INFECTION AND IMMUNITY, Aug. 1996, p. 3360–3368 Vol. 64, No. 8
0019-9567/96/$04.00+0

Distinct Characteristics of Initiation of the Classical and Alternative Complement Pathways by *Candida albicans*

THOMAS R. KOZEL,* LEANNE C. WEINHOLD, AND DAVID M. LUPAN

Department of Microbiology and Cell and Molecular Biology Program, University of Nevada, Reno, Nevada 89557

Received 22 March 1996/Returned for modification 30 April 1996/Accepted 15 May 1996

Candida albicans **is a potent activator of the complement system. The objective of this study was to characterize factors that influence the kinetics for activation of C3 and binding of C3 fragments to *C. albicans*. Factors that were examined included the surface properties of the yeast and contributions of the classical and alternative complement pathways. The results showed that incubation of hydrophobic, hydrophilic, or germinating yeast cells in normal human serum (NHS) containing radiolabeled C3 led to immediate accumulation of C3 on all three cell types, although the rate of accumulation of C3 on germinating cells was lower. An examination of the sites for early C3 binding showed that classical pathway initiation led to immediate, synchronous binding over the entire cell surface. A blockade of the classical pathway by absorption of putative classical pathway initiators or by chelation of calcium limited activation to the alternative pathway. Binding of C3 solely via the alternative pathway was characterized by a significant lag in the initial binding kinetics. In the absence of classical pathway initiation, the early cellular sites for C3 binding appeared as random, asynchronous foci of C3 that appeared to expand with time. The factor(s) mediating rapid deposition of C3 that was characteristic of the classical pathway initiation was reciprocally cross-absorbed by hydrophilic and hydrophobic *C. albicans* but was not removed by absorption of NHS with *Saccharomyces cerevisiae*, encapsulated *Cryptococcus neoformans*, or nonencapsulated *C. neoformans*. Delayed binding of C3 produced by absorption of serum was largely reversed by addition to the absorbed serum of immunoglobulin G isolated from NHS, indicating a significant role for a naturally occurring anti-*C. albicans* immunoglobulin G in classical pathway initiation.**

Candida albicans yeast cells have been recognized for many years as potent activators of the complement system. Evidence for complement activation following incubation of *C. albicans* yeast cells with normal mammalian serum includes activation of C3 and binding of C3 fragments to the cells (26), generation of chemotactic cleavage fragments (39, 42), opsonization of the yeast to facilitate phagocytosis (33, 35), and the activation of C3 and factor B to generate cleavage products that can be identified by immunoelectrophoresis (39). Studies using animal models of candidiasis demonstrated the importance of the complement system in resistance to *C. albicans*. *C. albicans* infection has an increased mortality in guinea pigs that have been treated with cobra venom factor to deplete serum complement (8). Mice with congenital deficiencies in production of C5 show decreased resistance to disseminated candidiasis (19, 34).

Recent reports indicate that the candidal surface is dynamic in expression of various antigens and surface properties (reviewed in reference 3). For example, *C. albicans* yeast cells grown at room temperature have a hydrophobic surface, whereas cells grown at 37°C have a hydrophilic surface (17). In contrast, *C. albicans* germ tubes are uniformly hydrophobic, regardless of the initial surface hydrophobicity of the parent yeast cells (16, 31). In another example, yeast-to-hypha morphogenesis is accompanied by the surface expression of new antigens not found in blastoconidia (reviewed in reference 3). Previous studies of complement activation by candidal cells utilized yeast cells grown at 20 to 25°C (26, 39) or 37°C (35, 42). There have been few, if any, studies that examined complement activation by *C. albicans* germ tubes or hyphae. The possible contributions that variation in surface properties might make to initiation and regulation of the complement system have not been examined. This is a potentially important issue because expression of the candidal CR-3-like molecule (iC3b receptor) appears to be highly dependent on growth conditions. Surface expression of the iC3b receptor is greater on hyphae than on yeast cells (3, 4, 20). Other studies have found that expression of the iC3b receptor is temperature sensitive, with reduced expression by cells grown at 37 or 38.5°C (5).

The objectives of this study were to compare hydrophobic, hydrophilic, and germinating *C. albicans* yeast cells with regard to (i) the kinetics for activation of C3 and binding of C3 fragments, (ii) the cellular sites for early deposition of C3 fragments, and (iii) the role of the classical complement pathway in initiation of the complement cascade. The results showed that the classical pathway plays a prominent role in initiation of the complement system by all three cell types. This initiation appears to be due primarily to an anti-*C. albicans* antibody found in normal human serum (NHS).

MATERIALS AND METHODS

Yeast cells. *C. albicans* CA-1, a serotype A strain provided by Jim E. Cutler, Montana State University, was used for all studies. A stock culture of the yeast was maintained frozen at −20°C. When fresh yeast cells were required, a sample was taken from the frozen stock and used to inoculate a starter medium consisting of 10 ml of 0.3% yeast extract, 1.0% peptone, and 2.0% dextrose (YEPD) which was then incubated in a gyratory incubator at room temperature.

Yeast cells (hydrophilic and hydrophobic) and germinating yeast cells were examined in this study. Hydrophobic cells were produced by inoculation of 25 ml of YEPD and incubation in a gyratory incubator at 150 rpm at room temperature. The culture was passaged twice at 24-h intervals. The cells were collected by

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

centrifugation, washed four times with cold, sterile distilled water, treated overnight at 4°C with 1% formaldehyde, washed with distilled water, and stored at 10^9 cells per ml at 4°C until use. Hydrophilic blastoconidia were prepared the same way as hydrophobic blastoconidia except the culture was done at 37°C. The hydrophobic or hydrophilic nature of the blastoconidia was assessed by the hydrophobic microsphere assay of Hazen et al. (14) using hydrophobic polystyrene microspheres (0.825-μm diameter; stock code P0008251PN) obtained from Bangs Labs, Inc. (Carmel, Ind.). The criterion for designation of a cell as hydrophobic was the binding of three or more hydrophobic microspheres to the cell. Populations of hydrophobic cells routinely contained >80% hydrophobic yeast cells. Populations of hydrophilic yeast cells routinely contained <10% hydrophobic cells. Germinating yeast cells were prepared from cultures passaged at room temperature. Yeast cells were harvested, washed as described above, and resuspended in cold M199 (pH 7.2, without bicarbonate; GIBCO, Grand Island, N.Y.). These yeast cells were used to inoculate 1 liter of prewarmed (37°C) M199 at a density of 1×10^6 to 2×10^6 cells per ml. This suspension was incubated in a gyratory incubator at 175 rpm at 37°C for 45 to 60 min, or until the cell population demonstrated >80% conversion of yeast cells to germ tubes. Formaldehyde (1%) was added to the culture flasks, and they were stored overnight at 4°C. Formalin-treated yeast cells with germ tubes were harvested, washed, counted, and stored as described above.

Cryptococcus neoformans 388 is an encapsulated strain of serotype A. *C. neoformans* 602 is a stable, nonencapsulated strain. *C. neoformans* cells were grown in a liquid synthetic medium (2) on a gyratory shaker at 100 rpm for 72 h at 30°C. *Saccharomyces cerevisiae* BC159 was provided by William Courchesne from the University of Nevada, Reno. *S. cerevisiae* was cultured under conditions identical to those used for hydrophobic yeast cells of *C. albicans*. The yeast cells were treated overnight at 4°C with 1% formaldehyde, washed with sterile 0.01 M phosphate-buffered 1.27 mM saline (PBS) (pH 7.3), and stored at 4°C.

Serum and serum proteins. Peripheral blood samples were collected from 5 to 10 healthy donors after their informed consent was obtained. The sera were pooled and stored at −85°C. This pool was used as the source of NHS. Serum was heated for 30 min at 56°C for studies requiring heat-inactivated serum. C3 was isolated from frozen human plasma as previously described (27, 41) and stored at −85°C until use. C3 was labeled with ^{125}I by use of Iodo-Gen reagent (Pierce Chemical Co., Rockford, Ill.) (7). Immunoglobulin G (IgG) was isolated from the NHS pool by differential precipitation with caprylic acid and ammonium sulfate (12).

Several experiments used NHS that had been absorbed with yeast cells to remove potential initiators of the classical pathway. To produce once-absorbed serum, NHS was incubated for 60 min at 0°C with 10^8 yeast cells per ml of serum. The cells were removed by centrifugation; the serum was filtered through a 0.45-μm-pore-size filter and frozen at −85°C. In the case of twice-absorbed serum, the absorption procedure was repeated with an identical number of cells under identical conditions.

Immunofluorescence analysis of C3 binding patterns. A 1.0-ml reaction mixture was prepared, consisting of (i) 40% NHS or 40% absorbed serum, (ii) GVB^{2+} (5 mM sodium Veronal-buffered saline [142 mM], pH 7.3, containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$) or GVB-Mg-EGTA [5 mM sodium Veronal-buffered saline (142 mM), pH 7.3, containing 0.1% gelatin, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 5 mM $MgCl_2$], and (iii) 6.0×10^5 *C. albicans* cells. The mixtures were incubated at 37°C, and 200-μl samples were withdrawn after 1, 2, 4, and 8 min. The reaction was stopped by addition of 900 μl of ice-cold PBS containing 10 mM EDTA, and unbound C3 was removed by three washes with PBS. The cells were incubated for 1 h at 4°C in 250 μl of fluorescein-conjugated antiserum to human C3 (Kent Laboratories Inc., Redmond, Wash.) diluted 1:20 in PBS containing 1% bovine serum albumin (Sigma). The cells were washed twice in PBS, suspended in a 9:1 (vol/vol) glycerol-PBS solution, and applied to microscope slides that had been precoated with poly-L-lysine (Sigma). Preliminary experiments found that the polyclonal fluorescein-conjugated antiserum contained antibodies that were reactive with the *C. albicans* cell wall. As a consequence, the anti-C3 serum was absorbed with *C. albicans* cells (10^8 cells per ml of antiserum) before use.

The pattern of C3 deposition was determined by confocal microscopy with a Bio-Rad MRC-600 Confocal Imaging System. Images were scanned at 1-μm intervals through individual cells and projected onto a single plane.

Kinetic analysis of C3 binding. Activation of C3 and binding of C3 fragments to the various fungal cells were done in 1.5-ml reaction mixtures consisting of (i) 40% NHS, 40% absorbed NHS, or 40% heat-inactivated NHS; (ii) GVB^{2+} or GVB-Mg-EGTA; (iii) ^{125}I-labeled C3 sufficient to provide a specific activity of 50,000 cpm/μg of C3 for the mixture of labeled and unlabeled C3 in the serum (assuming that NHS contains 1,200 μg of C3 per ml); and (iv) 3.0×10^6 yeast cells. In the case of encapsulated cryptococci, the number of yeast cells in the reaction mixture was reduced to 6×10^5 because the use of larger numbers of yeast cells was found to consume an excessive amount of serum complement and to lead to erroneous estimates of activation kinetics (45). The tubes were incubated at 37°C, and 50-μl samples were withdrawn in duplicate at various intervals and added to 200 μl of a stop solution consisting of PBS containing 0.1% sodium dodecyl sulfate (SDS) and 20 mM EDTA. The particles were washed four times with PBS-SDS (PBS containing 0.1% SDS) by using Millipore MABX-N12 filter plates fitted with BV 1.2-μm-pore-size filter membranes. The membranes were removed, and the amount of radioactivity bound to cells collected on the membranes was determined with a Packard Auto-Gamma gamma counter. Specific binding was determined by subtracting the radioactivity of samples which used heat-inactivated serum from the total binding observed with NHS.

Binding data are reported as plots of molecules of C3 per yeast cell versus incubation time and as a first-order rate plot. In the first-order rate plot, $\ln A/A_0$ is plotted against incubation time where A_0 is the maximum number of apparent sites available for C3 binding and A is the number of unoccupied sites at each time interval. The slope of the first-order rate plot was used to calculate k', the first-order rate constant, which reflected the rate of accumulation of C3 on the yeast cells. The application of first-order rate plots to analysis of the kinetics for C3 binding has been described previously (29). Many of the kinetics experiments revealed a significant lag before logarithmic accumulation of C3 occurred on the yeast cells. This lag was calculated as the intercept on the x axis where $x = 0$ of the linear portion of a plot of $\ln A/A_0$ versus time (e.g., see Fig. 1). This intercept, termed t_0, and a 95% confidence interval on the intercept were determined by linear regression with the assistance of a graphing and analysis program (SigmaPlot; Jandel Scientific, San Rafael, Calif.). Statistical comparisons of results obtained from two groups were done with the t test; multiple-group comparisons were done by analysis of variance.

RESULTS

Kinetics for accumulation of C3. An initial experiment evaluated the contribution of the classical complement pathway to the kinetics for activation of C3 and binding of C3 fragments to hydrophilic, hydrophobic, and germinating yeast cells. The role of the classical pathway was assessed by two procedures. First, antibodies or other initiators that might contribute to classical pathway initiation were removed by absorption of NHS one or two times with 10^8 candidal cells of each type (homologous cells) per ml of serum. Second, Mg-EGTA was incorporated into the incubation medium to chelate Ca^{2+}, which is required for classical pathway activity (6, 37). The results of one such experiment (Fig. 1), representative of five similar experiments, showed that incubation of each cell type with NHS produced a rapid accumulation of C3 fragments on the cells. Absorption of the serum or incorporation of Mg-EGTA into the buffer introduced a substantial lag into the kinetics for accumulation of C3 on all cell types. A quantitative analysis of the effects of each treatment on the initial lag (t_0) is summarized in Table 1. The results show that absorption of serum once with the homologous cells introduces a substantial lag into the initiation kinetics. A second absorption produces a lag that is indistinguishable from the lag observed when NHS is treated with Mg-EGTA.

The rate of accumulation of C3 on the cells (k') was influenced by both the cell type and the serum treatment. An analysis of results from five replicate experiments (data not shown) showed a significantly ($P \leq 0.005$) greater rate of accumulation of C3 on hydrophilic and hydrophobic yeast cells than on germinating yeast cells. The rates of accumulation of C3 were similar ($P = 0.47$) on hydrophilic and hydrophobic cells incubated in NHS. Treatment of serum by absorption with the homologous yeast cells or by addition of Mg-EGTA generally effected a significant ($P \leq 0.05$) reduction in k' for both hydrophilic and hydrophobic yeast cells. The exception was the effect of serum absorption on the rate of accumulation of C3 on hydrophobic yeast cells ($P = 0.29$). The effect of serum treatment on k' was subtle and required several replications of the experiment before an unequivocal pattern emerged. The failure of absorption with hydrophobic cells to significantly alter the rate of accumulation on the cells is consistent with the results shown in Table 1 which indicate that serum absorption is less effective, in terms of effects on t_0, when done with hydrophobic cells. Treatment of serum by absorption or incorporation of Mg-EGTA did not significantly ($P > 0.05$) reduce the rate of accumulation of C3 on germinating cells. Taken together, these results indicate that classical pathway initiation

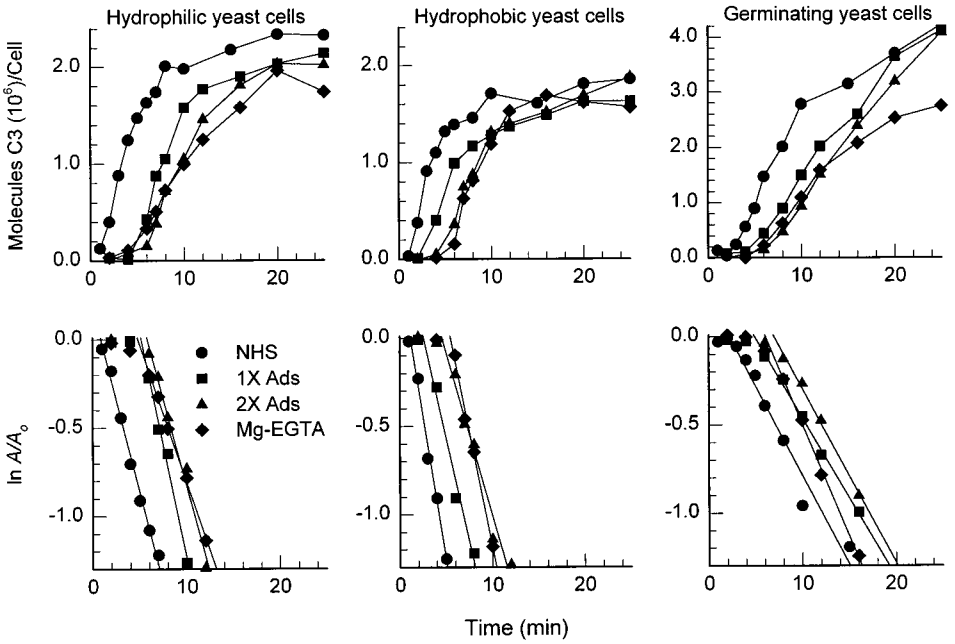

FIG. 1. Effect of serum treatment on the kinetics for activation of C3 and binding of C3 fragments to hydrophilic, hydrophobic, or germinating yeast cells in an incubation medium containing NHS (●), NHS that had been absorbed once with the homologous cell type (■), NHS that had been absorbed twice with the homologous cell type (▲), or NHS that had been treated with Mg-EGTA (◆). The lower panels are first-order rate plots of the consumption of available sites for C3 binding, where A_0 is the maximum number of apparent sites available for C3 binding and A is the number of unoccupied sites at each time interval (see Materials and Methods).

greatly accelerates early activation and binding of C3 to *C. albicans.*

Cellular sites for early C3 binding. An examination of the cellular sites for early deposition of C3 fragments onto candidal cells of each type showed that incubation of *C. albicans* yeast cells with NHS led to rapid and synchronous binding of C3 fragments over the entire cell surface (Fig. 2 to 4). A diffuse pattern was readily discernible after 2 min of incubation. Cells incubated for longer times with NHS showed an increase in the apparent density of bound C3, but there was no obvious change in the location of bound C3. The pattern of NHS-mediated early C3 binding to germinating yeast cells differed somewhat from the pattern observed with hydrophobic or hydrophilic yeasts. There was a rapid and diffuse accumulation of C3 on the surface of the mother cell; binding of C3 to the germ tube was also diffuse but appeared to proceed more slowly. A blockade of the classical pathway by treatment of the serum with Mg-EGTA or by absorption of the serum to remove classical pathway initiators produced a significant alteration in the cellular sites for early deposition of C3 on all three cell types (Fig. 2 to 4). Little or no bound C3 was noted after incubation for 2 min with the treated serum, a result that is in good agreement with results from the kinetics experiments (Fig. 1). After incubation for 4 min with the treated serum, focal deposition of C3 fragments was noted at sites distributed over the entire cell surface. The sites for alternative pathway-mediated formation of early-binding foci were largely random, although several cells showed rapid accumulation of C3 fragments near the site of budding. Alternative pathway-mediated activation of C3 and binding of C3 fragments to germinating yeast cells (Fig. 4) were characterized by the focal initiation pattern observed with hydrophilic and hydrophobic cells. The rate of alternative pathway-mediated accumulation of C3 fragments on the germinating yeast cells (Fig. 4) appeared to be lower than the rate of accumulation on hydrophilic or hydrophobic cells, an observation consistent with results from the kinetic analysis (Fig. 1).

TABLE 1. Effect of serum treatment on the lag before rapid accumulation of C3 fragments on hydrophilic, hydrophobic, and germinating yeast cells

Serum treatment	t_0[a]		
	Hydrophilic yeast cells	Hydrophobic yeast cells	Germinated yeast cells
Normal serum	0.9 (0.2–1.4)	1.2 (0.0–1.8)	2.3 (−0.3–3.9)
Absorbed once	5.2 (4.0–5.9)	2.6 (NA[b])	4.9 (4.2–5.6)
Absorbed twice	5.8 (4.9–5.5)	4.6 (2.5–5.7)	6.9 (5.8–7.9)
Mg-EGTA treated	4.8 (4.5–5.2)	5.5 (4.7–6.0)	6.1 (5.1–6.8)

[a] Data are reported as the t_0 in minutes with the 95% confidence interval shown in parentheses.

[b] NA, statistical analysis not possible because of the limited number of points on line.

Contribution of antibody to early activation kinetics. The observation that treatment of NHS by addition of Mg-EGTA and by absorption with homologous cells produced similar alterations in early activation kinetics suggested that serum contains an absorbable initiator of the classical pathway. A likely candidate for such initiation was antibody, particularly in view of reports that sera from healthy adults contain antibodies of the IgG class that are reactive with *C. albicans* mannan (10, 23, 30). Accordingly, an experiment was done to evaluate the contribution of IgG antibody in NHS to early activation kinetics. Hydrophilic, hydrophobic, or germinating yeast cells were incubated for various times with (i) NHS, (ii) NHS that had been absorbed to remove the putative classical pathway initiator, (iii) absorbed serum that had been supplemented with 480 μg of IgG isolated from NHS per ml, and (iv) NHS treated with Mg-EGTA. The amount of IgG added to the absorbed NHS was calculated to be equivalent to the amount of IgG in

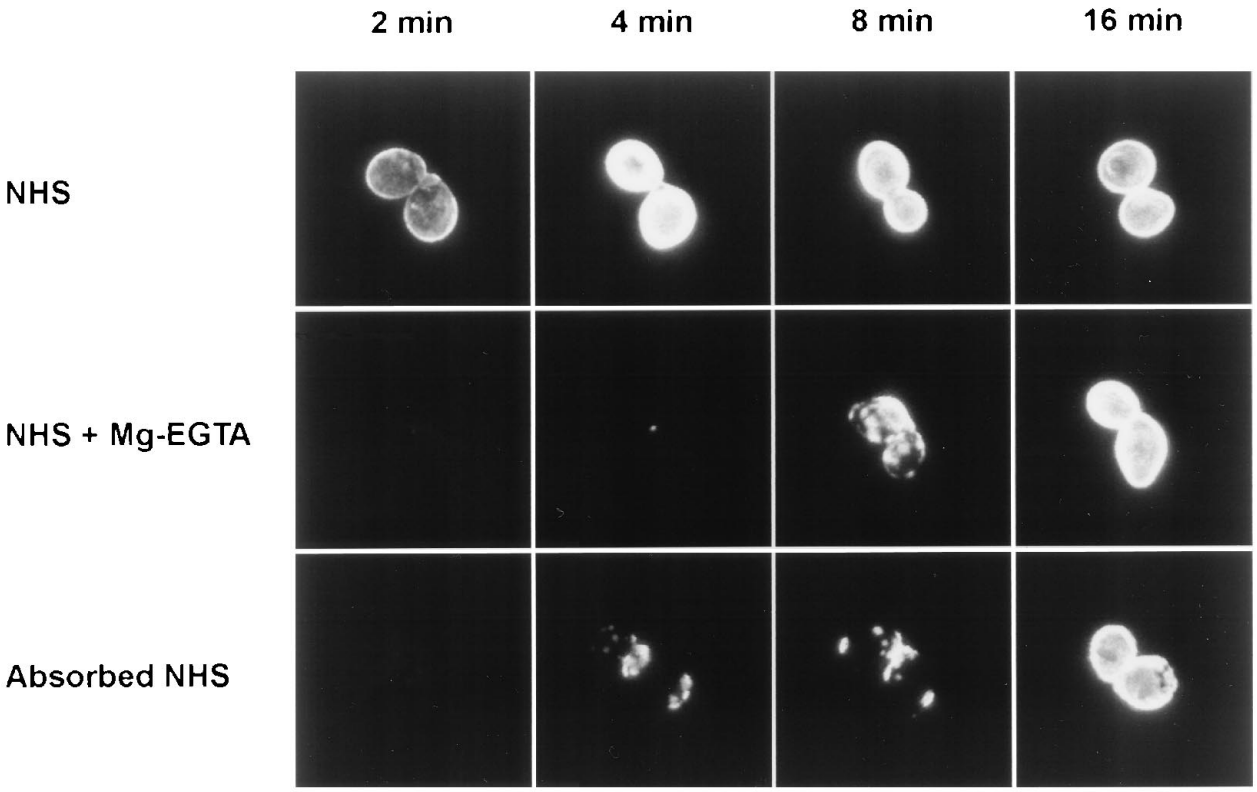

FIG. 2. Sites for deposition of C3 fragments on hydrophilic *C. albicans* yeast cells after incubation for the indicated times with 40% NHS, 40% NHS treated with Mg-EGTA, or 40% NHS that had been absorbed twice with the homologous yeast cells. Sites of C3 deposition were determined by use of fluorescein-labeled antiserum to C3 and examination by confocal microscopy.

40% NHS, assuming 12 mg of IgG per ml of NHS. Data from one of two replicate experiments with similar results are shown in Table 2. As in previous experiments (Fig. 1), absorption influenced both t_0 and k'. The results showed that treatment of NHS by addition of Mg-EGTA or by absorption with homologous yeast cells introduced a significant lag into the early activation kinetics, as shown by a prolongation of t_0 for all three cell types. The effect of absorption on t_0 was almost completely reversed by addition to the absorbed serum of IgG that had been isolated from NHS. In most cases, there was little or no difference between the t_0 observed with NHS and that observed with absorbed serum to which IgG had been added. In all cases, the t_0 observed with absorbed serum supplemented with IgG was significantly shorter than the t_0 observed with absorbed NHS or NHS treated with Mg-EGTA. In contrast to the ability of isolated IgG to largely restore t_0 to levels observed with NHS, addition of IgG did little to restore the reduced rate of C3 accumulation that is produced when serum is absorbed.

Specificity of serum factor mediating classical pathway initiation. An experiment was done to determine the specificity of the serum factor(s) which initiate the classical pathway. Pooled NHS was absorbed with cells of hydrophilic *C. albicans*, hydrophobic *C. albicans*, *S. cerevisiae*, encapsulated *C. neoformans*, or nonencapsulated *C. neoformans*. The kinetics for activation of C3 and binding of C3 fragments to each cell type were then determined for each of the absorbed sera. The results are shown in Fig. 5 as plots of molecules C3 bound per cell versus time. The quantitative effects of serum absorption on t_0 are shown in Table 3.

The results showed that hydrophilic or hydrophobic *C. albicans* cells incubated with sera absorbed with hydrophilic or hydrophobic *C. albicans* exhibited a significantly longer t_0 than similar cells incubated with NHS or NHS absorbed with *S. cerevisiae*, nonencapsulated *C. neoformans*, or encapsulated *C. neoformans*. Absorption of NHS with hydrophilic or hydrophobic *C. albicans* and *S. cerevisiae* cells significantly prolonged t_0 for activation of C3 and binding of C3 fragments to *S. cerevisiae* cells. Absorption of NHS with nonencapsulated cryptococci significantly increased t_0 for activation of C3 and binding of C3 fragments to nonencapsulated cryptococci, a result that is in agreement with previous studies (44); absorption of serum with all other yeast cells had little or no effect on t_0 for nonencapsulated *C. neoformans*. Finally, absorption of NHS with hydrophobic *C. albicans*, *S. cerevisiae*, nonencapsulated cryptococci, or encapsulated cryptococci had no effect on t_0 for activation of C3 and binding of C3 fragments to encapsulated cryptococci. Absorption of NHS with hydrophilic *C. albicans* significantly increased t_0 for encapsulated cryptococci. This was an effect that was observed in some replications of the experiment but not in others (not shown).

DISCUSSION

The objective of this study was to characterize factors that influence the kinetics for activation of C3 and binding of C3 fragments to *C. albicans*. One of the factors to be examined was the effect that variation in growth conditions had on the ability of the candidal surface to initiate the complement system. Conditions that were examined included growth at room temperature to produce a hydrophobic cell surface, growth at 37°C to produce a hydrophilic yeast surface, and germination

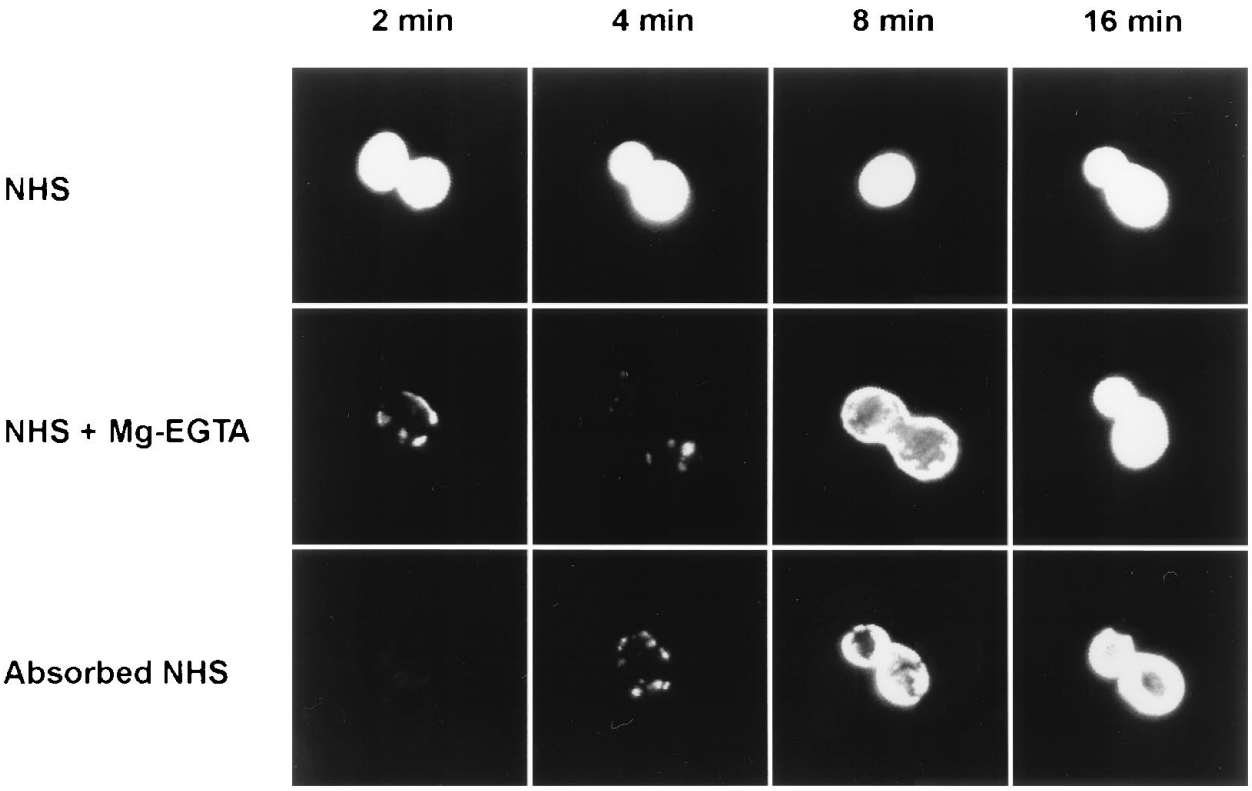

FIG. 3. Sites for deposition of C3 fragments on hydrophobic *C. albicans* yeast cells after incubation for the indicated times with 40% NHS, 40% NHS treated with Mg-EGTA, or 40% NHS that had been absorbed twice with the homologous yeast cells. Sites of C3 deposition were determined by use of fluorescein-labeled antiserum to C3 and examination by confocal microscopy.

of *C. albicans* blastoconidia. A series of studies by Hazen and coworkers (13, 15, 17, 18) found that hydrophilic cells are covered with long and densely arranged fibrillar mannoproteins. Such fibrils are either absent, sparse, or greatly shortened on the surface of hydrophobic cells. The long fibrils of hydrophilic cells cover a layer of hydrophobic proteins which are constitutively exposed on hydrophobic cells. Thus, a comparison of the complement-activating properties of hydrophobic and hydrophilic cells examines two very different cell surfaces.

A comparison of the kinetics for activation of C3 and binding to hydrophilic, hydrophobic, and germinating *C. albicans*

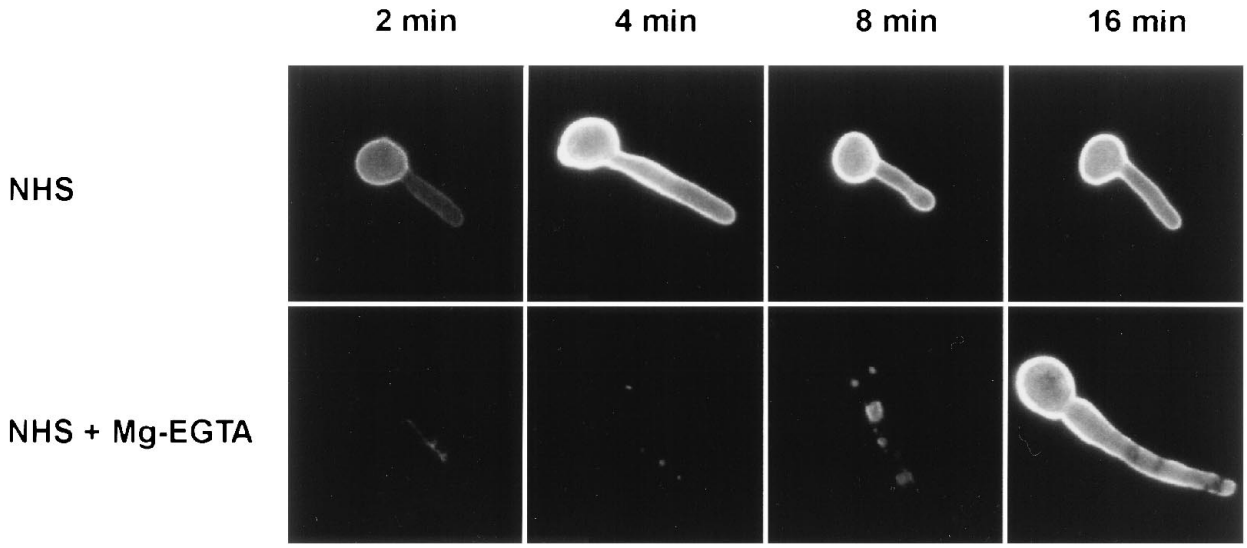

FIG. 4. Sites for deposition of C3 fragments on germinating *C. albicans* yeast cells after incubation for the indicated times with 40% NHS or 40% NHS treated with Mg-EGTA. Sites of C3 deposition were determined by use of fluorescein-labeled antiserum to C3 and examination by confocal microscopy.

TABLE 2. Effect of human IgG on the lag in initial activation kinetics and rate of accumulation of C3 produced by absorption of serum with *C. albicans* cells

Serum treatment	Hydrophilic yeast cells		Hydrophobic yeast cells		Germinating yeast cells	
	t_o[a]	k'[b]	t_o	k'	t_o	k'
Normal serum	1.6[a] (1.1–1.9)	0.27	2.1 (1.5–2.5)	0.25	2.7 (1.5–2.7)	0.13
Absorbed serum	9.2 (7.9–10.1)	0.11	6.8 (4.5–8.4)	0.13	7.2 (5.7–8.5)	0.06
Absorbed serum + purified IgG	3.2 (2.5–3.8)	0.13	2.2 (1.4–2.8)	0.13	3.4 (2.1–4.3)	0.08
Mg-EGTA	8.7 (8.2–9.3)	0.18	7.9 (5.9–8.9)	0.14	7.5 (6.3–8.3)	0.23

[a] Data are reported as the t_o in minutes with the 95% confidence interval shown in parentheses.
[b] Data are reported as the first-order rate constant for amplification of C3 deposition.

cells showed several similarities between the three cell types. The lags before logarithmic accumulation of C3 were similar in the case of hydrophilic and hydrophobic yeast cells ($t_o \approx 1$ to 2 min). The lag was also short for germinating yeast cells but was significantly longer than that observed with hydrophilic or hydrophobic yeast cells ($t_o \approx 2$ to 4 min). Once past the initial lag, the rates of accumulation of C3 on hydrophilic and hydrophobic yeast cells were similar ($k' \approx 0.2$ to 0.3), whereas the rate of accumulation of C3 on germinating yeast cells was significantly lower ($k' \approx 0.10$ to 0.15). The maximum numbers of C3 fragments bound to hydrophobic and hydrophilic yeast cells were similar (approximately 2×10^6 C3 fragments per cell); more C3 molecules (3×10^6 to 4×10^6 C3 fragments per cell) accumulated on germinating yeast cells. The larger numbers of C3 fragments binding to germinating *C. albicans* most likely reflect the larger surface area available for binding.

Rapid initiation of the complement system by all three cell types was due to the action of the classical complement pathway. This result is consistent with a previous report from our laboratory (26). A blockade of the classical pathway by absorption of serum to remove classical pathway initiators or by treatment of serum with Mg-EGTA (6, 37) introduced a significant lag before logarithmic accumulation of C3 occurred on the cells. An examination of the data in Tables 1 and 2 shows that absorption of NHS twice with homologous cells increased t_o by approximately 5 min. The increase in t_o was not appreciably influenced by the cell type or the procedure used to block the classical pathway.

The cellular sites for early deposition of C3 fragments depended on whether the classical pathway was operative. Incubation of all three cell types in NHS produced rapid, synchronous deposition of C3 fragments at sites distributed over the entire cell surface. Hydrophobic and hydrophilic yeast cells showed no apparent preference in sites for early classical pathway-mediated binding. The germ tubes of germinating yeast cells exhibited diminished early binding of C3 relative to that of the mother cell (Fig. 4); however, this difference was less apparent after 4 min of incubation. A decrease in very early classical pathway-mediated binding of C3 to the germ tubes may account for the slight delay in t_o and the lower rate of accumulation of C3 observed with germinating yeast cells (Tables 1 and 2). A blockade of the classical pathway by absorption of NHS with homologous candidal cells or by treatment with Mg-EGTA produced a striking alteration in the pattern

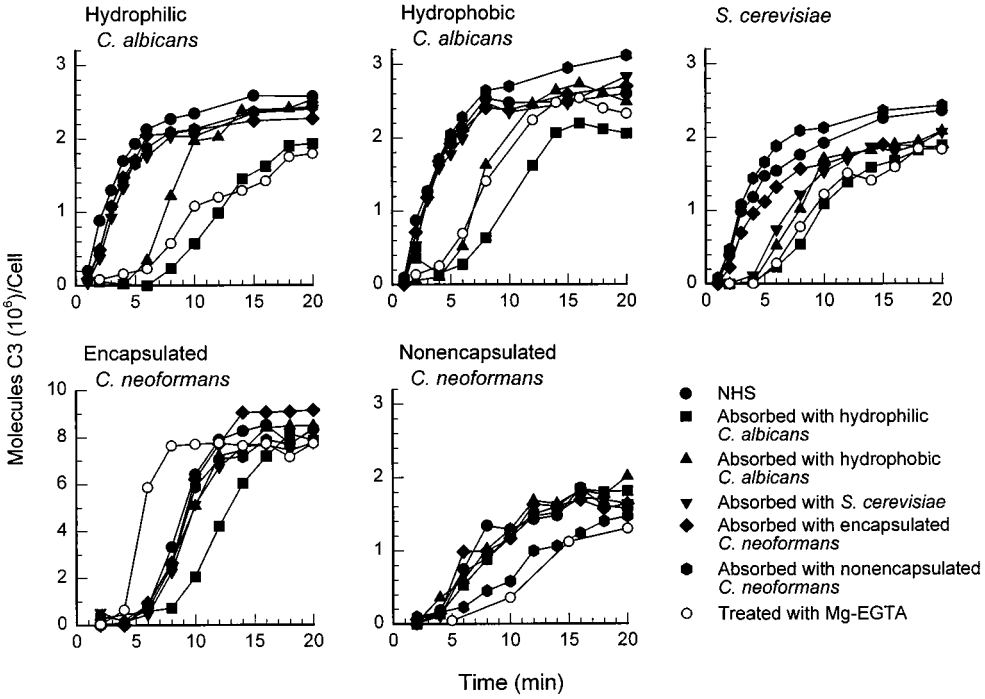

FIG. 5. Effect of cross-absorption on the kinetics for activation of C3 and binding of C3 fragments to hydrophilic *C. albicans*, hydrophobic *C. albicans*, *S. cerevisiae*, encapsulated *C. neoformans*, and nonencapsulated *C. neoformans.*

TABLE 3. Effect of cross-absorption on the lag in initial activation of C3 and binding of C3 fragments to hydrophobic *C. albicans*, hydrophilic *C. albicans*, *S. cerevisiae*, encapsulated *C. neoformans*, and nonencapsulated *C. neoformans*

Yeast cells used to absorb serum[a]	t_0 with the indicated target cell[b]				
	C. albicans		*S. cerevisiae*	*C. neoformans*	
	Hydrophilic	Hydrophobic		Encapsulated	Nonencapsulated
None (NHS)	0.8 (0.5–1.0)	0.9 (0.6–1.1)	1.0 (0.4–1.4)	6.4 (NA[c])	1.9 (0.0–4.7)
C. albicans					
Hydrophilic	6.4 (2.8–7.9)	5.8 (NA)	5.5 (4.4–6.2)	9.6 (8.6–10.3)	3.9 (2.9–4.6)
Hydrophobic	5.8 (2.7–7.3)	5.5 (4.9–5.9)	4.4 (1.9–5.7)	6.1 (3.9–7.4)	3.5 (0.1–5.5)
S. cerevisiae	1.2 (0.5–1.2)	0.9 (0.5–1.2)	3.6 (3.3–3.9)	6.4 (NA)	3.9 (1.5–5.2)
C. neoformans					
Encapsulated	1.0 (0.6–1.4)	1.1 (0.5–1.5)	1.1 (0.5–1.5)	6.2 (2.5–7.6)	3.5 (0.3–5.4)
Nonencapsulated	1.0 (0.6–1.3)	1.1 (0.6–1.5)	2.0 (1.5–2.4)	6.1 (3.7–7.5)	6.6 (5.0–7.7)

[a] Serum was absorbed twice with the indicated yeast cells.
[b] Data are reported as the t_0 in minutes with the 95% confidence interval shown in parentheses.
[c] NA, statistical analysis not possible because of the limited number of points on line.

for early binding of C3 fragments to the cells. There was little binding after 2 min of incubation, a result consistent with the quantitative kinetic analysis (Fig. 1). Once past the lag, alternative pathway-mediated binding was characterized by asynchronous appearance of focal sites for C3 deposition that appeared to expand with increased incubation time to eventually cover the cells. In some cases, the sites for earliest alternative pathway-mediated deposition appeared to occur at the site of budding, but this was not a pattern that was consistently observed with all cells in a given preparation.

All available evidence indicates that an IgG antibody found in NHS is a significant contributor to classical pathway initiation. The increased t_0 produced by absorption of NHS was largely restored to control levels by incorporation of IgG purified from NHS into the incubation medium. In every case (Table 2), the t_0 observed with IgG-restored serum was slightly greater than the t_0 observed with NHS; however, the differences in t_0 for NHS and IgG-restored serum for the most part did not fall outside the reciprocal 95% confidence intervals. Conversely, the t_0 for absorbed serum with added IgG was significantly shorter than the t_0 for absorbed serum. Our studies do not identify the epitope specificity of the antibody responsible for classical pathway initiation; however, the results of cross-absorption of NHS provide some clues. The ability of hydrophilic and hydrophobic *C. albicans* to reciprocally absorb the classical pathway initiator indicates that the epitope is expressed on the surface of both cell types. One possibility is that the antibody is specific for *C. albicans* mannan. Mannoprotein is found at the surface of *C. albicans* yeast cells (17, 38, 43). Moreover, surveys of sera from healthy and hospitalized adults have demonstrated the presence of antimannan antibodies in virtually all sera tested, although there is a considerable range in the levels of the antibodies from different donors (9, 10, 23, 30). Of particular interest for our studies are the observations that these antibodies are primarily of the IgG class (10, 23, 30) and can be removed by absorption with whole *C. albicans* yeast cells (23). The failure of absorption with *S. cerevisiae* to alter t_0 for *C. albicans* indicates that the epitope recognized by the antibody that initiates the classical pathway on *C. albicans* is absent on *S. cerevisiae*. In contrast, serum absorbed with either hydrophobic or hydrophilic *C. albicans* had an increased t_0 for activation and binding of C3 to *S. cerevisiae*. The lack of reciprocal cross-absorption suggests that NHS contains an additional initiator that binds to both *C. albicans* and *S. cerevisiae* and that this initiator is responsible for classical pathway activation by *S. cerevisiae*. One possibility is an antibody specific for a common epitope of yeast mannan. This explanation is consistent with previous studies of the immunochemistry of *C. albicans* and *S. cerevisiae* mannans. Summers et al. used quantitative immunoprecipitation to determine that mannans from *S. cerevisiae* produce relatively weak cross-reactions with antisera raised against whole *C. albicans* cells (40). Moreover, the acetolysis patterns of *C. albicans* mannans are more complex than those of *S. cerevisiae* strains (25). Taken together, these studies indicate the presence of epitopes on *C. albicans* mannans that are absent on *S. cerevisiae.*

Our results raise the possibility that NHS contains a classical pathway initiator in addition to IgG. This possibility is indicated by the failure of purified IgG to restore the kinetics for rapid accumulation of C3 to levels observed with untreated NHS. One possible explanation is a contribution by mannan-binding protein (MBP) to classical pathway initiation by *C. albicans*. MBP binds to yeast mannan and is able to activate complement via the classical pathway (21, 32, 36). However, data on cross-absorption with other yeast cells (Table 3) argue against a significant role for MBP in activation of the classical pathway by *C. albicans*. Absorption of serum with *S. cerevisiae* cells had no effect on t_0 for hydrophobic or hydrophilic *C. albicans*. MBP is known to bind to zymosan particles and *S. cerevisiae* mannan (21, 32, 36), and a shift in t_0 would have been expected if MBP played a significant role in initiation of the classical pathway by *C. albicans*. The slight delay in t_0 and the reduction in k' that could not be restored by IgG could be due to depletion of one or more complement components during the absorption process. For example, early studies of properdin used absorption of serum with zymosan particles as a means for depleting serum of properdin (1). In the present study, serum was absorbed at 0°C in an effort to prevent loss of complement proteins. This procedure appeared to be successful in the case of hydrophobic *C. albicans*, because absorption of NHS with hydrophobic cells had no effect on the kinetics for activation of C3 and binding of C3 fragments to encapsulated *C. neoformans*, a process that occurs strictly via the alternative pathway (28, 44). In some cases, absorption of serum with hydrophilic cells slightly increased the t_0 observed with encapsulated *C. neoformans*. The fact that this shift in t_0 did not occur in all instances argues that depletion of alternative pathway proteins by absorption with hydrophilic *C. albicans* is not a major effect.

In previous studies, we found that the classical complement pathway greatly influences early events in activation of C3 and

binding of C3 fragments to nonencapsulated *C. neoformans* (44). An IgG antibody found in NHS was responsible for the classical pathway initiation. Removal of the antibody by absorption with nonencapsulated cryptococci increased t_0 and changed the pattern of binding from a sudden and synchronous binding pattern similar to that found when *C. albicans* cells are incubated in NHS to a focal and asynchronous pattern similar to that observed when *C. albicans* cells are incubated in absorbed serum or serum treated with Mg-EGTA. The crucial difference between classical pathway initiation by *C. albicans* and that by nonencapsulated cryptococci is the specificity of the antibody mediating initiation. Absorption of NHS with hydrophilic or hydrophobic *C. albicans* had little or no effect on the kinetics for activation of C3 and binding to nonencapsulated cryptococci. Similarly, absorption of NHS with nonencapsulated cryptococci had no effect on the kinetics for activation of C3 and binding of C3 fragments to *C. albicans*. NHS contains high levels of antiglucan antibodies that bind to nonencapsulated cryptococci and most likely initiate the classical pathway at the cryptococcal cell wall (24). Moreover, the cell wall of nonencapsulated cryptococci has a high glucan content, with mannan as only a transient constituent (22). Thus, the surfaces and epitopes presented by *C. albicans* and nonencapsulated cryptococci are quite different.

The importance of the classical pathway in resistance to candidiasis is not known. Similar numbers of C3 fragments are eventually bound to the cells, regardless of the mechanism for initiation. However, the much more rapid accumulation of C3 fragments via the classical pathway may confer an advantage to the host. A significant role for the classical pathway in host resistance is not supported by studies of experimental candidiasis which found similar courses of infection in normal guinea pigs and guinea pigs with a C4 deficiency (8). However, the sera of the guinea pigs were not tested for the presence of anti-*C. albicans* antibodies, and the kinetics for activation and binding of C3 from normal or C4-deficient sera were not established in this model. More recently, Han and Cutler reported that passive immunization of mice with antimannan antibodies protects against disseminated candidiasis (11). The effector mechanism by which immunization increases resistance to infection is not known. It is attractive to speculate that antimannan antibodies increase the rate at which the yeast is coated with opsonic C3 fragments or alter the quantity or location of opsonic fragments.

ACKNOWLEDGMENTS

This study was supported in part by National Institutes of Health grants AI 14209 and AI 37194.

REFERENCES

1. **Blum, L.** 1964. Evidence for immunological specificity of the properdin system. Demonstration, isolation and properties of a serum factor which interacts with zymosan and other polysaccharides at 0°C. J. Immunol. **92:** 61–72.
2. **Cherniak, R., E. Reiss, and S. H. Turner.** 1982. A galactoxylomannan antigen of *Cryptococcus neoformans* serotype A. Carbohydr. Res. **103:**239–250.
3. **Cutler, J. E., and T. Kanbe.** 1993. Antigenic variability of *Candida albicans* cell surface. Curr. Top. Med. Mycol. **5:**27–47.
4. **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.
5. **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.
6. **Fine, D. P., S. R. Marney, Jr., D. G. Colley, J. S. Sergent, and R. M. Des Prez.** 1972. C3 shunt activation in human serum chelated with EGTA. J. Immunol. **109:**807–809.
7. **Fraker, P. J., and J. C. Speck, Jr.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. **80:**849–857.
8. **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.
9. **Greenfield, R. A., M. J. Bussey, J. L. Stephens, and J. M. Jones.** 1983. Serial enzyme-linked immunosorbent assays for antibody to *Candida* antigens during induction chemotherapy for acute leukemia. J. Infect. Dis. **148:**275–283.
10. **Greenfield, R. A., J. L. Stephens, M. J. Bussey, and J. M. Jones.** 1983. Quantitation of antibody to *Candida* mannan by enzyme-linked immunosorbent assay. J. Lab. Clin. Med. **101:**758–771.
11. **Han, Y., and J. E. Cutler.** 1995. Antibody response that protects against disseminated candidiasis. Infect. Immun. **63:**2714–2719.
12. **Harlow, E., and D. Lane.** 1996. Antibodies: a laboratory manual, p. 283–318. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Hazen, K. C.** 1990. Cell surface hydrophobicity of medically important fungi, especially *Candida* species, p. 249–295. *In* R. J. Doyle and M. Rosenberg (ed.), Microbial cell surface hydrophobicity. American Society for Microbiology, Washington, D.C.
14. **Hazen, K. C., D. L. Brawner, M. H. Riesselman, M. A. Jutila, and J. E. Cutler.** 1991. Differential adherence of hydrophobic and hydrophilic *Candida albicans* yeast cells to mouse tissues. Infect. Immun. **59:**907–912.
15. **Hazen, K. C., and P. M. Glee.** 1994. Hydrophobic cell wall protein glycosylation by the pathogenic fungus *Candida albicans*. Can. J. Microbiol. **40:**266–272.
16. **Hazen, K. C., and B. W. Hazen.** 1987. A polystyrene microsphere assay for detecting cell surface hydrophobicity heterogeneity within *Candida albicans* populations. J. Microbiol. Methods **6:**289–299.
17. **Hazen, K. C., and B. W. Hazen.** 1992. Hydrophobic surface protein masking by the opportunistic fungal pathogen *Candida albicans*. Infect. Immun. **60:** 1499–1508.
18. **Hazen, K. C., and B. W. Hazen.** 1993. Surface hydrophobic and hydrophilic protein alterations in *Candida albicans*. FEMS Microbiol. Lett. **107:**83–88.
19. **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.
20. **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.
21. **Ikeda, K., T. Sannoh, N. Kawasaki, T. Kawasaki, and I. Yamashina.** 1987. Serum lectin with known structure activates complement through the classical pathway. J. Biol. Chem. **262:**7451–7454.
22. **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.
23. **Jones, J. M.** 1980. Quantitation of antibody against cell wall mannan and a major cytoplasmic antigen of *Candida* in rabbits, mice, and humans. Infect. Immun. **30:**78–89.
24. **Keller, R. G., G. S. T. Pfrommer, and T. R. Kozel.** 1994. Occurrences, specificities, and functions of ubiquitous antibodies in human serum that are reactive with the *Cryptococcus neoformans* cell wall. Infect. Immun. **62:**215–220.
25. **Kocourek, J., and C. E. Ballou.** 1969. Method for fingerprinting yeast cell wall mannans. J. Bacteriol. **100:**1175–1181.
26. **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.
27. **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.
28. **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.
29. **Kozel, T. R., M. A. Wilson, and W. H. Welch.** 1992. Kinetic analysis of the amplification phase for activation and binding of C3 to encapsulated and nonencapsulated *Cryptococcus neoformans*. Infect. Immun. **60:**3122–3127.
30. **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.
31. **Liebert, R. E., and K. C. Hazen.** 1988. Relationship of cell surface hydrophobicity to morphology of monomorphic and dimorphic fungi. Mycologia **80:**348–355.
32. **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.
33. **Morelli, R., and L. T. Rosenberg.** 1971. The role of complement in the phagocytosis of *Candida albicans* by mouse peripheral blood leukocytes. J. Immunol. **107:**476–480.
34. **Morelli, R., and L. T. Rosenberg.** 1971. Role of complement during experimental *Candida* infection in mice. Infect. Immun. **3:**521–523.

35. **Morrison, R. P., and J. E. Cutler.** 1981. In vitro studies of the interaction of murine phagocytic cells with *Candida albicans*. J. Reticuloendothel. Soc. **29:**23–34.
36. **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.
37. **Platts-Mills, T. A. E., and K. Ishizaka.** 1974. Activation of the alternative pathway of human complement by rabbit cells. J. Immunol. **113:**348–357.
38. **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.
39. **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.
40. **Summers, D. F., A. P. Grollman, and H. F. Hasenclever.** 1964. Polysaccharide antigens of *Candida* cell wall. J. Immunol. **92:**491–499.
41. **Tack, B. F., J. Janatova, M. L. Thomas, R. A. Harrison, and C. H. Hammer.** 1981. The third, fourth, and fifth components of human complement: isolation and biochemical properties. Methods Enzymol. **80:**64–101.
42. **Thong, Y. H., and A. Ferrante.** 1978. Alternative pathway of complement activation by *Candida albicans*. Aust. N. Z. J. Med. **8:**620–622.
43. **Tronchin, G., J. P. Bouchara, and R. Robert.** 1989. Dynamic changes of the cell wall surface of *Candida albicans* associated with germination and adherence. Eur. J. Cell Biol. **50:**285–290.
44. **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.
45. **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.

Editor: D. H. Howard