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Production, Characterization, and Antibody Specificity of a Mouse Monoclonal Antibody Reactive with *Cryptococcus neoformans* Capsular Polysaccharide

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Two monoclonal immunoglobulin G1 antibodies reacting with *Cryptococcus neoformans* capsular polysaccharide (CNPS) were produced in mice by using a carefully defined procedure for immunization with unmodified CNPS purified from *C. neoformans* serotype A. Since the antibodies were found to have the same pattern of specificity, only one of them (E_1) is described. This anti-CNPS monoclonal antibody reacted with the glucuronoxylomannan component of CNPS but not with the constituent monosaccharides or with the mannose α(1→3)-linked oligosaccharide structures present on CNPS. E_1 appeared to be specific for *C. neoformans* serotype A by agglutination of whole cells; it was specific for soluble CNPS A by gel immunoprecipitation. However, indirect immunofluorescence and competitive-binding enzyme-linked immunosorbent assay experiments showed low levels of cross-reactivity with serotypes B and D but not with serotype C. Concentrations 10,000 times higher for serotypes B and D cells than for serotype A cells were required for a 50% inhibition of E_1 anti-CNPS A activity as measured by enzyme-linked immunosorbent assay. Among the other yeasts tested, a cross-reaction was only detected with *Trichosporon beigelii*. The four serotypes of *C. neoformans* could be distinguished based on intensities and patterns of fluorescence in an indirect immunofluorescence assay using the monoclonal anti-CNPS A antibody. Monoclonal anti-CNPS A antibodies could be useful for fundamental studies on the glucuronoxylomannan structure, as well as for clinical applications such as serotyping and possibly the serological diagnosis of cryptococcosis.

Cryptococcus neoformans is a yeastlike encapsulated fungus responsible for life-threatening infections in immunocompromised hosts (16, 21). Anti-*C. neoformans* antibodies, raised in rabbits after active immunization and then properly absorbed, are used for the serotyping of *C. neoformans* clinical isolates by agglutination (28) or by indirect immunofluorescence assay (IIFA) (14). The latex agglutination test for the diagnosis of cryptococcosis, which detects soluble *C. neoformans* capsular polysaccharide (CNPS) in the sera and cerebrospinal fluids of infected patients, is also routinely performed with polyclonal anti-CNPS antibodies (8). Finally, anti-CNPS antibodies contribute, with the techniques of carbohydrate chemistry, to the characterization of *C. neoformans* capsular components. The precise structure of the capsular mannan polysaccharides, including the epitope involved in serotype recognition, has not been totally elucidated, but subtle changes in sugar composition seem to be associated with serotype differences (25). Monoclonal anti-CNPS antibodies might improve the results obtained with immune sera in the three areas cited above. In this paper, we report the production and characterization of, as well as the first results obtained with, a monoclonal mouse anti-CNPS antibody.

MATERIALS AND METHODS

CNPS purification. CNPS was purified by the method of Kozel and Cazin (17) from a 5-day-old culture of a well-encapsulated strain of *C. neoformans* serotype A (kindly donated by E. Drouhet, Institut Pasteur, Paris) on yeast extract dialysate medium. After centrifugation at 1,000 × *g*, the supernatant was sterilized by filtration (0.45-μm [pore size] filter; Millipore Corp., Bedford, Mass.) and concentrated by ultrafiltration to a viscous syrup in a Dia-flow pressure cell (Amicon Corp., Lexington, Mass.) equipped with a PM30 membrane filter (Millipore). Sodium acetate (10%) and glacial acetic acid (1%) were added to the concentrate, and the polysaccharide was precipitated by adding dropwise 2.5 volumes of 95% ethanol with constant stirring. The precipitate was sedimented by centrifugation and dissolved in distilled water containing 10% sodium acetate and 1% acetic acid. Deproteinization was performed by repeated extractions with chloroform and butyl alcohol (1:1 [vol/vol]). The polysaccharide was again precipitated in 95% ethanol and retrieved by centrifugation; it was then dissolved in water, dialyzed against distilled water, and lyophilized. The protein concentration, measured by the Coomassie blue dye method, was less than 1 μg of protein per mg of CNPS. The CNPS molecular weight was estimated to be 550,000 by gel permeation chromatography on Sepharose-6B. The purified polysaccharide and a reference serotype A CNPS (generously provided by J. E. Bennett, National Institutes of Health, Bethesda, Md.), diluted at 3 mg/ml in phosphate-buffered saline (PBS; pH 7.4), gave a reaction of identity by gel immunoprecipitation (Ouchterlony) with a rabbit anti-serotype A reference serum (also a gift from J. E. Bennett).

Immunization procedures. To overcome the low level of humoral response elicited in mice after CNPS immunization (19), we conducted preliminary studies designed to define the immunization procedures resulting in the highest level of

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antibody production. The following parameters were studied: route of administration, presence of adjuvant, number of immunizations, CNPS concentration, and strain and age of mice. Anti-CNPS antibody titers were determined by an IIFA 9 days after immunization. After the preliminary studies, BALB/c and C3H/HeJ mice aged 3 months or more were injected intravenously with 1 μg of CNPS. Antibody-producing cells, estimated by rosette-forming cells (4) with CNPS-coated sheep erythrocytes (18), were counted in the spleens 3 to 7 days after primary immunization. Fusions were performed the day before the peak of anti-CNPS-secreting cell proliferation.

Production of hybridomas. The procedure used for the production of hybridomas was derived from the protocol described by Kohler and Milstein (15). The spleens were aseptically removed from the immunized mice, minced through a sterile screen, washed in minimal essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.), and centrifuged at 400 × *g* for 5 min. The cells were resuspended in 10 ml of MEM, and counts of viable cells were made after Trypan blue dye exclusion. Spleen cells and murine myeloma cells (P3X63-Ag8 653) were mixed (10:1) and centrifuged for 10 min at 400 × *g*. The pellet was resuspended in a drop of MEM, and 0.8 ml of a 45% solution of sterile polyethylene glycol 1000 was added for 1 min with gentle shaking. After a 3-min incubation at room temperature, the reaction was stopped by dilution with 10 ml of MEM for 5 min. The cells were centrifuged for 5 min at 400 × *g*, suspended in MEM supplemented with 10% horse serum, 1% glutamine, 1% pyruvate (GIBCO), gentamicin (100 μg/ml), penicillin (100 U/ml), 1% hypoxanthine (Sigma Chemical Co., St. Louis, Mo.), and 1% azaserine (Sigma), and plated at 10^6 cells in 0.75 ml of medium per well of a 24-well culture plate (Becton Dickinson Labware, Oxnard, Calif.).

Hybridomas were detected microscopically after 10 days of culture at 37°C in a 10% CO_2 incubator. The supernatants were screened by an enzyme-linked immunosorbent assay (ELISA) for anti-CNPS antibody production. Selected hybridomas were cloned by limiting dilution (0.5 cell per well in microplates; Becton Dickinson), and all positive hybridomas and clones were expanded in petri culture dishes (Becton Dickinson) and frozen at −80°C. Some clones were injected into pristane-primed mice for production of ascites.

Detection of anti-CNPS antibodies. Anti-CNPS antibodies were detected in supernatants from hybridomas by ELISA. The procedures used were modifications of the original procedure described by Engvall and Perlman (9). Polystyrene 96-well microtiter plates (A/S NUNC, Roskilde, Denmark) were used for the solid phase. A 100-μl sample of CNPS antigen per well, diluted to 10 μg/ml in 10 mM PBS (pH 7.4), was incubated at 37°C for 1 h. The plates were washed twice with PBS and once with PBS containing 3% bovine serum albumin (Sigma; PBS-BSA). Unbound sites were reacted for 1 h with 100 μl of PBS-BSA. The plates were then washed twice with PBS containing 0.1% Tween 20 (Sigma; PBS-Tween) and once with PBS-BSA-Tween. Duplicate samples (50 μl) of undiluted supernatants were incubated for 1 h at 37°C. After the plates were washed as described above, 50 μl of a horseradish peroxidase-labeled goat immunoglobulin G (IgG) anti-mouse immunoglobulin (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted 1:5,000 in PBS-BSA-Tween was added. After the plates were washed, *o*-phenylenediamine diluted at 4 mg/ml in citrate buffer (pH 5.6) was used as a substrate; 100 μl of the solution was incubated at room temperature for 10 min, and the reaction was stopped by the addition of 20 μl of 2 N sulfuric acid. The optical density was measured at 492 nm with an enzyme immunoassay reader (Titertek Uniskan; Flow Laboratories, Inc., McLean, Va.). Supernatants were considered positive for anti-CNPS antibody when the absorbance was at least twice that of negative controls.

Immunological characteristics of monoclonal anti-CNPS antibodies. (i) Antibody purification and biochemical characterization. Monoclonal antibodies were purified from ascitic fluids after affinity chromatography with a *Staphylococcus aureus* protein A-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden). Immunoglobulins were eluted by pH gradient in citrate buffer and monitored at 280 nm with a spectrophotometer. The monoclonal antibodies were then dialyzed against PBS for 24 h and stored at −30°C at a concentration of 1 mg/ml. The purified monoclonal antibodies were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing on polyacrylamide gel.

(ii) Isotype determination. Isotypes were identified by ELISA with rabbit anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM conjugated with horseradish peroxidase (Miles Diagnostics, Kankakee, Ill.).

(iii) Antibody activity. Five methods were used to assess anti-CNPS activity. The first method, agglutination, was performed as follows. Agglutination was performed in V-shaped wells of microtiter plates (Linbro Scientific Co., Hamden, Conn.) by the method of Murphy and Cozad (24). Cells from *C. neoformans* serotypes A (IP 960-67), B (IP 961-67), C (IP 962-67), and D (B3501; provided by J. E. Bennett) were used with 10-fold dilutions of monoclonal antibodies. A reference rabbit anti-CNPS A immune serum was run in parallel. Plates were read after 24 h.

The second method, precipitation, was performed as follows. CNPS A (45 μl) and purified polysaccharides from *C. neoformans* serotypes B, C, and D (a gift from J. E. Bennett) diluted at 2 mg/ml in PBS were added to the peripheral wells of agarose immunodiffusion plates with antibodies (80 μl in PBS) in the central well.

The third method, IIFA, was performed as follows. The following twelve strains of *C. neoformans* were used: six serotype A strains, including strains 1 and 271 (J. E. Bennett), IP 960-67 (E. Drouhet), RV 43-182 (De Vroe, Antwerp, Belgium); two serotype B strains (IP 961-67 and NIH 112B); two serotype C strains (IP 962-67 and NIH 18C); and two serotype D strains (B 3501 and NIH 52D). IIFA was performed by the method of Bindschadler and Bennett (3). Briefly, cells were suspended at 10^7/ml after four washings in PBS, and 10 μl of the suspension was incubated in each well of 10-well immunofluorescence slides. The slides were then air dried, heat fixed for 30 min, and stored at −30°C. Sera or monoclonal antibodies (25 μl) diluted in PBS were incubated at room temperature for 30 min in a moist chamber. The slides were washed three times in PBS. Anti-CNPS antibodies were detected by using fluorescein-labeled goat immunoglobulins (Nordic) that were specific for mouse polyvalent immunoglobulins. Fluoresceinated antibodies were diluted 1:100 in PBS and incubated for 30 min at room temperature. The slides were then washed, dried, covered with buffered glycerol, and examined under a Zeiss epifluorescence microscope. Control wells incubated with normal mouse serum were consistently negative. Serial 10-fold dilutions were tested, and the minimum antibody concentration at which fluorescence could be clearly observed was recorded. The patterns of fluorescence with the four serotypes were studied with monoclonal antibodies diluted in PBS at 100 μg/ml. All

slides were read by an observer with no previous knowledge of the serotypes of the cells.

The fourth method, ELISA, was performed as follows. Serial 10-fold dilutions of the monoclonal antibodies were tested as described above on plates coated with 10 μg of CNPS of the A, B, C, or D serotype per ml, and the minimum antibody concentration giving a positive reaction was recorded.

The fifth method, competitive-binding assays, was performed as follows for soluble antigens. Antibody specificity was studied by competitive-binding assays in which increasing concentrations of various antigens diluted in PBS-BSA-Tween were incubated (vol/vol) for 1 h at 37°C with 20 ng of antibody per ml. The ELISA was then performed in duplicate as described above in wells coated with CNPS A. The percentage of inhibition of anti-CNPS A antibody activity was calculated by the following formula: % inhibition = (max − exp)/(max − min) × 100, where max is the anti-CNPS A activity of unabsorbed antibody, exp is the residual anti-CNPS A activity of absorbed antibody, and min is the nonspecific binding to plastic. All values were expressed as arbitrary units of absorbance. Competitive-binding studies were performed with CNPS A, B, C, and D, constituent monosaccharides (mannose, xylose, galactose, and glucuronic acid), glucuronoxylomannan (GXM) purified from the four serotypes (GXM A, GXM B, GXM C, and GXM D), galactoxylomannan (GalXM), and mannoprotein purified from J67, an acapsular mutant (purified GXM from the four serotypes, GalXM, and mannoprotein were gifts from R. Cherniak, Atlanta, Ga.). A mannan from *Saccharomyces cerevisiae* (Sigma) was also tested because it contains α(1→3) diamannoside structures (5), as does the mannan backbone of *C. neoformans* GXM.

For yeast cells, competitive-binding assays were also performed with *C. neoformans* strains from the different serotypes and other yeasts that have been reported to cross-react with CNPS (1, 2, 5, 11, 22), i.e., *Cryptococcus laurentii* (IP 1276-81), *Candida albicans* (IP 884), *Trichosporon beigelii* (IP 1557), *Histoplasma capsulatum* (IP 1052), and *Histoplasma duboisii* (IP 1098). The cells were harvested from Sabouraud slants; they were heat killed at 80°C for 1 h and washed three times in PBS. They were suspended in PBS-BSA-Tween at 10^9 cells per ml (for *Histoplasma* cells, the concentration was adjusted according to the A_{650} given by a suspension of 10^9 cells per ml or *C. neoformans* serotype A). Serial 10-fold dilutions were incubated (vol/vol) with 20 ng of antibody per ml. After 1 h of incubation at 37°C and one centrifugation (20 min at 1,000 × *g*), 50 μl of supernatant was deposited in duplicate into wells coated with CNPS A, and anti-CNPS A residual activity was measured. The percentage of inhibition was calculated as described above for soluble antigens.

TABLE 1. Direct binding of anti-CNPS serotype A monoclonal antibody detected with *C. neoformans* (agglutination and IIFA) or purified CNPS (precipitation and ELISA) from the four serotypes

C. neoformans serotype	Minimum antibody concn (μg/ml) detected by:			
	Precipitation	Agglutination	IIFA	ELISA
A	10	1	0.001	0.001
B	>1,000	>100	1	0.1
C	>1,000	>100	>1,000	>1,000
D	>1,000	>100	1	1

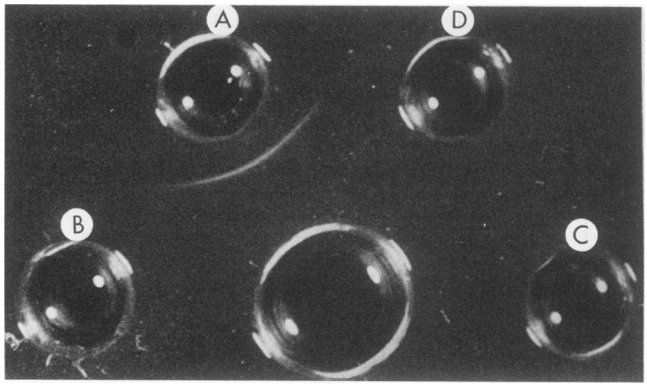

FIG. 1. Immunoprecipitation study of E_1 anti-CNPS antibody with E_1 at 1 mg/ml in the central well and CNPS from the four serotypes (A, B, C, and D) at 2 mg/ml in the peripheral wells.

RESULTS

Isolation of hybrid cell lines producing anti-CNPS antibodies. Hybridization was performed by fusion of P3X63-Ag8 653 myeloma cells with splenic lymphocytes from either C3H/HeJ or BALB/c mice immunized with CNPS A. A total of 44 plates were seeded after eight fusions (five with C3H/HeJ cells and three with BALB/c cells). Fusion efficiency was nearly 100%. Supernatants were tested by ELISA for anti-CNPS antibody production, and 21 hybridomas produced specific immunoglobulins (yield of 0.4% with splenocytes from C3H/HeJ and 2% with cells from BALB/c). Of the 21 hybridomas, 7 were IgG- and 14 were IgM-secreting cells. Six hybridomas with the highest level of antibody production (A_{492} on CNPS-coated wells of over 1.0) were cloned. Two stable clones (E_1 and G_1) producing monoclonal antibodies at significant levels were obtained from the C3H/HeJ cell fusions.

Characteristics of monoclonal antibodies. E_1 and G_1 monoclonal antibodies were eluted from a protein A-Sepharose column at pH 3.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed entire IgG with different isoelectric patterns (data not shown). The isotypes were IgG1 in both cases.

Antibody activity. Since results were similar for E_1 and G_1, only the experiments performed with E_1 will be presented in detail. Antigen-binding studies involving whole *C. neoformans* cells of the different serotypes (agglutination and IIFA) or purified CNPS (precipitation and ELISA) were performed (Table 1). E_1 could precipitate with CNPS A by immunodiffusion (Fig. 1) and could agglutinate *C. neoformans* A cells. A 1-ng amount of antibody per ml could be detected by IIFA or ELISA. E_1 was specific for *C. neoformans* A cells by IIFA when used at 0.1 μg/ml or less. Cross-reactivity with *C. neoformans* B and D cells could be detected by IIFA by using E_1 at a concentration of 1 μg/ml of more, but fluorescent patterns were distinct for the four serotypes (Fig. 2). A classical homogeneous pattern with a rim reinforcement was observed on all *C. neoformans* A cells. The fluorescent intensity was lower and also differed from cell to cell on smears of *C. neoformans* B and D cells; some cells were negative, whereas others were as brightly stained as A cells were. Positive cells were exclusively of the serotype A homogeneous pattern on *C. neoformans* D smears, whereas fluorescence appeared speckled on B smears. Fluorescence was negative with all *C. neoformans* C cells. Results obtained with six A, two B, two C, and two serotype D strains

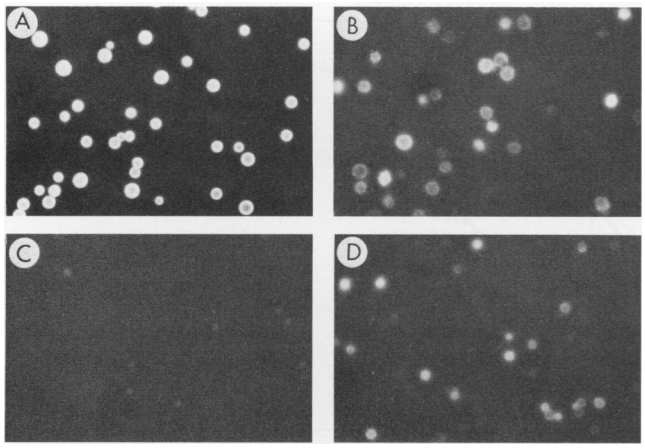

FIG. 2. Patterns of immunofluorescence with 100 μg of E_1 anti-CNPS A antibody per ml on smears of *C. neoformans* strains of serotypes A, B, C, and D.

were consistent with the patterns described above and were also obtained on smears made from isolated colonies.

To better define the structure involved in antibody recognition on CNPS A, we performed competitive-binding assays in which residual anti-CNPS A activity was measured by ELISA after incubation of E_1 with soluble antigens. As indicated in Fig. 3a, E_1 reacted with GXM but not with GalXM. No inhibition was detected with mannoprotein, with the constituent monosaccharides of CNPS, or with an *S. cerevisiae* mannan containing α(1→3) dimannoside structures (data not shown).

Competitive-binding studies allowed the evaluation of the specificity of E_1 for *C. neoformans* A, CNPS A, and GXM A. Studies performed with soluble antigens showed (Fig. 3b) that E_1 was specific for CNPS A; 50% inhibition of anti-CNPS activity required less than 1 μg of CNPS A per ml, whereas 7% or less inhibition was achieved with CNPS from the other serotypes at 1 mg/ml. However, studies with purified GXM showed that GXM D was a better inhibitor of E_1 anti-CNPS A activity than CNPS D was. Little or no inhibition was observed with GXM B (Fig. 3b) or with GXM C (data not shown). Tests performed with whole yeasts (Fig. 4) confirmed that E_1 was specific for *C. neoformans* serotype A; 50% inhibition of anti-CNPS A activity required 10^5 *C. neoformans* A cells per ml, compared with 10^9 cells per ml or more for the other serotypes and for *T. beigelii*. No inhibition was observed with *Candida albicans*, *C. laurentii*, *H. capsulatum*, and *H. duboisii* (data not shown).

DISCUSSION

Numerous applications can be developed with monoclonal anti-*C. neoformans* antibodies in biochemical studies as well as in clinical medicine. To date, there has been one report on monoclonal anti-*C. neoformans* antibodies dealing with unpurified hybridoma supernatants (11). The paucity of reports can be due in part to the poor anti-CNPS antibody response elicited in mice (19) and humans (12) after specific immunization. For this reason, we conducted preliminary immunization studies (data not shown) testing the influence of strain and age of mice, total dose and number of injections of antigen, route of administration, and presence of adjuvant on the level of antibody response. We selected one intravenous injection of 1 μg of CNPS in at least 3-month-old BALB/c and C3H/HeJ mice as the best protocol, giving an anti-CNPS antibody serum titer over 1:1,000 by IIFA 9 days after immunization. Closely related conditions for immunization have been used by others with dextran (26). Fusions were performed during the phase of rapidly proliferating antibody-secreting cells in the spleen; antibody-producing cells were estimated as rosette-forming cells with CNPS-coated sheep erythrocytes. Although we used carefully primed cells for fusion with mouse myeloma cells, the yield of positive hybridomas was low, i.e., 0.4% with C3H cells and 2% with BALB/c cells. Two clones, purified from the two hybridomas obtained with C3H cells, were expanded in ascites. The corresponding antibodies were purified on a protein A-Sepharose column and analyzed for isoelectric forms, isotypes, and antibody activities. The two antibodies had different isoelectric patterns but had the same IgG1 isotype and similar in vitro activities. Therefore, only the results obtained with one of them (E_1) are discussed in this paper.

CNPS purified from *C. neoformans* serotype A consists of 88% GXM, 11% GalXM, and approximately 1% mannoprotein (6, 27). The GXM backbone consists of a linear α(1→3)-linked mannan substituted at 2-*o* positions by single residues of either xylose or glucuronic acid (5, 23). The GXM structure has been analyzed by biochemical, immunological, and nuclear magnetic resonance studies (25). The most likely GXM hapten configuration reported (5) is a tetrasaccharide consisting of xylose and glucuronic acid, both linked to a mannose α(1→3) disaccharide. Subtle differences involving the relative proportion of xylose and glucuronic acid residues, the degree of mannan substitution, and the percentage of *o*-acetyl attachments (20) seem to be responsible for the distinctions among the four serotypes described with immune sera (28). Monoclonal antibodies could give some insight into the GXM structure and the epitope configuration involved in serotype definition. E_1 reacted with the GXM component of CNPS but not with GalXM and mannoprotein, since only GXM was able to inhibit anti-CNPS A activity. Anti-CNPS A activity could not be inhibited by the constituent monosaccharides or a mannan purified from *S. cerevisiae*. This suggests that E_1 does not react with the mannose α(1→3) disaccharide, since the same structure is also present on *S. cerevisiae* mannan (10). Further studies are warranted to better assess the precise target of E_1 activity.

To evaluate whether E_1 was specific for CNPS purified from *C. neoformans* serotype A, studies were performed using whole cells from the four serotypes (agglutination and IIFA) as well as the corresponding purified CNPS (precipitation and ELISA). Competitive-binding studies were also performed as more sensitive techniques. Agglutination studies suggested that E_1 is specific for *C. neoformans* serotype A, and immunodiffusion experiments indicated that E_1 is specific for CNPS A. However, more sensitive techniques revealed a certain extent of cross-reactivity; binding to serotype B and D cells was detected by IIFA at an E_1 concentration 1,000 times higher than that needed to label serotype A cells. Competitive-binding studies confirmed IIFA results, showing that high concentrations of CNPS B and CNPS D, but not of CNPS C, could inhibit less than 10% E_1 anti-CNPS A activity. Comparison of the ability of GXM from the four serotypes to inhibit E_1 anti-CNPS A activity showed that the highest level of inhibition was obtained with GXM A, whereas no inhibition was observed with GXM C. It remains unclear why GXM D was a better inhibitor of E_1

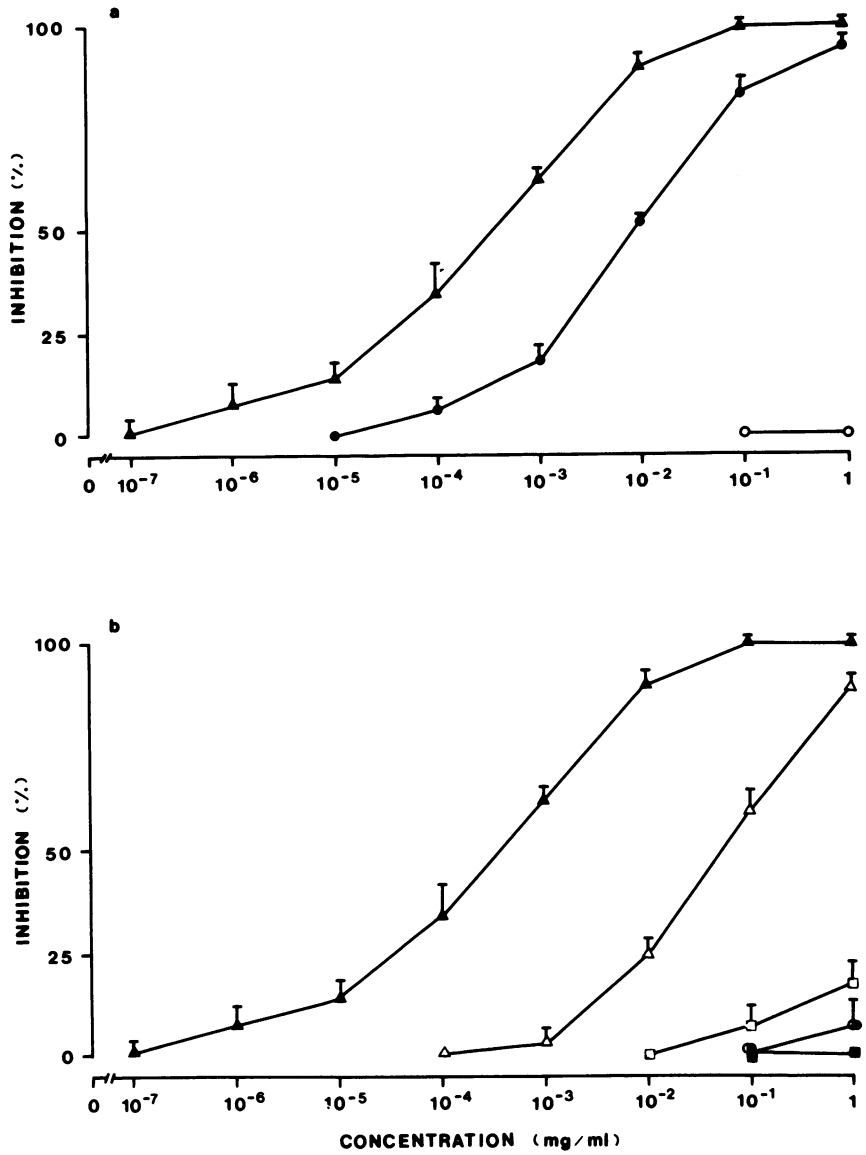

FIG. 3. Competitive-binding studies on antigenic structure (a) and antibody specificity (b) with soluble antigens. E_1 (20 ng/ml) anti-CNPS A antibody activity was measured by ELISA after incubation with (a) CNPS serotype A (▲), GXM purified from *C. neoformans* serotype A (●), and GalMX (○). (b) Incubations were with CNPS serotypes A (▲), B (○), C (■), D (●), and GXM purified from *C. neoformans* serotypes B (□) and D (△). Values are the means of at least three experiments ± the standard deviation.

anti-CNPS A activity than CNPS D was. Inhibition studies performed with *C. neoformans* cells from the four serotypes confirmed the results obtained by IIFA studies; i.e., concentrations of serotypes B and D cells 10,000 times higher than that of serotype A were needed for a 50% inhibition of E_1 anti-CNPS A activity. *C. neoformans* cells of serotypes A, D, and to a lesser extent B are known to exhibit a certain degree of cross-reactivity with polyclonal immune serum raised after immunization with A cells (13, 14, 28). Studies performed with properly absorbed immune serum showed that some serotype specificities are shared by two or more serotypes, whereas the others are serotype specific. Ikeda et al. (13) identified an antigen, designated antigen 2 and shared by serotypes A, B, and D, which could be the epitope binding E_1. The results of our inhibition studies performed with a monoclonal antibody suggest that such specificities shared by different serotypes can be diversely expressed. Other yeasts were tested in competitive-binding assays because they have been reported to cross-react with CNPS (1, 2, 5, 11). No inhibition was observed with *C. laurentii*, *Candida albicans*, *H. capsulatum*, or *H. duboisi*, but *T. beigelii* competed with CNPS A to the same extent as *C. neoformans* D and B cells did. Interestingly, false-positive reactions to the serological test for cryptococcosis have been reported in the case of infections with *T. beigelii* (22).

C. neoformans serotyping requires properly absorbed anti-CNPS immune serum (14, 28). Monoclonal anti-CNPS antibodies could be of value for two reasons. First, E_1 was specific for serotype A without requiring absorption when used at 100 μg/ml or less for agglutination or at 0.1 μg/ml or less for IIFA. Second, distinct patterns of fluorescence were observed with cells from the four serotypes incubated with E_1 at 10 to 100 μg/ml; all serotype A cells appeared brightly fluorescent, whereas serotype C cells were consistently

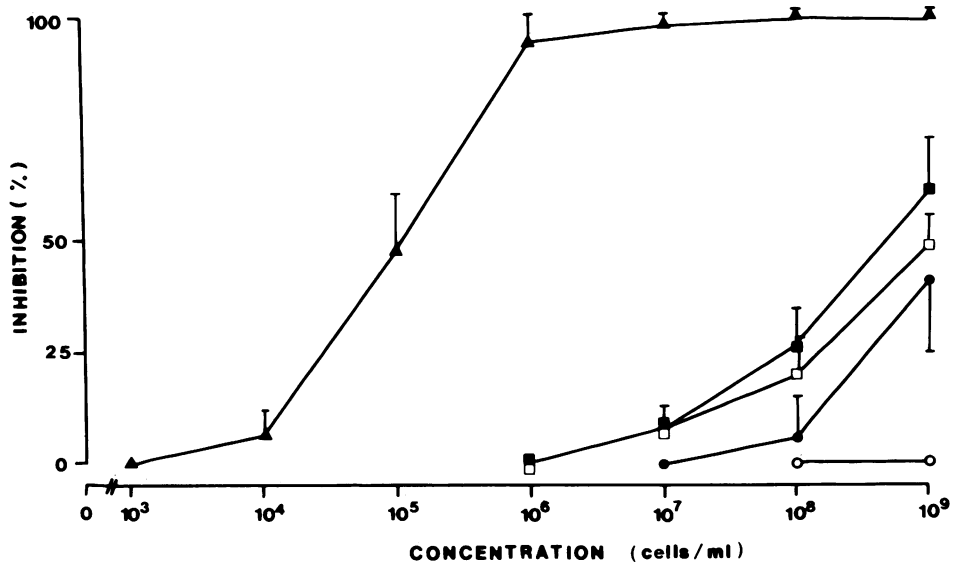

FIG. 4. Competitive-binding studies of antibody specificity with yeasts. E_1 binding to *C. neoformans* serotypes A (▲), B (●), C (○), and D (■) and *T. beigelii* (□) was tested as described in the legend to Fig. 4.

negative. The overall fluorescent intensity on B and D smears was lower than that on A smears, and the amount of fluorescence was highly variable from one cell to another, from brightly fluorescent to negative cells. Such fluorescent diversity was not observed on serotype A smears, even when E_1 was diluted, and did not result from contamination, since the same pattern was observed on smears made from isolated colonies. Serotype D cells could be readily distinguished from serotype B cells, since positive D cells had a homogeneous aspect, with a rim reinforcement identical to that observed with A cells, whereas B cells appeared speckled.

Immune anti-CNPS antibodies are routinely used for the serological diagnosis of cryptococcosis. When coating latex particles, they give a positive agglutination reaction in the presence of soluble CNPS in sera or cerebrospinal fluids from infected patients. Commercial kits are available and give a positive reaction for a CNPS concentration above 0.02 μg/ml (29). Monoclonal anti-CNPS antibodies reacting with the four serotypes might be preferred to polyclonal immune sera because they would allow a better standardization of the test. Moreover, preliminary results suggest that approximately 1 ng of CNPS A per ml can be detected by a sensitive test (competitive-binding enzyme immunoassay) using E_1.

In conclusion, monoclonal anti-CNPS antibodies should be developed because they may serve several purposes, i.e., a better knowledge of the structure of CNPS, a simple serotyping method, and possibly an improvement in cryptococcosis diagnostic procedures. Finally, mouse monoclonal antibodies could contribute to the treatment of experimental murine cryptococcosis, and this topic is addressed in the accompanying paper (7).

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LITERATURE CITED

1. **Atkinson, A. J., and J. E. Bennett.** 1968. Experience with a new skin test antigen prepared from *Cryptococcus neoformans*. Am. Rev. Respir. Dis. **97**:637–643.
2. **Bennett, J. E., and H. F. Hasenclever.** 1965. *Cryptococcus neoformans* polysaccharide: studies of serologic properties and role in infection. J. Immunol. **94**:916–920.
3. **Binschadler, D. D., and J. E. Bennett.** 1968. Serology of human cryptococcosis. Ann. Intern. Med. **69**:45–52.
4. **Biozzi, G., C. Stiffel, D. Mouton, M. Liacopoulos-Briot, C. Decreusefond, and Y. Bouthillier.** 1966. Etude du phénomène de l'immuno-cyto-adhérence au cours de l'immunisation. Ann. Inst. Pasteur (Paris) **110**(Suppl. 3):7–32.
5. **Cherniak, R., E. Reiss, M. E. Slodki, R. O. Plattner, and S. O. Bulmer.** 1980. Structure and antigenic activity of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. Mol. Immunol. **17**:1025–1032.
6. **Cherniak, R., E. Reiss, and S. H. Turner.** 1982. A galactoxylomannan antigen of *Cryptococcus neoformans* serotype A. Carbohydr. Res. **103**:239–250.
7. **Dromer, F., J. Charreire, A. Contrepois, C. Carbon, and P. Yeni.** 1987. Protection of mice against experimental cryptococcosis by anti-*Cryptococcus neoformans* monoclonal antibody. Infect. Immun. **55**:749–752.
8. **Drutz, D. J.** 1986. Antigen detection in fungal infections. N. Engl. J. Med. **314**:115–117.
9. **Engvall, E., and P. Perlman.** 1971. Enzyme-linked immunosorbent assay (ELISA): quantitative assay of immunoglobulin G. Immunochemistry **8**:871–874.
10. **Fleet, G. H.** 1985. Composition and structure of yeast cell walls. Curr. Top. Med. Mycol. **1**:24–56.
11. **Hall, N. K., and R. Blackstock.** 1981. Production of specific antibody to *Cryptococcus neoformans* by hybridomas *in vitro*. Sabouraudia. **19**:157–160.
12. **Henderson, D. K., J. E. Bennett, and M. A. Huber.** 1982. Long-lasting specific immunologic unresponsiveness associated with cryptococcal meningitis. J. Clin. Invest. **69**:1185–1190.
13. **Ikeda, R., T. Shinoda, Y. Fukazawa, and L. Kaufman.** 1982. Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. J. Clin. Microbiol. **16**:22–29.
14. **Kaplan, W., S. L. Bragg, S. Crane, and D. G. Ahearn.** 1981. Serotyping *Cryptococcus neoformans* by immunofluorescence. J. Clin. Microbiol. **14**:313–317.
15. **Kohler, G., and C. Milstein.** 1975. Continuous culture of fused

cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
16. **Kovacs, J. A., A. A. Kovacs, M. Polis, C. Wright, V. J. Gill, C. U. Tuazon, E. P. Gelman, H. C. Lane, R. Longfield, G. Overtuf, A. M. Macher, A. S. Fauci, J. E. Parillo, J. E. Bennett, and H. Mazur.** 1985. Cryptococcosis in the acquired immunodeficiency syndrome. Ann. Intern. Med. **103**:533–538.
17. **Kozel, T. R., and J. Cazin, Jr.** 1971. Nonencapsulated variant of *Cryptococcus neoformans*. I. Virulence studies and characterization of soluble polysaccharide. Infect. Immun. **3**:287–294.
18. **Kozel, T. R., and J. Cazin, Jr.** 1972. Immune response to *Cryptococcus neoformans* soluble polysaccharide. I. Serological assay for antigen and antibody. Infect. Immun. **5**:35–41.
19. **Kozel, T. R., and J. Cazin, Jr.** 1974. Induction of humoral antibody response by soluble polysaccharide of *Cryptococcus neoformans*. Mycopathologia **54**:237–244.
20. **Kozel, T. R., and C. A. Hermerath.** 1984. Binding of cryptococcal polysaccharide to *Cryptococcus neoformans*. Infect. Immun. **43**:879–886.
21. **Lewis, J. L., and S. Rabinovich.** 1972. The wide spectrum of cryptococcal infections. Am. J. Med. **53**:315–322.
22. **McManus, E. J., and J. M. Jones.** 1985. Detection of *Trichosporon beigelii* antigen cross-reactive with *Cryptococcus neoformans* capsular polysaccharide in serum from a patient with disseminated *Trichosporon* infection. J. Clin. Microbiol. **21**:681–685.
23. **Merrifield, E. H., and A. M. Stephen.** 1980. Structural investigations of two capsular polysaccharides from *Cryptococcus neoformans*. Carbohydr. Res. **86**:69–76.
24. **Murphy, J. W., and G. C. Cozad.** 1972. Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assay by the hemolytic plaque technique. Infect. Immun. **5**:896–901.
25. **Reiss, E., M. Huppert, and R. Cherniak.** 1985. Characterization of protein and mannan polysaccharide antigen of yeasts, moulds and actinomycetes. Curr. Top. Med. Mycol. **1**:172–208.
26. **Sharon, J., E. A. Kabat, and S. L. Morrison.** 1981. Studies on mouse hybridomas secreting IgM or IgA antibodies to α(1-6)-linked dextran. Mol. Immunol. **18**:831–836.
27. **Turner, S. H., R. Cherniak, and E. Reiss.** 1984. Fractionation and characterization of galactoxylomannan from *Cryptococcus neoformans*. Carbohydr. Res. **125**:343–349.
28. **Wilson, D. E., J. E. Bennett, and J. W. Bailey.** 1968. Serological grouping of *Cryptococcus neoformans*. Proc. Soc. Exp. Biol. Med. **127**:820–823.
29. **Wu, T. C., and S. Y. Koo.** 1983. Comparison of three commercial cryptococcal latex kits for detection of cryptococcal antigen. J. Clin. Microbiol. **18**:1127–1130.