Contribution of Epitope Specificity to the Binding of Monoclonal Antibodies to the Capsule of *Cryptococcus neoformans* and the Soluble Form of Its Major Polysaccharide, Glucuronoxylomannan

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Cryptococcus neoformans* is a pathogenic yeast that may produce a life-threatening meningitis, particularly in immunocompromised individuals such as people with AIDS. *C. neoformans* is surrounded by a polysaccharide capsule that is primarily composed of glucuronoxylomannan (GXM). GXM is a linear (1→3)-α-D-mannopyranosyl with β-D-xylopyranosyl, β-D-glucopyranosyluronic acid, and 6-O-acetyl substituents. *C. neoformans* is divided into four primary serotypes (A, B, C, and D) and eight chemotypes (2, 6, 24). Differences in xylose substitution on the mannose backbone and O acetylation are the primary structural determinants of capsular serotype.

Antibodies are important components of host resistance to encapsulated microorganisms, including *C. neoformans*. Anti-GXM antibodies are directly opsonic for phagocytosis of *C. neoformans* in vitro (13, 20, 25), initiate the classical pathway leading to accelerated deposition of opsonic fragments of C3 onto the capsule (12), and are protective in murine models of cryptococcosis (9, 16, 19). A protein conjugate GXM vaccine has been shown to induce high levels of anti-GXM antibodies and is reactive with soluble GXM. Production of a capsular quellung-type reaction is one means to assess antibody-capsule interaction (18). Other immunochromical assays that can measure binding of antibody to the cryptococcal capsule include whole-cell agglutination and immunofluorescence. It is also possible to assay the interaction of antibody with soluble GXM. Available procedures for assessment of antibody-GXM interactions include enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation.

In our earlier studies of the interactions between anti-GXM monoclonal antibodies (MAbs) and the cryptococcal capsule, we found that antibodies having different epitope specificities produced distinct capsular quellung-type reactions (15). Importantantly, the ability to produce a particular capsular reaction was associated with biological consequences of the antibody-capsule interaction. One reaction, termed rim, is associated with activation of the classical pathway, suppression of overall C3 deposition via the alternative pathway, potent opsonization for phagocytosis by macrophages, and protection in a murine model of cryptococcosis. A second capsular reaction, termed puffy, is associated with a failure to initiate the classical pathway, no impact on activation and binding of C3 via the alternative pathway, limited opsonic activity, and a failure to produce protection in a murine model of cryptococcosis. The ability of an antibody to produce a particular capsular reaction is determined by the epitope specificity of the MAb and the serotype of the cryptococcal cell.

Production of a capsular quellung-type reaction is one means to assay antibody-capsule interaction (18). Other immunochromical assays that can measure binding of antibody to the cryptococcal capsule include whole-cell agglutination and immunofluorescence. It is also possible to assay the interaction of antibody with soluble GXM. Available procedures for assessment of antibody-GXM interactions include enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation.

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Given the close association between the capsule reaction and a variety of biological activities, including protection, we wanted to determine the extent to which immunochromical assays such as agglutination and ELISA, etc., are predictive of rim or puffy capsular reactions. In the present study, we examined the activities of two families of antibodies in several immunochromical assays. One group of antibodies, termed group II, is reactive with an epitope that is shared by GXMs of...
TABLE 1. Serotypes, chemotypes, and GXM structures of *C. neoformans* strains

| Strain   | Serotype | Chemotype | Structure reporter group composition<sup>a</sup> | M1 | M2 | M3 | M4 | M5 | M6 |
|----------|----------|-----------|-------------------------------------------------|----|----|----|----|----|----|
| MU-1     | A        |           |                                                 | 4  | 100|     |     |     |    |
| CN6      | A        |           |                                                 | 5  | 67 | 17 | 15 |    |    |
| 9375B    | D        |           |                                                 | 1  | 100|     |     |     |    |
| M0024    | D        |           |                                                 | 1  | 26 |     |     |     | 74 |

<sup>a</sup> Serotypes, chemotypes, and structure reporter groups are from reference 6 and R. Cherniak, personal communication.

<sup>b</sup> The structure reporter group composition is the percentage of repeating units in GXM with the indicated M group structural motif.

MATERIALS AND METHODS

*C. neoformans* and GXM. *C. neoformans* strains of serotype A (strains MU-1 and CN6) and serotype D (strains 9375B and M0024) were provided by R. Cherniak (Georgia State University, Atlanta). The chemotypes and structural components of these polysaccharides as defined by Cherniak et al. (6) are summarized in Table 1. Immunochromatographic analyses that examined binding of MAbs to whole cells were done with yeast cells that were grown under conditions that induce production of large capsules (11). Brieﬂy, *C. neoformans* cells were incubated in 20 ml of synthetic medium (5) supplemented with 24 mM sodium bicarbonate and 25 mM HEPES in Nunc T-25 culture flasks (Fisher Scientiﬁc, Pittsburgh, Pa.) with gentle rocking at 37°C for 5% CO<sub>2</sub>. After 4 days of growth, the cell density had reached approximately 10<sup>8</sup> cells/ml, at which time the cells were killed by addition of formaldehyde to the culture medium to a ﬁnal concentration of 1% followed by overnight incubation at room temperature. The formalin-killed cells were washed with phosphate-buffered saline (PBS) and stored at 4°C. GXM was isolated from supernatants of each strain. Yeast cells were grown for 4 days at 30°C on synthetic medium (5) and killed by treatment overnight with formaldehyde, and the GXM was isolated and puriﬁed by differential precipitation with ethanol and hexadecyltrimethylammonium bromide as described previously (4).

MAbs, Anti-GXM MAbs 471, 3C2, 302, and 1326 have been described previously (1, 10, 17, 22). The characteristics of these MAbs are summarized in Table 2. MAbs 3C2, 1326, and 302 were produced by in vitro culture in a Tecnomouse system (Integra Biosciences, Ijamsville, Md.). MAb 471 was puriﬁed from mouse ascites liquid by differential precipitation with caprylic acid and ammonium sulfate followed by immunoadfinity chromatography with a GXM-Sepharose column and afﬁnity chromatography with protein A. MAbs produced by in vitro culture were isolated by protein A afﬁnity chromatography. Concentrations of MAbs were determined by UV spectrophotometry, using an optical density of 1.43 at 280 nm for 1 mg of immunoglobulin G/ml (21).

**ELISA.** The ELISA was a variation of an assay described by Leinonen and Frasch for detection of antibody to meningococcal polysaccharide (14). Brieﬂy, microtiter plates were coated for 5 h with poly-l-lysine at a concentration of 5 µg/ml of PBS, washed with PBS, and incubated overnight with GXM (4 µg/ml of PBS). The plates were washed with PBS-Tween and blocked with a blocking solution (5% skim milk and 0.5% Tween 20 in PBS [pH 7.4]). After the blocking step, the plates were washed with blocking solution, and 100 µl of serial twofold dilutions of anti-GXM MAbs in blocking solution (starting with a 1:5,000 dilution) was added and incubated for 90 min at room temperature. After incubation with anti-GXM MAbs, the plates were washed with blocking solution and incubated for 90 min at room temperature with 100 µl of horseradish peroxidase-labeled secondary antibody speciﬁc for mouse IgG heavy chains (Southern Bio-technology, Inc., Birmingham, Ala.) at a 1:5,000 dilution in blocking solution.

After incubation with secondary antibody, the plates were washed with PBS-Tween and incubated for 30 min at room temperature with 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md). Stop solution (1 M H<sub>2</sub>PO<sub>4</sub>) was added to each well, and the absorbance was read at 450 nm.

**Competitive-inhibition ELISA.** The ELISA was modiﬁed to assess competitive inhibition of the binding of MAbs to puriﬁed GXM. Antigen (10 µg/ml) was incubated with MAb (12.5 ng/ml) was incubated for 1.5 h with serial twofold dilutions of puriﬁed GXM in blocking solution. After the incubation, the GXM-MAb solution was added to plates coated with MU-1 GXM, and MAb binding was assessed in the same manner as for the ELISA. The concentration of GXM that produced 50% inhibition was calculated from the inhibition curves by using a sigmoidal Hill four-parameter curve-ﬁt that was calculated with the assistance of SigmaPlot (SPSS, Inc., Chicago, Ill.).

**Quantitative precipitin assay.** A ﬁxed amount of MAb (200 µg) was mixed with various amounts of GXM (0 to 250 µg) in PBS containing 0.04% sodium azide in a ﬁnal reaction volume of 0.6 ml. The tubes were incubated for 2 h at room temperature and then for 48 h at 4°C. The precipitate was collected by centrifugation, washed twice with cold PBS, and dissolved in 0.75 ml of 0.1 N NaOH. The amount of antibody in each precipitate was determined by UV spectrophotometry.

**Capsular (quelling) reaction.** Differential-interference contrast imaging of capsular quelling-type reactions was done as described by MacGill et al. (15). Brieﬂy, *C. neoformans* cells (5 × 10<sup>5</sup>) were mixed with MAbs (50 µg/ml of PBS) in a 50-µl reaction volume and incubated for 5 min at 37°C. The cell suspensions were transferred to microscope slides, and capsular reactions were examined with a Nikon Eclipse E800 microscope with a 100× oil immersion objective by using differential-interference contrast microscopy. Digital images were captured with a SPOT RT color digital camera (Diagnostic Instruments, Inc., Sterling, Mich.) that was controlled by SimplePCI software (Compix Inc., Cranberry Township, Pa.). All image analysis and processing were done with SimplePCI. Capsular reactions were classiﬁed as rim or puffy (15). The rim pattern is characterized by a sharp increase in the optical gradient at the capsular edge followed directly by a decrease in the optical gradient. The hallmarks of the puffy pattern are an increase in the optical gradient at the capsular surface and the absence of the immediate decrease that is characteristic of the rim pattern.

**Agglutination assay.** Serial twofold dilutions of MAbs (starting at 400 µg/ml) were mixed with 8 × 10<sup>6</sup> yeast cells in a 500-µl reaction volume in PBS. The mixture was incubated for 2 h at 37°C and overnight at 4°C. The agglutination patterns of cells that had settled to the bottom of the tubes were recorded on a scale of 0 (no agglutination) to +4 (23). The highest dilution of antibody producing an unambiguous agglutination (+4; smooth mat with edges somewhat ragged) was reported as the end point agglutination titre. Statistical analysis of the abilities of group II and group IV MAbs to agglutinate cells of serotypes A and D was done by the Mann-Whitney rank sum test.

**Flow cytometry.** MAbs were coupled to Alexa Fluor 488 by using an Alexa Fluor 488 protein labeling kit (Molecular Probes, Eugene, Oreg.) according to the manufacturer’s directions. Yeast cells (5 × 10<sup>5</sup> cells/ml in PBS) were incubated with labeled MAbs (0.5 µg/ml) for 30 min at room temperature, washed twice with PBS, and resuspended in PBS, and antibody binding was assessed by flow cytometry. Ten thousand cells per sample were counted, and data are reported as mean fluorescence intensity.
RESULTS

Binding of MAbs to whole yeast cells. An initial experiment determined the capsular reaction of group II (anti-serotype A, B, C, and D) and group IV (anti-serotype A and D) antibodies. Our previous study found that group II antibodies produced a rim pattern with cells of serotype A and a puffy pattern with cells of serotype D. In contrast, the group IV MAb 302 produced a puffy pattern with cells of both the serotype A and serotype D strains. The results (Fig. 1) confirmed this initial observation and showed that similar reactions also occur with additional strains and that the reactivity of MAb 302 is identical to that of MAb 1326, an antibody that has a serotype specificity characteristic of group IV. Serotype D strain 9375B failed to produce a large capsule under capsule induction conditions. As a result, capsule reactions done with 9375B were equivocal.

Agglutination titers were determined for group II and IV MAbs and cells of serotypes A and D. The results (Table 3) showed that group II MAbs produced high agglutination titers with cells of both serotypes A and D ($P > 0.05$). Since the group II MAbs produced a rim pattern with cells of serotype A and a puffy pattern with cells of serotype D, this result indicates that reactivity as shown by whole-cell agglutination is not predictive of the capsule reaction. MAbs of group IV produced high agglutination titers with cells of serotype D and lower titers with cells of serotype A ($P = 0.03$). As with the group II antibodies, there was a dissociation between the qualitative capsular reaction, where both group IV antibodies produced a puffy pattern with cells of both serotypes, and the agglutination reaction, where titers were generally higher with cells of serotype D.

Binding of MAbs to whole cryptococci was further examined by use of immunofluorescence. Each MAb was directly labeled with a fluorescent tag, and their binding to whole cryptococci was visualized. The results showed that MAb 3C2 produced a rim pattern, whereas MAb 302 produced a puffy pattern, as expected. These results confirmed the specificity of the MAbs for their respective serotypes.

![FIG. 1. Capsule reactions produced by binding of group II (3C2 and 471) or group IV (1326 or 302) MAbs to serotype A or serotype D cryptococci. One capsular reaction was the rim pattern (e.g., with MAb 3C2 and cells of serotype A), which is characterized by a precipitous increase in the refractive index at the capsular edge followed directly by a decrease in the refractive index. The second capsular reaction was the puffy pattern (e.g., with MAb 302 and cells of serotype A), which is characterized by a gradual change in refractive index across the capsule.](image)

| Group | MAb  | Serotype A titer | Serotype D titer |
|-------|------|-----------------|-----------------|
|       |      | MU-1 | CN6 | 9375B | M0024 |
| II    | 3C2  | 1/1,024 | 1/2,048 | 1/8,192 | 1/2,048 |
|       | 471  | 1/512  | 1/1,024 | 1/1,024 | 1/1,024 |
| IV    | 1326 | 1/1   | 1/64  | 1/512  | 1/256  |
|       | 302  | 1/4   | 1/64  | 1/1,024 | 1/128  |

*Titers are reported as the highest dilution of MAb (initial concentration of 400 μg/ml) that produced a 2+ agglutination.*
with Alexa Fluor 488, and binding was quantitatively assessed by flow cytometry. The antibodies showed various labeling efficiencies; consequently, a quantitative comparison of binding between antibodies is not possible. However, a relative assessment of the binding activity of a given labeled MAb for cells of various strains was not compromised by variability in labeling efficiency. The results (Table 4) showed that production of the rim capsular reaction, i.e., group II antibodies and serotype A cells, was associated with a marked reduction in binding of antibody as shown by flow cytometry. In addition, MAb 1326 showed limited binding to cells of one strain of serotype A (MU-1) but substantial binding to cells of another strain of serotype A having a different chemotype (CN6), a result that parallels the reactivity of MAb 1326 with these yeasts in the agglutination assay (Table 3).

**Binding of MAb 1326 to purified GXM.** The quantitative precipitin curve is the classic means to assess binding of antibodies to soluble antigens. Group II MAbs produced readily measurable precipitation of polysaccharides of both serotypes A and D (Fig. 2). Maximum precipitation occurred with lower concentrations of serotype A GXM than serotype D GXM. The group II MAb 302 also produced readily measurable precipitation of GXM from both serotypes. In contrast, MAb 1326 was a good precipitating antibody for GXM of serotype D but produced no detectable precipitation of serotype A GXM.

ELISA analysis was done with each MAb by using microtiter plates coated with GXM that had been isolated from culture supernatant fluids (Fig. 3). Group II MAbs 3C2 and 471 showed similar titration curves with all polysaccharides. Group IV MAb 1326 showed good binding to plates coated with

**TABLE 4. Binding of Alexa Fluor 488-labeled group II and group IV antibodies to encapsulated serotype A and serotype D *C. neoformans***

| Group | MAb   | Binding* to:          |
|-------|-------|-----------------------|
|       |       | Serotype A cells | Serotype D cells |
|       |       | MU-1 | CN6 | 9375B | M0024 |
| II    | 3C2   | 55   | 53  | 460   | 110   |
|       | 471   | 26   | 65  | 140   | 94    |
| IVb   | 1326  | 32   | 190 | 230   | 130   |

* Binding of Alexa Fluor 488-labeled MAb was assessed by flow cytometry. Data are reported as mean fluorescence intensity for 10,000 cells.
* Immuno fluorescence data could not be obtained for MAb 302 because the antibody consistently failed to provide satisfactory labeling with fluorescein isothiocyanate.

**FIG. 2. Quantitative precipitin reactions between group II (3C2 and 471) and group IV (302 and 1326) MAbs and soluble GXM of serotypes A (MU-1 and CN6) and D (9375B and M0024).**
serotype A MU-1 GXM and serotype D 9375B but very limited binding to serotype A CN6 GXM. Surprisingly, MAb 302 showed good binding to one GXM of serotype A and serotype D and little or no binding to another GXM of serotypes A and D.

Since the ability of MAb 302 to bind to one GXM of serotypes A and D but not to another GXM of each serotype was somewhat unexpected, we used a competitive-inhibition ELISA to confirm the reactivity patterns of MAb 302. The results showed that serotype A MU-1 and serotype D 9375B were potent inhibitors of the binding of MAb 302 to MU-1 GXM in the solid phase, with 0.11 and 0.029 μg/ml, respectively, producing 50% inhibition. In contrast, serotype A CN6 and serotype D M0024 GXM required exceptionally high concentrations (13 and 14 μg/ml, respectively) to produce 50% inhibition of binding.

DISCUSSION

The overall goal of our study was to assess the reactivities of two families of anti-GXM MAbs with the cryptococcal capsule and soluble GXM across a spectrum of immunochemical assays. Such an analysis is important because anti-GXM antibodies have a variety of biological activities that correlate with capsular quellung-type reactions, and identification of immunochemical assays that are predictive of the capsular reaction would aid efforts aimed at active or passive immunization for control or prevention of infection. Our study has four primary findings. First, the capsular reaction is a qualitative consequence of binding of antibody to the capsule that cannot be reliably predicted on the basis of immunochemical assays. Second, assessment of antibody activity in one assay is not necessarily predictive of reactivity in another assay. Third, there is a disconnect between results from assays that examine binding of antibody to the capsule versus soluble GXM that suggests differences in antigenic structure between capsular and soluble GXM. Finally, the ability of at least one MAb to react with soluble GXM is blocked by the presence of the M6 structure reporter group in soluble GXM.

Our previous studies found that the ability of a MAb to produce a rim or puffy capsular reaction was independent of antibody concentration (15). In the present study, we demonstrate that the ability of an antibody to produce the rim or puffy pattern is also independent of antibody titer as measured by several immunochemical assays. The best example is found in a comparison of agglutination titers produced by group II antibodies. Both MAbs 3C2 and 471 produced similar agglu-
tination titers with cells of serotypes A and D; however, both antibodies produced prominent rim patterns with cells of serotype A, little or no capsular reaction with serotype D strain 9375B, and a puffy pattern with cells of serotype D strain M0024. In another example, group IV MAbs 1326 and 302 produced prominent capsular reactions with cells of serotype A strain MU-1 but had little ability to agglutinate the same cells. The ability of an antibody to produce the rim pattern correlated with limited binding of antibody as shown by flow cytometry. This reduced binding of rim pattern MAbs is most likely due to exclusion of antibody from the capsular interior due to cross-linking of the capsular surface by antibody (15).

Variability in the reactivities of MAbs in different assay formats is illustrated by a comparison of results from quantitative precipitin assays and ELISAs. For example, MAb 1326 showed no precipitin activity with serotype A GXM but had a high ELISA titer with GXM from serotype A strain MU-1, which was indistinguishable from the titer found with serotype D GXM. In another example, MAb 302 produced readily measurable precipitation of GXM from serotype A strain CN6 and serotype D strain M0024 but had no reactivity in the ELISA format with plates coated with the same polysaccharides. Failure to react with these polysaccharides in the ELISA format is not due to a failure of the GXM to bind to the plates because other MAbs showed a high level of reactivity with these polysaccharides in the ELISA assay.

Our results raise the possibility that epitope expression by shed (soluble) GXM is different than epitope expression by capsular GXM. Such a disconnect is suggested by a comparison of results from agglutination assays and ELISAs. For example, MAb 1326 had little ability to agglutinate cells of serotype A strain MU-1; however, the ELISA titers were quite high and were indistinguishable from titers obtained with GXM from serotype D strains 9375B and M0024. Conversely, MAb 302 readily agglutinated cells of serotype A strain CN6 and serotype D strain M0024 but had no reactivity with GXM from these same cells in the ELISA format. It is possible that such differences are due solely to idiosyncrasies of the assay format rather than structural differences between capsular and soluble GXM. A rigorous assessment of this question will require removal of GXM from whole cells and a direct comparison of the structural and antigenic properties of soluble GXM isolated from culture filtrates and GXM isolated from whole cells.

The high titers of MAb 302 with GXM from serotype A strain MU-1 and serotype D strain 9375B in the ELISA and the complete lack of reactivity with GXM from serotype A strain CN6 and serotype D strain M0024 were unexpected. As a consequence, we confirmed this reactivity pattern by using a competitive inhibition assay. The pattern of the results was identical; GXM from serotype A strain MU-1 and serotype D strain 9375B were potent inhibitors of the ELISA, whereas GXM from serotype A strain CN6 and serotype D strain 9375B were 100- to 500-fold less effective inhibitors.

An explanation for the anomalous behavior of GXM from serotype A strain CN6 and serotype D strain 9375B most likely lies in the presence of the M6 structure reporter group in these polysaccharides. The M6 reporter group was absent in the polysaccharides for which MAb showed high reactivity in the ELISA. The M6 structure reporter group consists of three mannose residues in the backbone, one of which is substituted with β-D-glucopyranosyluronic acid, with two unsubstituted manno-pyranosyl residues (6). This creates a stretch in the backbone that lacks the β-D-xylopyranosyl residue found in the M1 and M2 structural motifs that characterize serotype D and serotype A GXMs. It is possible that this unsubstituted stretch induces a conformational change, e.g., a twist in the backbone, that prevents antibody binding. Alternatively, the presence of this stretch in a region adjacent to the antibody binding site may introduce a gap that reduced binding affinity or prevented bivalent binding by antibody with a consequent loss of avidity.

In summary, our study found that anti-GXM MAbs may have markedly different results when assessed by different immunological assays. As a consequence, considerable caution should be exercised in interpreting positive or negative results from assays for antibody to cryptococcal polysaccharide. This limitation is likely to be of greatest concern when dealing with MAbs. Ideally, an assessment of levels of anti-GXM antibodies should utilize multiple assay formats.

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