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Opsonization of Encapsulated *Cryptococcus neoformans* by Specific Anticapsular Antibody

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Antisera prepared in rabbits against either whole encapsulated cells of *Cryptococcus neoformans* or purified cryptococcal polysaccharide were opsonic for the encapsulated yeast. The opsonic activity was removed by absorption with whole cryptococci and was inhibited by free polysaccharide. As little as 0.13 μg of cryptococcal polysaccharide produced a 50% inhibition of opsonization. Various degrees of neutralization by polysaccharides from the four cryptococcal serotypes suggested that the opsonins were type specific. Fractionation of antiserum on Bio-Gel A-5m (Bio-Rad Laboratories) and diethylaminoethyl cellulose showed that the opsonins were antibodies of the immunoglobulin G class. These opsonizing antibodies did not require heat-labile serum components for optimal phagocytosis of the yeast. Inhibition studies using 2-deoxy-D-glucose demonstrated that ingestion of encapsulated cryptococci opsonized with anticapsular antibody was a 2-deoxy-D-glucose-inhibitable process. This result differed from similar studies with non-encapsulated cryptococci which showed that ingestion of non-encapsulated cryptococci opsonized with normal serum was not inhibited by 2-deoxy-D-glucose.

Anticapsular antibody is produced during cryptococcal disease (4, 15, 25) and is induced by appropriate immunization procedures in experimental animals (11, 16). The role of anticapsular antibody in phagocytosis of encapsulated *Cryptococcus neoformans* is not well defined. Diamond et al. (9) observed that serum obtained from individuals who developed positive skin tests after injection of cryptococcin had no effect on rate of ingestion or killing of cryptococci by neutrophils or monocytes. In a later study (17), we found that antiserum prepared against whole cryptococci slightly enhanced phagocytosis of *C. neoformans* by macrophages.

We report in this paper (i) that cryptococcal antiserum is opsonic for phagocytosis of encapsulated cryptococci by macrophages, (ii) that the opsonic activity is due to specific anticapsular antibody, and (iii) that the opsonic antibody is immunoglobulin G (IgG) that mediates phagocytosis via a 2-deoxy-D-glucose-inhibitable process.

MATERIALS AND METHODS

Yeast strains and soluble polysaccharide. *C. neoformans* 613 is a moderately encapsulated isolate of cryptococcal serotype D. *C. neoformans* 602 is a non-encapsulated isolate. The characteristics of these strains have been described elsewhere in detail (13, 14). Cultures of *C. neoformans* serotypes A, B, C, and D were obtained from John E. Bennett, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Yeasts used in phagocytosis assays were Formalin killed (17) and used as a suspension in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) containing antibiotics (100 U of penicillin and 100 μg of streptomycin per ml; GIBCO) and buffered with sodium bicarbonate to pH 7.2.

The procedure for purification of cryptococcal polysaccharide has been described previously (14). Polysaccharide was prepared for use as a saline solution.

Phagocytosis assays. Unstimulated peritoneal macrophages were obtained from 8- to 12-week-old Swiss mice (Microbiological Associates, Walkersville, Md.). The procedure for collection and culture of macrophages has been described previously (17). Monolayers were prepared in four-chamber tissue culture chamber/slides (Lab-Tek Products, Div. Miles Laboratories Inc., Westmont, Ill.; model 4804) and incubated for 24 to 48 h at 37°C in 2.6% CO_2 before use. Each monolayer contained approximately 2.5×10^5 macrophages.

For phagocytosis assays, the culture medium was decanted, and each monolayer was washed two times with warm (37°C) Hanks balanced salt solution. One milliliter of a test yeast suspension was added to each chamber. The yeast suspensions consisted of (i) 10^6 yeast cells; (ii) unless otherwise noted, 10% normal bovine serum; (iii) a dilution of cryptococcal antiserum in Hanks balanced salt solution; (iv) when required by an experimental protocol, cryptococcal polysaccharide in saline; and (v) enough Hanks balanced salt solution to give a final volume of 1 ml. All encapsulated cryptococci were washed extensively with saline before use to ensure an absence of free polysaccharide. Normal human serum was substituted for bovine serum when cryptococci were opsonized with antiserum prepared against a methylated bovine serum albumin conjugate of cryptococcal polysaccharide.

Phagocytosis of yeast cells was determined after incubation of yeast cells with macrophages for 1 h at

37°C. After incubation, the slides were washed, fixed, and stained as previously described (17). Slides were examined microscopically, and 200 macrophages per monolayer were observed for ingested yeasts. Previous studies (17) have shown that the large size of the yeast permits a clear microscopic distinction in stained preparations between attached and ingested yeasts. These results have been confirmed by scanning electron microscopy and by interference-contrast microscopy. Results are presented as mean values from at least four monolayers and are reported as the percentages of macrophages with ingested yeasts (percent phagocytosis). Analysis of variance was used for statistical analysis of data. Probit analysis was used to determine 50% endpoints.

Serum, antiserum, and serological assays. Bovine serum (GIBCO, lot no. A790120) or human serum drawn from normal healthy donors was incorporated into media used for phagocytosis assays. Cryptococcal antiserum was prepared in rabbits against whole cells of *C. neoformans* 613 (15). Anti-whole cell serum had a passive hemagglutination titer of 1:512 and an indirect fluorescence antibody titer of 1:320. Antiserum was prepared in rabbits against purified cryptococcal polysaccharide by use of a methylated bovine serum albumin conjugate of the polysaccharide. Bovine serum albumin was methylated by the procedure of Kozel and Cazin (16). A saline solution containing 2 mg of cryptococcal polysaccharide was mixed with a saline solution containing 2 mg of methylated bovine serum albumin. The precipitate formed by this mixture was collected by centrifugation, suspended in saline, and emulsified with an equal volume of Freund complete adjuvant. The immunization schedule was a variation of the protocol used by Apicella (1) for preparation of meningococcal antiserum. Briefly, rabbits were given three weekly subcutaneous injections of an antigen consisting of 250 μg of polysaccharide conjugated to an equal amount of protein and emulsified in adjuvant. The animals were given a single intravenous injection of 50 μg of polysaccharide in saline 3 weeks after the last subcutaneous injection. One week after the intravenous injection, all rabbits were bled, and the serum was collected. IgG was isolated from immune rabbit serum by diethylaminoethyl cellulose chromatography (26).

Passive hemagglutination titers were determined by previously described techniques (15). Indirect fluorescence antibody titers were assessed by the techniques of Goren and Warren (12). A fluorescein-conjugated IgG fraction of goat anti-rabbit immunoglobulins (IgA plus IgG plus IgM; Cappel Laboratories, Downingtown, Pa.) was used for the indirect fluorescence antibody assays. Heavy chain-specific antiserum to rabbit IgG was obtained from Michael Apicella, University of Nevada, Reno.

Gel filtration. Cryptococcal antiserum (1.5 ml) was fractionated on a column (1.7 by 90 cm) of Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, Calif.). The buffer was 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0. The column was run at a 4-ml/h flow rate, and fractions were collected every 30 min. Fractions were assayed by Ouchterlony diffusion for IgM and IgG. Protein concentrations were determined by absorbancy at 280 nm.

Reagents. 2-Deoxy-D-glucose was obtained from Sigma Chemical Co. (St. Louis, Mo.). Minimal essential medium with Earle base was obtained from K. C. Biological, Inc., Lenexa, Kans., and was buffered to pH 7.2 with sodium bicarbonate before use.

RESULTS

Opsonization by cryptococcal antiserum. Antiserum prepared in rabbits against whole encapsulated *C. neoformans* cells was opsonic for the homologous yeast. Cells of strain 613 were incubated for 30 min at 37°C with 10% bovine serum and various amounts of antiserum. The opsonized yeasts were then added to monolayers of macrophages. The results (Fig. 1) showed a dose-dependent enhancement of phagocytosis in the presence of cryptococcal antiserum. Normal nonimmune serum was not opsonic for the encapsulated yeast.

Antiserum used in the previous experiment had been prepared against whole cryptococci; therefore, it was possible that the opsonic antibody might be directed against some antigen other than the capsule. Accordingly, we examined the opsonic activity of antiserum prepared against a methylated bovine serum albumin conjugate of the capsular polysaccharide. Cells of strain 613 were incubated for 30 min at 37°C with 10% human serum and various amounts of antiserum. The opsonized yeasts were then added to monolayers of macrophages. The results (Fig. 2) showed that the antiserum was markedly opsonic for cells of strain 613. Antiserum prepared against whole cryptococci was chosen for further study because the whole cell most closely resembles the antigen encountered in cryptococcal disease.

Specificity of opsonic antibody. The opsonic activity of cryptococcal antiserum could be removed by absorption with whole cryptococci. One milliliter of cryptococcal antiserum was absorbed for 30 min at 0°C with 0.25 ml of packed cryptococcal cells. The absorbed antiserum was then examined for opsonic activity. The possibility existed that a solid-phase absorption might

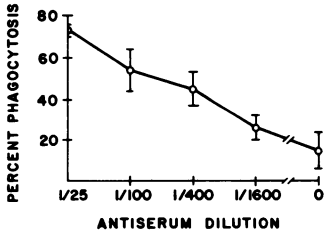

FIG. 1. *Opsonization of encapsulated cryptococci by antiserum prepared against whole encapsulated cryptococci. Data are given as the mean ± standard deviation.*

not occur and that free polysaccharide might be washed from the cells to inhibit any phagocytosis that might take place. Such a phenomenon has been described for non-encapsulated cryptococci (13). As a consequence, we included a control in which 1 ml of saline was absorbed with 0.25 ml of packed cryptococcal cells. The absorbed saline was then diluted in a manner identical to that used for the absorbed serum, and a similar dilution of unabsorbed antiserum was added. Cells of strain 613 were then added and incubated for 30 min at 37°C with 10% bovine serum. All opsonized yeasts were then added to monolayers of macrophages. The results (Table 1) showed that absorption of antiserum with whole cryptococci removed all opsonic activity from the antiserum. This decreased phagocytosis could not be attributed to inhibition by free polysaccharide because saline absorbed with cryptococci had no effect on opsonization by cryptococcal antiserum.

An additional experiment was done to determine whether the opsonic activity was inhibitable by free polysaccharide. Cryptococcal antiserum (1:25 dilution) was preincubated with various amounts of purified cryptococcal polysaccharide for 30 min at room temperature. Cryptococcal cells and bovine serum were added, and the opsonized cryptococci were added to monolayers of macrophages. The results (Fig. 3) showed a 50% inhibition of opsonization by 0.13 μg of homologous polysaccharide. These data support previous results (Fig. 2) demonstrating that opsonic activity was due to anticapsular antibody.

Anticapsular antibody exhibited type specificity with regard to ease of neutralization by free polysaccharide. Cryptococcal antiserum (1:25 dilution) was preincubated for 30 min at room temperature with various amounts of polysaccharide isolated from the four cryptococcal serotypes. Cells of strain 613 and bovine serum

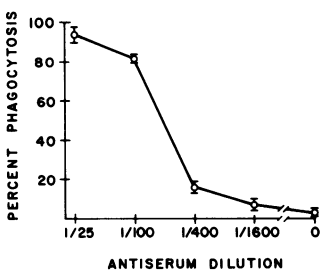

FIG. 2. *Opsonization of encapsulated cryptococci by antiserum prepared against a methylated bovine serum albumin conjugate of cryptococcal polysaccharide. Data are given as the mean ± standard deviation.*

TABLE 1. *Absorption of opsonic activity by whole encapsulated cryptococci*

Antiserum treatment	% Phagocytosis[a]
Unabsorbed antiserum (1:25)	78 ± 4
Strain 613 absorbed antiserum (1:25)	4 ± 1
Strain 613 absorbed saline (1:25) plus unabsorbed antiserum (1:25)	74 ± 13

[a] Cryptococcal cells were opsonized with treated antiserum and 10% normal bovine serum. Results are expressed as the mean ± standard deviation.

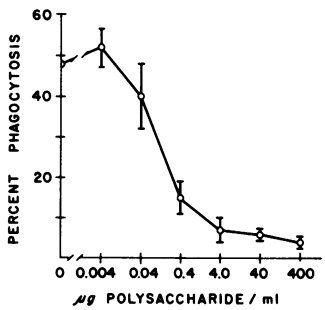

FIG. 3. *Inhibition of the opsonic activity of a 1:25 dilution of cryptococcal antiserum by homologous cryptococcal polysaccharide. Data are given as the mean ± standard deviation.*

were added, and the opsonized cryptococci were added to monolayers of macrophages. The results (Fig. 4) showed that the homologous polysaccharide was most effective at neutralization, followed in order of decreasing effectiveness by polysaccharides of serotypes D, A, B, and C.

Immunoglobulin class of opsonic antibody. Cryptococcal antiserum was fractionated on Bio-Gel A-5m to determine the immunoglobulin class of the opsonic antibody. Fractions were assayed (i) for IgM by immunodiffusion against specific anti-rabbit heavy mu chain antiserum, (ii) for IgG by immunodiffusion against specific anti-rabbit heavy gamma chain antiserum, (iii) by passive hemagglutination for anticapsular antibody, (iv) by indirect immunofluorescence for anti-cryptococcal antibody, and (v) for opsonic activity for encapsulated cryptococci. The results (Fig. 5) showed (i) that all antibody detectable by passive hemagglutination was of the IgM class, (ii) that all antibody observed by indirect immunofluorescence was of the IgG class, and (iii) that all opsonic activity was found in fractions of antiserum containing IgG. This result was confirmed when IgG isolated from cryptococcal antiserum by diethylaminoethyl cellulose chromatography was found to contain high levels of opsonic activity (data not shown).

Characteristics of IgG-mediated opsonization. An experiment was done to determine

the need for heat-labile opsonins in phagocytosis of opsonized cryptococci. Encapsulated cryptococci were preincubated for 30 min at 37°C with cryptococcal antiserum at a final concentration of 1:25 and either (i) 10% normal bovine serum or (ii) 10% heat-inactivated bovine serum. Phagocytosis experiments (Table 2) showed similar levels of phagocytosis in the presence of either normal or heat-inactivated serum. Thus, heat-labile opsonins were not needed to optimally opsonize encapsulated *C. neoformans* for phagocytosis by unstimulated resident macrophages.

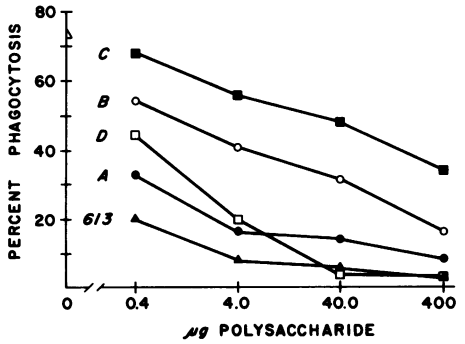

FIG. 4. *Inhibition of the opsonic activity of a 1:25 dilution of cryptococcal antiserum by homologous polysaccharide (▲), type A polysaccharide (●), type B polysaccharide (○), type C polysaccharide (■), or type D polysaccharide (□).*

Previous studies in our laboratory showed that IgG found in normal serum promotes phagocytosis of non-encapsulated cryptococci by a 2-deoxy-D-glucose-resistant process (18). Similar experiments in the present study with encapsulated strain 613 showed that anti-capsular IgG promotes phagocytosis of the encapsulated yeast via a 2-deoxy-D-glucose-inhibitable process. Macrophage monolayers were preincubated for 2 h at 37°C with minimal essential medium or minimal essential medium containing 50 mM 2-deoxy-D-glucose. Cells of strain 602 were preincubated for 30 min at 37°C with 10% normal bovine serum in the presence or absence of 50 mM 2-deoxy-D-glucose. Cells of strain 613 were preincubated with cryptococcal antiserum at a final concentration of 1:25 and 10% normal bovine serum in the presence or absence of 50 mM 2-deoxy-D-glucose. The opsonized yeast cells were added to the macrophages and incubated for 1 h at 37°C. The results (Table 3) showed that 2-deoxy-D-glucose significantly ($P < 0.01$) reduced ingestion of opsonized encapsulated cryptococci (strain 613), but had no effect on ingestion of opsonized non-encapsulated cryptococci (strain 602). Thus, in contrast to phagocytosis of non-encapsulated cryptococci, anticapsular antibody mediates ingestion of encap-

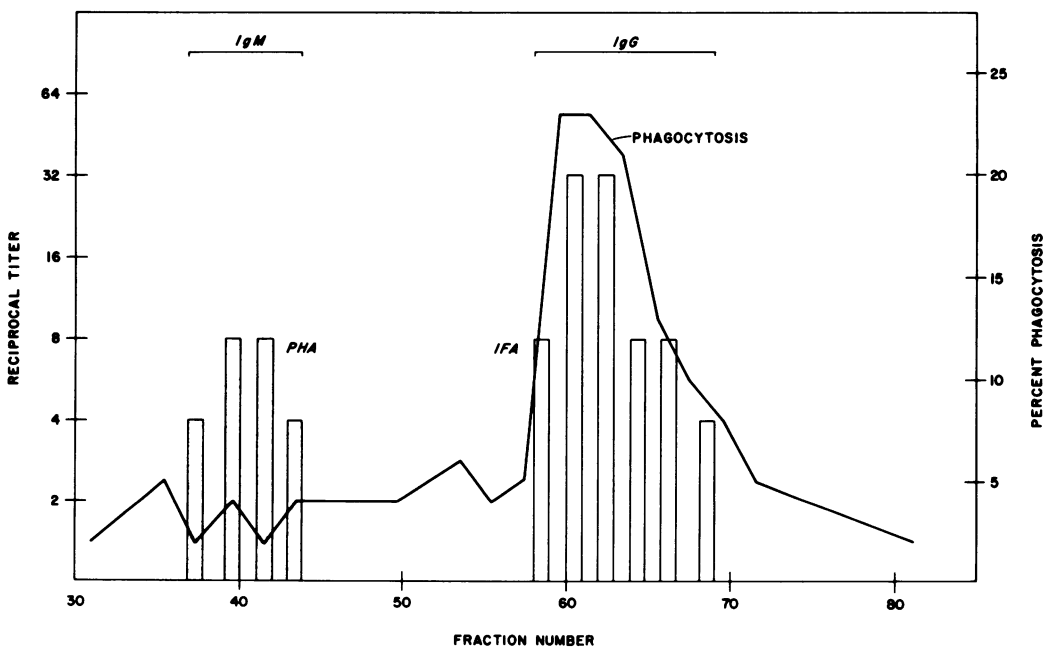

FIG. 5. *Fractionation of rabbit anti-cryptococcal serum on Bio-Gel A-5m. Fractions were assayed (i) for opsonic activity for encapsulated cryptococci, (ii) for IgG and IgM by immunodiffusion, (iii) for passive hemagglutination activity (shaded bars), and (iv) for indirect immunofluorescence activity (open bars).*

TABLE 2. *Opsonization of encapsulated cryptococci by homologous antiserum in the presence of normal or heat-inactivated bovine serum*

Serum	Antiserum[a]	% Phagocytosis[b]
Normal bovine serum	Present	67 ± 5
Heat-inactivated bovine serum[c]	Present	73 ± 7
Normal bovine serum	Absent	14 ± 1

[a] Cryptococcal antiserum was 1:25 dilution.
[b] Mean ± standard deviation.
[c] Bovine serum was inactivated at 56°C for 30 min.

TABLE 3. *Effect of 2-deoxy-D-glucose on phagocytosis of strain 602 opsonized with normal serum or strain 613 opsonized with immune serum*

Yeast strain	% Phagocytosis[a]	
	Untreated	2-deoxy-D-glucose treated
602[b]	92 ± 2	93 ± 2
613[c]	84 ± 7	41 ± 3

[a] Mean ± standard deviation.
[b] Non-encapsulated strain 602 was opsonized with 10% normal bovine serum.
[c] Encapsulated strain 613 was opsonized with cryptococcal antiserum (1:25) and 10% normal bovine serum.

sulated cryptococci via a 2-deoxy-D-glucose-inhibitable process.

DISCUSSION

Encapsulated cryptococci differ from other pathogenic fungi because they have an antiphagocytic capsule at the cell surface (13, 20). Several studies have shown that encapsulated isolates are not opsonized by normal nonimmune serum (9, 20), but the opsonizing activity of anticapsular antibody has received little attention. Schneerson-Porat et al. (23) found that in vivo formation of histiocyte rings around the yeast is dependent upon the presence of immune serum. Diamond et al. (9) examined the opsonic activity of serum from individuals who formed antibody to *C. neoformans* in response to skin testing. The immune serum was positive in a bentonite flocculation assay but negative by the indirect fluorescence antibody assay (R. Diamond, personal communication). These authors noted that immune serum did not significantly increase ingestion or killing by neutrophils or monocytes. In a previous study (17) we found that cryptococcal antiserum was only marginally opsonic for encapsulated cryptococci. There are at least two possible reasons for our previous failure to demonstrate the levels of opsonization by anticapsular antibody reported in the present study. First, the antiserum used in our previous study was rich in anticapsular IgM and relatively poor in immune IgG (unpublished observations). Thus, the antiserum probably lacked the appropriate opsonic immunoglobulins. Second, no effort was made in the previous study to extensively wash the encapsulated yeasts before use. It is possible that free polysaccharide may have blocked any potential opsonic activity.

In the present study, we found that antiserum prepared against whole encapsulated cryptococci and antiserum prepared against a methylated bovine serum albumin conjugate of cryptococcal polysaccharide were opsonic for the encapsulated yeast. This opsonic antibody was blocked by purified cryptococcal polysaccharide. Further, various degrees of neutralization by polysaccharides from the four cryptococcal serotypes showed that the opsonins were type specific. Thus, all available evidence indicates that opsonization of encapsulated cryptococci was due to type-specific antibody directed against the capsular polysaccharide.

Cryptococcal opsonins were antibodies of the IgG class that did not require heat-labile serum components for optimal phagocytosis of the yeast by resident peritoneal macrophages. It could be argued that the experimental conditions did not permit adequate activation of heat-labile opsonins at the cell surface; however, the experimental conditions were identical to conditions used previously to demonstrate that thioglycolate-elicited macrophages utilize heat-labile opsonins for optimal phagocytosis of non-encapsulated cryptococci (24). The possibility should not be excluded that heat-labile opsonins might play a significant role in phagocytosis of the encapsulated yeast by phagocytes that have a greater dependence on the C3b receptor such as neutrophils (8) or thioglycolate-elicited macrophages (24).

Inhibition studies with 2-deoxy-D-glucose suggest that the function of anticapsular IgG in phagocytosis of encapsulated cryptococci differs markedly from the role of IgG from unimmunized individuals in phagocytosis of non-encapsulated *C. neoformans*. Ingestion of encapsulated cells opsonized with anticapsular serum was blocked by 2-deoxy-D-glucose, whereas ingestion of non-encapsulated cells opsonized with non-immune serum was not blocked by 2-deoxy-D-glucose. Previous studies have shown that 2-deoxy-D-glucose has little effect on Fc-mediated attachment, but inhibits Fc-mediated ingestion (18, 19). Therefore, it is likely that the IgG in normal serum that opsonizes non-encapsulated cryptococci and anticapsular antibody differ in their respective roles in attachment and ingestion of the yeast.

Opsonic cryptococcal antiserum was extremely sensitive to neutralization by free polysaccharide. Approximately 0.13 μg of polysaccharide produced a 50% inhibition of the opsonic activity (Fig. 3). This amount of polysaccharide is at the low end of the range of results reported for neutralization of antibody opsonic for encapsulated bacteria. Meningococcal polysaccharide concentrations of 10 μg/ml were required to block opsonic meningococcal antibody (22), and opsonic antibody to group B streptococci was blocked by 0.4 to 4.0 μg of group B polysaccharide (2).

Our results illustrate one mechanism by which cryptococcal antibody can enhance host resistance. A second antibody-dependent resistance mechanism was reported by Diamond and Allison (6), who found that nonphagocytic cell-mediated killing of *C. neoformans* was dependent on the presence of cryptococcal antibody. As with our report of opsonic antibody, nonphagocytic cell-mediated killing of *C. neoformans* was blocked by cryptococcal polysaccharide (5). The level of polysaccharide needed for 50% inhibition was approximately 0.47 μg of polysaccharide per ml (R. Diamond, personal communication). Given these potential killing mechanisms, a significant role for anticapsular antibody in resolution of cryptococcal disease would be expected; however, there is little experimental evidence for protective action by antibody. Monga et al. (21) found that the virulence of *C. neoformans* was identical for normal and B-cell-deficient mice. Goren (10) reported that immunization with cryptococcal polysaccharide not only fails to protect but may actually predispose to experimental cryptococcosis. Clinical studies have shown that the presence of cryptococcal antibody in the serum of patients with cryptococcosis was correlated with eventual cure (4, 7); however, there is no evidence for a causal relation between the presence of antibody and recovery from cryptococcosis. The extreme sensitivity of opsonic antibody to neutralization by free polysaccharide may account, at least in part, for the observed failure of antibody to enhance resistance. Serum levels of polysaccharide in both human and experimental cryptococcosis (3, 4) may exceed the levels of polysaccharide needed to inhibit opsonization (Fig. 3) or the levels needed to inhibit nonphagocytic cell-mediated killing. Thus, antibody-dependent killing mechanisms may be blocked by the levels of polysaccharide encountered in active cryptococcal disease.

ACKNOWLEDGMENT

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