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Serological, Electrophoretic, and Biological Properties of *Cryptococcus neoformans* Antigens

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We compared a cryptococcal culture filtrate antigen referred to as CneF with chemically defined cryptococcal antigen fractions isolated by Cherniak and co-workers by using double immunodiffusion gels, polyacrylamide gel electrophoresis, immunoblots, and footpad reactivity of immunized mice. The three previously described components of cryptococcal culture filtrates are a high-molecular-weight glucuronoxylomannan (GXM), which is the major constituent, a galactoxylomannan (GalXM), and a mannoprotein (MP). In this study we demonstrated that CneF contained components which were serologically and electrophoretically similar to the three previously described cryptococcal culture filtrate fractions. The MP fraction elicited significantly stronger delayed-type hypersensitivity responses than did the GXM or GalXM fraction when used in mice immunized either with the CneF in complete Freund adjuvant or whole heat-killed *Cryptococcus neoformans* yeast cells. These findings were confirmed when the footpads of immunized mice were challenged with GalXM and MP preparations from a culture filtrate of a *C. neoformans* acapsular mutant that does not produce GXM. Thus, we concluded that the MP was the primary component recognized by the anticryptococcal cell-mediated immune response in mice.

Cell-mediated immunity (CMI), which can be assessed by determining the level of delayed-type hypersensitivity (DTH), is an important host defense mechanism in cryptococcosis. The cryptococcal antigen preparations used for detection of DTH are heterogeneous mixtures of relatively high-molecular-weight (>50,000) polysaccharides and proteoglycans. One such preparation, which specifically detects CMI responses to *Cryptococcus neoformans* and has been extensively studied in the murine cryptococcosis model, is a culture filtrate antigen referred to as CneF.

When injected subcutaneously into mice, CneF (200 μg of carbohydrate) in complete Freund adjuvant (CFA) induces an anticryptococcal DTH response (15). However, if given intravenously, CneF will induce antigen-specific immunological suppression (15). CneF can be separated electrophoretically in 3% polyacrylamide gels into two components which stain with periodic acid-Schiff reagent (PAS) (16). One component migrates only a short distance into the gel, is glycosidic, and does not elicit significant skin test reactions in *C. neoformans*-sensitized guinea pigs (16). The other component is a fast-migrating fraction which moves with the tracking dye. The rapidly moving component stains for both carbohydrate and protein and elicits positive skin test responses in sensitized guinea pigs (16).

Recently, Cherniak and co-workers (4, 5, 19) have fractionated *C. neoformans* culture filtrates which were prepared in a manner similar to that used for the culture filtrates from which CneF was obtained. They characterized three major components, i.e., glucuronoxylomannan (GXM), a high-molecular-weight, serotype-specific polysaccharide; galactoxylomannan (GalXM), a polysaccharide with a molecular mass of approximately 275 kilodaltons; and a mannoprotein (MP).

It was not known how the CneF fractions compare with those isolated by Cherniak and co-workers (4, 5, 19); therefore, one objective of this study was to compare CneF with GXM, GalXM, and MP fractions by using Ouchterlony double immunodiffusion (ID) in a gel, polyacrylamide gel electrophoresis, and immunoblots. Since previous studies have not addressed whether all of the components of the culture filtrate antigen can elicit a CMI response such as DTH or whether only certain constituents are immunologically reactive, a second goal of this work was to assess which of the component(s) could elicit a DTH response in mice sensitized with either CneF in CFA or heat-killed *C. neoformans* cells.

MATERIALS AND METHODS

Organisms. Three different *C. neoformans* isolates and one acapsular mutant were used in these investigations. Isolates 184A (serotype A) and B2550 (NIH 371A, a serotype A variant) were previously described by Murphy and Cozad (13) and Cherniak et al. (4; R. Cherniak, R. J. Jones, and E. Reiss, Carbohydr. Res., in press), respectively. Isolate B-3501 (serotype D) and an acapsular mutant, J67, derived therefrom have been described by Jacobson et al. (11) and Fromtling et al. (9).

Preparation of antigens. The cryptococcal culture filtrate antigen CneF-184A was prepared by using *C. neoformans* 184A in a manner similar to that described by Murphy and Pahlavan (16). Supernatant fluids from 4-day-old cultures of *C. neoformans* were subjected to ultrafiltration by using a 50,000-molecular-weight-exclusion hollow fiber cartridge (Amicon Corp., Danvers, Mass.). After being washed with 10 volumes of physiological saline, the retentate was concentrated to 1/10 the original volume, filter sterilized, and stored at −20°C. The culture filtrate antigen prepared by using isolate 184A (CneF-184A) contained 2 mg of carbohydrate per ml, as determined by the phenol-sulfuric acid

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method (6), and 4.1 mg of protein per ml, based on a protein assay of Lowry et al. (12).

Procedures for preparation of the ethanol-precipitated antigen, GXM, GalXM-N, GalXM, and MP have been described in detail by Cherniak and co-workers (4, 5, 19). Briefly, supernatant liquors from 4-day-old cultures were dialyzed and concentrated 10-fold before the polysaccharides were precipitated with cold, absolute ethanol. The precipitated material was dried by washing sequentially with ethanol, acetone, and diethyl ether. This product was the ethanol-precipitated antigen used in this study and was further fractionated into a cetyltrimethylammonium bromide (CTAB) precipitate, GXM, and a CTAB supernatant, GalXM-N (5). The GalXM-N was then separated into two fractions by concanavalin A (ConA) affinity chromatography. The ConA column filtrate was designated as GalXM, and the eluate was the MP (19). Each of these fractions was lyophilized and then reconstituted in physiological saline to contain 2 mg/ml (wt/vol).

Antibodies. Three different antibody preparations were used for ID and immunoblots. One was a polyclonal antibody prepared against whole formaldehyde-killed *C. neoformans* yeast cells (5) and is referred to as anti-A1. Anti-A1 had an anticryptococcal antibody titer of 1/20,480 in an enzyme-linked immunosorbent assay. It was used undiluted for ID. An ammonium-sulfate-precipitated preparation of the rabbit polyclonal anticryptococcal serum was used for immunoblots.

The second polyclonal antibody, designated anti-MP, was prepared by immunizing New Zealand White rabbits with purified MP. A 5-ml portion of a mixture of equal parts CFA (Sigma Chemical Co., St. Louis, Mo.) and MP (20 μg/ml in physiological saline) were injected into each rabbit; 2 ml were given intramuscularly, 2 ml were injected intraperitoneally, and 1 ml was injected subcutaneously in the abdominal area. Three weeks later, the rabbits were boosted with 5 ml of a mixture of equal parts Freund incomplete adjuvant (Sigma) and MP (40 μg/ml in physiological saline) by injection of 2 ml intramuscularly, 2 ml intraperitoneally, and 1 ml intradermally spread among 10 sites along the back. At week 5, the rabbits were boosted a second time by intravenous injection of 10 μg of MP per ml in physiological saline. The rabbits were then boosted intravenously twice weekly 1, 2, and 3 weeks later with 15, 20, and 25 μg of MP, respectively. The rabbits were bled 3 days after the last injection, and the sera were collected and stored at −20°C. The enzyme-linked immunosorbent assay titer of the anti-MP antibody was 1/20,000.

The third antibody was a monoclonal anti-GXM antibody designated MAb 471. This antibody was produced by a hybridoma which was derived from mice immunized with serotype A polysaccharide. The procedures for immunization and preparation of hybridomas have been described (7). MAb 471 was reactive with serotypes A, B, C, and D in ID, whole-cell agglutination, and opsonophagocytic assays. The isotype of the anti-GXM antibody was immunoglobulin G1 (IgG1), and the stock concentration used in these studies was 3.5 mg of IgG per ml.

Double ID. Double ID gels were prepared on microscope slides by using 1% agarose (Pharmacia, Inc., Piscataway, N.J.) in Tris-Veronal (Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.) buffer (pH 8.6) (20). The anti-A1, anti-MP, and anti-GXM antibodies were used undiluted. The concentration of CneF used was 2 mg of carbohydrate per ml, and the other antigen preparations were assayed at a concentration of 2 mg/ml (wt/vol). The ID slides were incubated in moist chambers for 24 h before assessment for precipitation bands.

Gel electrophoresis. The cryptococcal antigens were analyzed by anodic polyacrylamide gel electrophoresis through 10% nonreducing polyacrylamide slab gels in a Hoeffer SE500 electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, Calif.). All reagents were obtained from Sigma. Samples were mixed with glycerol and bromophenol blue to give final concentrations of 10% (vol/vol) and 0.001%, respectively, and then a 30-μl sample containing 50 μg of the designated antigen preparation was layered onto duplicate gels. The gels were electrophoresed at a 90-mA constant current with 0.05 M Tris–0.04 M glycine (pH 8.3) as the buffer. When the dye front was within 1 cm of the bottom of the gel, the slabs were removed from the electrophoresis apparatus, and the gels were fixed and stained for carbohydrate with either PAS (16, 22) or a silver stain (18).

Immunoblotting. Polyacrylamide slab gels were electrophoretically transferred onto nitrocellulose paper in 25 mM Tris–192 mM glycine buffer (pH 8.3) containing 10% (vol/vol) methanol at 1 A for 1 h. The nitrocellulose blots were incubated for 1 h at room temperature in 1% bovine serum albumin in Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl [pH 7.4]) to block nonspecific binding sites. The blots were then placed in one of the following for 1 h at room temperature: (i) a 1/50 (80 μg of protein per ml) dilution of monoclonal anti-GXM antibody in TBS plus 0.05% (vol/vol) Tween 20 (TBST), (ii) TBST, (iii) a 1/40 dilution of polyclonal rabbit anticryptococcal antibody in TBST, or (iv) normal rabbit serum diluted 1/40 in TBST. After being washed three times for 10 min with TBST, the blots which had been reacted with TBST or the monoclonal antibody were incubated in 25 ml of a 1/500 dilution of biotin-labeled goat IgG anti-mouse IgG (1.4 μg/ml; Sigma) in TBST for 1 h at room temperature and then washed three times with TBST. Next, the blots were incubated in a 1/1,000 dilution of streptavidin-horseradish peroxidase (0.5 μg/ml; Sigma) in TBST for 1 h at room temperature and again washed three times. Finally, the blots were developed with the substrate-chromogen consisting of 0.03% hydrogen peroxide in 1.67 mM 3,3-diaminobenzidine tetrahydrochloride–50 mM Tris (pH 7.6). After sufficient color had developed, the reactions were stopped by rinsing the blots with distilled water. When normal rabbit serum or rabbit anticryptococcal antibody was used as the primary reagent, biotin-labeled goat IgG anti-rabbit IgG (Vector Laboratories, Burlingame, Calif.) diluted 1:1,000 (1.5 μg/ml) was used as the secondary antibody, followed by incubation in a 1/1,000 dilution of avidin D (Vector; 10 μg/ml), a 1/1,000 dilution of biotin-horseradish peroxidase (Vector; 5 μg/ml), and finally substrate-chromogen. All reagents were diluted in TBST, three washes were applied between each reagent, and the incubation times and temperatures were the same as those indicated above for blots treated with the monoclonal antibody.

Induction and detection of DTH. Inbred CBA/J female mice were purchased from Jackson Laboratory, Bar Harbor, Maine, and used between the ages of 7 and 10 weeks. Mice were immunized either by the injection of 0.1 ml of an emulsion of CneF-184A antigen in CFA (CneF-CFA) at each of two sites at the base of the tail (15) or by injecting 10^7 heat-killed NIH 371A cells in CFA at two subcutaneous sites. Six days after CneF-CFA immunization or 8 days after injection with heat-killed cryptococci, five mice were footpad tested as previously described (3) with 30 μl of one of the cryptococcal antigen preparations at a concentration of 2 mg/ml. Immunizations using the two protocols were done on

staggered days, and the footpads of all groups were challenged on the same day. DTH-negative control groups in these studies were untreated mice which were footpad challenged with the appropriate antigen preparation.

Statistical analyses. Means, standard errors of the means, and unpaired Student's *t* tests were used to analyze the data.

RESULTS

Double ID gels. The results of reacting polyclonal anti-A1 antibody with the various cryptococcal antigen preparations are shown in Fig. 1A. Two precipitation bands formed between the polyclonal anti-A1 antibody well (center well) and the wells containing CneF (wells 1, 3, and 5). A single precipitation line was seen between the antibody and the GXM (well 2). The GXM band fused with one of the two bands formed with CneF, indicating that the wide heavy band close to the CneF well represented the anti-GXM antibody reaction with GXM. This interpretation was confirmed by the fact that the monoclonal anti-GXM antibody (Fig. 1B, center well) reacted only with the GXM fraction (well 2) and CneF (wells 1, 3, and 5), resulting in the heavy bands near the GXM antigen well (well 2) and the CneF wells (wells 1, 3, and 5). The fusion of the bands near the GXM well (well 2) with the bands near the CneF wells (wells 1 and

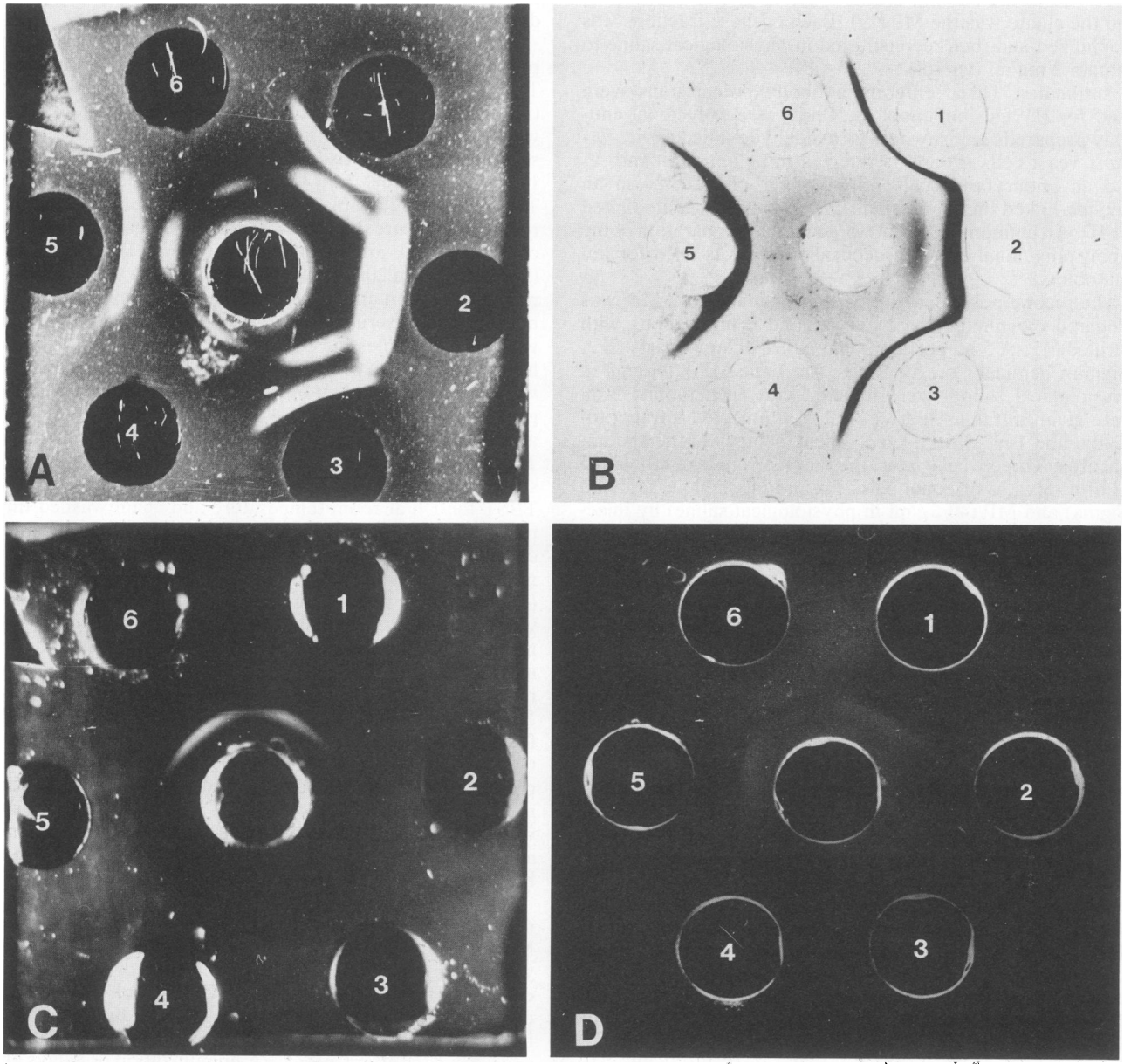

FIG. 1. Double ID gels showing precipitation reactions. (A) Rabbit polyclonal anticryptococcal antibody (center well) with CneF (wells 1, 3, and 5), GXM (well 2), GalXM-N (well 4), and MP (well 6). (B) Monoclonal anti-GXM antibody (center well) with CneF (wells 1, 3, and 5), GXM (well 2), GalXM (well 4), and MP (well 6). (C) Rabbit anti-MP antibody (center well) with CneF (well 1), GXM (wells 2 and 4), and MP (well 6). Wells 3 and 5 were empty. (D) Rabbit anti-MP antibody (center well) with CneF (wells 1, 3, and 5), GXM (well 2), GalXM (well 4), and MP (well 6).

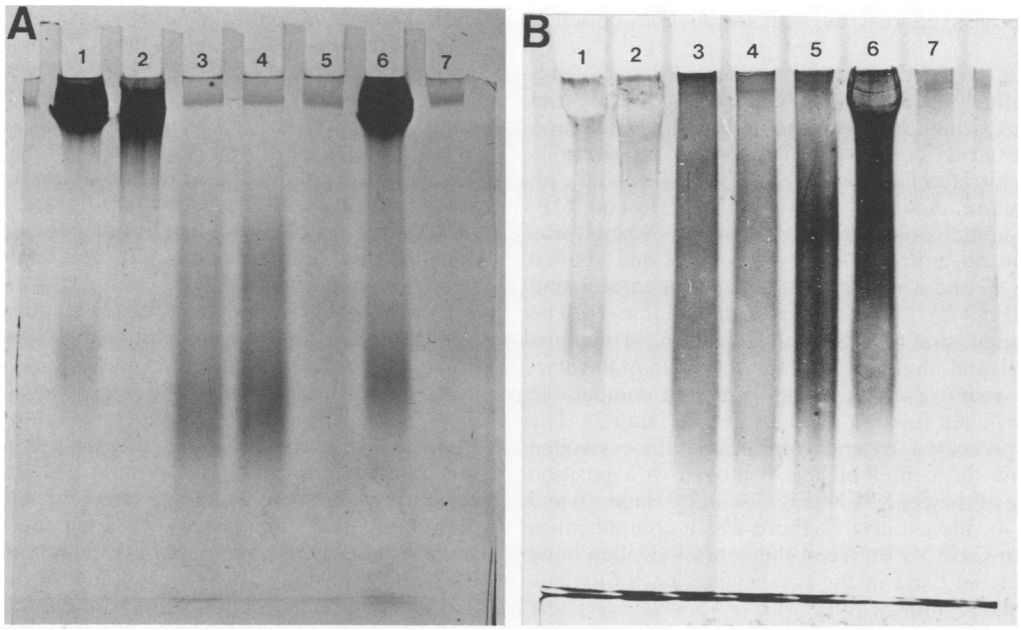

FIG. 2. PAS-stained polyacrylamide gel (A) and silver-stained gel (B) of an ethanol precipitate of the culture filtrate from *C. neoformans* NIH 371A (lane 1), CTAB precipitate of NIH 371A culture filtrate or GXM (lane 2), CTAB supernatant or GalXM-N (lane 3), ConA effluent or GalXM (lane 4), ConA eluate or MP (lane 5), and CneF-184A (lane 6). Lane 7 was empty. An interpretation of the results is given in Table 1.

3) indicate that the bands near the CneF were formed by the reaction of the anti-GXM antibody with GXM in CneF. The second band observed between the anti-GXM antibody and GXM wells (center well and well 2) was lost when the reagents were diluted. It is likely that this band was due to a low concentration of low-molecular-weight fragments of GXM which may have resulted from shearing of the GXM during the preparation.

A single band precipitated with the polyclonal anti-A1 antibody (Fig. 1A, center well) and GalXM-N (well 4) or MP (well 6). These bands were near the antibody well and formed a line of identity with the second of the two bands observed between the antibody well and the CneF wells (wells 1, 3, and 5). Since the GalXM fraction did not precipitate with the polyclonal anti-A1 antibody (data not shown), the bands near the antibody well in Fig. 1A were considered to represent the reactions between anti-MP antibodies and MP. This conclusion was supported by the fact that anti-MP antibody (Fig. 1C and D, center wells) gave a single band with CneF (Fig. 1C, well 1, and Fig. 1D, wells 1, 3, and 5) which was near the antibody well and which fused with the band between the anti-MP antibody and MP wells (Fig. 1C and D, wells 6). Furthermore, the monoclonal anti-GXM antibody (Fig. 1B, center well) did not react with MP (well 6) or form bands near the antibody well with CneF (wells 1, 3, and 5).

The GalXM fraction (wells 4) did not precipitate with the anti-GXM antibody (Fig. 1B, center well) or anti-MP antibody (Fig. 1D, center well). Moreover, the GalXM fraction did not react with the polyclonal anti-A1 antibody (results not shown). Several other rabbit polyclonal anticryptococcal sera, prepared in a manner similar to that used for anti-A1 sera and having anticryptococcal agglutination titers ranging from 1/1,280 to 1/5,120, showed precipitation patterns similar to those obtained with anti-A1 sera when reacted with the various antigen fractions and CneF (data not shown). None

TABLE 1. Interpretation of results shown in Fig. 2

Gel lane	Prepn[a]	Components thought present	Staining with:	
			PAS	Silver
1	EtOH prec, NIH 371A	GXM, GalXM, and MP	Summation of patterns in lanes 2, 4, and 5	Summation of patterns in lanes 2, 4, and 5
2	CTAB prec, NIH 371A	GXM	Upper, bulging, intense	Upper, bulging, intense
3	CTAB sup, NIH 371A	GalXM and MP	Summation of patterns in lanes 4 and 5	Summation of patterns in lanes 4 and 5
4	ConA effluent, NIH 371A	GalXM	Middle, swollen, moderate intensity	Middle, swollen, faintly stained
5	ConA eluate, NIH 371A	MP	Middle, very faint	Middle, intense
6	CneF-184A	GXM, GalXM, and MP	Similar to lane 1	Similar to lane 1

[a] EtOH, Ethanol; prec, precipitate; sup, supernatant.

of the antisera assayed reacted with the GalXM-enriched fraction.

Polyacrylamide gel electrophoresis of CneF-184A, ethanol-precipitated culture filtrate, GXM, GalXM-N, GalXM, and MP. CneF-184A, which has been shown to induce and elicit DTH responses, was compared with antigen preparations from a culture filtrate of *C. neoformans* NIH 371A, i.e., an ethanol precipitate, GXM, GalXM-N, GalXM, and MP. CneF-184A contained at least three components when it was electrophoresed on a 10% polyacrylamide gel and stained with PAS (16, 22) or a silver stain for carbohydrates and proteins (18) (Fig. 2A and B, lanes 6, and Table 1). The uppermost bulging band (Fig. 2A and B, lanes 6), which just entered the gel and thus was probably a high-molecular-weight and/or weakly charged component, ran comparable to the GXM-enriched fraction (Fig. 2A and B, lanes 2). The component represented by the somewhat diffuse swollen band just below the center of lane 6 moved to a position similar to those of the GalXM-N (Fig. 2A and B, lanes 3) and GalXM (lanes 4), indicating that there was a component in CneF similar to GalXM. Between the central swollen band and the top bulging band in the CneF lane (lanes 6) was a region that stained faintly pink with PAS (Fig. 2A) and intensely dark with silver (Fig. 2B). Similar staining patterns were observed with PAS (Fig. 2A) and silver (Fig. 2B), respectively, in the lanes containing GalXM-N (lanes 3) and MP (lanes 5). These results suggest that the CneF contained an MP-like component in addition to GXM and GalXM. There was also evidence of a fast-migrating fraction at the dye front (Fig. 2A and B, lanes 6). The only band which stained with amido black on identical gels was the fast-migrating band in lane 6 (results not shown). The bands at the tops of lanes 3, 4, and 5 in Fig. 2A and 2B are considered to be artifacts, since they are similar to the band at the top of lane 7, which was an empty lane.

The ethanol precipitate of the isolate NIH 371A culture filtrate separated into two visible fractions (Fig. 2A and B, lanes 1), one similar to the CTAB precipitate, GXM (lanes 2), and the other similar to the diffuse bands of the CTAB supernatant, GalXM-N (lanes 3), and the ConA effluent, GalXM (lanes 4). With the silver stain (Fig. 2B), the region between the top and central bands in the lane containing the ethanol precipitate of the NIH 371A culture filtrate (lane 1) showed some dark staining indicative of MP, whereas the same region on the PAS-stained gel did not stain. The absence of color on the PAS-stained gel may have resulted from having a low concentration of MP in the ethanol precipitate of the NIH 371A culture filtrate. The lower stain band in lane 6, containing CneF, was not evident in the NIH 371A culture filtrate (lanes 1) in gels stained with PAS, silver, or amido black.

Immunoblots. Electrophoretic gels identical to the stained gels discussed above were blotted to nitrocellulose membranes and reacted with either monoclonal anti-GXM antibody or polyclonal rabbit anticryptococcal antibody. The blot developed with the monoclonal anti-GXM antibody (Fig. 3A) showed a band at the top of lanes 1 (ethanol precipitate of isolate NIH 371A culture filtrate) and 2 (GXM-enriched fraction). We had two concerns with regard to this blot. First, the staining was weak on the two bands that stained, and second, the uppermost band in lane 6, containing CneF, did not stain, yet we knew from double ID gels that CneF contained a component that precipitated with the monoclonal anti-GXM antibody. Since we suspected that the GXM had not transferred to the nitrocellulose membrane, we did a PAS stain of the same acrylamide gel that had been blotted and found that the only spots that stained were the uppermost bands in lanes 1, 2, and 6 (results not shown). The diffuse staining center bands were completely absent in all lanes on the PAS-stained blotted gels, indicating there was satisfactory transfer of those components to the blots. There was no evidence of nonspecific staining in that a control blot similar to the one discussed above but treated with TBST instead of monoclonal anti-GXM antibody displayed no bands (results not shown).

The immunoblot reacted with the polyclonal rabbit anticryptococcal antibody is shown in Fig. 3B. Although this antibody was shown by ID to contain antibodies that precipitate GXM, there was no staining of the uppermost bands in lanes 1, 2, and 6, which had been shown by PAS and silver

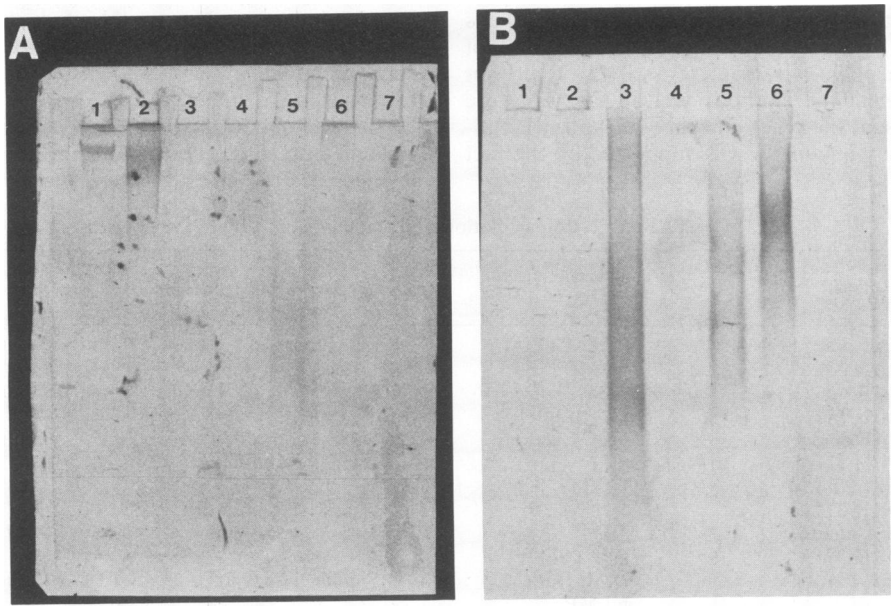

FIG. 3. Immunoblots of gels identical to those shown in Fig. 2A and B probed with monoclonal anti-GXM antibody (A) and rabbit polyclonal anticryptococcal antibody (B).

staining to have polysaccharide material at that spot in the gel. However, the polyclonal antibody did react with the broad smear in lanes 3 (GalXM-N), 5 (MP-enriched fraction), and 6 (CneF), indicating that the MP component was the serologically reactive material. This deduction was supported by the fact that the GalXM-enriched fraction (lane 4) showed no staining with the polyclonal antibody. We also noted that the MP isolated from isolate NIH 371A moved further into the gel than did the putative MP component of CneF (compare stained portions of Fig. 3B, lanes 5 and 6). An identical blot to that shown in Fig. 3B but reacted with normal rabbit serum instead of the rabbit anticryptococcal antibody did not have any areas stained (results not shown), indicating that the anti-MP antibody specifically detected cryptococcal antigens.

Elicitation of DTH with the various antigen preparations. To discern which of the antigenic components might be responsible for eliciting the DTH response, we immunized mice with either CneF-CFA or heat-killed *C. neoformans* NIH 371A cells in CFA and 6 and 8 days later, respectively, tested footpads with the various culture filtrate fractions or CneF. Unimmunized control groups were also included in the study. Both immunized groups of mice were sensitized to *C. neoformans* antigens, as indicated by the strongly positive responses elicited by CneF-184A compared with the responses to CneF-184A in the unimmunized controls ($P < 0.0005$) (Fig. 4). The ethanol-precipitated material from the NIH 371A culture filtrate also elicited positive footpad reactions in both immunized groups of animals (compared with the unimmunized controls; $P < 0.0005$); however, the reactions were not as high as those elicited by CneF-184A. The CTAB precipitate of the ethanol-precipitated material, GXM, stimulated very weak but significantly positive reactions in the CneF-CFA-immunized group (compared with the unimmunized controls; $P < 0.005$) and insignificant reactions in the NIH 371A-immunized group ($P < 0.25$). On the other hand, the CTAB supernatant or GalXM-N elicited strongly positive footpad swelling in immunized mice compared with the responses in the control animals ($P < 0.001$). The component of the GalXM-N which has an affinity for ConA, i.e. MP, stimulated positive footpad responses in the immunized mice; however, the fraction of GalXM-N which does not bind to ConA or the GalXM-enriched fraction triggered only weak DTH responses in the group immunized with NIH 371A cells ($P < 0.05$) and insignificant reactivity in the CneF-184A-immunized mice ($P < 0.25$).

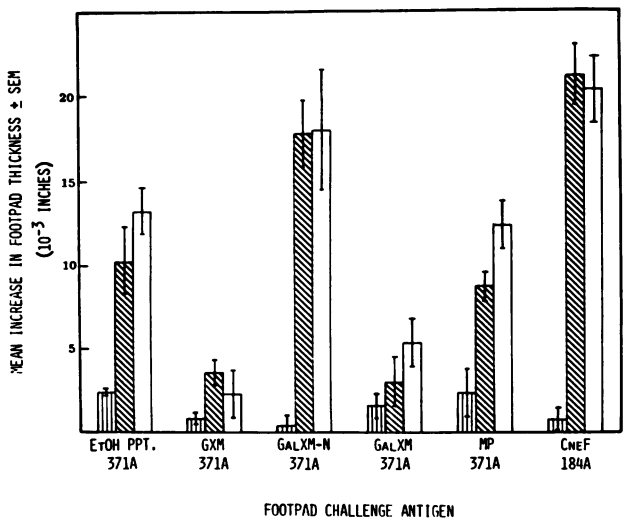

FIG. 4. DTH reactions of untreated mice (negative controls) (▮), mice immunized with CneF-184A in CFA (▨), and mice immunized with heat-killed *C. neoformans* NIH 371A (□) after footpad challenge with the ethanol precipitate of a culture filtrate from *C. neoformans* NIH 371A, with NIH 371A GXM, GalXM-N, GalXM, or MP, or with CneF-184A. SEM, Standard error of the mean.

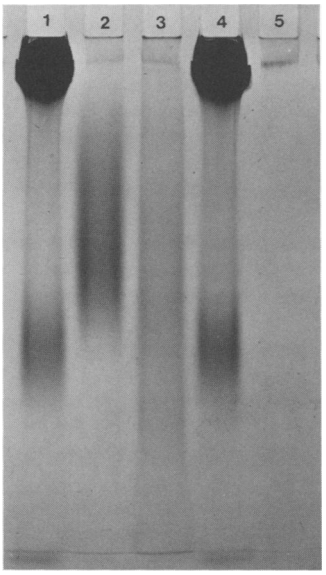

FIG. 5. PAS stain of a 10% polyacrylamide electrophoretic gel with CneF (lanes 1 and 4), GalXM isolated from the acapsular mutant J67 (lane 2), and MP isolated from J67 (lane 3). Lane 5 was empty.

Comparison of CneF with GalXM and MP isolated from J67, an acapsular mutant. The data discussed above showed the MP-enriched component elicited stronger DTH responses than did the GalXM-enriched or GXM-enriched fraction (Fig. 4). To confirm that the MP is the main component recognized in the DTH response, GalXM and MP fractions from J67, a *C. neoformans* mutant which does not make GXM (9, 11), were compared with CneF in polyacrylamide electrophoretic gels stained with PAS and silver and in their abilities to elicit DTH responses. A PAS-stained gel is shown in Fig. 5. As expected, the J67 fractions were free of GXM, which is indicated by the absence of the upper dense band. This was confirmed with the silver stain (results not shown). The J67 GalXM (lane 2) did not run as fast and showed more smearing and trailing than did the putative GalXM in the unfractionated CneF-184A (center portion of gel, lanes 1 and 4). The J67 MP fraction (lane 3) stained more intensely with PAS than did the NIH 371A MP (Fig. 2A, lane 5). On the silver-stained gel (not shown), the J67 MP displayed an intense dark smear over the same area of the gel that stained with PAS.

When J67 GalXM and MP fractions were used to elicit footpad responses in mice immunized with either CneF-184A or heat-killed *C. neoformans* NIH 371A, the J67 MP-enriched fraction elicited strong responses in both groups of immunized mice ($P < 0.0005$ when compared with the negative controls), but the responses to the J67 GalXM fraction were not significantly different from those of the negative controls (Fig. 6).

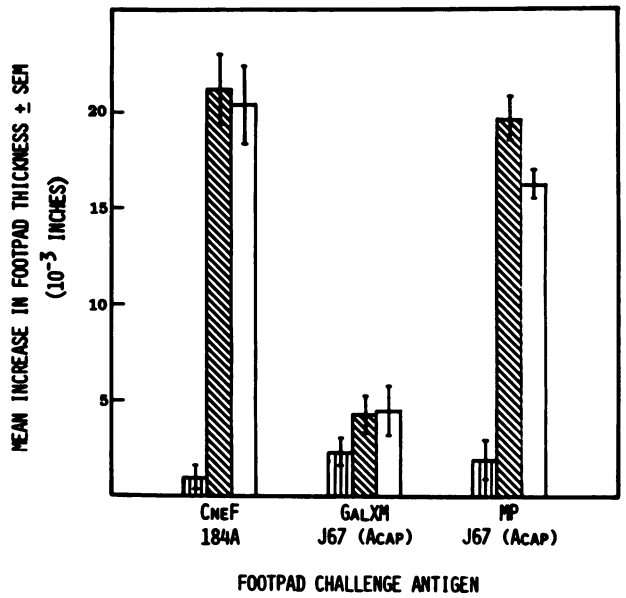

FIG. 6. DTH reactions in unimmunized mice (▥), mice immunized with CneF-184A in CFA (▨), and mice immunized with heat-killed *C. neoformans* NIH 371A (□) after footpad challenge with CneF-184A, GalXM isolated from the acapsular (Acap) mutant J67, or MP from J67. SEM, Standard error of the mean.

DISCUSSION

Clearly, previous studies show that CneF specifically detects CMI responses to *C. neoformans* (14, 16). However, CneF is not a homogeneous material but is composed of at least three and possibly more constituents, as was demonstrated by the electrophoretic patterns on PAS-stained (Fig. 2A, lane 6, and Fig. 5, lanes 1 and 4) and silver-stained (Fig. 2B, lane 6) 10% polyacrylamide gels. By comparing the components in CneF-184A with the relatively well-characterized heteroglycans and glycoprotein, GXM, GalXM, and MP, isolated from culture filtrates of *C. neoformans* NIH 371A, we found that CneF-184A contained similar components. Moreover, GalXM and MP prepared from an acapsular mutant, J67, which does not produce GXM, had migration patterns in 10% gels comparable to those of two of the CneF-184A constituents (Fig. 5).

When polyspecific and monospecific rabbit polyclonal antisera and a mouse monoclonal antibody were reacted with CneF in ID gels, only two serologically reactive components were identifiable in CneF-184A (Fig. 1A). One component was considered to be GXM, based on the observation that the band it formed with anti-GXM antibody fused with the precipitation band formed by the monoclonal anti-GXM antibody and the GXM-enriched fraction (Fig. 1B), and the other component was regarded as MP because of lines of identity with MP in ID (Fig. 1C and D). None of the anticryptococcal antibodies used in this investigation precipitated GalXM-enriched fractions. This finding is in agreement with the report of Reiss et al. (17), who indicated that sera from cryptococcosis patients contain antibodies to the mannose-enriched portion, i.e., MP of the GalXM complex (GalXM-N), but do not contain antibodies which react in a definitive manner with the galactoxylo component, GalXM.

When CneF and the various heteroglycan and glycoprotein fractions were used as footpad antigens in mice sensitized either to CneF-184A or to whole heat-killed *C. neoformans* NIH 371A yeast cells, the serotype-specific, high- molecular-weight, CTAB-precipitable polysaccharide or GXM-enriched fraction which was slow in migrating on 10% gels elicited weak footpad reactions (Fig. 4). Murphy and Pahlavan (16) showed that the slow-migrating polysaccharide fractions on 3% polyacrylamide electrophoretic gels of CneF prepared with three isolates of *C. neoformans* with different serotypes did not elicit significant skin test responses in guinea pigs sensitized with viable cryptococci of the homologous serotype. These combined results from our laboratory indicate that GXM is only weakly active as a reagent for eliciting a DTH response in mice sensitized to either cryptococci or soluble cryptococcal antigen. Moreover, our findings are consistent with those of Bennett (1), who used a different cryptococcal antigen preparation in a guinea pig model. The skin test antigen used by Bennett was prepared by extracting viable *C. neoformans* cells with 11.6 M urea in 0.1 M borate buffer (pH 9). The high-molecular-weight component of the urea extract antigen which was the serologically active polysaccharide did not elicit positive skin test reactions in sensitized guinea pigs (1).

In contrast to the GXM, we found that the CTAB-soluble material, GalXM-N, which contained GalXM and MP, generated strong footpad responses in sensitized animals (Fig. 4). However, when the GalXM-N was separated into GalXM- and MP-enriched fractions, most of the footpad reactivity was associated with the MP-enriched fraction (Fig. 4), suggesting that the MP was the predominant moiety recognized by the CMI response induced by either CneF-184A or whole blastoconidia of isolate NIH 371A. Similar results were obtained with a culture filtrate from an acapsular mutant of *C. neoformans* that does not produce GXM. The GalXM- and MP-enriched fractions from the mutant were prepared without CTAB and were free of GXM, as demonstrated by electrophoretic patterns on 10% gels (Fig. 5). The MP-enriched fraction of the acapsular mutant elicited strong DTH reactions, whereas the GalXM-enriched fraction had no effect (Fig. 6), thus confirming that the MP is the primary component recognized in the cryptococcal CMI response. These results are consistent with those of Murphy and Pahlavan (16), who showed that the fast-moving glycoproteinlike material isolated by electrophoresis of CneF on 3% polyacrylamide gels elicited strongly positive skin tests in cryptococcus-sensitized guinea pigs. The glycoproteinlike component from the 3% gels is comparable to the GalXM-N used in this study in that it separates on 10% gels into two fractions equivalent to the intermediate- and fast-moving components (unpublished data). The MP used in this study had physical, chemical, and functional characteristics which are similar to the active skin test component that Bennett prepared from the urea extract antigen (1). The biologically active material of the antigen used by Bennett was a low-molecular-weight (approximately 10,000) proteinaceous component which migrated anodally in a single band on 7% disc gels (1). Bennett (1) also noted faint staining of the migrating band when electrophoretic gels were stained for carbohydrate with PAS, suggesting there may be some polysaccharide in his skin-test-active material.

In the present study, MP prepared from an acapsular mutant, J67, of a serotype D isolate of *C. neoformans* (B3501) elicited similar levels of DTH responses as did antigen prepared from a serotype A isolate (184A) in mice sensitized with the serotype A antigen (Fig. 6). Thus, the MP fractions from the various serotypes and the acapsular mutant have at least one common epitope that is recognized by the CMI system. This concept is supported by the study

of Murphy and Pahlavan (16), who demonstrated that the glycoproteinlike antigen from serotype A, B, or C isolates of *C. neoformans* elicited equivalent DTH reactions in guinea pigs sensitized with a serotype A isolate. There is considerable serological cross-reactivity between the four serotypes of cryptococci (2, 8, 10, 21), and the MP may be responsible, in part, for this cross-reactivity. The MP of *C. neoformans* apparently stimulates a good humoral response, since Reiss et al. (17) have shown that the antibody titers against the MP, as measured with an indirect enzyme immunoassay, are higher than titers obtained by using other cryptococcal antigens such as GalXM.

In summary, the cryptococcal polysaccharide GXM, which is the serotype-specific antigen, does not appear to be the primary recognition component in the cryptococcal CMI response. In contrast, the protein-rich moiety of cryptococcal antigen preparations, MP, is the immunodominant component for the CMI response. Furthermore, the MP fractions isolated from the various cryptococcal serotypes and the acapsular mutant are recognized equally by the CMI response and thus appear to have a common epitope(s).

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