INFECTION AND IMMUNITY, May 1997, p. 1849–1855 Vol. 65, No. 5
0019-9567/97/$04.00+0

Activation, Binding, and Processing of Complement Component 3 (C3) by *Blastomyces dermatitidis*

MASON X. ZHANG AND BRUCE KLEIN*

Departments of Pediatrics, Internal Medicine, and Medical Microbiology and Immunology and the Comprehensive Cancer Center, University of Wisconsin Medical School, University of Wisconsin Hospital and Clinics, Madison, Wisconsin 53792

Received 13 November 1996/Returned for modification 19 December 1996/Accepted 18 February 1997

Complement plays a key role in phagocyte recognition and killing of *Blastomyces dermatitidis*, but little is known about how complement components interact with the yeast. We report the characteristics of activation, binding, and processing of C3 by *B. dermatitidis*. In pooled normal human serum (NHS), deposition of C3 on the yeast was detectable within 2 min, whereas in NHS containing MgEGTA, deposition was delayed by 6 min, indicating that the yeast activates C3 by both classical and alternative pathways. When both pathways were operative, maximal binding of 4.5×10^6 C3 molecules/cell was achieved in less than 30 min. In the absence of the classical pathway, yeast cells bound 80% of the maximum C3, indicating that the yeast intrinsically activates the alternative pathway. Delayed deposition of C3 in NHS-MgEGTA was similar to that in NHS preabsorbed by the yeast or by immobilized protein A/G to remove serum immunoglobulin. Purified immunoglobulin restored C3 binding to NHS preabsorbed by the yeast, suggesting that antibody in nonimmune serum initiates the classical pathway. β-Glucan absorption of NHS abolished the classical pathway, suggesting that cell wall β-glucan is a target of initiating antibodies. Hydroxylamine treatment of NHS-opsonized yeast cells showed that 76% of C3 was bound to yeast cells by ester linkage, supporting a role for hydroxyl groups on cell wall polysaccharides. Hydroxylamine-cleaved fragments were chiefly C3b and iC3b; 70% of hydroxylamine-sensitive C3b was converted to iC3b within 1 min of opsonization, and the ratio was stable over 1 h. Our data predict that C3b and iC3b on opsonized yeast cells direct binding to CR1 and CR3 receptors on human phagocytes, which, in turn, may influence the fate of this host-pathogen interaction.

Blastomyces dermatitidis is a thermal dimorphic fungus and the causal agent of blastomycosis, which is one of the principal endemic systemic mycoses of humans and other mammals. Inhaled conidia of *B. dermatitidis* initiate the infection, and at body temperature, they convert to invasive yeast forms that produce a chronic, progressive pneumonia and often disseminate to extrapulmonary organs (9).

The principal host defense mechanisms against *B. dermatitidis* have not been clearly defined (9). The complement system has been shown to be one of the important host defense mechanisms against fungal pathogens (14). Activation of the complement system by fungi via either the classical or the alternative pathway results in the release of free complement fragments as anaphylatoxins and chemotactic molecules and the deposition of complement opsonins onto the activating fungal surfaces. The major complement opsonins are peptide fragments derived from C3, such as C3b, iC3b, C3dg, and C3d, which facilitate recognition and killing of these organisms by complement receptor-bearing phagocytes. Drutz and Frey (3) showed that complement promotes attachment of human phagocytes to *B. dermatitidis* and is required for neutrophil-mediated killing of the yeast. However, the mechanisms of activation of the complement system and binding and processing of C3 at the yeast surface have not been described.

In this study, we investigated the features associated with activation, binding, and processing of C3 by *B. dermatitidis*. We show that *B. dermatitidis* activates and binds C3 avidly via both classical and alternative pathways, that naturally occurring antibodies reactive with β-glucan are largely responsible for activating complement through the classical pathway, and that conversion of bound C3b to iC3b is rapid but incomplete. These results shed new light on a key host defense mechanism against *B. dermatitidis*. They will permit detailed investigation of complement-mediated interaction between *B. dermatitidis* and phagocytes and of how yeast cell surface components may modulate these events.

* Corresponding author. Mailing address: University of Wisconsin Hospitals and Clinics, 600 Highland Ave., K4/434, Madison, WI 53792. Phone: (608) 263-9217. Fax: (608) 263-0440.

MATERIALS AND METHODS

Fungal strains and zymosan particles. *B. dermatitidis* 26199 was purchased from the American Type Culture Collection (Rockville, Md.) and maintained in its yeast form on Middlebrook 7H10 agar medium containing oleic acid-albumin complex (Sigma Chemical Co., St. Louis, Mo.) as described previously (10). This *B. dermatitidis* strain is a virulent, wild-type isolate that has been studied extensively (9). The yeast measures approximately 8.9 μm in diameter. *Histoplasma capsulatum* 186AR and 186AS were the gifts of William Goldman (Washington University, St. Louis, Mo.) and Jon Woods (University of Wisconsin, Madison). These are a genetically related pair of rough (186AR) and smooth (186AS) strains with a structural difference mainly in the expression of surface α-1,3-glucan. Strain 186AS contains 1,000-fold less of the polymer than strain 186AR (11). *H. capsulatum* strains were maintained on HMM agar in their yeast forms as described previously (28).

For experiments reported here, the yeasts were grown on the medium described for 72 h at 37°C, collected, and washed three times in phosphate-buffered saline (PBS; 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl [pH 7.2]). The yeasts were killed by heat at 60°C for 1 h (23), washed once in PBS, and stored in PBS containing 0.05% azide at 4°C.

The effect of heat treatment on the expression of surface components of *B. dermatitidis* yeast cells was evaluated. Analysis with immunofluorescence staining and flow cytometry revealed similar quantitative displays of two cell surface molecules, WI-1 (10) and α-1,3-glucan (5), in the live and heat-killed yeasts. Furthermore, initial experiments showed that the heat-killed yeasts bound C3 at nearly the same rate as live ones. Consequently, the heat-killed yeasts were used throughout this study.

Zymosan was purchased from Fluka Chemie AG (Buchs, Switzerland), treated, and stored as described previously (19).

Human serum, serum absorption, and C3 protein. Normal human serum (NHS) was prepared by the method of Newman and Mikus (22) from blood samples donated by healthy adults who had no history of blastomycosis. Individual sera were screened for antibodies to *B. dermatitidis* A antigen by a commercial enzyme immunoassay kit (Meridian Diagnostics, Inc., Cincinnati, Ohio). Sera that were nonreactive with *B. dermatitidis* A antigen were pooled. Each pooled serum preparation was derived from the blood of at least four donors. To obtain heat-inactivated NHS (HINHS), NHS was incubated at 56°C for 30 min to abolish complement activity and filtered through a 0.45-μm-pore-size sterile cellulose acetate filter (Corning Glass Works, Corning, N.Y.).

To remove potential initiators of the classical pathway, serum was incubated with yeast cells, zymosan, or cell wall polysaccharides on ice for 60 min, with mixing every 10 min. The absorbed serum was separated from absorbing materials by centrifugation at 4°C and filtered through 0.45-μm-pore-size sterile cellulose acetate filters. The absorbing materials and their concentrations per milliliter of NHS were as follows: 10^8 *B. dermatitidis* 26199 yeast cells; 5×10^8 *H. capsulatum* 186AS or 186AR yeast cells; 5×10^8 zymosan particles; and cellulose (Sigma C6413), chitin (Sigma C3641), baker's yeast glucan (Sigma G5011), or *B. dermatitidis* 26199 yeast glucan in amounts ranging from 2 to 60 mg. The *B. dermatitidis* 26199 yeast glucan was isolated by the method of Kanetsuna and Carbonell (7), with minor modifications as follows. Fragmented cell walls were treated with trypsin before alkali extraction, and alkali-soluble water-insoluble glucan was precipitated by dialysis against distilled water at 4°C. The alkali-extractable glucan of *B. dermatitidis* has been shown to consist of 95% α-1,3-glucan (7). Prior to absorption, yeast particles and cell wall polysaccharides were washed twice in PBS. Absorbed sera were used fresh or stored at −80°C.

Purified C3 was a generous gift of Thomas Kozel (University of Nevada, Reno). C3 molecules were radiolabeled with ^{125}I to a specific activity of approximately 2×10^5 cpm per μg by using Iodobeads (Pierce Chemical Co., Rockford, Ill.), which carry an immobilized oxidizing reagent. Briefly, 10 Iodobeads were incubated for 10 min with 1 mCi of ^{125}I at room temperature in 200 μl of 0.125 M sodium phosphate buffer (pH 7.2). After the reaction mixture was chilled on ice, 1 mg of C3 was added. Iodination of the C3 was allowed to proceed on ice for 10 min. Iodinated C3 was separated from free ^{125}I by filtration through a 10DG desalting column (Bio-Rad, Richmond, Calif.). The eluted, iodinated C3 was added to 10% of its volume with a 5× gelatin-Veronal-saline buffer (GVB; see below), then filtered through a 0.45-μm-pore-size sterile cellulose acetate filter, and stored at 4°C.

Kinetics analysis of C3 binding. The C3 binding assay was adapted from the method of Kozel et al. (16). Briefly, to each 1.5-ml microtube, the following reagents were added in sequence: GVB (1% gelatin, 5 mM sodium Veronal, 142 mM NaCl [pH 7.3]), ^{125}I-C3 at 2.4×10^7 cpm per ml of binding reaction mixture, and serum (NHS, HINHS, or absorbed NHS) at 40% of the final reaction volume. To study complement activation and binding through both classical and alternative pathways, the reaction mixture was supplemented with 1 mM $MgCl_2$ and 0.15 mM $CaCl_2$. To study the activity of the alternative pathway alone, 10 mM EGTA and 5 mM $MgCl_2$ (MgEGTA) were added to the reaction since EGTA chelates Ca^{2+} effectively and binds Mg^{2+} to a lesser extent (4). Chemicals were purchased from Sigma unless otherwise specified. Reaction tubes were placed in a heating block and warmed at 37°C for 3 min. Complement was activated by the addition of yeast cells in 50 μl of PBS to yield a final concentration of 10^6 yeast cells per ml of reaction mixture. Duplicate samples of 50 μl each were transferred at serial time points to wells of a 96-well MultiScreen filtration plate (filtration porosity, 1.2 μm; Millipore MABV-N12; Millipore, Marlborough, Mass.), each of which had been filled with 200 μl of PBS containing 0.1% sodium dodecyl sulfate (SDS) and 20 mM EDTA. Yeast cells were collected onto the membrane filter in each well by using a Millipore MultiScreen manifold and washed five times with PBS containing 0.1% SDS. The membranes were air dried and punched out into 1.2-ml microtubes (National Scientific Supply Company, Inc., San Rafael, Calif.) by using Millipore MultiScreen disposable punch tips. Radioactivity (counts per minute) of ^{125}I-C3 on each membrane filter was measured in a gamma counter. Variation within an assay was small between samples taken either from a single reaction tube or from multiple, identical reaction tubes. Therefore, unless otherwise specified, the observation for each time point reported here is the average of duplicate samples taken from one reaction tube.

The amount of ^{125}I-C3 specifically bound to yeast cells was calculated by subtracting nonspecifically bound counts for HINHS-opsonized yeast cells from the total counts for NHS-opsonized or absorbed NHS-opsonized yeast cells. The specific binding of ^{125}I-C3 was converted to the number of C3 molecules bound per yeast cell, based on the specific activity of ^{125}I-C3, the molecular weight of C3 (183,000), and the ratio of labeled C3 to total C3 (labeled plus nonlabeled) in the reaction mixture. The amount of labeled C3 in each binding reaction was estimated based on the total number of counts of radioactivity added to the reaction and the specific activity of ^{125}I-C3. The concentration of nonlabeled C3 was assumed to be 1.2 mg/ml of human serum (12).

Removal and purification of antibodies from NHS. Immobilized protein A/G (Pierce) binds human immunoglobulins and was used as specified by the manufacturer to absorb and remove antibodies from NHS. Prior to absorption of NHS with protein A/G, the beads were washed with a binding buffer of 20 mM sodium phosphate (pH 7.2). To compensate for the dilution effect of binding buffer retained by the beads, the last wash of the beads was done with NHS. Absorption was performed on ice, using 1 ml of packed beads per ml of NHS for 60 min, with mixing every 10 min. Absorbed serum was separated from the protein A/G beads by centrifugation in an Eppendorf centrifuge (model 5415C) at 5,000 rpm for 3 min at 4°C, filtered through a 0.45-μm-pore-size cellulose acetate filter (Corning) and either used fresh for binding assays or stored at −80°C.

To elute immunoglobulins from serum-treated protein A/G, the beads were washed once with the binding buffer and packed in a column. The packed beads were washed with the binding buffer until the A_{280} of the flowthrough reached the background level. Bound immunoglobulins were eluted from the beads in 0.1 M glycine-HCl (pH 2.5) and immediately neutralized with 1 M Tris (pH 7.5) as recommended by the manufacturer (Pierce). The eluate was desalted with PBS and concentrated with CentriprepR-10 concentrators (Amicon, Beverly, Mass.). The concentration of eluted antibodies was estimated by measurement of the A_{280}. The purity and integrity of immunoglobulin in the eluate was assessed by SDS-polyacrylamide gel electrophoresis (PAGE), using a PhastSystem (Pharmacia, Uppsala, Sweden). The eluted immunoglobulin was used in C3 binding assays to attempt to restore C3 binding activity of the classical pathway to absorbed serum. Yeast cells (10^6) were incubated for 60 min on ice in 400 μl of PBS alone or in 400 μl of PBS containing 2 to 8 mg of the affinity-purified immunoglobulins, pelleted, and washed twice with PBS. The immunoglobulin-treated yeast cells were suspended in 1 ml of binding reaction medium containing 40% yeast-absorbed serum. The PBS-alone-treated yeast cells were suspended in binding medium containing either 40% NHS (positive control) or 40% yeast-absorbed NHS (negative control). Kinetics of C3 binding to the immunoglobulin-treated yeast cells was compared to that for the PBS-alone-treated cells.

Immunofluorescence and flow cytometry analysis of yeast surface β-glucan. Two million *B. dermatitidis* yeast cells or 3×10^6 zymosan particles were washed in PBS and incubated at 37°C for 60 min in 200 μl of PBS containing a 1:200 dilution of rabbit polyclonal antiserum against β-1,3-glucan or rabbit nonimmune serum as a control. The antiserum was raised against a laminariheptaose-human transferrin hapten conjugate and reacts specifically with β-1,3-glucan (24). Rabbit sera were the generous gift of D. M. Schmatz (Merck Laboratories, Rahway, N.J.). After three washes with PBS, the serum-treated yeast cells and zymosan particles were incubated at 37°C for 60 min in 100 μl of PBS containing a 1:100 dilution of goat anti-rabbit immunoglobulin G (IgG) antibodies conjugated with fluorescein isothiocyanate (GAR-FITC; Immunotech, Inc., Westbrook, Maine). GAR-FITC-stained yeasts and zymosan particles were washed three times in PBS and suspended in 0.9 ml of PBS containing 1% bovine serum albumin. Stained yeast cells and zymosan were analyzed by single-color flow cytometry with a four-decade log amplification, using the nonimmune rabbit serum-treated yeast cells or zymosan particles to set the background fluorescence. Five thousand events were acquired. Histograms of the number of events versus fluorescence intensity were constructed. In addition, the immunofluorescence patterns of β-glucan distribution on the surface of the yeast cells or zymosan particles were examined microscopically.

Kinetics of conversion of C3 to other fragments. Individual C3 binding reactions for 1 to 60 min were performed with GVB, 1 mM $MgCl_2$, 0.15 mM $CaCl_2$, 40% NHS, and 2.4×10^7 cpm of ^{125}I-C3 and 10^6 yeast cells per ml of reaction mixture. To minimize spontaneous loss and degradation of bound C3 fragments, reactions with the longest incubation time were started first. At the end of each incubation, ice-cold PBS–20 mM EDTA (final EDTA concentration, >10 mM) was added to stop the reaction. Yeasts were pelleted and washed three times with PBS–0.1% SDS. Pellets were resuspended in 15 μl of PBS–0.1% SDS and 15 μl of 1 M hydroxylamine in 0.2 M $NaHCO_3$ (pH 10.5) and incubated for 1 h at 37°C. Treated yeasts were separated from released C3 fragments by centrifugation at 14,000 rpm for 10 min in an Eppendorf centrifuge (model 5415C), and the supernatant was collected. The pellet was resuspended in 20 μl of 0.5 M sodium phosphate buffer (pH 7.2) and centrifuged, and the supernatant was pooled with the previous one. Radioactivity in the pellet and the supernatant was determined. Supernatant samples for the various time points were adjusted to equivalent counts per minute of radioactivity, mixed with an equal volume of 2× SDS sample buffer (0.125 M Tris-Cl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol [pH 6.8]), and incubated at 100°C for 2 min. C3 fragments in the samples were separated by SDS-PAGE and analyzed by either autoradiography or Western blotting. For autoradiography, 5,000 cpm per sample was loaded onto each lane. Gels were dried at 80°C for 1 h and exposed to X-ray films. Autoradiographs were scanned with a Hoefer GS300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, Calif.) for quantitative analysis of C3 bands. For each gel lane, the density of a band was expressed as percentage of the total density of all bands in the lane representing C3 fragments. For Western blot analysis, 3,000 cpm was loaded in each lane of the gel. C3 fragments in gels were blotted onto 0.2-μm-pore-size Protran nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) and detected by goat anti-human C3 antibodies (Sigma C-7761) followed by rabbit anti-goat IgG conjugated with alkaline phosphatase (Sigma A4187).

Pulse-chase analysis of conversion of bound C3. The method described above analyzes the composition of a dynamic population of C3 molecules at each time point at which new C3b may continuously be bound to the surface of the yeast. To analyze how a population of C3b molecules bound synchronously are processed to other C3 fragments over time, a pulse-chase method was used. Yeast cells were opsonized for 30 min in a binding reaction with ^{125}I-C3 as described

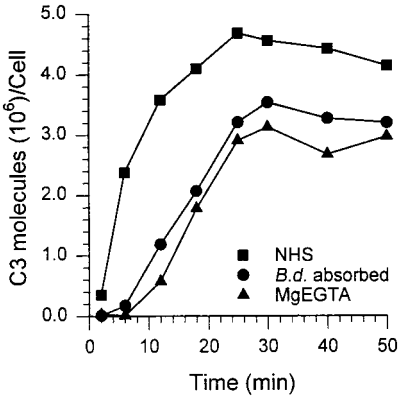

FIG. 1. Kinetics of C3 binding to *B. dermatitidis*. For comparison, yeast cells were opsonized in 40% NHS, NHS containing MgEGTA, or NHS preabsorbed with yeast cells of *B. dermatitidis* 26199 (*B.d.* absorbed).

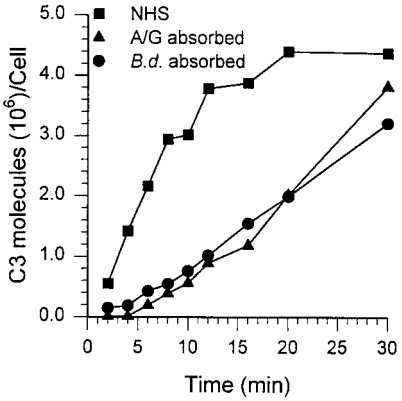

FIG. 2. Effect of protein A/G absorption of serum on the kinetics of C3 binding to *B. dermatitidis* yeast cells. Kinetics of C3 binding was compared for yeast cells opsonized in 40% NHS, protein A/G-absorbed NHS, or *B. dermatitidis* yeast-absorbed NHS (*B.d.* absorbed).

above, washed twice with PBS, and resuspended and incubated in GVB containing 1 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 40% serum without labeled C3. Aliquots of these yeasts were transferred at serial time points to tubes containing 1 ml of ice-cold PBS–20 mM EDTA. C3 fragments were eluted, separated, and analyzed as described above.

RESULTS

Kinetics of C3 binding to *B. dermatitidis* via the classical and alternative pathways. Serum at 40% of the physiological concentration was used to opsonize 10^6 yeast cells (2,500 yeast cells per ml of serum) in experiments reported here. Initial experiments determined that this ratio of yeast cells to serum provided a nonsaturating condition for binding complement. Under these conditions, the kinetics of C3 binding to yeast cells opsonized in NHS, yeast-absorbed NHS, and NHS-MgEGTA was compared over a 50-min period (Fig. 1). Binding of C3 to the yeast in NHS was rapid and detectable within 2 min of adding the cells to the reaction medium, and it reached the maximum of 4.5×10^6 C3 molecules per yeast cell at 25 min. Approximately 80% of the total deposition of C3 occurred during the first 10 min of opsonization. Similar kinetics patterns were observed in 10 independent binding assays. If one assumes that the yeast cells are perfect spheres approximately 8.9 μm in diameter, the density of C3 molecules on the yeast surface is calculated to be 12,000 to 18,000 molecules per μm^2, based on these assays.

In contrast to the pattern of C3 binding in NHS, a more typical sigmoidal kinetics of C3 binding was observed in yeast cells incubated in NHS containing 10 mM EGTA or in NHS preabsorbed by *B. dermatitidis* 26199. Under these conditions, C3 deposition was detectable only after a 6-min delay and reached a level of about 80% of the maximum binding observed for yeast cells in NHS (Fig. 1). Because initiation of the classical pathway requires Ca^{2+} and the concentration of EGTA used here removes it from serum (4), the data in Fig. 1 demonstrate that both the classical and alternative pathways are operative in C3 binding by *B. dermatitidis*. Furthermore, the similarity of kinetics curves between NHS containing 10 mM EGTA and NHS preabsorbed by *B. dermatitidis* 26199 suggested that preabsorbing serum with the yeast cells removed the factors that initiate the classical pathway. We investigated this hypothesis as described below.

Role of natural antibodies in activation of the classical pathway. IgM and some subclasses of IgG may initiate the classical pathway. For nonencapsulated strains of *Cryptococcus neoformans*, natural IgG antibodies directed against surface components initiate the classical pathway and C3 binding to the yeast (27). We investigated the role of such natural antibodies in activating the classical pathway and C3 binding to *B. dermatitidis*. We used protein A/G, which binds human IgG avidly and IgM weakly, to absorb naturally occurring antibodies from NHS. C3 binding to *B. dermatitidis* in NHS that had been preabsorbed with protein A/G exhibited a lag of 8 to 12 min compared to C3 binding in untreated NHS, but it was quantitatively and qualitatively similar to C3 binding in NHS that had been preabsorbed with the yeast (Fig. 2). These observations suggest that absorbing NHS with either protein A/G or yeast cells removed immunoglobulins from serum. When analyzed by SDS-PAGE under reducing conditions, the protein A/G eluate indeed contained two distinctive bands of 50 and 23 kDa (data not shown), compatible with the sizes of heavy and light chains of human IgG.

We next determined whether antibodies eluted from protein A/G could restore the early binding activity to serum that had been preabsorbed by *B. dermatitidis*. Yeast cells were preincubated for 60 min in PBS either alone as a control or together with the affinity-purified antibodies in increasing concentrations. Preincubated yeast cells were collected, washed, and used in C3 binding reactions with NHS, NHS-MgEGTA, or yeast-absorbed NHS. The protein A/G-eluted immunoglobulins effectively restored, in a concentration-dependent manner, the early and rapid C3 binding activity to the yeast-absorbed NHS to the level observed for NHS (Fig. 3).

Evidence for β-glucan as the yeast ligand of natural antibodies. We sought to define the yeast cell wall components that are ligands of natural antibodies during initiation of the classical pathway. The cell wall of *B. dermatitidis* contains structural proteins, chitin, and α-linked glucan as the predominant glucan component (26). The surface protein adhesin WI-1 is the principal target of specific antibodies in patients with blastomycosis (10), but pooled human serum in this study excluded immune patient serum. We therefore investigated the role of cell wall components other than WI-1 as possible ligands of naturally occurring antibodies. To test their role, NHS was preabsorbed with the component and the absorbed serum was investigated for loss in ability to promote C3 binding to *B. dermatitidis* 26199 through the classical pathway.

C3 binding to *B. dermatitidis* in serum preabsorbed with baker's yeast glucan, which is chiefly β-glucan (1), was delayed significantly and reduced in a concentration-dependent manner (Fig. 4A). The kinetics of C3 binding in sera treated with

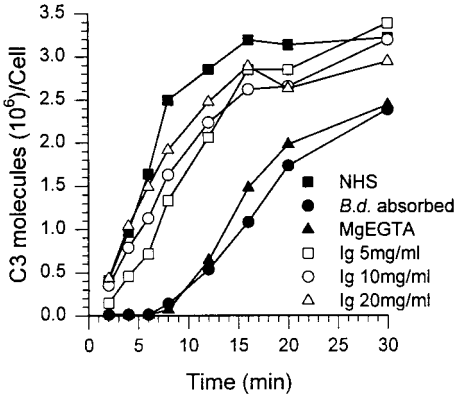

FIG. 3. Restoration of early C3 binding activity to yeast-absorbed opsonic serum by purified serum antibodies. One million yeast cells were incubated on ice for 60 min in 400 μl of PBS containing 2, 4, or 8 mg of protein A/G-purified immunoglobulin (Ig 5mg/ml, Ig 10mg/ml, or Ig 20 mg/ml), washed, and transferred to 1 ml of binding cocktail containing 40% yeast-absorbed normal human serum to initiate C3 binding. As positive and negative controls, 10^6 yeast cells incubated in PBS alone for 60 min were transferred to the binding cocktail containing 40% NHS, NHS absorbed with yeasts (*B.d.* absorbed), or NHS containing MgEGTA.

30 mg of baker's yeast glucan or more per ml of serum was nearly identical to that in NHS absorbed with 26199 yeast cells, indicating that the classical pathway had been abolished. In contrast, α-glucan isolated directly from the cell wall of *B. dermatitidis* 26199 produced relatively little effect on the binding kinetics when used to absorb NHS at concentrations as high as 30 mg per ml of serum (Fig. 4B). This observation was unexpected because α-glucan has been shown to make up about 95% of the yeast cell wall glucan (7) and is displayed over the surface of *B. dermatitidis* yeast cells (5), whereas the expression of β-glucan on the yeast surface had not been reported.

To confirm our observation on the relative lack of influence of surface α-glucan on C3 binding to *B. dermatitidis*, we compared the kinetics of C3 binding to 26199 in sera preabsorbed by either one of two *H. capsulatum* strains that differ 1,000-fold in the expression of surface α-1,3-glucan (11). Although absorption of serum with either strain somewhat inhibited the binding of C3 to *B. dermatitidis*, there was no discernible difference between the effects of the two strains on the kinetics of C3 binding (Fig. 4C). Lastly, sera absorbed by chitin or cellulose at concentrations up to 30 mg per ml did not produce a noticeable effect on the kinetics of C3 binding to the yeast (data not shown).

We next sought to confirm that β-glucan is displayed on the surface of *B. dermatitidis* 26199 yeast cells. We used a rabbit polyclonal antiserum against β-1,3-glucan to investigate surface expression of the polymer by flow cytometry and fluorescence microscopy. Flow cytometry revealed clear expression of the polysaccharide on the surface of 26199 yeast cells. The mean channel fluorescence of *B. dermatitidis* treated with the β-glucan antiserum is 96, compared to 12 for the yeast cells treated with rabbit nonimmune serum. The fluorescence intensity for *B. dermatitidis* was similar to that observed for zymosan particles, which are composed mainly of β-glucan (1, 2). The mean channel fluorescence values for the zymosan particles were 59 for staining with immune serum and 5 for the nonimmune serum. Consistent with this analysis by flow cytometry, fluorescence microscopy showed that only the β-glucan antiserum-treated yeast cells and zymosan particles fluoresced. The surfaces of both the yeasts and zymosan particles were uniformly and evenly stained.

Kinetics of conversion of C3 to other fragments. C3b bound to the yeast surface may be further processed to iC3b, C3d, or C3dg. Each of these C3 opsonins has preference for a different complement receptor on phagocytes. Consequently, the outcome of C3 processing may influence the interaction between the yeast and phagocyte. To gain further insight about the ligands that may bind opsonized *B. dermatitidis* to phagocytes, we analyzed the molecular forms of C3 bound to *B. dermatitidis*. We first assessed the ability of hydroxylamine to release C3 fragments bound via an ester linkage directly to components on the yeast surface. In four independent experiments, an average of 76% ± 2% (standard error of the mean) of bound C3 molecules were released after hydroxylamine treatment, indicating that much of the C3 is linked to surface hydroxyl groups. In each experiment, this value did not vary more than 5% over the course of 60 min of incubation of C3 with yeast cells, demonstrating a consistent preference of ester over amide linkage between C3 and yeast surface components.

C3 fragments released by hydroxylamine were separated by SDS-PAGE and characterized by autoradiography. The 110-kDa α-chain of C3b (α_{C3b}), the 68-kDa α-chain of iC3b (αi_{C3b}), and the 75-kDa β-chain common to all C3 molecules

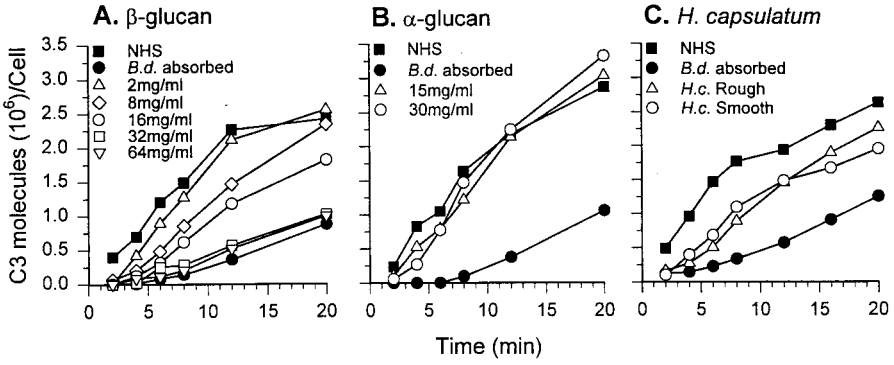

FIG. 4. Effect of α- or β-glucan absorption of serum on the classical pathway of C3 binding to *B. dermatitidis*. In each panel, C3 binding to *B. dermatitidis* in 40% absorbed serum is compared to binding in 40% NHS or in NHS absorbed with *B. dermatitidis* yeasts (*B.d.* absorbed). (A) Effect of absorbing NHS with baker's yeast glucan as a source of β-glucan at 2 to 64 mg per ml of serum (2mg/ml, 8mg/ml, 16mg/ml, 32mg/ml, and 64mg/ml). (B) Effect of absorbing NHS with *B. dermatitidis* yeast glucan as a source of α-glucan at 15 or 30 mg per ml of serum (15mg/ml and 30mg/ml). (C) Effect of absorbing NHS with 5×10^8 yeast cells per ml of serum of either *H. capsulatum* rough strain (*H.c.* Rough) or smooth strain (*H.c.* Smooth). Yeast cells of the rough strain display α-glucan, whereas yeast cells of the smooth strain do not.

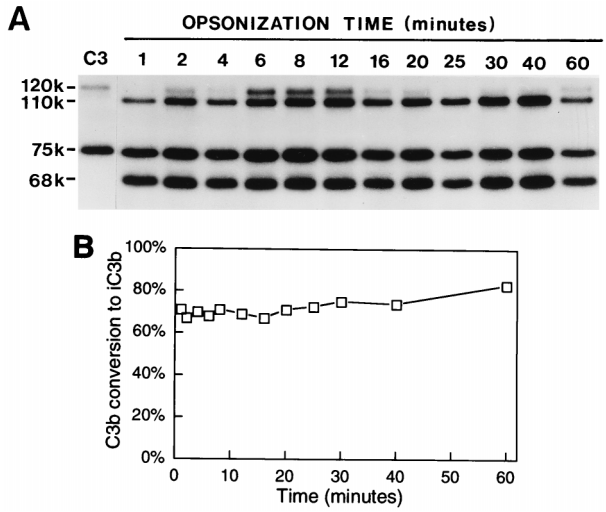

FIG. 5. Time course of release of C3 fragments from *B. dermatitidis* by hydroxylamine. Yeast cells were opsonized for 1 to 60 min in 40% NHS containing ^{125}I-C3. Yeasts opsonized for various durations were treated simultaneously with EDTA and then with hydroxylamine to release bound C3 fragments. Hydroxylamine-released C3 fragments were separated by SDS-PAGE, detected by autoradiography, and analyzed by scanning densitometry. (A) Molecular forms of hydroxylamine-released C3 fragments. Lane C3 contains ^{125}I-C3. (B) Kinetics of conversion of C3b to iC3b over 60 min. The relative densities of the α-chain of C3b (110 kDa) and α-chain of iC3b (68 kDa) within each lane are used to represent the relative amounts of C3b and iC3b in the total eluted C3 fragments. The kinetics of conversion of C3b to iC3b is expressed as percent change of C3b over time.

appeared in all the samples taken from yeasts incubated in NHS for 1 to 60 min (Fig. 5A). The conversion of C3b to iC3b was extremely rapid but incomplete, as both forms were present on the yeast opsonized in NHS for as short as 1 min and as long as 60 min. Approximately 70% of α_{C3b} converted to α_{iC3b} within 1 min after C3b was bound to the yeast surface (Fig. 5B). This percentage remained relatively stable for up to 40 min, followed by a further 10% conversion of α_{C3b} to α_{iC3b} over the next 20 min. Molecular forms of C3 corresponding to C3dg were not observed. The identity of the C3 bands was confirmed by Western blot analysis.

Pulse-chase analysis of conversion of bound C3. The kinetics of conversion of C3b to iC3b shown in Fig. 5 is based on a population of C3 sampled at serial time points where new C3b could continue to bind to the yeast cell surface. The lack of further conversion beyond 1 min, as revealed in Fig. 5, could result from the influx of newly bound C3b. To address this possibility, a pulse-chase method was used to study C3 conversion. Yeast cells were incubated for 30 min in NHS containing ^{125}I-C3 and then collected, washed, and reincubated in NHS without ^{125}I-labeled C3 for 0 to 60 min. This pulse-chase approach showed that C3b and iC3b were still the two major types of C3 fragments (Fig. 6A). Extended incubation during the chase for up to 60 min did not promote further conversion of C3b to iC3b. Approximately 70% of hydroxylamine-released C3 fragments were in the form of iC3b on the yeast surface throughout the entire time (Fig. 6B). This observation supports our previous one that C3 conversion is rapid but incomplete.

DISCUSSION

Little has been known about how *B. dermatitidis* yeast cells interact with the complement system, despite the fact that recognition and killing of the yeast cells by human phagocytes is enhanced by complement opsonins (3). In this study, we investigated the characteristics of activation, binding, and processing of complement component C3 by *B. dermatitidis*. We found that the yeast possesses a surface that strongly activates the complement system. In NHS at 40% of the physiological concentration, the yeast cells bind up to 4.5 million C3 molecules per cell in less than 30 min. The number of C3 molecules bound per square micrometer of *B. dermatitidis* yeast cells was estimated to be 12,000 to 18,000. This number agrees with 12,000 to 17,000 C3 molecules per μm^2 for nonencapsulated *C. neoformans* (calculated from data in reference 27), which is a strong activator of the complement system.

The binding of C3 to *B. dermatitidis* is mediated by both classical and alternative pathways, as shown by the rapid deposition of C3 on yeast cells incubated in NHS and by the delayed deposition on yeast cells incubated in NHS containing MgEGTA or in NHS preabsorbed with either yeast cells or immobilized protein A/G (Fig. 1 and 2). This dual property of activating both pathways in NHS has also been reported for nonencapsulated *C. neoformans* and *Candida albicans* (16, 27). The lag in early C3 binding when the alternative pathway is operative alone reflects the random access of small amounts of fluid metastable C3b to binding sites on the yeast surface, as revealed by immunofluorescence analysis (13). In the presence of factor D, initially bound C3b and factor B form C3 convertase of the alternative pathway, which initiates amplification and rapid deposition of C3b (17).

The amount of C3 bound to *B. dermatitidis* via the alternative pathway was found to be 80% of that bound when both pathways were operative, indicating an important role for the alternative pathway. In contrast to *B. dermatitidis*, however, nonencapsulated *C. neoformans* and zymosan have been shown to bind similar numbers of C3 molecules when either the alternative pathway alone or both pathways are operative (27).

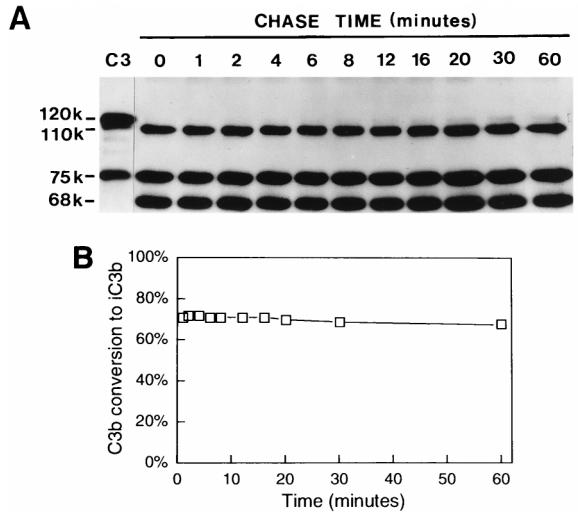

FIG. 6. Pulse-chase analysis of the conversion of C3b to iC3b on *B. dermatitidis*. Yeast cells were opsonized for 30 min in 40% NHS containing ^{125}I-C3, washed, and incubated with 40% NHS with no ^{125}I-C3 for 0 to 60 min. Hydroxylamine-released C3 fragments were separated by SDS-PAGE, detected by autoradiography, and analyzed by scanning densitometry. (A) Molecular forms of hydroxylamine-released C3 fragments. Lane C3 contains ^{125}I-C3. (B) Kinetics of conversion of C3b to iC3b over 60 min. The relative densities of the α-chain of C3b (110 kDa) and α-chain of iC3b (68 kDa) within each lane are used to represent the relative amounts of C3b and iC3b in the total eluted C3 fragments. The kinetics of conversion of C3b to iC3b is expressed as percent change of C3b over time.

The nonsaturable binding observed with *B. dermatitidis* yeast-absorbed serum is not likely due to impairment of the alternative pathway activity from absorption because similar observations were made when serum containing MgEGTA was used. Furthermore, the binding capacity of yeast-absorbed serum could be restored with the antibodies eluted off of serum-treated protein A/G. One possibility for the reduction in maximum binding when the alternative pathway is solely operative is that some of the binding sites for C3 via the classical pathway are topographically isolated from those for binding via the alternative pathway. In other words, C3b molecules produced by the alternative pathway cannot find more sites to bind in their vicinity within the time that they are functional. An alternative explanation is that antibody itself provides additional binding sites.

Naturally occurring antibodies were found to initiate the classical pathway. The kinetics of C3 binding in serum that had been absorbed with either immobilized protein A/G or 26199 yeast cells were almost identical, exhibiting a lag during early binding compared to the rapid early binding in NHS. Normal binding could be fully restored by antibodies eluted from serum-treated protein A/G. These observations together indicate that naturally occurring antibodies initiate the classical pathway, which is largely responsible for the early, rapid binding of C3 to the yeast surface. This situation is similar to that for nonencapsulated strains of *C. neoformans* (27) and for *C. albicans* (16). Initiation of the classical pathway can also be mediated by mannan-binding protein (6, 20, 21). Our data demonstrate a role for natural antibody in initiating the classical pathway but do not exclude a possible contribution of mannan-binding protein.

We investigated the yeast ligand for these natural antibodies. Absorption of NHS with baker's yeast glucan abolished classical pathway activity, whereas other cell wall polysaccharides did not, thereby implicating β-glucan as the ligand. Analyses with flow cytometry and immunofluorescence microscopy confirmed the presence of β-glucan on the yeast surface. Our results suggest that natural antibodies to β-glucan on the yeast cell wall initiate binding of C3 to *B. dermatitidis* via the classical pathway. Our findings are similar to those described for initiation of the classical pathway in nonencapsulated *C. neoformans*. Keller et al. (8) demonstrated that 11 randomly selected sera contained IgG antibodies reactive with *C. neoformans* nonencapsulated yeasts and that absorption by β-glucan from baker's yeast removed the antibodies. The prevalence of natural antibodies reactive with β-glucan and the failure of our *B. dermatitidis* cell wall preparation of nearly 95% α-glucan (7) to inhibit the classical pathway together suggest little involvement of α-glucan in initiating the classical pathway. However, the role of α-glucan as an acceptor for C3b or as an activating surface for initiating the alternative pathway was not examined.

Lastly, we examined the nature of the bond through which C3b binds to the yeast surface, the molecular forms of bound C3 fragments, and the kinetics of conversion of C3b to other fragments. We observed that C3b binds to acceptor molecules through both a hydroxylamine-sensitive ester bond and through a nonsensitive amide bond. Hydroxylamine treatment of yeasts released about 76% of bound ^{125}I-C3, indicating that the ester bond is the chief linkage (18). In encapsulated cryptococci, C3 molecules bind to a polysaccharide capsule almost exclusively through a hydroxylamine-sensitive ester bond (15). This contrasts with nonencapsulated cryptococci, where about 80% of C3 molecules are associated with the cell surface through an ester linkage (12a), and with *C. albicans*, where about 50% of bound C3 is released by hydroxylamine treatment (15). From our results, the situation for *B. dermatitidis* most closely resembles that for nonencapsulated cryptococci, where glucan also is the major component governing interaction of C3 with the cell surface and where most but not all bound C3 molecules associate with the surface through an ester linkage. Although the biological consequence of linkage choice is unknown, the preference for one type over another may reflect differences in cell wall surface chemistry and acceptors for metastable C3b or different regulating mechanisms in these fungi for interaction with complement.

We did not investigate an influence of the complement pathway on the nature of C3 linkage to the yeast surface. However, the nearly constant percentage of ^{125}I-C3 counts released by hydroxylamine-treated yeasts opsonized in NHS for up to 60 min suggests that the preferred linkage does not change from the early binding period, which is largely a function of the classical pathway, to the late binding period, which is more a function of the alternative pathway.

The kinetic analysis of conversion of bound C3 fragments revealed that C3b and iC3b were two types of C3 fragments bound to the yeast surface. It also showed that 70% conversion of C3b to iC3b took place within 1 min after C3b was bound and that this rapid conversion was never completed. This type of analysis is limited by the fact that influx of newly bound C3b may obscure the kinetics of conversion of C3b to iC3b. To assess the kinetics of conversion of a uniform population of C3b to other forms, Pfrommer et al. (25) first coated *C. neoformans* with C3b and then introduced purified factors H and I, which are required to convert C3b. They observed that nonencapsulated *C. neoformans* converts 60% of bound C3b within 2 min of incubation and 20% more over the next 30 min. We used a pulse-chase method as an alternative approach to address the molecular fate of a population of C3 fragments bound synchronously to *B. dermatitidis*. Our analysis revealed a constant ratio over time of 30% of C3 fragments as C3b to 70% as iC3b. Thus, the kinetics of C3b conversion by *B. dermatitidis* is similar to that for nonencapsulated *C. neoformans* (25). The reason for the incomplete conversion of C3b to iC3b by either organism is not known. We did not analyze the distribution and identity of C3 fragments bound to the yeast through an amide linkage. Nevertheless, our results predict that CR1 and CR3 receptors for C3b and iC3b, respectively, on human phagocytes should play a key role in promoting recognition, phagocytosis, and killing of opsonized *B. dermatitidis*.

ACKNOWLEDGMENTS

We thank Tom Kozel and his research laboratory for technical advice on the assays used for this study, and we thank Robert Audet for technical assistance.

This work was supported by grants from the USPHS. B. Klein is the recipient of an NIH Research Career Development Award and a Scholar Award in Molecular Pathogenic Mycology from the Burroughs Wellcome Fund.

REFERENCES

1. **Bacon, J. S. D., V. C. Farmer, D. Jones, and I. F. Taylor.** 1969. The glucan components of the cell wall of Baker's yeast (*Saccharomyces cerevisiae*) considered in relation to its ultrastructure. Biochem. J. **114:**557–567.
2. **DiCarlo, F. J., and J. V. Fiore.** 1958. On the composition of zymosan. Science **127:**756–757.
3. **Drutz, D. J., and C. L. Frey.** 1985. Intracellular and extracellular defenses of human phagocytes against *Blastomyces dermatitidis* conidia and yeasts. J. Lab. Clin. Med. **105:**737–750.
4. **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.
5. **Hogan, L. H., and B. S. Klein.** 1994. Altered expression of surface α-1,3-glucan in genetically related strains of *Blastomyces dermatitidis* that differ in virulence. Infect. Immun. **62:**3543–3546.

6. **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.
7. **Kanetsuna, F., and L. M. Carbonell.** 1971. Cell wall composition of the yeastlike and mycelial forms of *Blastomyces dermatitidis*. J. Bacteriol. **106:** 946–948.
8. **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.
9. **Klein, B. S.** 1992. Immunology of blastomycosis, p. 133–163. *In* Y. Al-Doory and A. F. DiSalvo (ed.), Blastomycosis. Plenum Medical Book Company, New York, N.Y.
10. **Klein, B. S., and J. M. Jones.** 1990. Isolation, purification, and radiolabeling of a novel 120-kD surface protein on *Blastomyces dermatitidis* yeasts to detect antibody in infected patients. J. Clin. Invest. **85:**152–161.
11. **Klimpel, K. R., and W. E. Goldman.** 1988. Cell walls from avirulent variants of *Histoplasma capsulatum* lack α-(1,3)-glucan. Infect. Immun. **56:**2997–3000.
12. **Kohler, P. F., and H. J. Muller-Eberhard.** 1967. Immunochemical quantitation of the third, fourth and fifth components of human complement: concentrations in the serum of healthy adults. J. Immunol. **99:**1211–1216.

12a. **Kozel, T.** Personal communication.

13. **Kozel, T., M. Wilson, and J. Murphy.** 1991. Early events in initiation of alternative complement pathway activation by the capsule of *Cryptococcus neoformans*. Infect. Immun. **59:**3101–3110.
14. **Kozel, T. R.** 1996. Activation of the complement system by pathogenic fungi. Clin. Microbiol. Rev. **9:**34–46.
15. **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.
16. **Kozel, T. R., L. C. Weinhold, and D. M. Lupan.** 1996. Distinct characteristics of initiation of the classical and alternative complement pathways by *Candida albicans*. Infect. Immun. **64:**3360–3368.
17. **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.
18. **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.
19. **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.
20. **Lu, J., S. Thiel, H. Wiedemann, R. Timpl, and K. B. M. Reid.** 1990. Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme C1r2C1s2 complex, of the classical pathway of complement, without involvement of C1q. J. Immunol. **144:**2287–2294.
21. **Matsushita, M., and T. Fujita.** 1992. Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. J. Exp. Med. **176:**1497–1502.
22. **Newman, S. L., and L. K. Mikus.** 1985. Deposition of C3b and iC3b onto particulate activators of the human complement system. J. Exp. Med. **161:** 1414–1431.
23. **Newman, S. L., S. Chaturvedi, and B. S. Klein.** 1994. WI-1 antigen on *Blastomyces dermatitidis* yeasts mediates binding to human macrophage CR3 (CD11b/CD18) and CD14. J. Immunol. **154:**753–761.
24. **Nollstadt, K. H., M. A. Powles, H. Fujioka, M. Aikawa, and D. M. Schmatz.** 1994. Use of β-1,3-glucan-specific antibody to study the cyst wall of *Pneumocystis carinii* and effects of pneumocandin B_0 analog L-733,560. Antimicrob. Agents Chemother. **38:**2258–2265.
25. **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.
26. **Sugar, A. M.** 1992. Taxonomy and biology of *Blastomyces dermatitidis*, p. 9–29. *In* Y. Al-Doory and A. F. DiSalvo (ed.), Blastomycosis. Plenum Medical Book Company, New York, N.Y.
27. **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.
28. **Worsham, P. L., and W. E. Goldman.** 1988. Quantitative plating of *Histoplasma capsulatum* without addition of conditioned medium or siderophores. J. Med. Vet. Mycol. **26:**137–143.

Editor: R. E. McCallum