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Early Events in Initiation of Alternative Complement Pathway Activation by the Capsule of *Cryptococcus neoformans*

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The capsule of *Cryptococcus neoformans* is a powerful activator of the alternative complement pathway. This study examined the manner in which the cryptococcal capsule influences initiation of and early events in complement activation by *C. neoformans*. These studies examined the effects of the classical and alternative pathways on the kinetics and early sites for deposition of C3 fragments on encapsulated cryptococci, nonencapsulated cryptococci, and zymosan. The results showed that nonencapsulated cryptococci and zymosan are qualitatively and quantitatively similar in the manner in which they initiate complement activation. Both utilize the classical and alternative pathways. Initiation via the classical pathway occurs suddenly and simultaneously at sites distributed over the entire cell surface. Initiation of the alternative pathway by zymosan and nonencapsulated cryptococci is characterized by a lag of 6 to 8 min before appreciable amounts of C3 accumulate on the cells. Alternative pathway initiation by zymosan and nonencapsulated cryptococci occurs at a limited number of focal initiation sites that expand with alternative pathway amplification to cover the cell surface. Presence of the cryptococcal capsule blocks classical pathway initiation, which would normally occur at the cryptococcal cell wall, and produces an initiation that is dependent solely on the alternative pathway. Initiation of the alternative pathway by the cryptococcal capsule is characterized by a lag in C3 accumulation and the appearance of a limited number of focal initiation sites which resemble those observed when the alternative pathway is activated by zymosan and nonencapsulated cryptococci.

Yeast cell walls are potent activators of the alternative complement pathway. Many of the earliest studies of complement activation used zymosan, a cell wall product of *Saccharomyces cerevisiae*, as the prototype complement activator. Incubation of zymosan in normal human serum leads to the deposition of 3×10^6 to 4×10^6 C3 molecules per zymosan particle (26). Although the specific molecular architecture responsible for alternative pathway activation has not been determined for all yeasts known to activate the alternative pathway, available evidence indicates that the active constituent of zymosan is an insoluble form of its β-(1→3) and/or β-(1→6) glucan (4, 5).

Cryptococcus neoformans is a pathogenic yeast that has emerged as a leading opportunistic pathogen in patients with AIDS. The organism is unusual among the pathogenic yeasts because it is surrounded by a polysaccharide capsule. One of the most striking features of the cryptococcal capsule is its ability to serve as a site for activation and deposition of opsonic fragments of C3. Incubation of the yeast in normal human serum (NHS) leads to the binding of 1×10^7 to 3×10^7 C3 molecules per yeast cell (17). The capsule itself is the site for C3 deposition, and the C3 is almost entirely in the form of iC3b (17). Incubation of encapsulated cryptococci in an alternative pathway reconstituted from purified factors B, D, H, I, and C3 and properdin produces deposition of C3 in the capsule that is quantitatively and qualitatively identical to that observed when the yeast is incubated in normal serum (18). Thus, activation of the complement system by the cryptococcal capsule is initiated, amplified, and regulated entirely via the alternative pathway.

The cryptococcal cell wall consists almost entirely of β-(1→3) and β-(1→6) glucans (12). The presence of polysaccharides in the cryptococcal cell wall with structures similar to those of the alternative pathway-activating polysaccharides of zymosan suggests that nonencapsulated cryptococci can activate the alternative pathway in a manner similar to that of zymosan. Thus, it is not surprising that nonencapsulated cryptococci have been shown to activate the complement system (21). The cryptococcal capsule has a structure that differs markedly from those of the complement-activating glucans of zymosan or the glucans found in the cryptococcal cell wall. The capsular polysaccharide has a backbone of α-(1→3)-linked mannose with single-unit side branches of xylose and glucuronic acid residues (1). The dissimilarity in the structures of the sites where activation occurs with encapsulated and nonencapsulated yeasts raises the question of the extent to which the cryptococcal capsule effects a quantitative and qualitative alteration in complement activation that would occur in the absence of a capsule.

The purpose of this study was to examine in greater detail the manner in which the cryptococcal capsule influences initiation of complement activation by *C. neoformans*. Our results show that nonencapsulated cryptococci and zymosan are qualitatively and quantitatively similar in the manners in which they initiate complement activation. Both utilize the classical and alternative pathways. Initiation via the classical pathway occurs suddenly and simultaneously at sites distributed over the entire cell wall. Initiation of the alternative pathway by zymosan and nonencapsulated cryptococci occurs at a limited number of focal sites that expand with alternative pathway amplification to cover the cell wall. In contrast, presence of the cryptococcal capsule blocks initi-

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ation events that would normally be mediated by the cryptococcal cell wall and produces an initiation that is dependent solely on the alternative pathway. Initiation of the alternative pathway by the cryptococcal capsule involves focal sites that resemble those observed when the alternative pathway is activated by zymosan and nonencapsulated cryptococci.

MATERIALS AND METHODS

Yeast cells and zymosan. *C. neoformans* 388 is an encapsulated isolate of serotype A that was provided by K. J. Kwon-Chung. Nonencapsulated *C. neoformans* 602 has already been described (13). The yeast cells were cultured on a liquid synthetic medium (3) on a gyratory shaker at 100 rpm for 72 h at 37°C. All fungi were killed with 0.33% formaldehyde before use. Zymosan A (Sigma Chemical Co., St. Louis, Mo.) was boiled and treated with 0.1 N NaOH and 1% sodium dodecyl sulfate (SDS) as described by Law et al. (20). Zymosan particles were suspended to 5.6×10^8/ml in 0.15 M NaCl containing 0.02% sodium azide and stored at 4°C.

C. neoformans isolate 184A was used for electron microscopy studies. These yeast cells were grown for 3 days at room temperature on modified Sabouraud agar slants. The cells were harvested, washed three times, and diluted to 5×10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cryptococcal cell suspensions were incubated for 12 h at 37°C in an atmosphere of 7% CO_2. The yeast cells were washed three times with serum-free RPMI 1640 medium, counted, and adjusted to 4×10^5/ml.

Serum and isolation of serum proteins. Peripheral blood was collected from 5 to 10 volunteers after their informed consent was obtained. The sera were pooled and stored at −70°C. This pool was used as the source of NHS. Serum was heated at 56°C for 30 min for studies requiring heat-inactivated serum. Complement proteins were isolated from frozen human plasma. C3 (17, 31) and factor H (18, 29, 31) were isolated as previously described. C3 was labeled with ^{125}I by the Iodogen (Pierce Chemical Co., Rockford, Ill.) procedure (18). Radiolabeled C3 was separated from free iodine by filtration through Sephadex G-25. Typically, 1 mg of C3 was labeled to a specific activity of 4×10^5 cpm/μg.

Activation and binding kinetics. Activation and binding of C3 to cryptococci and zymosan was done in 1.2-ml reaction mixtures consisting of (i) 40% NHS or 40% heat-inactivated serum, (ii) GVB^{2+} (sodium Veronal [5 mM]-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 (sodium Veronal [5 mM]-buffered saline [142 mM], pH 7.3, containing 0.1% gelatin, 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 10 mM MgCl), (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, and (iv) 2.4×10^6 cryptococcal cells or zymosan particles. The tubes were incubated at 37°C, and 100-μl samples were withdrawn at 1-, 2-, 4-, 6-, 8-, 12-, 16-, 32-, and 64-min intervals. The reaction was stopped by addition of the sample to phosphate-buffered saline (PBS)-SDS (10 mM phosphate, 127 mM saline [pH 7.3], 0.1% SDS) containing 10 mM EDTA. The particles were washed five times in PBS-SDS, and the amount of bound radioactivity was determined. Specific binding was determined by subtracting the radioactivity of samples which used heat-inactivated serum from the total binding observed with NHS. Each experiment was done at least four times. Data from representative experiments are shown in the results.

Immunofluorescence analysis of early C3 binding. Cryptococcal cells or zymosan particles (3.5×10^5) were incubated in a 1.75-ml reaction mixture of 40% NHS or heat-inactivated serum in GVB^{2+}. The tubes were incubated at 37°C, and 250-μl samples were withdrawn at various times. The reaction was stopped by addition of the sample to an excess of ice-cold PBS containing 10 mM EDTA, and unbound C3 was removed by three washes with PBS. The particles 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 (BSA; Sigma). The particles were washed twice in PBS, suspended in a 9:1 (vol/vol) glycerol-PBS solution, and applied to microscope slides coated with poly-L-lysine (Sigma).

The pattern of C3 deposition was determined by epifluorescence microscopy and confocal microscopy. Standard fluorescence microscopy was done with a Leitz Orthoplan microscope equipped with a Ploemopak vertical illuminator which used a Leitz wide-band blue H filter block cube. Confocal microscopy was done with a Bio-Rad MRC-600 Confocal Imaging System. The system was equipped with a 25-mW argon ion laser with excitation peaks at 488 and 514 nm. Specimens were excited at 488 nm with approximately 0.9 mW. A single gain setting was used for illumination of cells collected at all incubation intervals except 8 min. Use of a single gain setting allowed comparison of the relative amount of fluorescence in cells at each incubation time. The gain was reduced for cells collected after 8 min of incubation to show greater detail in the pattern of C3 deposition on these brightly fluorescing cells. Images were scanned at 1-μm intervals through individual cells. In most cases, all images collected from individual cells were projected onto a single plane. In some cases, images collected at the 1-μm intervals were used to generate a stereoscopic image of an entire cell.

Ultrastructural analysis of early C3 binding. Cells of *C. neoformans* 184A (4×10^5) were incubated for 5 min at 37°C with 1 ml of 20% NHS or 20% heat-inactivated (56°C for 30 min) human serum diluted in RPMI 1640. The reaction was stopped by addition of cold RPMI 1640; the cells were washed twice in cold RPMI 1640 medium, followed by two washes with PBS containing 0.5% BSA (PBS-BSA). The cryptococcal cells were then either prepared for scanning electron microscopy (SEM) or subjected to immunogold labeling procedures before being prepared for transmission electron microscopy (TEM).

Cells to be labeled with immunogold were preblocked by incubation for 10 min at 4°C with 10 μl of 1 mg of goat immunoglobulin per ml. After blocking, the cells were treated for 30 min at 4°C with 20 μl (50 μg/ml) of mouse anti-human iC3b (immunoglobulin G2b [IgG2b]; Quidel, San Diego, Calif.), an irrelevant mouse IgG2b, or PBS-BSA. The cells were washed three times with PBS-BSA and incubated for 45 min at 4°C in 40 μl of a 1:20 dilution of goat anti-mouse IgG to which 10-nm-diameter gold particles were conjugated (E-Y Labs, Inc., San Mateo, Calif.). The cells were washed with PBS and prepared for TEM.

The serum-treated or serum-treated and immunogold-labeled cryptococcal cells were adhered to Kodacel film (Eastman Kodak Co., Rochester, N.Y.) that had been precoated with 0.1% poly-L-lysine (Sigma) by incubating the cell suspension on the film for 1 h at 4°C. The cells were fixed for 30 min at 4°C with 3% (vol/vol) glutaraldehyde, 6%

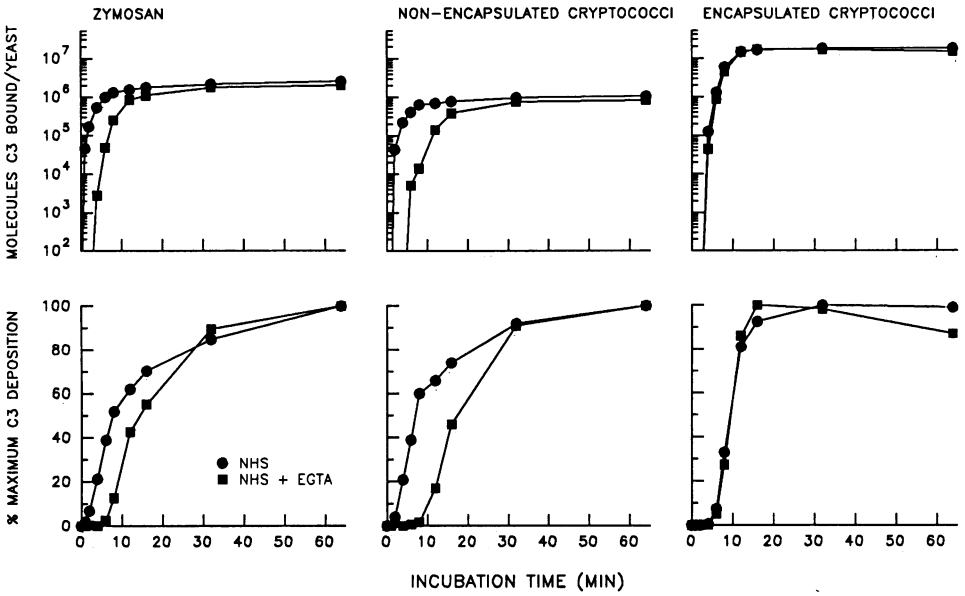

FIG. 1. Activation and binding of C3 fragments to zymosan, nonencapsulated cryptococci, or encapsulated cryptococci. Yeast cells were incubated in 40% NHS or 40% NHS treated with Mg-EGTA. Binding of C3 fragments was determined by incorporation of trace amounts of ^{125}I-labeled C3 into the reaction mixture. Data are reported as numbers of C3 molecules bound per yeast cell (upper panels) or as percentages of the maximum level of binding by each target cell during the 64-min reaction time (lower panels).

(vol/vol) paraformaldehyde, and 0.2% (wt/vol) tannic acid in 0.1 M sodium cacodylate buffer (pH 7.4). The fixed cells were washed with three changes of 0.1 M sodium cacodylate buffer at 4°C and postfixed for 30 min at 4°C with 1% (vol/vol) osmium tetroxide and 0.07% (wt/vol) ruthenium red diluted in 0.1 M sodium cacodylate buffer. Following three washes in 0.1 M sodium cacodylate buffer at 4°C, the cells were dehydrated in a graded series of ethanol. Cells to be examined by SEM were critical point dried in an Autosamdri 814 using liquid CO_2 and then coated thinly with gold-palladium (60:40) in a Hummer VI before examination on a JEOL JSM-880 SEM at 15 to 20 kV. For TEM, the preparations were infiltrated with Spurr's low-viscosity resin (30). After flat embedding, the cells were serially sectioned into 100-nm sections with a diamond knife on a Sorvall MT-6000 or a Reichart Ultracut ultramicrotome, and the thin sections were collected by the method of Galey and Nilsson (8). Thin sections were mounted on Formvar-and-carbon-coated copper slot grids, stained with 0.5% (wt/vol) uranyl acetate and Reynold's lead citrate (28), and examined on a Zeiss 10A electron microscope at 40 or 60 kV.

RESULTS

Kinetics for activation and binding of C3 via the classical and alternative pathways. An initial experiment was done to determine the kinetics for activation and binding of C3 to encapsulated cryptococci (strain 388), nonencapsulated cryptococci (strain 602), and zymosan under conditions in which the classical pathway was active or was blocked by EGTA. Yeast cells were incubated in 40% NHS containing ^{125}I-labeled C3 for intervals that ranged from 1 to 64 min, and the average number of C3 molecules bound per cell was determined. The role of the classical pathway was determined by incorporation of Mg-EGTA into the reaction mixture (NHS-Mg-EGTA). Magnesium-supplemented EGTA chelates calcium, which is required for classical pathway activation. The alternative pathway, in contrast, requires Mg^{2+} but not Ca^{2+} (7, 27). The data are also plotted as the percentage of the maximum observed binding. This type of data analysis facilitated direct comparison of the pattern of activation and binding of C3 to each particle. The results (Fig. 1) showed no qualitative differences in the patterns of activation and binding of C3 to nonencapsulated cryptococci and zymosan. This is particularly evident when the data are plotted as the percentage of maximum binding. The only apparent difference between nonencapsulated cryptococci and zymosan was the number of C3 molecules bound to each yeast. Zymosan bound approximately four times more C3 than did nonencapsulated cryptococci.

The classical complement pathway exerted a strong influence on initiation of activation by both nonencapsulated cryptococci and zymosan. Both cell types showed immediate accumulation of C3 following incubation in NHS. This is most apparent when the data are plotted as the percentage of maximum binding (lower panel of Fig. 1). In six experiments, it required a mean ± the standard error of the mean of only 1.2 ± 0.2 min before 5% of the maximum binding was observed on zymosan particles incubated in NHS. Similarly, it required only 1.8 ± 0.4 min before 5% of maximal binding was observed on nonencapsulated cryptococci incubated in NHS. In contrast, incubation in NHS-Mg-EGTA produced a prominent lag before measurable binding of C3 was observed. Once again, this is most evident when the data are plotted as the percentage of maximum binding (lower panel of Fig. 1). In six experiments, it required a mean ± the standard error of the mean of 5.0 ± 0.3 min before 5% of the maximum binding was observed on zymosan incubated in NHS-Mg-EGTA. Similarly, in six experiments, it required 7.8 ± 0.3 min before 5% of the maximum binding was observed with nonencapsulated cryptococci incubated in NHS-Mg-EGTA. This lag before the rapid-accumulation phase was not an artifact of plotting data as a percentage of maximum binding because the same lag was apparent when

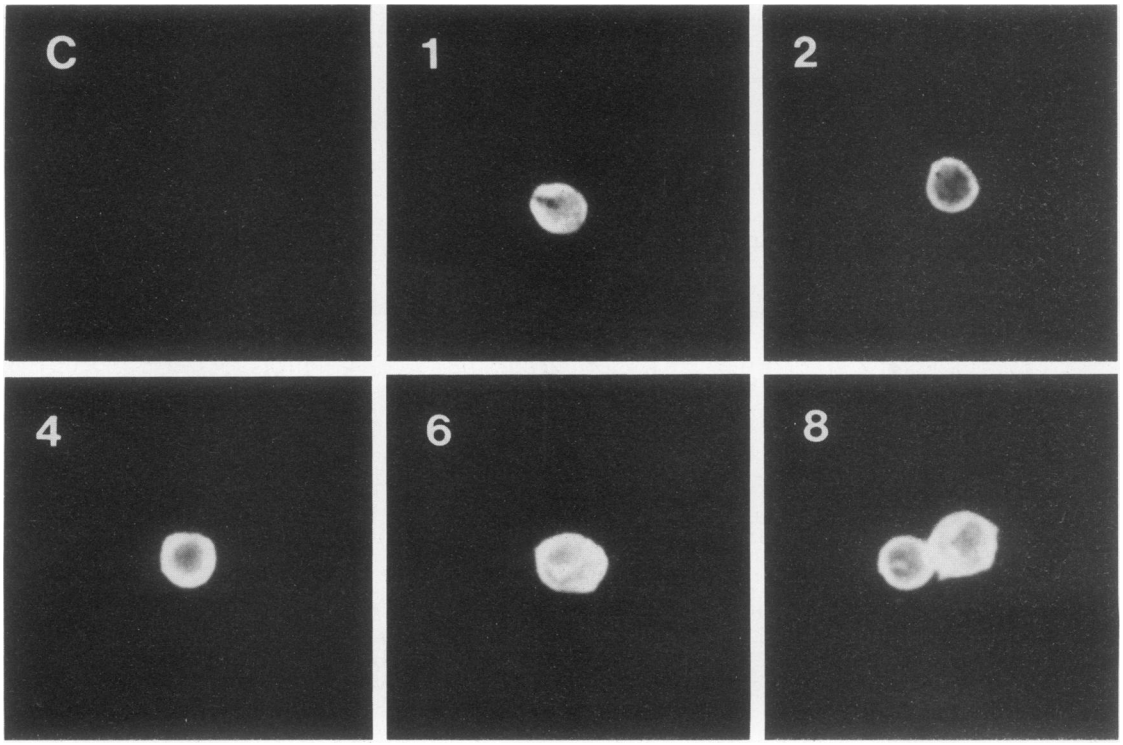

FIG. 2. Sites for deposition of C3 fragments on nonencapsulated cryptococci incubated in 40% heat-inactivated serum (panel C), or 40% NHS for 1 (panel 1), 2 (panel 2), 4 (panel 4), 6 (panel 6), or 8 (panel 8) min. Sites of C3 deposition were determined by use of fluorescein-labeled antiserum to C3 and examination by confocal microscopy.

the data were plotted on a linear scale rather than the log scale used in the upper panel of Fig. 1 (data not shown).

Incubation of encapsulated cryptococci in NHS and NHS-Mg-EGTA produced identical patterns for activation and binding of C3 to the yeast (Fig. 1). Plotting the data as the percentage of maximum binding (lower panel of Fig. 1) showed that a lag of 4 to 6 min occurred before rapid accumulation of C3 was observed on encapsulated cryptococci. In four individual experiments, the mean ± the standard deviation of the time required before 5% of maximum binding occurred was 5.3 ± 0.3 min. As with zymosan and nonencapsulated cryptococci, this lag was not an artifact of the manner of data presentation since the same lag was observed when the data were plotted on a linear scale rather than the log scale used in the upper panels of Fig. 1 (data not shown). The immediate activation and binding of C3 observed with nonencapsulated cryptococci and zymosan incubated in NHS was not observed with encapsulated cryptococci incubated in NHS. These results suggested that the early classical pathway activation by the yeast cell wall observed with nonencapsulated cryptococci and zymosan is blocked by the presence of the capsule.

There were two additional differences between the kinetics for activation and binding of C3 to encapsulated cryptococci and the activation pattern exhibited by both nonencapsulated cryptococci and zymosan. (i) Once past the initial lag, C3 accumulated at a much greater rate on encapsulated cryptococci than on either nonencapsulated cryptococci or zymosan, regardless of the presence or absence of Mg-EGTA. This rapid accumulation of C3 occurred between 6 and 16 min of incubation. (ii) There was an abrupt and early termination in the amplification of C3 on encapsulated cryptococci that occurred after 12 to 16 min of incubation. In contrast, C3 continued to accumulate on both nonencapsulated cryptococci and zymosan throughout the 64 min of incubation.

Visualization by immunofluorescence microscopy of early events in complement activation on encapsulated and nonencapsulated yeasts. Immunofluorescence microscopy was used to visualize the early patterns of activation and binding of C3 to encapsulated and nonencapsulated yeasts. Encapsulated (strain 388) and nonencapsulated (strain 602) cryptococci and zymosan particles were incubated in 40% NHS or 40% NHS-Mg-EGTA for 1 to 8 min and stained with fluorescein isothiocyanate-labeled anti-C3 to identify the sites of C3 activation and binding. Two distinct patterns were observed. Nonencapsulated cryptococci (Fig. 2) and zymosan particles (data not shown) incubated in NHS showed sudden and simultaneous activation and binding of C3 at sites that completely covered the surface of the yeast cells. This was readily apparent at the earliest time (1 min). Staining at later times showed an increase in the intensity of C3 deposition, but the site of deposition was unchanged.

Activation and binding of C3 to encapsulated cryptococci incubated in NHS (Fig. 3) followed a pattern different from that observed with nonencapsulated cryptococci or zymosan incubated in NHS. No C3 was observed on the cells after 1 min of incubation, a result consistent with data from use of radiolabeled C3 (Fig. 1). After 2 min of incubation, there were a limited number of focal points at which C3 was visible. Four minutes of incubation found an increase in the number of apparent initiation sites. Some activation foci were the small point foci characteristic of the 2-min samples; other sites of C3 binding were much larger. Cells incubated

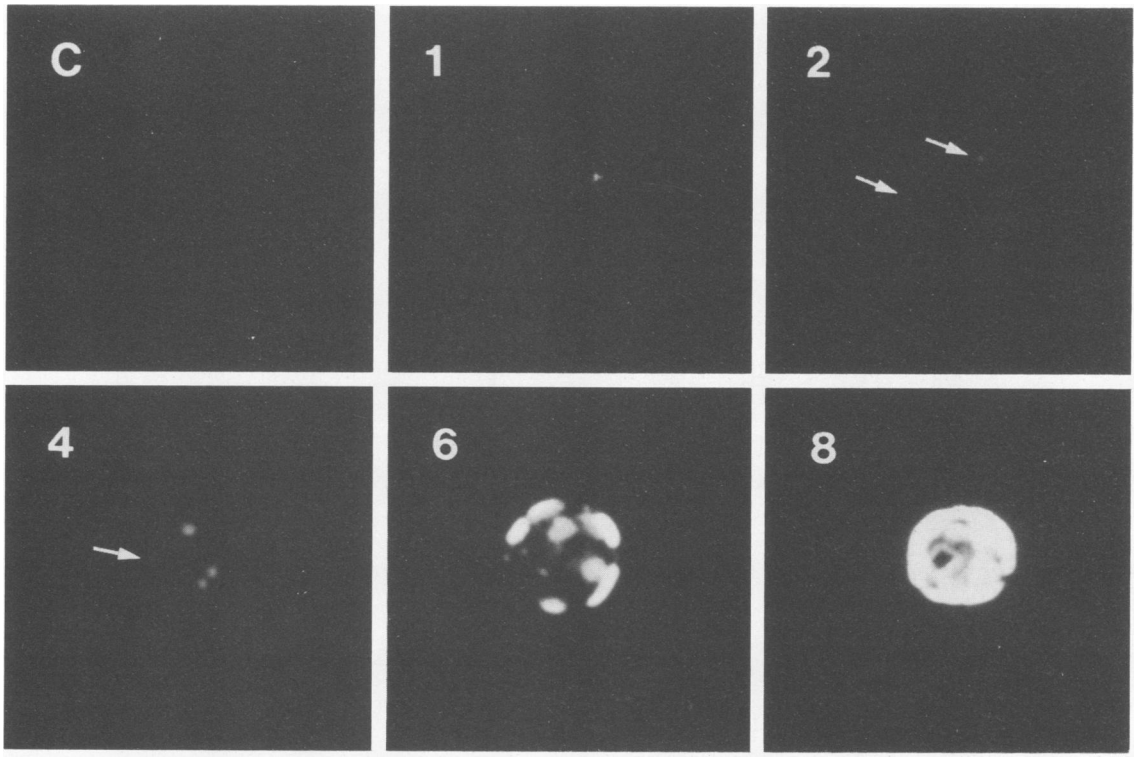

FIG. 3. Sites for deposition of C3 fragments on encapsulated cryptococci incubated in 40% heat-inactivated serum (panel C), or 40% NHS for 1 (panel 1), 2 (panel 2), 4 (panel 4), 6 (panel 6), or 8 (panel 8) min. Sites of C3 deposition were determined by use of fluorescein-labeled antiserum to C3 and examination by confocal microscopy. Early sites of C3 deposition are characterized by minute foci (arrows), which appear to expand in size.

in serum for 6 min showed a marked increase in the size of the foci, as well as the presence of additional small sites. Cells incubated for 8 min had very large patches of C3 that were beginning to coalesce. The focal nature of the activation sites is readily apparent in the stereoscopic view of cells incubated for 6 min (Fig. 4, top). Both large and small sites are visible at apparently random sites around the capsule. The larger patches appear to have reached a limit for spread into the capsule, presumably at the cell wall, and have spread laterally around the cell. The stereoscopic view of cells incubated for 8 min (Fig. 4, bottom) showed some areas that were not covered with C3, but most portions of the capsule exhibited appreciable accumulation of C3.

An examination of the pattern of C3 deposition on nonencapsulated cryptococci or zymosan incubated in NHS-Mg-EGTA (data not shown) showed the focal pattern of deposition observed with encapsulated cryptococci incubated in NHS. No binding was observed after 2 min, minute foci of bound C3 were seen after 4 min, and additional incubation produced an apparent lateral spread of the bound C3 until the cells were completely covered with C3 after 12 min of incubation.

Visualization by SEM and TEM of the early sites for deposition of C3 in the cryptococcal capsule. The focal nature of C3 deposition in the cryptococcal capsule was confirmed by an ultrastructural examination of cryptococci incubated for 5 min in NHS. Encapsulated cryptococcal cells were examined by TEM and SEM. Cryptococci examined by TEM had been stained for the presence of C3 fragments by incubation with murine antibodies specific for iC3b, followed by incubation with goat anti-mouse IgG coupled to 10-nm-diameter gold particles. The results (Fig. 5) showed dense accumulation of C3 at discrete sites in the capsule. This pattern of deposition was also observed by SEM, which made apparent sites of C3 deposition visible as prominent blebs on the capsular surface. Cells incubated in heat-inactivated NHS, C3-depleted serum (data not shown), or normal human IgG (data not shown) showed no deposition of C3 by TEM, and no blebs were apparent by SEM (Fig. 5).

Regulation by factor H of initiation of the alternative pathway. In the alternative pathway, the transition from deposition of the initial metastable C3b molecules on a particle to amplification depends on the ability of the particle to influence the interaction between particle-bound C3b and factor H. Factor H has a high affinity for C3b on nonactivators and a relatively low affinity for C3b on activating particles (6, 9, 23). If factor H binds to C3b, it serves as a cofactor for factor I, leading to conversion of C3b to the inactive iC3b. Thus, factor H may influence the extent of the lag observed before amplification, as well as the number of C3 fragments bound to particulate activators (26). As a consequence, we examined the influence of factor H concentration on the kinetics for activation and binding of C3 fragments to cryptococci.

The effect of additional factor H on initiation of activation by encapsulated and nonencapsulated cryptococci was determined by incubation of the yeast cells with (i) 40% NHS containing no additional factor H beyond that found in the NHS, (ii) 40% NHS supplemented with 225 μg of factor H per ml, and (iii) 40% NHS supplemented with 450 μg of factor H per ml. The amount of added factor H was calculated from the reported concentration of factor H in

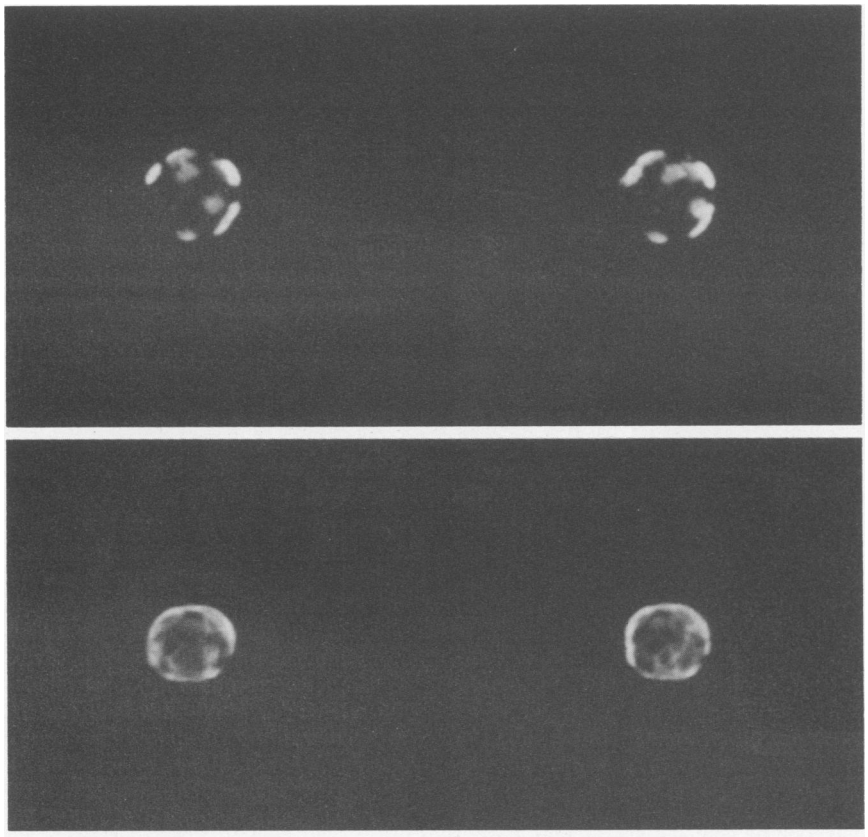

FIG. 4. Stereoscopic image of sites for deposition of C3 in the capsule of *C. neoformans*. Encapsulated cryptococci were incubated in 40% NHS for 6 (upper panel) or 8 (lower panel) min. Sites of C3 deposition were determined by use of fluorescein-labeled antiserum to C3. Imaging was done by confocal microscopy using sections taken at 1-μm intervals.

NHS of 560 μg/ml (22). On the basis of this estimate, 40% NHS contains approximately 225 μg of factor H per ml. Thus, addition of 225 μg of purified factor H per ml to the reaction mixture will increase the factor H concentration by 100%, while addition of 450 μg of factor H per ml will increase the factor H concentration by 200%. A kinetic analysis of binding of C3 to the yeast cells was determined by use of radiolabeled C3 as described in the legend to Fig. 1. The data are reported as percentages of the maximum binding that occurred in the absence of additional factor H.

Addition of factor H to nonencapsulated cryptococci which were incubated with 40% NHS had little effect on the activation and binding of C3. As already noted (Fig. 1), the classical pathway is operative under these conditions. The only apparent effect was a slight diminution in the number of C3 fragments that bound at each time interval.

Incorporation of excess factor H into a reaction mixture in which the classical pathway is blocked by the presence of Mg-EGTA had a marked effect on the kinetics of C3 deposition on nonencapsulated cryptococci (Fig. 6). Additional 225 or 450 μg of factor H per ml prolonged the lag from 8 to 16 min. There was eventual recovery from the initial inhibition, and the amount of bound C3 approached that observed on yeasts incubated with 40% NHS without additional factor H.

The effect of excess factor H on activation and binding of C3 to encapsulated cryptococci incubated in NHS resembled some aspects of the effect observed when factor H was added to nonencapsulated cryptococci incubated with NHS-Mg-EGTA. Increasing the concentration of factor H by 100% doubled the lag from 4 to 6 min to approximately 8 to 10 min. Increasing the concentration of factor H by 200% completely blocked activation and binding of C3 to encapsulated cryptococci. Thus, activation and binding of C3 to encapsulated cryptococci showed much greater susceptibility to the regulatory action of factor H than did alternative pathway-mediated activation and binding of C3 to nonencapsulated cryptococci.

DISCUSSION

The present study was undertaken to examine initiation of the alternative pathway by the cryptococcal capsule. We used activation and binding of C3 fragments to zymosan and nonencapsulated cryptococci as reference points. Analysis of the kinetics of C3 deposition showed several differences between nonencapsulated cryptococci and zymosan on the one hand and encapsulated cryptococci on the other. (i) Incubation of nonencapsulated cryptococci or zymosan in NHS led to rapid accumulation of C3 on these yeast cells. In contrast, there was a 4- to 6-min lag before appreciable amounts of C3 bound to encapsulated cryptococci that were incubated in NHS. The immediate accumulation of C3 fragments on zymosan and nonencapsulated cryptococci was due to the action of the classical pathway. Incubation of zymosan or nonencapsulated cryptococci in NHS-Mg-EGTA produced a lag similar to that observed with encapsulated cryptococci. Addition of Mg-EGTA to NHS had no

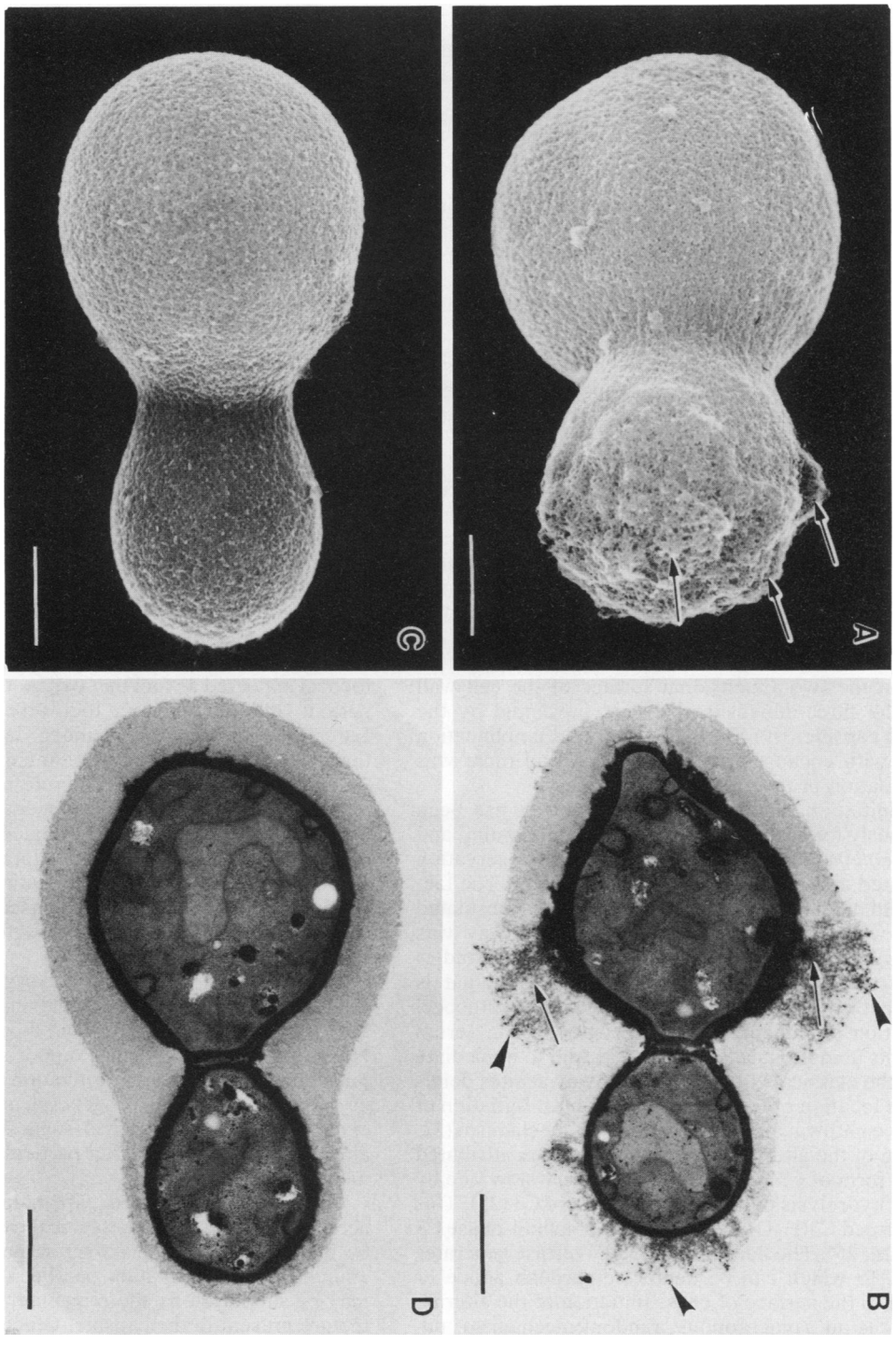

FIG. 5. SEM (A and C) and TEM (B and D) of *C. neoformans* treated with NHS (A and B) or heat-inactivated human serum (C and D). Arrows indicate the areas of C3 fragment binding. Gold particles can be seen in panel B (arrowheads) as black spheres in the areas of C3 fragment binding on specimens treated with mouse anti-iC3b IgG antibody, followed by goat anti-mouse IgG labeled with 10-nm-diameter gold particles and indicate the presence of iC3b. Bars, 1 μm.

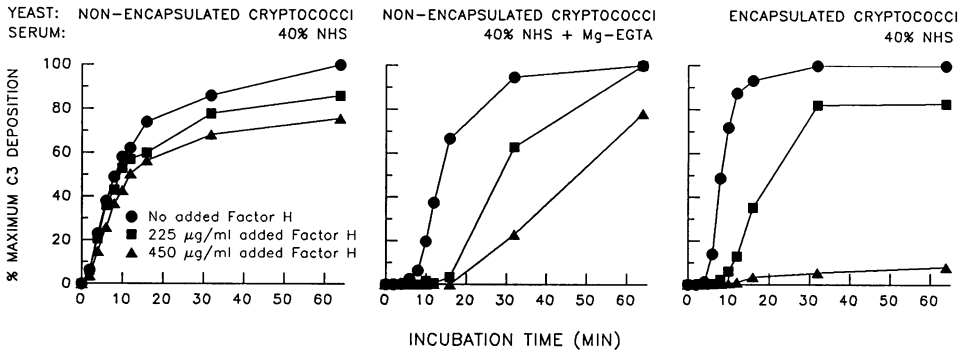

FIG. 6. Effect of an increased concentration of factor H on the activation and binding of C3 fragments to nonencapsulated cryptococci incubated in 40% NHS, nonencapsulated cryptococci incubated in 40% NHS treated with Mg-EGTA, or encapsulated cryptococci treated with 40% NHS. Additions of 225 and 450 μg of factor H per ml were calculated to increase the concentration of factor H in 40% NHS by 100 and 200%, respectively.

qualitative or quantitative effect on the kinetics of C3 deposition on encapsulated cryptococci, supporting our earlier report that activation and binding of C3 to encapsulated cryptococci is dependent solely on the alternative pathway (18). (ii) The second difference between complement activation by zymosan or nonencapsulated cryptococci and encapsulated cryptococci is the apparent rate of amplification. Once rapid deposition of C3 began, there was much more vigorous accumulation of C3 fragments on encapsulated cryptococci than on nonencapsulated cryptococci or zymosan. (iii) More C3 fragments bound to encapsulated cryptococci than to zymosan or nonencapsulated cryptococci. This most likely represents the limited area that is available for C3 deposition on the two-dimensional surface of the cell wall relative to the three-dimensional matrix presented by the cryptococcal capsule. (iv) The phase of rapid amplification was shorter with encapsulated cryptococci, and there was abrupt termination of the amplification phase.

A lag in initiation of the alternative pathway has been noted previously for activators such as zymosan, inulin, and *Escherichia coli* 04 (26). Our studies extend this observation to encapsulated and nonencapsulated cryptococci. The fact that the lag did not occur with zymosan and nonencapsulated cryptococci when the classical complement pathway was operative suggests a mechanism for the lag. Previous studies have shown that NHS contains an IgG antibody that is reactive with the surface of nonencapsulated cryptococci (15). Incubation of nonencapsulated cryptococci in serum containing this ubiquitous antibody would lead to immediate initiation of the classical complement pathway at sites determined by the location of the relevant antigen(s). Initiation of the alternative pathway occurs by a different mechanism (22, 24). Initiation of the alternative pathway has been attributed to an altered form of C3 that is formed at a very slow rate by spontaneous hydrolysis of the thioester bond of C3 (25). This molecule, termed C3(H_2O), becomes part of a fluid-phase C3 convertase (24, 25). The fluid-phase C3 convertase generates metastable C3b which can be deposited through amide or ester linkage to the surface of cells. Initiation of the alternative pathway is an asynchronous, random mechanism, unlike the synchronous, nonrandom initiation of the classical pathway by antibody (24).

Activation via the classical pathway produces immediate deposition of C3b at all sites at which complement-activating antibodies have bound. In contrast, activation via the alternative pathway is intermittent, random, and governed in part by the slow rate of formation of C3(H_2O) (estimated at 0.005%/min) (24, 25). Examination via immunofluorescence microscopy, immunoelectron microscopy, and SEM of the early sites of C3 deposition produced results that are consistent with this hypothesis. Incubation of nonencapsulated cryptococci and zymosan in NHS led to sudden and simultaneous activation and binding of C3 fragments at sites that were distributed over the entire cell surface. The pattern of C3 deposition on encapsulated cryptococci was quite different. No bound C3 was evident at the very early times at which zymosan and nonencapsulated cryptococci incubated in NHS were coated with readily apparent amounts of C3. Early alternative pathway activation on encapsulated cryptococci appeared as foci that expanded during the incubation period. Only a few minute foci were apparent at 2 to 3 min. Later time points found much larger foci, presumably through amplification of the limited number of early foci. There also were additional minute foci, suggesting the formation of new sites for subsequent amplification. The lag probably represents the time needed for formation of sufficient numbers of foci needed to support measurable levels of amplification. The rapid-accumulation phase with encapsulated cryptococci would be a consequence of expansion of existing foci with addition of new foci which, in turn, are expanded.

Incubation of nonencapsulated cryptococci or zymosan in NHS-Mg-EGTA produced focal initiation sites similar to those observed with encapsulated cryptococci incubated in NHS. This similarity further supports the exclusive action of the alternative pathway in activation and binding of C3 to the cryptococcal capsule and generalizes our observation with encapsulated cryptococci to suggest that activation of the alternative pathway by other particulate activators is focal in nature.

There are at least two explanations for the paucity of activation foci in the cryptococcal capsule. (i) Initiation may be due to a molecular activator that is present in limiting amounts. Once activation has been initiated, amplification may be supported by the remainder of the molecular architecture present in the capsule. Glucuronoxylomannan is the primary structural component in the capsule (2). It is likely that lesser amounts of galactoxylomannan or mannoprotein are also present in the capsule (32). Galactoxylomannan or mannoprotein could be an initiator of alternative pathway activation, with amplification supported primarily by glucuronoxylomannan. (ii) An alternative explanation is that sites

needed for initiation of the alternative pathway are present in unlimited amounts but initiation is a rare event. In this latter case, the abundance of glucuronoxylomannan would make it a strong candidate for the component of the capsule responsible for initiation of the alternative pathway.

Our data do not provide an unequivocal answer to this question, but the appearance of new foci throughout the activation process favors the latter hypothesis. If the molecular activator is present in limiting amounts but the activation events are relatively unregulated, immunofluorescence microscopy would be expected to show synchronous appearance and expansion of the focal activation sites. In contrast, if activation is a relatively rare event, appearance and expansion of the sites would be asynchronous, occurring whenever initiation has occurred. Asynchronous appearance of new foci throughout the incubation period is, therefore, consistent with the notion that initiation is a rare event.

Activation of the alternative pathway has been described as occurring in four phases: initiation, deposition of initial metastable C3b, discrimination, and amplification (25). The first two stages occur on both activators and nonactivators. The discrimination phase occurs when C3b deposited on nonactivators is rapidly inactivated by control proteins factors H and I. Control is reduced on activators because of the decreased affinity of factor H for C3b bound to activating particles (6, 9, 23). Transition from the lag phase to the amplification phase requires resistance to the action of factors H and I. We speculated that the lag phase would be extended by incorporation of additional factor H into the reaction mixture. The results (Fig. 6) showed that a 100% increase in the factor H concentration doubled the lag period for deposition of C3 fragments on nonencapsulated cryptococci incubated in NHS-Mg-EGTA. This is similar to results reported previously for the effect of an increased factor H concentration on activation and binding of C3 fragments to rabbit erythrocytes (26). In contrast, an increase in the concentration of factor H had little effect on activation and binding of C3 fragments to nonencapsulated cryptococci incubated in NHS, where the classical pathway was operative. The most striking effect of the increased factor H concentration occurred with encapsulated cryptococci. A 100% increase in the factor H concentration increased the lag by two- to threefold. A 200% increase in the factor H concentration blocked transition to the amplification phase for activation and binding of C3 fragments to encapsulated cryptococci.

The increased susceptibility of encapsulated cryptococci to the inhibitory action of factor H relative to nonencapsulated cryptococci incubated in NHS-Mg-EGTA may explain several characteristics of complement activation by encapsulated cryptococci. The abrupt termination in the amplification phase observed with encapsulated cryptococci (Fig. 1) may be due to increased susceptibility to the action of factors H and I. Similarly, previous studies have noted that almost all C3 bound to encapsulated cryptococci is in the form of iC3b (16, 18). These results differ from those of studies with zymosan, which showed a preponderance of C3b and only limited conversion to iC3b (19). Such results are consistent with our observation that activation and binding of C3 to encapsulated cryptococci is more susceptible to the inhibitory action of factor H than is activation and binding of C3 to nonencapsulated yeast cells.

Capsules can play at least three roles with regard to activation of the complement system. (i) They can be neutral, with the capsule having no apparent direct activating properties yet allowing activation and binding of C3 fragments to the cell wall, as has been described for the capsule of *Staphylococcus aureus* (34). (ii) Capsules may be nonactivators and also block activation by the cell wall, as has been described for the K-1 capsule of *E. coli* (10, 33). (iii) The capsule itself can also act as a site for activation and binding of C3 fragments, as has been suggested for the capsule of *Streptococcus pneumoniae* (11). Previous studies from our laboratory have shown that the cryptococcal capsule acts as a site for activation of the alternative pathway (14, 18). Results from the present study suggest that the capsule also prevents the classical pathway activation that would normally occur at the cell wall when nonencapsulated cryptococci are incubated in NHS. The immediate accumulation of C3 seen when zymosan or nonencapsulated cryptococci were incubated in NHS was absent when encapsulated cryptococci were incubated in NHS. Similarly, immunofluorescence studies showed that the sudden and synchronous deposition of C3 observed when zymosan or nonencapsulated cryptococci were incubated in NHS was absent in the case of encapsulated cryptococci. Available data do not provide an explanation, but several alternatives are possible. The capsule may prevent binding of IgG to the cell wall, prevent access of components of the classical pathway to activation sites at the cell wall, or influence regulation of classical pathway activation at the cell wall.

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