation of the anticomplementary protein from cobra venom and its mode of action on C3. J. Immunol. **107:**1666–1672.
76. **Munk, M. E., and W. Dias Da Silva.** 1992. Activation of human complement system *Paracoccidioides brasiliensis* and its deposition on the yeast form cell surface. J. Med. Vet. Mycol. **30:**481–484.
77. **Munk, M. E., A. Kajdacsy-Balla, G. Del Negro, L. C. Cuce, and W. Dias Da Silva.** 1992. Activation of human complement system in paracoccidioidomycosis. J. Med. Vet. Mycol. **30:**317–321.
78. **Nilsson, U. R., and H. J. Müller-Eberhard.** 1967. Deficiency of the fifth component of complement in mice with an inherited complement defect. J. Exp. Med. **125:**1–16.
79. **Ohta, M., M. Okada, I. Yamashina, and T. Kawasaki.** 1990. The mechanism of carbohydrate-mediated complement activation by the serum mannan-binding protein. J. Biol. Chem. **265:**1980–1984.
80. **Ollert, M. W., E. Wadsworth, and R. A. Calderone.** 1990. Reduced expression of the functionally active complement receptor for iC3b but not for C3d on an avirulent mutant of *Candida albicans.* Infect. Immun. **58:**909–913.
81. **Pangburn, M. K., and H. J. Müller-Eberhard.** 1983. Kinetic and thermodynamic analysis of the control of C3b by the complement regulatory proteins factors H and I. Biochemistry **22:**178–185.
82. **Pangburn, M. K., and H. J. Müller-Eberhard.** 1984. The alternative pathway of complement. Springer Semin. Immunopathol. **7:**163–192.
83. **Pangburn, M. K., R. D. Schreiber, and H. J. Müller-Eberhard.** 1977. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein β1H for cleavage of C3b and C4b in solution. J. Exp. Med. **146:**257–270.
84. **Pfrommer, G. S. T., S. M. Dickens, M. A. Wilson, B. J. Young, and T. R. Kozel.** 1993. Accelerated decay of C3b to iC3b when C3b is bound to the *Cryptococcus neoformans* capsule. Infect. Immun. **61:**4360–4366.
85. **Pillemer, L., L. Blum, I. H. Lepow, O. A. Ross, E. W. Todd, and A. C. Wardlaw.** 1954. Properdin system and immunity: demonstration and isolation of new serum protein, properdin, and its role in immune phenomena. Science **120:**279–285.
86. **Poulain, D., G. Tronchin, J. F. Dubremetz, and J. Biguet.** 1978. Ultrastructure of the cell wall of *Candida albicans* blastospores: study of its constitutive layers by the use of a cytochemical technique revealing polysaccharides. Ann. Microbiol (Inst. Pasteur) **129:**141–153.
87. **Ratnoff, W. D., J. M. Pepple, and J. A. Winkelstein.** 1980. Activation of the alternative complement pathway by *Histoplasma capsulatum*. Infect. Immun. **30:**147–149.
88. **Ray, T. L., and K. D. Wuepper.** 1976. Activation of the alternative (properdin) pathway of complement by *Candida albicans* and related species. J. Invest. Dermatol. **67:**700–703.
89. **Ray, T. L., and K. D. Wuepper.** 1978. Experimental cutaneous candidiasis in rodents. II. Role of the stratum corneum barrier and serum complement as a mediator of a protective inflammatory response. Arch. Dermatol. **114:** 539–543.
90. **Rhodes, J. C.** 1985. Contribution of complement component C5 to the pathogenesis of experimental murine cryptococcosis. Sabouraudia **23:**225–234.
91. **Rhodes, J. C., L. S. Wicker, and W. J. Urba.** 1980. Genetic control of susceptibility to *Cryptococcus neoformans* in mice. Infect. Immun. **29:**494–499.
92. **Rosenberg, L. T., and D. K. Tachibana.** 1962. Activity of mouse complement. J. Immunol. **89:**861–867.
93. **Sahu, A., T. R. Kozel, and M. K. Pangburn.** 1994. Specificity of the thioester-containing reactive site of human C3 and its significance to complement activation. Biochem. J. **302:**429–436.
94. **Sohnle, P. G., M. M. Frank, and C. H. Kirkpatrick.** 1976. Deposition of complement components in the cutaneous lesions of chronic mucocutaneous candidiasis. Clin. Immunol. Immunopathol. **5:**340–350.
95. **Sohnle, P. G., and C. H. Kirkpatrick.** 1976. Deposition of complement in the lesions of experimental cutaneous candidiasis in guinea pigs. J. Cutan. Pathol. **3:**232–238.
96. **Sturtevant, J., and J.-P. Latge.** 1992. Participation of complement in the phagocytosis of the conidia of *Aspergillus fumigatus* by human polymorphonuclear cells. J. Infect. Dis. **166:**580–586.
97. **Sturtevant, J. E., and J.-P. Latge.** 1992. Interactions between conidia of *Aspergillus fumigatus* and human complement component C3. Infect. Immun. **60:**1913–1918.
98. **Swan, J. W., M. V. Dahl, P. A. Coppo, and D. E. Hammerschmidt.** 1983. Complement activation by *Trichophyton rubrum*. J. Invest. Dermatol. **80:** 156–158.

99. **Tagami, H., N. Natsume, T. Aoshima, F. Inuoe, S. Suehisa, and M. Yamada.** 1982. Analysis of transepidermal leukocyte chemotaxis in experimental dermatophytosis in guinea pigs. Arch. Dermatol. Res. **273:**205–217.
100. **Thong, Y. H., and A. Ferrante.** 1978. Alternative pathway of complement activation by *Candida albicans*. Aust. N. Z. J. Med. **8:**620–622.
101. **Truelsen, K., T. Young, and T. R. Kozel.** 1992. In vivo complement activation and binding of C3 to encapsulated *Cryptococcus neoformans*. Infect. Immun. **60:**3937–3939.
102. **Van den Berg, C. W., P. C. Aerts, and H. van Dijk.** 1991. In vivo anticomplementary activities of the cobra venom factors from *Naja naja* and *Naja haje*. J. Immunol. Methods **136:**287–294.
103. **Vogel, C.-W., C. A. Smith, and H. J. Müller-Eberhard.** 1984. Cobra venom factor: structural homology with the third component of human complement. J. Immunol. **133:**3235–3241.
104. **von Dungern, E.** 1900. Beitrage zur Immunitatslehre. Muench. Med. Wochenschr. **47:**677–680.
105. **Washburn, R. G., B. J. Bryant-Varela, N. C. Julian, and J. E. Bennett.** 1991. Differences in *Cryptococcus neoformans* capsular polysaccharide structure influence assembly of alternative complement pathway C3 convertase on fungal surfaces. Mol. Immunol. **28:**465–470.
106. **Washburn, R. G., D. J. DeHart, D. E. Agwu, B. J. Bryant-Varela, and N. C. Julian.** 1990. *Aspergillus fumigatus* complement inhibitor: production, characterization, and purification by hydrophobic interaction and thin-layer chromatography. Infect. Immun. **58:**3508–3515.
107. **Washburn, R. G., C. H. Hammer, and J. E. Bennet.** 1986. Inhibition of complement by culture supernatants of *Aspergillus fumigatus*. J. Infect. Dis. **154:**944–951.
108. **Wilson, M. A., and T. R. Kozel.** 1992. Contribution of antibody in normal human serum to early deposition of C3 onto encapsulated and nonencapsulated *Cryptococcus neoformans*. Infect. Immun. **60:**754–761.
109. **Young, B. J., and T. R. Kozel.** 1993. Effects of strain variation, serotype, and structural modification on the kinetics for activation and binding of C3 to *Cryptococcus neoformans*. Infect. Immun. **61:**2966–2972.