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Production and Characterization of Monoclonal Antibodies Specific for *Cryptococcus neoformans* Capsular Polysaccharide

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***Cryptococcus neoformans* is surrounded by a capsular polysaccharide. There are at least four known serotypes of the polysaccharide. The objective of this study was to produce monoclonal antibodies (MAbs) that could be used to study the distribution of epitopes among the serotypes of *C. neoformans*. BALB/c mice were immunized with cryptococcal polysaccharides of serotype A or D that were coupled to sheep erythrocytes. Splenocytes were isolated, and hybridomas secreting MAbs specific for cryptococcal polysaccharides were isolated. Two hybridomas, designated MAbs 439 and 1255, were produced from mice immunized with serotype A polysaccharide. One hybridoma, designated MAb 302, was produced from mice immunized with serotype D polysaccharide. All three antibodies were of the immunoglobulin G1 isotype. MAb 302 showed a specificity for serotypes A and D in Ouchterlony diffusion, agglutination, and opsonophagocytosis assays. MAb 1255 was reactive with polysaccharides and cells of serotypes A, B, and D. MAb 439 was reactive with polysaccharides and cells of serotypes A, B, C, and D. The reactivity of these MAbs closely matched the distribution of epitopes among cryptococcal polysaccharides predicted in previous studies of polyclonal antibodies reactive with cryptococcal polysaccharides. The ability to produce a MAb against an epitope shared by all four serotypes may have value for the detection of cryptococcal antigens in body fluids.**

Cryptococcus neoformans is a pathogenic yeast that is surrounded by a polysaccharide capsule. The capsular polysaccharide has a linear α-(1→3)-linked mannose backbone that is substituted with nonreducing D-xylosyl and D-glucuronosyl acid groups (2–5, 8, 14). The mannose backbone is partially O acetylated (1).

Five serotypes have been reported for cryptococcal polysaccharide, serotypes A, B, C, and D and a newly described A-D serotype that is reactive with antisera specific for both serotypes A and D (15, 30). Serotype is assessed by use of polyclonal antibodies produced by immunization of rabbits with whole cells of the yeast. The antiserum is cross-adsorbed with whole yeast cells to produce a polyclonal antiserum specific for a single serotype. A detailed analysis of cross-adsorption of polyclonal antisera by the different cryptococcal serotypes suggested that eight distinct epitopes are found among the five serotypes (15). Some epitopes are unique to a single serotype, whereas other epitopes are shared by two or more serotypes. One epitope is found on all serotypes. Thus, the serotype can be defined by the presence or absence of one or more of the eight epitopes.

The present study was undertaken to produce monoclonal antibodies (MAbs) that would be suitable as probes for the distribution of epitopes among the various cryptococcal serotypes. We report the properties of three distinct MAbs that are reactive with cells of *C. neoformans*. These antibodies are characterized with regard to (i) reactivity by Ouchterlony diffusion against purified cryptococcal polysaccharides, (ii) serotype specificity in agglutination assays, and (iii) opsonic activity for phagocytosis by macrophages. The results showed a general pattern of agreement with the epitope distribution predicted by cross-adsorption of polyclonal antiserum (15).

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MATERIALS AND METHODS

Yeast cells and cryptococcal polysaccharide. *C. neoformans* isolates of serotypes A (ATCC 24064), B (ATCC 24065), C (ATCC 24066), and D (ATCC 24067) were obtained from the American Type Culture Collection (Rockville, Md.). All yeast cells were grown in a yeast extract dialysate medium (13) and killed with Formalin before use (21). Yeast cells used in agglutination assays were suspended in phosphate-buffered saline (PBS) at a concentration of 10^7 cells per ml. Yeast cells used in phagocytosis assays were suspended at a concentration of 10^6 cells per ml in Hanks balanced salt solution buffered with sodium bicarbonate to pH 7.2. Cryptococcal polysaccharides of all four serotypes were isolated from culture filtrates of the yeast grown in yeast extract dialysate medium. The procedures for isolation and purification of cryptococcal polysaccharides have been previously described (9, 16).

Several structural derivatives were prepared from serotype A and D polysaccharides. The polysaccharides were carboxyl reduced by a modification (20) of the procedure described by Taylor and Conrad (28). The carboxyl reduction procedure was repeated for each polysaccharide to ensure complete reduction of carboxyl groups. The polysaccharide was de-O-acetylated by treatment with 0.1 M NaOH for 1 h at room temperature (19). A polyalcohol was produced from the polysaccharide by oxidation of the polysaccharide with periodate followed by reduction with sodium borohydride. Reduction of the periodate-oxidized polysaccharide with sodium borohydride was done under pH control in which the pH was maintained below 8.0 by the addition of 4 N HCl with a pH-stat. pH control was essential to prevent alkaline hydrolysis of *O*-acetyl groups. Periodate oxidation cleaves the xylosyl and glucuronosyl side chains between vicinal hydroxyl groups while leaving the mannose backbone intact (2–5). The procedure for preparation of the poly-

alcohol has been described previously in detail (11, 20). Smith degradation was completed by hydrolysis of the polyalcohol with 0.1 N HCl. This procedure removes all side chains from the mannose backbone of cryptococcal polysaccharide (2–5). The procedure for preparation of the Smith product has already been described (11, 20).

Production of MAbs. Two separate experiments were done in which mice were immunized and spleen cells were fused with Sp2/0-Ag14 mouse myeloma cells. One group of 6- to 10-week-old BALB/c mice (Simonsen Laboratories, Gilroy, Calif.) was immunized with serotype D polysaccharide coupled to sheep erythrocytes. The procedure for coupling cryptococcal polysaccharide to sheep erythrocytes has already been described (17). Mice were primed 22 days before fusion by intravenous injection of 0.2 ml of a 0.1% suspension of sheep erythrocytes in saline. The mice were then given intravenous immunizations with 0.2 ml of a 1% suspension of sheep erythrocytes coupled to serotype D polysaccharide. Injections with the polysaccharide-coated erythrocytes were done 17 and 3 days before collection of splenocytes for fusion.

A second group of BALB/c mice were immunized with serotype A polysaccharide coupled to sheep erythrocytes. Mice were primed by intravenous injection of 0.2 ml of a 0.1% suspension of sheep erythrocytes 124 days before fusion. The mice received subsequent intravenous injections of 0.2 ml of 1% sheep erythrocytes in saline coupled to a serotype A polysaccharide 111, 48, 10, 3, and 2 days before collection of splenocytes for fusion. Serum obtained from mice immunized in this manner typically have enzyme-linked immunosorbent assay (ELISA) titers of at least 1/20,000.

Spleen cells (2.88×10^8) obtained from immune mice were fused with Sp2/0-Ag14 (ATCC CRL 1581) mouse myeloma cells at a 1:1 ratio of lymphocytes to myeloma cells with 2.5 ml of 50% polyethylene glycol (PEG 1450; Eastman Kodak Co., Rochester, N.Y.) buffered in PBS. The fusion procedure has already been described (22).

Peritoneal cells isolated from BALB/c mice were used as feeder cells. These feeder cells (10^4) were distributed in 100 μl of hypoxanthine-aminopterin-thymidine medium (24) into each well of 96-well microtiter plates. Feeder cells were incubated at 37°C under 6% CO_2 for 24 h before use. Cells from the fusion (1.16×10^5 in 100 μl of hypoxanthine-aminopterin-thymidine medium) were distributed into each well. Hybridoma supernatant fluids were tested for specific cryptococcal antibody by ELISA on day 11 after fusion. Hybridoma colonies secreting anticryptococcal antibody were cloned three times by limiting dilution.

Ascites tumors of each cell line were produced in female BALB/c mice primed with pristane as previously described (7). Immunoglobulin G (IgG) antibody was isolated from the ascites fluid by caprylic acid precipitation (27).

Isotype was determined by incubation of purified antibody (200 μl of a 1:1,000 dilution) with polysaccharide-coated polystyrene plates (see below). The plates were washed and incubated with rabbit antibody specific for murine IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM (Hyclone Murine Isotyping Kit; Hyclone Laboratories, Inc., Logan, Utah) for 90 min at 37°C. The plates were washed and incubated with affinity-purified, peroxidase-labeled goat antibody specific for rabbit immunoglobulins. The plates were incubated with substrate in the manner described below for the ELISA.

Immunochemical assays. An ELISA was used to select for hybridomas secreting antibody specific for cryptococcal polysaccharide. The ELISA was a variation of the assay reported by Leinonen and Frasch for detection of antibody to meningococcal polysaccharide (23). Polystyrene plates were precoated with 200 μl of poly-L-lysine (150,000 molecular weight; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 5 μg/ml in 0.05 M phosphate buffer, pH 7.0, for 4 h at room temperature. The plates were washed with 0.05 M phosphate buffer, pH 7.0, and cryptococcal polysaccharide (200 μl) at a concentration of 4 μg/ml in 0.05 M phosphate buffer, pH 7.0, was added to each well. The plates were incubated overnight at room temperature. Plates coated with serotype D polysaccharide were used to screen hybridomas prepared from mice immunized with serotype D. Plates coated with serotype A polysaccharide were used to screen hybridomas prepared from mice immunized with serotype A. The polysaccharide-coated plates were washed once with 0.05 M phosphate buffer, pH 7.0, and then blocked by incubation with 1.0% gelatin in 0.05 M phosphate buffer, pH 7.0, for 2 h at 37°C. The plates were washed three times in a washing buffer consisting of 0.05% Tween 20 and 0.5% gelatin in PBS (29). The supernatant fluids from hybridoma colonies were screened by adding 50 μl of supernatant and 150 μl of washing buffer to the polysaccharide-coated plates for 90 min at 37°C. The ELISA plates were washed three times with washing buffer and incubated for 90 min with a peroxidase-labeled affinity-purified second antibody specific for mouse IgG heavy chains. After incubation with the second antibody, the plates were washed three times with washing buffer and incubated for 30 min with the substrate *o*-phenylenediamine as previously described (29). The reaction was stopped by addition of 50 μl of 4 N H_2SO_4, and optical densities at 492 nm were determined.

Double diffusion in agar was used to asess the reactivity of MAbs isolated from ascites fluid (2 mg of IgG per ml). Purified cryptococcal polysaccharides at a concentration of 2 mg/ml were used as the soluble antigens. Immunodiffusion was done in plates prepared from 1% agarose in PBS. Reactivity patterns were determined after 16 h.

Formalin-killed yeast cells of each serotype were used as the antigens in agglutination assays. Yeast cells (4×10^6 cells in 0.4 ml of PBS) were incubated with an equal volume of dilutions of IgG isolated from ascites fluid. All antibody preparations were adjusted to 320 μg of IgG per ml before dilution. Data are reported as the reciprocal geometric mean of three separate assays.

Phagocytosis assays. Monolayers of peritoneal macrophages were prepared from Swiss Webster mice as previously described (21), and phagocytosis was assessed by a modification of previously described procedures (21). Briefly, cryptococcal yeast cells (5×10^6) were incubated with 1 ml of various dilutions of the MAbs for 1 h at 37°C. The opsonized yeast cells were collected by centrifugation and suspended in 5 ml of Hanks balanced salt solution. One milliliter of opsonized yeast cells (10^6 cells per ml) was incubated with macrophage monolayers for 1 h at 37°C under 6% CO_2. The monolayers were washed, fixed, and stained with Giemsa stain. The slides were examined microscopically, and the numbers of ingested yeasts per macrophage were determined. Data are reported as the mean number of ingested yeast cells per 100 macrophages (phagocytic index).

RESULTS

Data are reported from two hybridomas produced by immunization with serotype A polysaccharide and one hybridoma produced by immunization with serotype D polysaccharide (Table 1). IgG isolated from ascites fluid was used

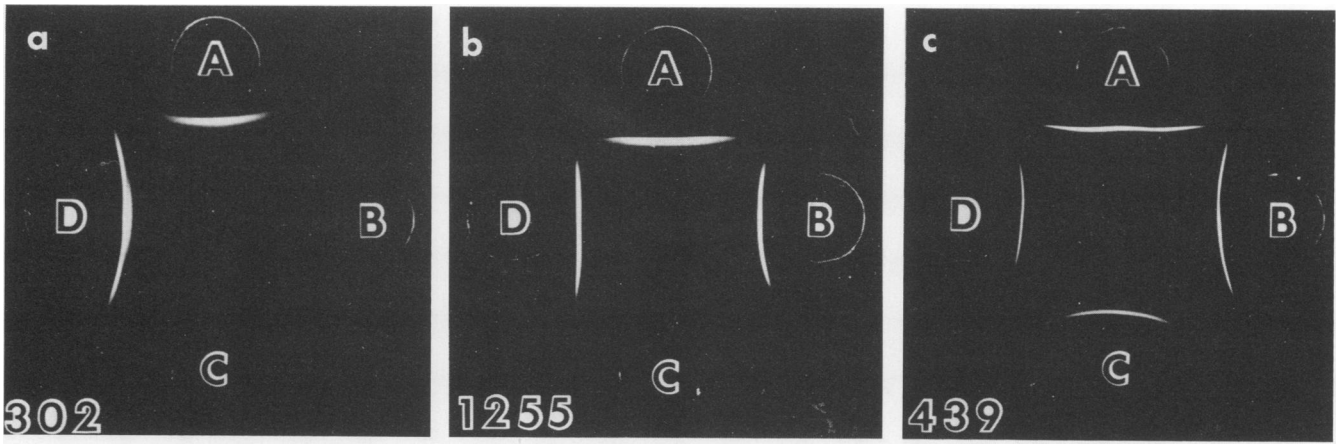

FIG. 1. Precipitation of cryptococcal polysaccharides by MAbs. MAbs were placed in the center wells as follows (panels): a, MAb 302; b, MAb 1255; c, MAb 439. Purified polysaccharides of serotypes A, B, C, and D were placed in the outer wells as indicated.

as the source of MAb in the studies reported here. Each of the antibodies was of the IgG1 isotype.

Ascites fluid from each cell line was assayed by immunodiffusion for reactivity with purified polysaccharides of the four serotypes. The results (Fig. 1) showed that MAb 302 was reactive with polysaccharides of serotypes A and D. MAb 1255 was reactive with polysaccharides of serotypes A, B, and D. MAb 439 was reactive with polysaccharides of serotypes A, B, C, and D.

An experiment was done to determine the effect of structural modification of cryptococcal polysaccharides on the reactivity of polysaccharides with each MAb. We examined the reactivity of the MAbs with polysaccharides of serotype A or D that were modified by (i) de-O-acetylation, (ii) reduction of carboxyl groups, (iii) oxidation with periodate followed by reduction to polyalcohols, and (iv) Smith degradation. The results are shown in Fig. 2. MAbs 439 and 1255 were reactive only with the de-O-acetylated derivative of the parent polysaccharides used for immunization. In contrast, MAb 302 was only reactive with the polyalcohol of serotype D. None of the antibodies were reactive with either the carboxyl-reduced polysaccharides or the Smith degradation products.

Agglutination assays were used to assess the reactivity of each MAb with whole yeast cells of serotypes A, B, C, and D. The results (Table 1) showed reactivity patterns for each MAb that largely paralleled the results of immunodiffusion assays. That is, MAb 302 agglutinated cells of serotypes A and D; MAb 1255 agglutinated cells of serotypes A, B, and D; and MAb 439 agglutinated cells of serotypes A, B, C, and D.

Phagocytosis assays were done with mouse peritoneal macrophages to assess the opsonic activity of the MAbs for cells of each serotype. Controls with nonopsonized yeast cells showed no phagocytosis (data not shown). Yeast cells of each serotype were preincubated with serial fourfold dilutions of each MAb. All MAbs were adjusted to 4 mg of IgG per ml before dilution. Results are shown only for MAb 1255. Once again, the specificity of the opsonic activity of each MAb was in good agreement with the serotype specificity seen in precipitation (Fig. 1) and agglutination (Table 1) assays. MAb 302 was highly opsonic for cells of serotypes A and D (data not shown). MAb 1255 was opsonic for cells of serotypes A, B, and D, but not those of serotype C (Fig. 3). MAb 439 was opsonic for cells of all four serotypes (data not shown).

TABLE 1. Agglutination titers of MAbs

MAb (serotype used for immunization)	Reciprocal of geometric mean agglutination titer with serotype[a]:			
	A	B	C	D
302 (D)	100	<12.5	<12.5	400
1255 (A)	635	200	<12.5	1,270
439 (A)	8,063	8,063	504	1,600

[a] The data are from three replicate experiments.

DISCUSSION

The objective of this study was to generate MAbs reactive with cryptococcal polysaccharides that could be used to study the distribution of epitopes among the serotypes of *C. neoformans*. The weak immunogenicity of cryptococcal polysaccharide in mice presented a particular problem for the study. Cryptococcal polysaccharide is poorly immunogenic (18), readily induces immunological unresponsiveness (25), and has been described as a T-independent antigen (6). We sought to produce MAbs of the IgG class because IgG antibodies have far greater utility than IgM antibodies. As a consequence, we coupled the polysaccharide to sheep erythrocytes in an attempt to improve the immunogenicity of the polysaccharide. We also used a labeled second antibody that was specific for IgG heavy chains to screen for antibody-producing clones. The small number of positive colonies identified is undoubtedly due to the poor immunogenicity of cryptococcal polysaccharide, and it emphasizes the need to screen large numbers of colonies.

Previous studies of the distribution of epitopes among the serotypes of cryptococcal polysaccharides used polyclonal antisera produced by immunization of rabbits with whole cryptococcal cells. Cross-adsorption of the antisera with cells of the four serotypes demonstrated the presence of at least eight distinct epitopes (15). Some of these epitopes were unique to a single serotype; others were found on two or more serotypes. One objective of our study was to determine whether MAbs could be produced with an epitope specificity similar to the specificity observed with adsorbed polyclonal antisera. We produced three distinct MAbs. In each case, the specificity of the MAbs matched an epitope specificity that would have been predicted by previous studies with polyclonal antibodies. MAb 439 was reactive

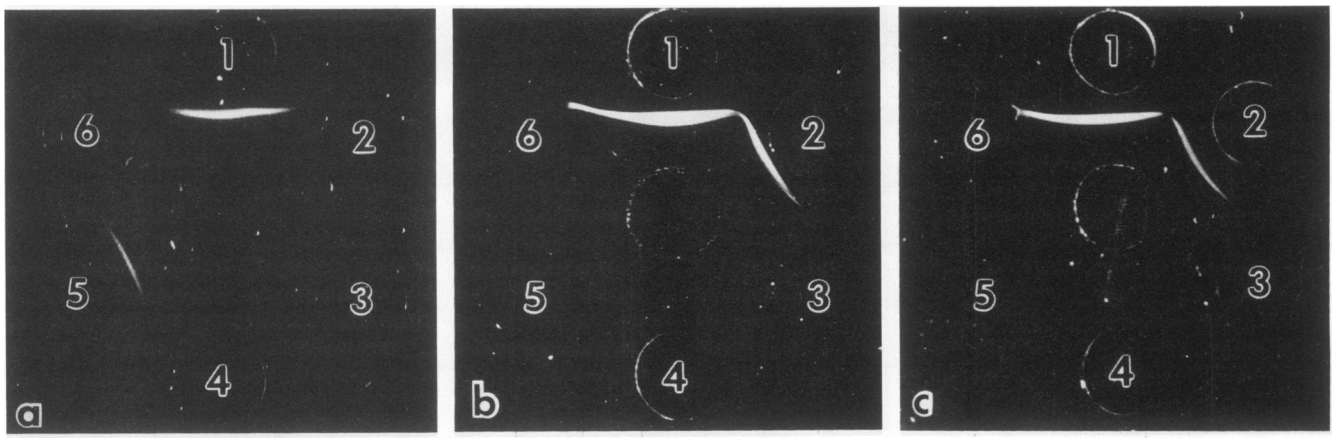

FIG. 2. Reactivity of MAbs with structural derivatives of cryptococcal serotypes A and D. MAbs were placed in the center wells as follows (panels): a, MAb 302; b, MAb 439; c, MAb 1255. Structural derivatives of serotype D were used in the outer wells of panel a; derivatives of serotype A polysaccharide were placed in the outer wells of panels b and c. Wells: 1, unmodified; 2, de-O-acetylated; 3, carboxyl reduced; 4, empty; 5, polyalcohol; 6, Smith product.

with polysaccharides of all four serotypes and therefore has an apparent specificity for antigen 1 described by Ikeda et al. (15). Similarly, MAb 1255 was reactive with polysaccharides of serotypes A, B, and D, which matches the distribution of antigen 2 (15). Finally, MAb 302 was reactive with polysaccharides of serotypes A and D. This corresponds to the distribution of antigen 3. Thus, our results show remarkable similarity to results demonstrated by use of adsorbed polyclonal antibodies, and they demonstrate the utility of MAbs for epitope mapping of cryptococcal polysaccharides.

Dromer et al. (10) have recently reported the production of two MAbs specific for cryptococcal polysaccharide. These antibodies were produced by immunization with type A polysaccharide. The antibodies were of the IgG1 isotype. These antibodies differed from antibodies in our current report because the antibodies described by Dromer et al. agglutinated and precipitated cells and polysaccharide, respectively, of serotype A alone. More sensitive assays such as immunofluorescence and competitive binding studies indicated very weak reactivity with cells and polysaccharide of serotypes B and D. Thus, the antibodies developed by Dromer et al. have an apparent specificity for antigen 2 or 7 described by Ikeda et al. (15).

An examination of the reactivity of the three MAbs with structural derivatives of the polysaccharides provided information on the structural requirements for the integrity of epitopes recognized by each antibody. (i) MAb 302 was not reactive with de-O-acetylated polysaccharide, whereas the remaining antibodies did not require the presence of the *O*-acetyl group for reactivity. Reduction of the periodate-oxidized polysaccharide with sodium borohydride was done under conditions which would preserve the *O*-acetyl group. Thus, reactivity of MAb 302 with the polyalcohol of serotype D polysaccharide is likely due to the *O*-acetyl group. MAb 302 was produced by immunization with serotype D polysaccharide. Serotype D is the most heavily O acetylated of the cryptococcal polysaccharides, and it has been shown that *O*-acetyl groups are important epitopes in the recognition of type D polysaccharide by polyclonal antisera (1, 3). (ii) The reactivity of MAbs 439 and 1255 required an intact carboxyl group. This may be due to the presence of the carboxyl group in the binding site, or it may indicate a key role for the carboxyl group in maintaining the conformation of the polysaccharide. (iii) The reactivity of epitopes on serotype A polysaccharide was more sensitive to periodate oxidation than was that of epitopes on serotype D polysaccharide. This is not surprising because serotype A polysaccharide has more xylosyl groups subject to periodate oxidation than does serotype D polysaccharide (3, 5). (iv) The reactivity of all MAbs with polysaccharide was lost if the polysaccharides were Smith degraded. This suggests that either the mannose backbone lacks epitopes reactive with antibody or mannose residues in the polysaccharide do not trigger antibody production (1). Alternatively, the xylosyl and glucuronosyl side chains may be essential for the conformation needed for the reaction by any putative backbone epitopes with antibody. Finally, our screening method was designed to detect only IgG-secreting clones. It is possible that antibodies directed against the mannose backbone are of the IgM class.

Previous studies from our laboratory have shown that antibodies reactive with different epitopes on the polysaccharide were equally opsonic for the yeast. These results were obtained by use of polyclonal antisera that were cross-adsorbed to produce antisera with differing epitope specificities. The results of the present study confirm these results. The potent opsonic activity of all three MAbs

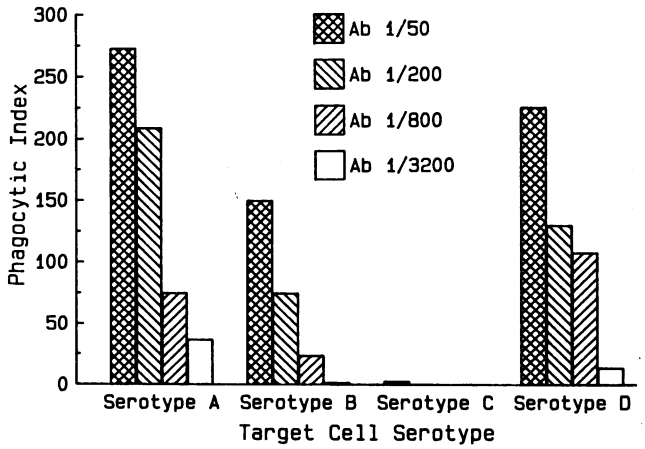

FIG. 3. Opsonization of cryptococcal cells of serotypes A, B, C, and D by MAb 1255.

confirms the conclusion that antibodies binding to any epitope on the capsular polysaccharide are opsonic if appropriate phagocyte receptors are available. These results are in marked contrast to the antiphagocytic streptococcal M protein, in which antibodies directed against some epitopes are opsonic, whereas antibodies directed against other epitopes are not opsonic (12).

The ability to produce MAbs of the IgG class that are reactive with all four serotypes (MAb 439) presents several opportunities for future study. One potential application is the latex agglutination assay for cryptococcal polysaccharide in body fluids. Ideally, such an assay would be free of bias toward one or more serotypes. This would require that the antibody have an identical affinity for polysaccharides of all cryptococcal serotypes. A MAb specific for a common epitope would be an ideal candidate for this application. Passive immunization is another possible application of MAbs. MAbs can be produced in large amounts, and use of isotype switching technologies (26) raises the possibility that antibodies with desirable biological properties can be produced in the laboratory.

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