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Activation of C3 and Binding to *Aspergillus fumigatus* Conidia and Hyphae

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Complement activation by *Aspergillus fumigatus* may play a crucial role in stimulating binding and killing of this organism by phagocytes. We examined the amount and type of C3 deposited on resting conidia, swollen conidia, and hyphae of *A. fumigatus* after incubation in pooled human serum. All three life forms of *A. fumigatus* were potent activators of the complement cascade, with deposition on the organisms of similar amounts of C3 per unit of surface area. The rate of deposition was slowest for resting conidia, although maximal deposition was still achieved within 40 min. The roles of the alternative and classical pathways were assessed by use of serum chelated with magnesium EGTA [magnesium ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and with an alternative pathway reconstituted from the six purified alternative-pathway proteins. Complement activation by resting conidia was mediated by the alternative pathway. In contrast, there was a progressive dependence on the classical pathway as the fungal particles matured into swollen conidia and then hyphae. Treatment with hydroxylamine, which disrupts ester linkages, removed 89 to 95% of the C3 bound to all three forms of *A. fumigatus*. This released C3 contained a mixture of C3b and iC3b, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. These data demonstrate that although all three forms of *A. fumigatus* are potent activators of the complement system, the transition from resting conidia to swollen conidia to hyphae results in progressive changes in the manner in which the fungal particles interact with the complement system. The lack of participation of the classical pathway in complement activation by resting conidia may have important implications regarding their ability to effectively stimulate phagocytes.

Phagocytes form a critical component of host defenses against infection with the ubiquitous fungus *Aspergillus fumigatus* (2, 26). Invasive aspergillosis generally begins after inhalation of resting conidia, which become metabolically active and swell. Eventually germination ensues and hyphae, the invasive form of the organism, grow (26). The host can prevent the development of aspergillosis by containing the resting conidia, swollen conidia, or hyphae. In vitro studies from several laboratories have demonstrated that monocytes and macrophages, but not neutrophils, are able to kill resting conidia (24, 27, 37, 41). In contrast, polymorphonuclear leukocytes are able to kill swollen conidia and hyphae (6, 27, 28, 37). In addition to being relatively resistant to killing by neutrophil oxidants and defensins, resting conidia, compared with swollen conidia and hyphae, are relatively poor stimulators of the polymorphonuclear leukocyte respiratory burst and degranulation (5, 27, 29).

Macrophages and polymorphonuclear leukocytes can attach to resting conidia and hyphae, respectively, in the absence of serum opsonins in vitro (6, 14). The situation in vivo is likely to be more complex, however, with complement components and perhaps immunoglobulins generally available to opsonize the organisms and thus to affect the manner in which phagocytes respond to the fungi. Both the type and amount of C3 deposited on the surface of a microorganism may have crucial bearing upon the extent that phagocytes are stimulated to bind, ingest, and kill targets. The C3 degradation products C3b and iC3b are recognized primarily by CR1 and CR3 receptors on phagocytes, respectively, and disparate phagocytic functional responses may be stimulated depending upon whether CR1 or CR3 is bound (10, 21, 36). Moreover, C3b, but not iC3b, functions as a convertase, further amplifying C3 deposition as well as cleaving C5 with the resultant generation of the potent chemotaxin C5a (21). Therefore, in the present study, we examined the amount and type of C3 as well as the mechanism for deposition of C3 on resting conidia, swollen conidia, and hyphae of *A. fumigatus* after incubation in pooled human serum. We found that all three life stages of *A. fumigatus* were potent activators of the complement system, with both C3b and iC3b deposited on the surface of the organisms. However, complement activation by resting conidia was mediated entirely by the alternative pathway, whereas both the classical and alternative pathways contributed to complement activation by swollen conidia and hyphae.

MATERIALS AND METHODS

Fungal cells, hyphae, and conidia. *A. fumigatus* was grown on Sabouraud dextrose agar slants at 23°C for 5 days, and the resting conidia were harvested as in previous studies (27, 29). Swollen conidia were prepared by incubating the resting conidia in Sabouraud broth until >95% of the conidia had swollen to almost twice their original diameter but had not yet germinated (about 4 h at 37°C). Hyphae were prepared as described for swollen conidia, except the incubation period was extended for an additional 2 h. At that time, the hyphal length averaged three to six times the diameter of swollen conidia. Resting and swollen conidia and hyphae were

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washed six times in Dulbecco phosphate-buffered saline (PBS) (GIBCO Laboratories, Grand Island, N.Y.), counted in a hemacytometer, and suspended at the desired concentration. *A. fumigatus* cells were gently sonicated to break up clumps into single cells.

Cryptococcus neoformans 388 was provided by K. J. Kwon-Chung. The yeast cells were cultured on a liquid synthetic medium (1) on a Gyrotory shaker at 100 rpm for 72 h at 37°C. All fungi were killed with formaldehyde before use (17).

Reagents and buffers. The following buffers were used: PBS (10 mM phosphate, 127 mM saline, pH 7.3), PBS-SDS (PBS containing 0.1% sodium dodecyl sulfate [SDS]), VBS (sodium Veronal [5 mM]-buffered saline [142 mM], pH 7.3), GVB (VBS containing 0.1% gelatin), GVB^{2+} (GVB containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$), GVB-Mg-EGTA (GVB containing 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and 10 mM MgCl), and GVB-EDTA (GVB containing 10 mM EDTA).

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. Serum was heated at 56°C for 30 min for studies requiring heat-inactivated serum.

Complement proteins were isolated from frozen human plasma. C3 was isolated as described previously (19, 40). Factor I was isolated by use of sodium sulfate precipitation to remove plasminogen (4), ion-exchange chromatography on DEAE-Sephacel (4), affinity chromatography on a column of Sepharose CL-4B coupled to C3b (31), removal of immunoglobulin G (IgG) with an anti-IgG affinity column, and molecular sieve chromatography on Sephacryl S-200. Factor H was isolated by differential precipitation with PEG 4000, plasminogen depletion on lysine–Sepharose CL-4B, ion-exchange chromatography on DEAE-Sephacel (11), molecular sieve chromatography on Sephacryl S-300, and chromatography on Urogel hydroxyapatite (38). Factor B was isolated by differential precipitation with PEG 4000 (11), plasminogen depletion on lysine–Sepharose CL-4B (11), ion-exchange chromatography on DEAE-Sephacel (11), ion-exchange chromatography on CM Sepharose CL-6B (15), and molecular sieve chromatography on Sephacryl S-300. Properdin was isolated by ion-exchange chromatography on QAE Sephadex A-50 (30) and Bio-Rex 70 (39) followed by removal of contaminating IgG by chromatography on protein A–Sepharose CL-4B and an anti-human IgG–Sepharose CL-4B affinity column. Factor D was purified as described previously (25).

The purity of the isolated proteins was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions with 7% polyacrylamide gels. Analysis by SDS-PAGE showed bands consistent with the reported molecular weights for each protein and the absence of contaminating fragments. The results of this analysis have been reported previously (20). All proteins lacked detectable contamination with IgG as assessed by double immunodiffusion with antiserum specific for human immunoglobulins (goat affinity-purified anti-human immunoglobulin, Southern Biotechnology Associates, Inc., Birmingham, Ala.; catalog no. 2010-01).

C3b was prepared by treatment of C3 with trypsin as described previously (40). iC3b was prepared by treating ^{125}I-labeled C3b (100 μg) with 1 mM PMSF (phenylmethylsulfonyl fluoride) followed by incubation with factor H (10 μg) and factor I (10 μg) for 60 min at 37°C.

C3 and C3b were labeled with ^{125}I by the Iodogen procedure (Pierce Chemical Co., Rockford, Ill.) (8). Radiolabeled proteins were separated from free iodine by filtration through Sephadex G-25. Typically, 1.0 mg of C3 was labeled to a specific activity of 4×10^5 cpm/μg, and 0.5 mg of C3b was labeled to a specific activity of 1×10^5 cpm/μg. Radiolabeled C3 remained functionally active for at least 7 days but was used within 4 days.

Assay for C3 fragments bound to cells. Activation of C3 and binding to resting and swollen conidia, hyphae, and yeast cells were assessed by a modification of a previously described procedure (18). Briefly, binding assays were done in 0.5-ml reaction volumes containing (i) 200 μl of either GVB, GVB-Mg-EGTA, or GVB-EDTA, (ii) ^{125}I-labeled C3 (3.2×10^6 cpm) in 50 μl GVB, (iii) 200 μl of normal human serum or heat-inactivated serum, and (iv) either 5×10^5 cryptococcal cells, 1×10^6 hyphae, or 5×10^6 resting or swollen conidia in 50 μl of GVB. The tubes were incubated for 30 min at 37°C, and the reaction was stopped by the addition of 200 μl of ice-cold 0.1 M EDTA. The cells were washed five times with PBS-SDS, and the amount of bound radioactivity was determined. The SDS in the wash buffer removed C3 fragments that were not covalently bound to the cells.

Tubes containing heat-inactivated serum served as controls for nonspecific binding of C3 to cells and tubes. The amount of nonspecific binding per particle was greater with hyphal fragments than with resting conidia, swollen conidia, or cryptococcal yeast cells, reflecting the larger surface area on the hyphal fragments. Specific binding was determined by subtracting the amount of nonspecific binding from the total binding in each tube. The number of C3 molecules bound to each cell was calculated from the specific activity of the radiolabeled C3, assuming a molecular weight of 187,500 for C3 (40).

Activation and binding kinetics. Cells were incubated in 0.6-ml reaction mixtures containing (i) 40% normal human serum or 40% heat-inactivated serum, (ii) GVB^{2+}, (iii) ^{125}I-labeled C3 sufficient to provide a specific activity of 30,000 cpm/μg of C3 for the mixture of labeled and unlabeled C3 in the serum, and (iv) 1.2×10^6 hyphae, 5×10^5 cryptococcal cells, or 6×10^6 resting or swollen conidia in 50 μl of GVB. The tubes were incubated at 37°C, and 100-μl samples were withdrawn at 5-, 10-, 20-, 40-, and 80-min intervals. Each sample was immediately placed on ice and quenched by addition of EDTA. The cells were washed five times with PBS-SDS, and the amount of cell-bound radiolabeled C3 was determined.

Kinetic assays with the alternative complement pathway reconstituted from isolated proteins were done in the same manner as kinetic assays with whole serum. A mixture of alternative-pathway components was used at 40% of their physiologic concentrations. Normal concentrations in serum of 1,200 μg of C3, 200 μg of factor B, 470 μg of factor H, 34 μg of factor I, 20 μg of properdin, and 2 μg of factor D per ml were the basis for these calculations (32, 33). C3 was preincubated with the factor H and factor I for 30 min at 37°C to inactivate any contaminating C3b. The additional three components were then added and used immediately.

Elution and analysis of bound C3. The proportion of C3 covalently bound to the cellular surface via an ester bond was determined by treatment of opsonized cells with different eluting agents. C3 fragments were activated and bound to the cells as described above, using a 30-min incubation and 200-μl reaction volumes. The cells were washed five times in PBS-SDS and incubated for 60 min at 37°C in 200 μl of (i) 1 M NH_2OH in 0.2 M $NaHCO_3$ (pH 10.0), (ii) 3.5 M NaSCN (pH 7.0), or (iii) PBS-SDS. After

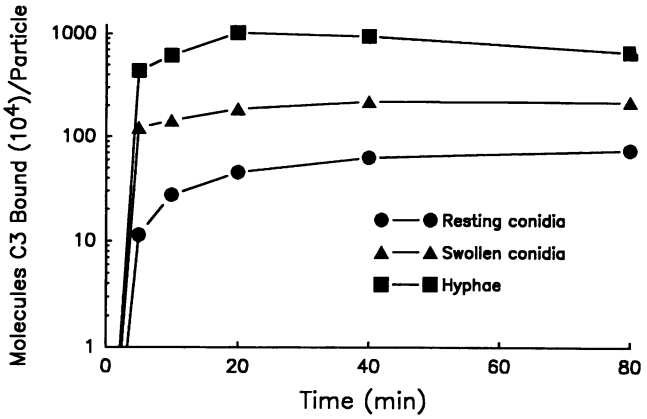

FIG. 1. Kinetics of ^{125}I-C3 deposition on resting conidia, swollen conidia, or hyphae incubated with 40% normal human serum.

treatment, the cells were washed two times in PBS-SDS. The pellets were counted, and the amount of C3 released by NH_2OH or NaSCN relative to the PBS-SDS treatment was calculated.

The molecular form of the bound C3 fragments was determined by elution of the fragments with hydroxylamine and analysis by SDS-PAGE under reducing conditions. Swollen spores (5×10^6 cells) and hyphae (1×10^6 cells) were incubated for 30 min at 37°C in 1 ml of a reaction mixture containing 40% serum, radiolabeled C3, and GVB^{2+}. Resting spores (1.5×10^7 cells) were incubated in 3 ml of the same mixture. ^{125}I-labeled C3 was included at a specific activity of 10^5 cpm/μg of C3. The cells were washed four times with PBS-SDS, suspended in 125 μl of 1 M NH_2OH in 0.2 M $NaHCO_3$ containing 0.1% SDS (pH 10.0), and incubated for 60 min at 37°C. The cells were removed by centrifugation, and the supernatant fluid containing the eluted fragments was evaluated under reducing conditions by SDS-PAGE with 7% polyacrylamide gels.

Radiolabeled C3, C3b, and iC3b were used as standards for the SDS-PAGE analysis. These proteins were diluted with 12.5 volumes of 1 M NH_2OH in 0.2 M $NaHCO_3$ (pH 10) and analyzed under reducing conditions in the same manner as the C3 fragments eluted from the yeast. The C3 fragment standards were treated with hydroxylamine to ensure that the hydroxylamine treatment itself had not altered the molecular form of the C3.

Statistical analysis. Data were analyzed by the Student *t* test. Results were considered significant at *P* values of <0.05 for a two-sided test.

RESULTS

An experiment was done to assess differences among life stages of *A. fumigatus* with regard to the kinetics for complement activation and the levels of C3 deposition. Resting and swollen conidia and hyphae were incubated with human serum containing radiolabeled C3, and the amount of C3 deposition was assessed at several time intervals. Figure 1 shows the results of one of three experiments. The results of the other two experiments exhibited an identical pattern (not shown). The total amount of C3 bound per particle was highest for hyphae; swollen conidia bound more C3 per cell than resting conidia (Fig. 1). Maximum binding was achieved by all three particles within 40 min. Early rates of activation and binding were slowest for resting conidia.

TABLE 1. Effect of chelating agents on activation of C3 from normal human serum and binding onto *A. fumigatus* conidia and hyphae and *C. neoformans*

Chelating agent	No. of C3 molecules (10^5)/cell[a]			
	A. fumigatus			*C. neoformans*
	Resting conidia	Swollen conidia	Hyphae	
None	5.0 ± 0.6	19 ± 6.5	79 ± 12	204 ± 48
Mg-EGTA (10 mM)	5.6 ± 0.5	17 ± 3	51 ± 0.1	266 ± 27
EDTA (10 mM)	0.05 ± 0.05	0	0.3 ± 0.02	1.2 ± 1.2

[a] Data are reported as the mean ± the standard error for two replicate experiments.

Chelation with Mg-EGTA was used to assess the importance of the classical complement pathway in activation of C3 and binding to *A. fumigatus*. Magnesium-supplemented EGTA chelates calcium, which is required for classical-pathway activation. The alternative pathway, in contrast, requires Mg^{2+} but not Ca^{2+} and is not inhibited by Mg-EGTA (7, 34). Resting conidia, swollen conidia, or hyphae of *A. fumigatus* were incubated with 40% human serum that was untreated or chelated with EDTA or Mg-EGTA. Cryptococci were used as a control for the effect of the inhibition because previous studies have shown that complement activation by encapsulated cryptococci is mediated entirely by the alternative pathway (20). Table 1 summarizes the results of two separate experiments. EDTA completely blocked complement activation by all fungal particles. Mg-EGTA had no effect on activation by cryptococcal cells or resting conidia and little effect on activation of C3 and binding to swollen conidia. In contrast, there was a 35% reduction in binding of C3 to hyphae. These results suggested that complement activation by resting conidia was mediated entirely by the alternative pathway, whereas the classical pathway is involved, at least in part, in complement activation by hyphae.

The role of the alternative pathway in C3 deposition was confirmed by incubating fungal cells in an alternative pathway reconstituted from purified factors B, D, H, and I, C3, and properdin. The fungal cells were incubated with either 40% normal serum or proteins of the alternative pathway at 40% of the concentrations found in normal serum. Encapsulated cryptococci were included as a positive control because previous studies demonstrated that a reconstituted alternative pathway can account for 100% of the binding of C3 from normal serum to the cryptococcal capsule (20). Figure 2 shows the results of one of two identical experiments. The results of the other experiment had an identical pattern (not shown). Normal serum and the reconstituted pathway produced similar maximum levels of C3 binding after 80 min of incubation; however, depending on the particle, there were marked differences between normal serum and the reconstituted pathway in the kinetics for activation and binding of C3. Incubation of resting conidia in the reconstituted pathway showed activation kinetics that were slightly retarded at the 10-min time interval. Swollen conidia showed significantly reduced binding via the reconstituted pathway at the 10-min ($P = 0.003$) and 20-min ($P = 0.003$) incubation times. Hyphae showed significantly reduced binding at the 10-min ($P = 0.003$), 20-min ($P = 0.006$), and 40-min ($P = 0.005$) time intervals. Differences between normal serum and the reconstituted pathway cannot be

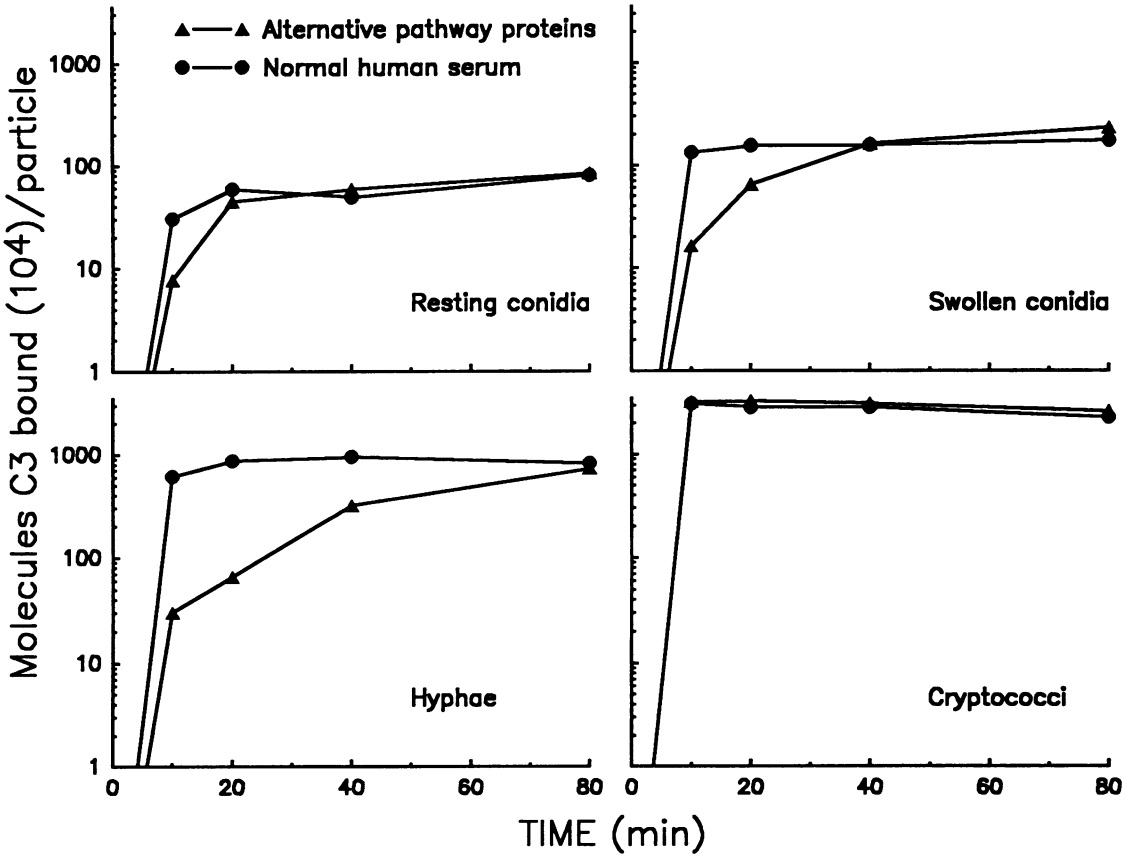

FIG. 2. Comparison of kinetics of ^{125}I-C3 deposition on resting conidia, swollen conidia, hyphae, or encapsulated cryptococci incubated with 40% normal human serum or with the alternative complement pathway reconstituted at 40% of the physiological concentrations of the six isolated proteins.

attributed to alternative-pathway proteins whose activities have been altered by the protein purification procedures, because cryptococcal cells showed identical kinetics for activation and binding of C3 in both normal human serum and the reconstituted pathway.

Previous studies demonstrated that C3 binds covalently to cellular surfaces by either ester or amide linkages (9, 22, 23). The sensitivity of ester bonds to hydrolysis with hydroxylamine was used to assess the role of ester bonds in binding of C3 to *A. fumigatus*. Fungal cells were first incubated in normal human serum containing radiolabeled C3. Cryptococcal cells were used as a control because previous studies have shown that essentially all C3 bound to the capsule is released by treatment with hydroxylamine (16, 18). The opsonized cells were washed, and C3-coated particles were treated with either NH_2OH or NaSCN. Treatment with NH_2OH released 89 to 95% of the C3 bound to the *A. fumigatus* cells (Table 2). Treatment with NaSCN showed its greatest effect on resting conidia, with a lesser effect on swollen conidia and no appreciable effect on C3 bound to hyphae or *C. neoformans*.

The molecular form of the C3 fragments attached to each of the fungal particles was determined by SDS-PAGE under reducing conditions. Fungal cells were incubated in 40% human serum, fragments were eluted by treatment with hydroxylamine, and the C3 fragments were analyzed by SDS-PAGE under reducing conditions. A mixture of C3b and iC3b was eluted from resting conidia, swollen conidia, and hyphae (Fig. 3). The M_r 102,000 fragment corresponds to the α' chain of C3b, and the M_r 58,000 fragment represents a breakdown product of the α' chain characteristic of iC3b (12).

DISCUSSION

These data establish that resting conidia, swollen conidia, and hyphae are potent activators of the complement system, with the resultant deposition of C3 fragments on their surfaces (Fig. 1). The absolute number of C3 molecules bound was greatest for hyphae and least for resting conidia. Swollen conidia are larger than resting conidia, and the hyphal fragments are relatively larger particles. Thus, if corrections are made to take into account particle size differences, the density of C3 on the surfaces of each of the

TABLE 2. Elution of C3 fragments from *A. fumigatus* and *C. neoformans* opsonized with normal human serum

Eluting agent	% of C3 removed from:			
	A. fumigatus			*C. neoformans*
	Resting conidia	Swollen conidia	Hyphae	
NH_2OH	89	95	94	100
NaSCN	30	14	0	5

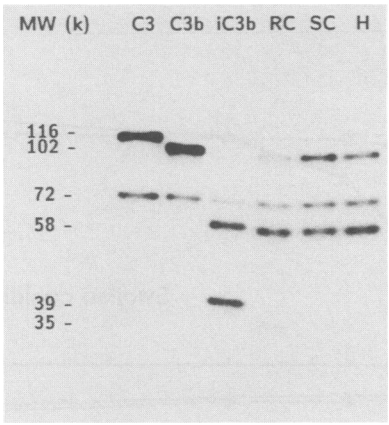

FIG. 3. Autoradiogram of SDS-PAGE analysis of ^{125}I-labeled C3 released by hydroxylamine-SDS treatment of resting conidia (RC), swollen conidia (SC), or hyphae (H) incubated with 40% normal human serum. ^{125}I-labeled C3, C3b, and iC3b were used as standards. MW (k), Molecular weight (thousands).

particles is similar. These results suggest that the failure of resting conidia to optimally stimulate the neutrophil's microbicidal apparatus (5, 27, 29) cannot be explained on the basis of diminished C3 deposition.

The three forms of *A. fumigatus* did differ with regard to the mechanism of complement activation. Chelation of serum with EGTA, a procedure that blocks the classical complement pathway (7, 34), produced a substantial inhibition of activation of C3 and binding to hyphae. This suggested that the role of the classical pathway was greater for activation by hyphae than for activation by swollen or resting conidia. This result was confirmed when the alternative pathway was reconstituted from the six purified proteins (Fig. 2). Kinetic studies demonstrated that hyphae incubated in the reconstituted pathway showed a considerable lag behind hyphae incubated in normal serum with regard to C3 deposition. Swollen conidia also showed a lag in activation kinetics, but the lag time was less than that seen with hyphae. Resting conidia showed little retardation. Thus, there was a progression toward increased dependence on the classical pathway as the fungal particles matured from resting conidia to swollen conidia to hyphae. The fact that activation by swollen conidia showed only limited inhibition by EGTA is likely explained by the kinetics curve shown in Fig. 2. Near-maximal activation and binding of C3 with the reconstituted pathway would be expected at the 30-min interval used when EGTA was an inhibitor (Table 1). Consequently, EGTA should show little inhibition of activation of C3 and binding to swollen conidia at 30 min.

It could be argued that the lag times observed in activation kinetics with the reconstituted pathway were due to possible inactivation of one of the alternative-pathway proteins. The fact that the reconstituted pathway produced activation kinetics for cryptococci that were identical to that observed with normal serum and produced near-identical kinetics for the resting conidia argue that the reconstituted pathway was fully functional.

An ester bond was the primary mechanism for binding of C3 to the fungal cells (Table 2). Sensitivity to hydrolysis by hydroxylamine is a characteristic of the ester bond that occurs between C3b and receptive surfaces (9, 22, 23). C3 fragments that were bound to resting conidia, swollen conidia, or hyphae did not differ markedly with regard to release by hydroxylamine. SDS-PAGE analysis of C3 fragments released from the particles showed that both C3b and iC3b are bound to the cells. The presence of the α' chain ($M_r \approx 102{,}000$) indicates the presence of C3b on resting conidia, swollen conidia, and hyphae. Similarly, the presence of a chain at $M_r \approx 58{,}000$ indicates that a portion of the α' chains was cleaved by the action of factors H and I (12). This latter result is consistent with the presence of iC3b. Thus, incubation of *Aspergillus* conidia and hyphae in normal serum leads to deposition of C3 fragments that can interact with phagocyte receptors CR1 and CR3 (36).

Elution of a portion of the bound C3 fragments from resting conidia with NaSCN is most likely due to a disruption of the spore surface by the chaotrope. Such a disruption might lead to a shedding of the material to which the C3 is bound. A similar, but lesser, effect was observed when opsonized swollen conidia were treated with the chaotrope. Previous studies have demonstrated that the surface of resting conidia consists of a smooth, waxy, hydrophobic layer (3, 13). This outer layer is likely shed as the resting conidia mature (3). It is attractive to speculate that C3 on the surface of resting conidia is similarly shed when growth ensues, an activity that could have clinical significance in areas of the host, such as the lungs, where complement and antibody levels are thought to be low (35). Moreover, *A. fumigatus* produces an inhibitor of alternative-pathway activation (42), which, if secreted by the growing fungus, could impede further complement deposition.

Our results show a consistent pattern in which the transition from resting conidia to swollen conidia to hyphae results in a progressive change in the manner in which the fungal particles interact with the complement system. The limited ability of resting conidia to activate complement via the classical pathway may have important implications regarding the failure of neutrophils to be effectively stimulated by and eventually kill this life stage of *A. fumigatus*. Classical-pathway activation, in general, occurs after deposition of immunoglobulins on the surface of a particle. Anti-*Aspergillus* antibodies present in normal human serum may thus be reactive primarily with swollen conidia and hyphae, and not with resting conidia. If there is little or no IgG on resting conidia, then these fungal forms will have little or no ability to stimulate phagocytes via their Fc receptors. Similarly, the presence of IgG, in addition to complement, on swollen conidia and hyphae may help account for their ability to potently stimulate neutrophils (6, 27). Future studies are needed to address these important issues.

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