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Kinetic Analysis of the Amplification Phase for Activation and Binding of C3 to Encapsulated and Nonencapsulated *Cryptococcus neoformans*

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Encapsulated and nonencapsulated cryptococci exhibit quantitative and qualitative differences in their activation of the complement system. We examined the kinetics for the rapid amplification phase in which C3 was activated and bound to encapsulated cryptococci, nonencapsulated cryptococci, and zymosan particles. Yeast cells were incubated in normal human serum containing ^{125}I-labeled C3, and bound C3 fragments were measured after 1 to 64 min of incubation. A kinetic analysis showed that the apparent first-order rate constant (k') for binding of C3 to nonencapsulated cryptococci did not differ significantly from k' for binding of C3 to zymosan particles ($P > 0.05$). However, the rate constant for binding of C3 to encapsulated cryptococci was significantly ($P < 0.001$) greater than k' for binding of C3 to nonencapsulated cryptococci and zymosan particles. A plot of C3 molecules bound to encapsulated cryptococci versus time cubed was nearly linear, suggesting that accumulation of C3 in the cryptococcal capsule follows the kinetics predicted by an expanding sphere. In contrast, the plot of C3 molecules bound to nonencapsulated cryptococci or zymosan particles against time was nearly linear, but those plots against time squared or time cubed were not. This result indicates that the rate-limiting step for the addition of C3 fragments to these latter yeast cells follows the kinetics of neither the perimeter of an expanding circle nor the surface of an expanding sphere. Taken together, the results indicate that the high rate of accumulation of C3 in the cryptococcal capsule is consistent with the expected geometry of an expanding sphere of bound C3 within the three-dimensional matrix of the capsule.

Yeast cells have been recognized for many years as powerful activators of the complement system. For example, incubation of zymosan particles, a cell wall product of *Saccharomyces cerevisiae*, in normal human serum (NHS) leads to the deposition of 3×10^6 to 4×10^6 C3 molecules per zymosan particle (9). In recent studies, we examined the activation and binding of C3 molecules to encapsulated and nonencapsulated cells of *Cryptococcus neoformans* (6, 12). These studies showed that activation and binding of C3 fragments to nonencapsulated cryptococci and zymosan particles are mechanistically and kinetically identical. Incubation of nonencapsulated cryptococci or zymosan in NHS leads to the immediate activation and binding of C3 to the yeast cells. Vigorous accumulation of C3 is readily evident as early as 1 min after incubation in NHS. An examination of these early events by immunofluorescence microscopy showed a pattern of C3 binding on the surface of the yeast cells that was immediate and synchronous and that occurred at sites distributed over the entire surface of the cell wall. This sudden and synchronous initiation of the complement cascade is mediated by the classical complement pathway. The antibody responsible for this classical pathway initiation is an immunoglobulin G antibody found in NHS which is cross-reactive between zymosan and nonencapsulated cryptococci (12).

Incubation of encapsulated cryptococci in NHS leads to activation and binding of C3 to the encapsulated cells in a manner which differs markedly in both mechanistic and kinetic perspectives from the patterns observed with zymosan and nonencapsulated cryptococci (6). Incubation of encapsulated cryptococci in NHS is characterized by a delay of 4 to 6 min before readily discernable amounts of C3 have bound to the cells. This initiation is solely dependent on the alternative complement pathway. Initiation is unaffected when the classical pathway is disrupted by the incorporation of ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) into the reaction mixture (2, 10). Moreover, the patterns of C3 deposition onto encapsulated cryptococci are identical when encapsulated yeast cells are incubated in NHS or when an alternative pathway is reconstituted from the six isolated proteins of the alternative pathway (7). Examination by immunofluorescence microscopy of the early sites for binding of C3 to the cryptococcal capsule showed a delayed and asynchronous pattern in which limited numbers of focal initiation sites were observed after incubation for 2 to 4 min. Continued incubation in NHS produced an apparent expansion from existing focal initiation sites and the formation of new focal sites that also appear to expand with time.

A striking difference between the kinetics for activation and binding of C3 to encapsulated cryptococci and that to nonencapsulated cryptococci is the rate of accumulation of C3 on the yeast cells once complement activation has proceeded beyond the initiation phase (6). Incubation of nonencapsulated cryptococci or zymosan in NHS leads to a gradual and prolonged accumulation of C3 fragments on the yeast cells during incubation periods as long as 1 to 2 h. In contrast, once the initial lag phase is past, activation and binding of C3 to encapsulated cryptococci proceeds at an extremely rapid rate over a 5- to 12-min incubation period. This rapid accumulation phase terminates abruptly after approximately 16 min.

Our previous studies focused on the molecular events in

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initiation of the complement cascade by zymosan, nonencapsulated cryptococci, and encapsulated cryptococci. The objective of the present study was to examine in more detail the phase that follows initiation in which there is a rapid accumulation of C3 fragments on these yeast cells. Our results show that the rate of accumulation of C3 on zymosan and nonencapsulated cryptococci is much lower than the rate of accumulation on encapsulated cryptococci. The rapid accumulation of C3 on encapsulated cryptococci is consistent with a three-dimensional expansion of initiation foci.

MATERIALS AND METHODS

Yeast cells and zymosan. *C. neoformans* 388 is the encapsulated isolate that was used throughout the study. This is a serotype A strain that was provided by K. J. Kwon-Chung. *C. neoformans* 602 was the nonencapsulated isolate used throughout the study (4). The yeast cells were grown in a liquid synthetic medium (1) on a gyratory shaker at 100 rpm for 72 h at 30°C. All fungi were killed with 1.0% formaldehyde, washed with sterile PBS (phosphate [0.01 M]-buffered saline [127 mM] [pH 7.3]), and stored at 4°C. Zymosan A (Sigma Chemical Co., St. Louis, Mo.) was boiled and treated with 0.1 N NaOH and 1% sodium dodecyl sulfate (SDS) by the procedure described by Law et al. (8). Zymosan particles were suspended at 2×10^9 particles per ml in 0.15 M NaCl containing 0.02% sodium azide and stored at 4°C.

Serum and isolation of C3. Peripheral blood samples were collected from 5 to 10 volunteers after their informed consents were 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. C3 was isolated from frozen human plasma as described previously (5, 11). C3 was labeled with ^{125}I by the Iodogen (Pierce Chemical Co., Rockford, Ill.) procedure (3). 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.

Kinetic analysis of C3 binding. The kinetics for activation and binding of C3 to cryptococci and zymosan were assessed in 1.8-ml reaction mixtures consisting of (i) 40% normal human serum or 40% heat-inactivated serum, (ii) GVB^{2+} (sodium Veronal [5 mM]-buffered saline [142 mM] containing 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% gelatin), (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) 3.6×10^6 cryptococcal cells or zymosan particles. The tubes were incubated at 37°C, and 100-μl samples were withdrawn at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 24, 32, and 64 min. The reaction was stopped by the addition of each sample to 1.25 ml of ice-cold PBS-SDS (PBS containing 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 incubated with heat-inactivated serum samples from the total binding observed with samples incubated with NHS. Nonspecific binding was consistently less than 1% of the amount of specific binding.

Statistical analysis. The numerical data were statistically compared by one-way analysis of variance. All experiments were done at least three times. The data shown in the figures are from representative experiments.

RESULTS

The kinetics for activation and binding of C3 fragments to encapsulated cryptococci (strain 388), nonencapsulated cryptococci (strain 602), and zymosan are shown in Fig. 1 (upper panels). The data shown in Fig. 1 are from a single experiment that was representative of three to four similar experiments. Yeast cells were incubated in 40% NHS containing ^{125}I-labeled C3 for time periods that ranged from 1 to 64 min, and the number of C3 molecules bound to each cell was determined. In agreement with previous reports from our laboratory (6, 12), there was an immediate accumulation of C3 molecules on both nonencapsulated cryptococci and zymosan particles. In contrast, there was a lag of approximately 5 min before a readily detectable accumulation of C3 was observed on encapsulated cryptococci. Once the early lag in initiation of complement activation was past, there was a phase of rapid activation and binding of C3 to encapsulated cryptococci. The rate of accumulation appeared to be much greater with encapsulated cryptococci than with nonencapsulated cryptococci or zymosan.

A simple kinetic analysis was used to determine apparent first- and second-order rate constants for activation and binding of C3 fragments to encapsulated cryptococci, nonencapsulated cryptococci, and zymosan. The process of activation and binding of C3 to each particle was viewed as an irreversible reaction with acceptor sites on the yeast cell. As the number of acceptor sites for C3 fragments is depleted, the observed velocity decreases. It is assumed in all following analyses that C3 binding sites are the single limiting reactant, i.e., the concentration of C3 is much greater than the total concentration of C3 binding sites. If t is defined as the elapsed time of the reaction, A is defined as the number of potential C3 binding sites at a given time t, C is defined as the concentration of C3, k' is the apparent first-order rate constant, and P is defined as the number of bound C3 fragments at any given time, then

$$-(dA/dt) = k[A][C] = k'[A] \quad (1)$$

and

$$\ln(A/A_o) = -k't \quad (2)$$

where A_o (the total number of possible C3 binding sites) is estimated as the maximum number of C3 molecules bound to each particle during a 64-min incubation period (Fig. 1; $A_o \equiv P_{max}$). The number of unoccupied binding sites at any time (t) was obtained by the difference, $A_o - P \equiv A$. The assumption that the amount of free (or unreacted) C3 was in excess throughout the course of the reaction is justified because the percentage of available C3 which bound to the yeast cells never exceeded 4%. Analysis of the data from the upper panels of Fig. 1 in this manner (Fig. 1, lower panels) produced plots of $\ln(A/A_o)$ which were essentially linear for at least two decades of available C3 binding sites for both zymosan and nonencapsulated cryptococci. Binding of C3 in both cases appeared to follow a simple pseudo-first-order rate law. Furthermore, the rate constants for binding of C3 to zymosan and nonencapsulated cryptococci were quite similar. From the concentration of free C3 (estimated to be 2.6×10^{-6} M), the apparent second-order rate constants in these representative experiments were 3.1×10^4 and 2.3×10^4 M^{-1} min^{-1} for activation and binding of C3 to nonencapsulated cryptococci and zymosan, respectively. Table 1 summarizes the results from several replications of the experiment. Also shown in Table 1 are the half-lives of the available binding sites for C3 on each particle and the apparent second-order rate constants for binding of C3.

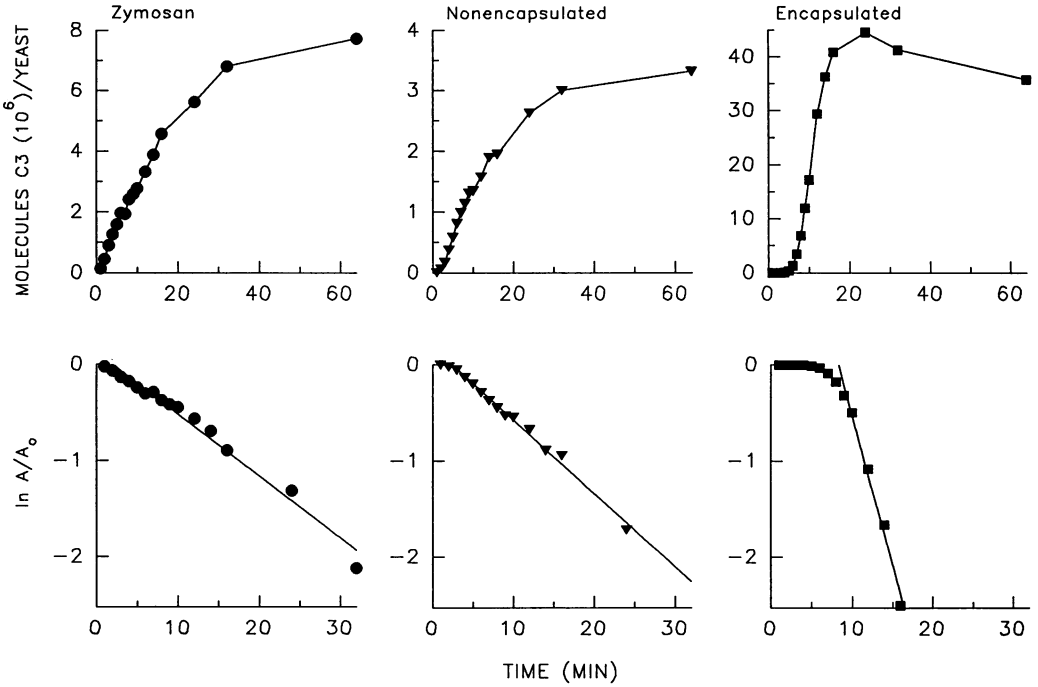

FIG. 1. Analysis of the kinetics for activation and binding of C3 fragments to zymosan (left panels), nonencapsulated cryptococci (center panels), and encapsulated cryptococci (right panels). The lower panels depict a kinetic analysis of the consumption of available sites for C3 binding to the yeast cells, where A_o is the maximum number of apparent sites available for C3 binding and A is the number of unoccupied sites at each time interval (see text).

A plot of equation 2 for binding of C3 to encapsulated cryptococci (Fig. 1, lower right panel) differed markedly from the plots for binding of C3 to zymosan and nonencapsulated cryptococci. Binding of C3 to encapsulated cryptococci showed a protracted lag. The latter phase of the reaction gave an excellent fit to an exponential rate law, with a significantly ($P < 0.001$) greater k' than that which occurred with either zymosan or nonencapsulated cryptococci. The apparent second-order rate constant for this representative experiment was $1.2 \times 10^5\ M^{-1}\ min^{-1}$. Results from three replicate experiments are summarized in Table 1.

Another analysis was used to assess the importance of geometry in the rate-limiting steps of amplification of C3 binding to each cell type. The cryptococcal capsule presents a three-dimensional matrix for amplification from initiation foci. The rate of C3 accumulation (velocity) would be expected to increase in a manner similar to the surface area of a growing sphere. In contrast, unimpeded amplification on the surface of nonencapsulated cryptococci is expected to occur in two dimensions, as does the perimeter of an expanding circle. Initially, consider a spherical geometry of propagation of C3 binding. If one assumes that, in the presence of a capsule, the predominant rate-limiting step is the addition of C3 fragments onto the surface of a sphere of previously attached C3, then we may write

$$(dP/dt) = k_s[C][S] = k_1[C][P] \quad (3)$$

where S is defined as the surface area of a sphere ($4\pi r^2$), r is defined as the radius of the sphere, P is the number of bound C3 fragments at any given time, and k_s and k_1 are the corresponding rate constants. The boundary condition is set when $t = 0$ because $S = P = 0$ when $r = 0$.

$$(dP/dt) = k_s[C](4\pi r^2) \quad (4)$$

At any time t, $P = (KV/N) = (K/N)(4/3)\pi r^3$, where K is a factor which relates the number of bound C3 to the volume

TABLE 1. Apparent first- and second-order rate constants for the rapid accumulation phase of the activation and binding of C3 fragments to encapsulated cryptococci, nonencapsulated cryptococci, and zymosan

Yeast cells or particles	No. of expts	k' (min^{-1})[a]	$t_{1/2}$ (min)	k ($M^{-1}\ min^{-1}$)[c]
Encapsulated cryptococci	3	0.29 ± 0.01	2.4 ± 0.10	$(1.1 \times 10^5) \pm (5.8 \times 10^3)$
Nonencapsulated cryptococci	4	0.11 ± 0.02[d]	6.7 ± 0.9[d]	$(4.2 \times 10^4) \pm (6.1 \times 10^3)$[d]
Zymosan	4	0.07 ± 0.01[e]	9.9 ± 1.0[e]	$(2.8 \times 10^4) \pm (3.0 \times 10^3)$[e]

[a] Mean ± standard error of the mean.
[b] Apparent half-life of available C3 binding sites (mean ± standard error of the mean), where $t_{1/2} = 0.693/k'$.
[c] Apparent second-order rate constant (mean ± standard error of the mean) where $k = k'/$[free C3]. The concentration of free C3 was estimated to be 2.6×10^{-6} M.
[d] P versus zymosan, >0.05; P versus encapsulated cryptococci, <0.001.
[e] P versus nonencapsulated cryptococci, >0.05; P versus encapsulated cryptococci, <0.001.

(V) of a sphere, N is the number of spheres in the reaction mixture, and $V = (4/3)\pi r^3$. Therefore,

$$r^3 = (N/K)(3/4\pi)[P]$$

$$r = (3N/4\pi K)^{1/3}[P]^{1/3}$$

Using the result to eliminate r in equation 4 and substituting the result into equation 3 yields an equation of the form

$$(dP/dt) = k_1'\,[C][P]^{2/3}$$

where $k_1' = k_1(4\pi)^{1/3}(3N/K)^{2/3}$. If $[C]$ is constant,

$$(dP/dt) = k_1''[P]^{2/3}$$

which after integration becomes

$$3[P]^{1/3} = k_1''t \quad (5)$$

In a similar manner, addition to the perimeter of an expanding circle can be shown to yield an equation of the form $2[P]^{1/2} = k_P t$, where k_P is the corresponding rate constant. The equation will hold until steric crowding of bound C3 prevents further addition of C3.

Equation 5 allows an assessment of the effect of geometry on amplification of C3 bound to each cell type. The number of bound C3 molecules was plotted against (i) the incubation time, (ii) the square of the incubation time, and (iii) the cube of the incubation time. If activation and binding of C3 occurred strictly by extension in two dimensions, a plot of C3 molecules bound against time would be concave upward, a plot against time squared would be linear, and a plot against time cubed would be concave downward. In contrast, activation and binding of C3 by extension in three dimensions should produce a plot of C3 molecules against time and time squared that is concave upward and a plot against time cubed that is linear.

Evaluation of the results was done early in the reaction, before the concentration of C3 binding sites became seriously depleted; i.e., initial velocity was determined. Tie lines were set between the number of bound C3 fragments when approximately 2% of maximal binding was observed and the time at which approximately 50% of maximal binding was observed. Analysis of data in this range avoided artifacts that would be introduced into the data analysis by the substantial lag observed with encapsulated cryptococci or by the inevitable deviation from linearity that would occur as potential binding sites became filled (Fig. 1). Activation and binding to both nonencapsulated cryptococci and zymosan showed the greatest linearity when plotted against time. The plots were concave downward when plotted against time squared or time cubed (Fig. 2). In contrast, a plot of C3 bound to encapsulated cryptococci was concave upward when plotted against both time and time squared, indicating that propagation was neither linear nor in two dimensions. A plot of C3 bound against time cubed was nearly linear.

DISCUSSION

Previous studies in our laboratory investigated the kinetics for activation and binding of C3 from NHS onto encapsulated cryptococci, nonencapsulated cryptococci, and zymosan (6, 12). These studies characterized the early events in initiation of complement activation. The current study focused on the kinetics of amplification once initiation had been firmly established.

Earlier studies suggested that the rapid amplification phase of C3 deposition proceeded at a much greater rate on encapsulated cryptococci than on nonencapsulated cryptococci or zymosan (6). A plot of C3 binding versus time (Fig. 1, upper panels) confirmed this earlier observation. A more detailed kinetic analysis of this process (Fig. 1, lower panels) provided evidence that amplification does indeed occur at a higher rate on encapsulated cryptococci, even when treated as a first-order rate process. In this analysis, activation and binding of C3 to each cell was assumed to be an irreversible reaction in which there was a high initial velocity of attachment of C3 to the cells and a subsequent slowing of the velocity as the number of acceptor sites for C3 fragments on each yeast cell was depleted. The number of acceptor sites was defined as the maximum number of C3 molecules bound during a 64-min incubation period. Thus, a plot of ln(potential C3 binding sites at any given time/maximal C3 binding) against time yields a line whose slope is k'. The results showed that k' for binding of C3 to nonencapsulated cryptococci did not differ significantly from k' for binding of C3 fragments to zymosan particles ($P > 0.05$). However, k' values for binding of C3 to both nonencapsulated cryptococci and zymosan were significantly lower than the rate constant for binding of C3 to encapsulated cryptococci ($P < 0.001$). In a similar manner, the half-life of available C3 binding sites was significantly shorter on encapsulated cryptococci than on nonencapsulated cryptococci or zymosan ($P < 0.001$), and the apparent second-order rate constant was significantly greater for encapsulated cryptococci than nonencapsulated cryptococci or zymosan ($P < 0.001$). In no case, however, did the observed rate constants approach those expected for diffusion controlled reactions.

There are at least two possible explanations for differences in the rate of binding of C3 to nonencapsulated cryptococci and zymosan on the one hand and encapsulated cryptococci on the other. The first explanation is the geometry of the rapid amplification phase. Binding of C3 to nonencapsulated cryptococci or zymosan occurs on the surface of each cell. This binding could be governed by one of two possible factors. First, because initiation occurs primarily by the classical pathway (6), the velocity of binding could be influenced by the rate and multiplicity of binding of immunoglobulin G from NHS to the cell surface (12). Alternatively, if there were only a limited number of initiation sites, the rapid accumulation of C3 on these cells would follow the kinetics for addition to the perimeter of an expanding circle. Examination by immunofluorescence of the binding of C3 from NHS onto nonencapsulated cryptococci and zymosan shows a pattern in which C3 is bound to sites distributed over the entire cell surface, with a gradual increase in fluorescence intensity with incubation time (6). These results suggest that accumulation of C3 on nonencapsulated cryptococci or zymosan will not follow the kinetics predicted by an expanding circle. In contrast, activation and binding of C3 to encapsulated cryptococci are focal in nature, with small initiation sites appearing to expand with time (6). In this case, the rate of initiation site formation is slow and the multiplicity of initiation site formation is limiting. After a period of time, the amplification phase of the alternative pathway in promoting the activation and binding of additional C3 becomes the rate-limiting step. Velocity is now determined by the number of C3 convertase complexes available for cleavage of free C3. Hence, even in time cubed plots, there will still be an observed lag phase before the geometric rate law dominates the observed velocity. The three-dimensional matrix provided by the cryptococcal capsule suggests that the applicable geometric rate law will be that predicted by the addition of C3 to the surface of an expanding sphere.

A plot of C3 molecules bound versus time, time squared,

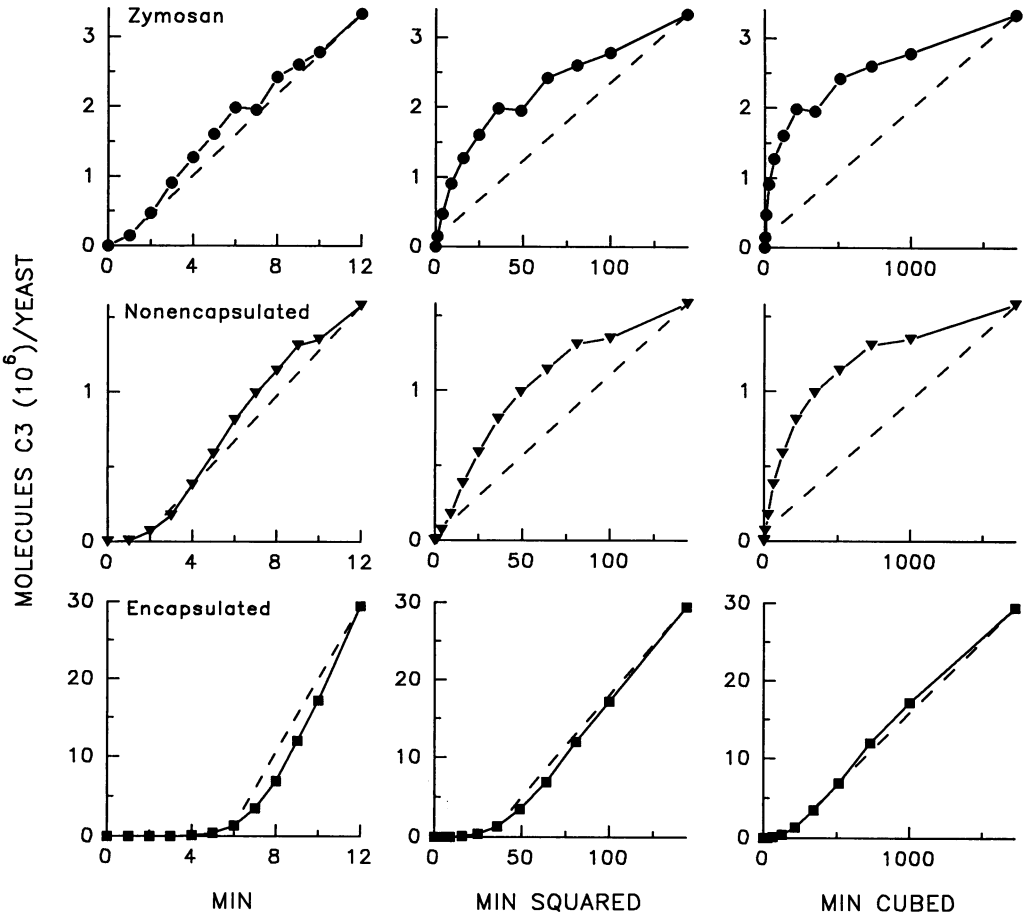

FIG. 2. Analysis of the importance of geometry in the rate-limiting steps of amplification of C3 bound to zymosan (top row of panels), nonencapsulated cryptococci (center row of panels), or encapsulated cryptococci (bottom row of panels). The number of C3 molecules is plotted against incubation time (left column of panels), the square of the incubation time (center column of panels), or the cube of the incubation time (right column of panels).

or time cubed was used to assess the effect of geometry on the rapid accumulation of C3 on encapsulated cryptococci, nonencapsulated cryptococci, and zymosan. The plot of C3 fragments bound to encapsulated cryptococci against time cubed was closest to linear. This supports our previous observation that amplification of bound C3 fragments in the cryptococcal capsule is focal in nature (6). The observation of focal initiation sites by immunofluorescence microscopy suggested that accumulation of C3 occurs as an expanding sphere of bound C3 (6). However, the expansion of a sphere could only be inferred from the earlier report because the immunofluorescence assays relied on examination of individual cells at a given point in time. The kinetic analysis in the present study provides additional independent evidence that amplification of C3 in the cryptococcal capsule does, indeed, follow the geometry of an expanding sphere. The plots against time of C3 fragments bound to both nonencapsulated cryptococci and zymosan were closest to linear. There was a substantial deviation from linearity when binding was plotted against time squared, and there was even greater deviation from linearity when it was plotted against time cubed. This result suggests that appreciable expansion from initiation foci plays a minimal role in the rapid accumulation of C3 on nonencapsulated cryptococci and zymosan. Once again, this result is consistent with the pattern of C3 binding observed when these cells are incubated in NHS. A three-dimensional matrix is not available in the absence of a capsule, and linearity with time cubed would not be expected. Incubation in NHS did not produce readily apparent focal initiation sites, and consequently, linearity with time squared also would not be expected. These results suggest that the rate of C3 binding is dependent on the rate at which immunoglobulin G molecules bind to the yeast cells and activate the classical pathway. Alternatively, the initial binding of immunoglobulin G may produce an abundance of initiation sites such that unimpeded expansion in two dimensions is impossible.

Alternative explanations for the rapid rate of accumulation of C3 fragments on encapsulated cryptococci should also be considered. For example, the cryptococcal capsule may provide a more favorable environment for binding of C3b than the environment provided by nonencapsulated cryptococci or zymosan. Alternatively, the enhanced amplification observed in the cryptococcal capsule could depend on the binding environment provided for factor B. Amplification of the alternative pathway is critically dependent on the binding of factor B to particle-bound C3b. Our data do not exclude the contributions of these alternative mechanisms to the rapid rate of amplification on encapsulated cryptococci. Nevertheless, our results indicate that the high rate of

accumulation of C3 in the cryptococcal capsule is consistent with the expected geometry of an expanding sphere of bound C3.

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