Monoclonal antibodies that react with the capsular polysaccharide, termed glucuronoxylomannan (GXM), of Cryptococcus neoformans have potential roles in the diagnosis, monitoring of disease progress, and immunotherapy of cryptococcosis. Monoclonal antibody 439 reacts with cryptococcal GXM of all four serotypes. A molecular model of the Fab fragment of monoclonal antibody 439 was constructed from the amino acid sequence and a template antibody molecule, Fab 4-4-20. A tryptophan is present on the surface between light chain CDR3 and heavy chain CDR3 in the putative binding site. This tryptophan residue proved to be a reporter group, and a fluorescence study of Fab 439 was performed to analyze the interaction between cryptococcal GXM and Fab 439. Binding of the polysaccharide enhanced the intrinsic fluorescence and caused a blue shift in the emission maximum, indicating that the environment of a tryptophan changes from a polar to less polar environment. This is consistent with the loss of water from the binding site caused by the binding of antigen. This interpretation was confirmed by acrylamide quenching, which showed that 1 less tryptophan was exposed to solvent in the Fab-GXM complex than in free Fab. Fluorescence titration was used to determine binding and dissociation constants (KD). The apparent KD values for serotypes A–C were approximately the same; the KD for serotype D GXM was 5–11-fold greater. De-O-acetylation of serotype A GXM produced a 31-fold increase in the KD, indicating that the O-acetyl groups are important, but not essential, for binding. Carboxylic groups appear to be essential for strong binding because the KD for carboxyl-reduced GXM was so large that it could not be determined.

Cryptococcus neoformans is an encapsulated yeast that causes a life-threatening meningitis. Infections occur most commonly in individuals who are immunocompromised by use of adrenal corticosteroids, occurrence of sarcoidosis and lymphoma, and more recently, the acquired immunodeficiency syndrome (AIDS) (1). Cryptococcosis is the fourth most commonly recognized life-threatening infection in people with AIDS (2, 3). C. neoformans produces large amounts of a polysaccharide composed of mannose, xylose, and glucuronic acid. This polysaccharide, termed glucuronoxylomannan (GXM), is the major constituent of the cryptococcal capsule (4). GXM occurs as four major serotypes (5), designated A–D (Scheme 1). All four serotypes have a linear α-1,3-linked mannosyl backbone with β-glucopyranosyluronic acid and β-xylopyranosyl substituents (6–9). In addition, the backbone is substituted with variable amounts of 6-O-acetyl groups, with serotype D being the most heavily O-acetylated and serotype C the least O-acetylated (10, 11). The discrete structures listed above are representative models for each serotype. Examination of large numbers of isolates has determined that individual strains of some serotypes show some variation in structure (12).

Protein interactions with homologous and heterologous proteins, polysaccharides, and enzymes with substrates have received much attention, whereas comparatively little is known about protein interactions with polysaccharides. Polysaccharides are important mediators of cell-cell communication, are associated with malignant transformations in human and animal tumors, are important in cell adhesion, are constituents of some hormones, are essential for sperm-to-egg binding, are implicated in lymphocyte homing, and have become important in recombinant therapeutics. The polysaccharide capsules surrounding pathogenic microorganisms are often virulence factors and strongly influence components of the immune system. Thus, the interaction of a monoclonal antibody with the capsular polysaccharide of C. neoformans is of interest both for applications in the diagnosis of opportunistic infections and as a model system to study protein-polysaccharide recognition.

At an applied level, immunoassays using antibodies specific for GXM are an important part of the diagnosis of cryptococcosis (13–16). Monoclonal antibodies reactive with GXM may have therapeutic value (17–19). Use of anti-GXM antibodies for diagnosis or treatment of cryptococcal infection will be greatly facilitated by an understanding of the precise epitope(s) recognized by anti-GXM antibodies. In particular, antibodies ideally suited for diagnostic or therapeutic applications should recognize an epitope that is shared by polysaccharides of all cryptococcal serotypes. mAb 439 is one such antibody. mAb 439 is reactive in both enzyme-linked immunosorbent assay and double immunodiffusion with polysaccharides from several representative strains of all four serotypes (20). Thus, this antibody is a useful probe for identification of an epitope that is shared by all four GXM serotypes.

In this paper, we describe the sequencing and construction of
**Scheme 1. Model structures of GXM of C. neoformans serotypes A-D.**

A-D.

a molecular model of the Fab portion of mAb 439 (Fab 439). We also report characteristics of the antigen-antibody interactions based on spectrofluorometric analysis.

**MATERIALS AND METHODS**

**Antibody Production and Purification**—The hybridoma secreting mAb 439 was produced from ascites obtained by BALB/c mice immunized with serotype A GXM coupled to sheep erythrocytes (21). Antibody was isolated from the ascites fluid and purified by differential precipitation with caprylic acid and ammonium sulfate, followed by hydrolysis with 0.1 M NaOH (27). Analysis by the Hestrin assay (see Ref. 28) of the Fab portion of mAb 439 (Fab 439). We showed that at least 98% of the Fab portion of mAb 439 (Fab 439) was reactive in enzyme-linked immunosorbent assay and double immunodiffusion with GXM isolated from serotypes A-D (19).

Serotype A GXM from strain 24064 was de-O-acetylated by alkaline hydrolysis with 0.1 M NaOH (27). Analysis by the Hestrin assay (see Ref. 28) of the degree of de-O-acetylation showed that at least 99% of the O-acetyl groups had been removed.

**Sequence of Variable Region**—First strand cDNA was prepared from total RNA isolated from murine hybridoma cells (31) using reverse transcriptase primed with a 3'-constant region degenerative primer at positions 348–366. The cDNA was amplified by polymerase chain reaction (PCR) (32, 33) using six different 5'-degenerative primers representing 95% of all mouse immunoglobulin leader regions (Novagen). Gel-purified PCR products were ligated into the PCR 1000 vector (In Vitrogen, San Diego, CA), cloned, and sequenced by the dideoxynucleotide chain termination method (34). Four independent clones were sequenced for each chain.

**Tryptophan and Tyrosine Determination**—The total number of tryptophan and tyrosine residues present in the intact Fab molecule was determined by the method of Edelhoch (35). Absorbance readings were taken at 280, 288, and 295 nm. The number of tryptophan residues/Fab molecule was determined by $M_{T} = \left(\varepsilon_{280}/\varepsilon_{295}\right) - \left(\varepsilon_{280}/\varepsilon_{295}\right)$, and the number of tyrosine by $M_{T} = \Delta A_{250}/2480$, where $\Delta A_{250}$ is the change in the molar absorptivity of the protein upon addition of KOH.

**Fluorescence Analysis of Antibody-Polysaccharide Complex**—All titrations were performed on a Spex 111 spectrofluorometer using photon counting. Binding was measured by adding small aliquots of the polysaccharide dissolved in PBS to a solution of Fab fragments (50 µg/ml) dissolved in PBS. The fluorescence intensity was measured using 285-nm excitation and 354-nm emission wavelengths and right angle geometry. Fluorescence data were corrected for excitation intensity. Four successive 60-s integration periods were averaged for each data point. The fluorescence yield was constant over this time period. Background fluorescence from a buffer blank was subtracted from all emission spectra. The emission spectra of all solutions were corrected for direct and a very small (0.1%) inner-filter effect as required.

Plots of $\Delta F$ (fluorescence) versus polysaccharide concentration were constructed. The $K_D$ in micrograms/milliliter and the $K_M$ in molar units were determined from the plots as the polysaccharide concentration at one-half $K_D$ max estimated either by inspection or by computer-aided direct fit to a hyperbolic binding isotherm. The $K_D$ was calculated from the $EC_{50}$ based on the molarity of repeating subunits of polysaccharide using basic structures reported previously (6–9).

**Fluorescence Quenching**—Small aliquots of quencher (2 mM acrylamide) were added to a solution of Fab fragments dissolved in PBS. The fluorescence intensity was measured at 288-nm excitation and 354-nm emission wavelengths as described above. The quenching data were analyzed by the Stern-Volmer relationship: $F/F_0 = 1 + K_{SV}[Q]$, where $K_{SV}$ is the dynamic quenching constant and $K_{SV}$ is the static quenching constant (36). The quenching data were fit to a non-linear least-squares algorithm (Jandel Scientific, San Rafael, CA), and the $K_{SV}$ and $K_{SV}$ constants of the Stern-Volmer relationship were estimated.

**Quantum Yields**—Fluorescence quantum yields were determined relative to quinine sulfate measured at 337-nm excitation and 458-nm emission wavelengths. A freshly prepared solution (0.005 mg of quinine sulfate (Eastman Kodak) dissolved in deionized H2SO4 (Fisher, Springfield, NJ) at 25 °C; Ca was used as the standard. A quantum yield of 0.546 at 25 °C was used for quinine sulfate (37).

**Molecular Modeling—SYBYL 5.51 running on Evans & Sutherland ESV50 and PS380 was used for all molecular modeling. The Kollman all-atom force field parameters were used with molecular dynamics and molecular mechanics for energy minimization of the antibody. The loop search algorithm from SYBYL was implemented to help construct complementarity-determining region (CDR) loops. The initial starting structure for construction of Fab 439 was the crystal structure of Fab 4-4-20 obtained from the Brookhaven data base (38–40). The residues were mutated to the correct sequence, and deletions or insertions were achieved by loop searches of the Brookhaven protein data base. An all-atom minimization of the entire Fab fragment was implemented to produce a low energy structure. Identification of the CDR regions was based on the methods of Kabat et al. (41) and Chothia et al. (42). Identity of CDR regions was confirmed by inspection of the loops extending from the framework region (β-barrel) of the derived molecular model. Construction of the CDR regions was aided by utilizing the unique $\phi$ and $\phi$ or $\chi$ angles of specific canonical loop conformations (43). The final step was the minimization of the entire Fab fragment.

**RESULTS**

**Isolation, Cloning, and Characterization of Variable Region cDNAs**—The heavy and light chain variable region cDNAs from mAb 439 were generated by priming total RNA with oligo(dT), followed by reverse transcription. The variable region was amplified by PCR using primers in the 3'-constant and 5'-leader regions. The PCR products were cloned and sequenced. The
amino acid sequences of the amino termini of both the heavy and light chains were sequenced directly to confirm the accuracy of the sequence predicted by the DNA. The nucleotide and predicted amino acid sequences of the heavy and light chain variable regions obtained from both sequencing methods are shown in Fig. 1. Based on the NH2-terminal sequence, the heavy chain was assigned to subgroup IIID, and the light chain was assigned to subgroup II as defined by Kabat et al. (41).

**Molecular Model of Fab 439**—Homology modeling was used to construct a plausible molecular model of Fab 439 (Fig. 2). It was based on homology to Fab 4-4-20, whose atomic coordinates are known (38-40). Only six mutations were made in the light chain. Four of the mutations were in framework regions, one in CDR2, and one in CDR3. Forty-eight mutations were made in the heavy chain: 27 in framework regions, 3 in CDR1, 10 in CDR2, and 10 in CDR3. The loops for CDR2 and CDR3 were mutated by changing the amino acid sequence and deleting 2 amino acids in CDR2 and inserting 5 amino acids into CDR3. The constant region was assumed to be identical to Fab 4-4-20.

Examination of the molecular model showed that 3 tryptophan residues were fully exposed and 1 residue was partially exposed on the surface of the antibody. One of the tryptophan residues (Trp-101) was located on the light chain immediately adjacent to CDR3 in a region of the light chain that is in close proximity to heavy chain CDR3. This area is in the putative binding site, suggesting that this tryptophan could be used as a reporter group for binding studies.

**Tryptophan and Tyrosine Determination**—Because only the variable regions of Fab 439 were sequenced, the total number of tryptophan residues present in the intact Fab molecule was determined by the method of Edelhoch (35). Based on this method, the number of measured tryptophan residues was 8.2, and the number of measured tyrosine residues was 19.4. This is in good agreement with our molecular model, which predicts 8 tryptophan (4 in variable regions and 4 in constant regions) and 20 tyrosine (13 in variable regions and 7 in constant regions) residues/Fab molecule.

**Quantum Yields**—Fluorescence quantum yields were measured for free N-acetyl-l-tryptophanamide, unbound Fab, and Fab complexed with serotype A GXM in PBS. The quantum yields were 0.147 for N-acetyl-l-tryptophanamide, 0.079 for Fab 439, and 0.088 for the Fab 439 and serotype A GXM complex. Therefore, the binding of Fab to GXM increased the quantum yield; however, the average tryptophan residue remained quenched relative to N-acetyl-l-tryptophanamide. The observed quantum yields are within the range (0.08-0.180) of quantum yields reported for other proteins (44-46).
Effects of Polysaccharide Binding on Emission Spectrum of Fab 439—As noted above, the presence of a tryptophan at the putative binding site suggested that the tryptophan could be a reporter for binding assays. As a consequence, we examined the effects of addition of the cryptococcal polysaccharide on the emission spectrum of Fab 439 at an excitation wavelength of 285 nm. The emission maximum of unbound antibody was recorded at 356 nm. The emission spectrum obtained by mixing 50 μg/ml Fab 439 with 11.3 μg/ml serotype A polysaccharide is shown in Fig. 3. An enhancement of the fluorescence intensity of the Fab fragment and a shift in the emission maximum to a shorter wavelength at 353 nm were seen. The blue shift was found consistently with all polysaccharides tested.

Acrylamide Quenching—The molecular model of Fab 439 (Fig. 2) showed a tryptophan in the putative binding site. Binding of ligand at this site could exclude solvent from this tryptophan, producing the observed enhancement of fluorescence and the change in the emission maximum. Fluorescence quenching by acrylamide was performed to monitor the accessibility of solvent to the tryptophan residues. Fig. 4 shows Stern-Volmer plots for mAb or Fab 439, mAb or Fab 439 bound to the serotype A polysaccharide, and N-acetyl-L-tryptophanamide alone. The linear portion indicates dynamic quenching, and the upward curvature at higher acrylamide concentrations indicates static quenching (47). The $K_{on}$ and $K_{q}$ constants were calculated using the Stern-Volmer relationship (Table I).

The acrylamide quenching data enabled us to calculate the number of tryptophan residues exposed to solvent in free Fab and Fab bound to the polysaccharide. Calculations were based on values in the linear portion of the Stern-Volmer plots (0.0333 M acrylamide). N-Acetyl-L-tryptophanamide was used as a standard for totally exposed tryptophan residues. The fraction of tryptophan residues exposed was 0.43 in free Fab and 0.32 when Fab was bound to the polysaccharide. Sequencing data...
and tryptophan determination by the method of Edelhoch (35) predict that the Fab fragment contains 8 tryptophan residues. Thus, quenching data predict ~3.5 residues exposed in unbound Fab and only 2.5 residues exposed in bound Fab. This experimental measure of the number of tryptophan residues exposed is in good agreement with results from a visual inspection of the molecular model, which shows 3.5 tryptophan residues exposed on the surface. Moreover, a reduction of 1 exposed tryptophan after binding is consistent with the presence of a single tryptophan in the putative binding site.

Fluorescence titrations were also performed with the serotype A polysaccharide and Fab 439 in PBS containing 0.033 M acrylamide to determine if the quencher affected binding. The apparent $K_D$ (see below) was the same with or without the acrylamide, indicating that acrylamide does not perturb the antigen-antibody reaction itself.

Influence of Serotype on Binding of GXM to Fab 439—Fab 439 at 50 µg/ml was mixed with various amounts of GXM, and the fluorescence was monitored at 354 nm. Fig. 5 shows the titration of Fab 439 with representative polysaccharides from cryptococcal serotypes A–D. Binding of each polysaccharide was characterized in terms of $\Delta F_{\text{max}}$, $K_{\text{D0}}$, and apparent $K_D$.

The $\Delta F_{\text{max}}$ was an indicator of the extent to which binding perturbed the microenvironment of the tryptophan residue. The concentrations of polysaccharide (micrograms/milliliter) producing one-half maximal $\Delta F$ were used to calculate the $EC_{50}$ and $K_D$ values, which serve as measures of relative affinity (Table II). The $EC_{50}$ was converted to molarity of repeating units to generate the $K_D$ values. Expression of affinity as $K_D$ allowed an approximate comparison with binding studies done with other polysaccharides. Enhancement of fluorescence emission was observed with all polysaccharides, reaching saturation at a value near 8 µg/ml for GXM of serotypes A–C and 50 µg/ml for serotype D GXM.

Fluorescence titrations were performed with serotype A polysaccharides that were carboxyl-reduced or de-O-acetylated. The apparent $K_D$ could not be determined for carboxyl-reduced GXM because saturation did not occur, even at a polysaccharide concentration of 500 µg/ml (Fig. 6). Titration of Fab 439 with de-O-acetylated serotype A GXM showed a marked reduction in $\Delta F_{\text{max}}$ (Table II) and an apparent $K_D$ of $5.3 \times 10^{-6}$ M (Fig. 6).

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{av}$</th>
<th>$K_{sv}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N$-Acetyl-L-tryptophanamide</td>
<td>9.7</td>
<td>9.6</td>
</tr>
<tr>
<td>Fab 439 only</td>
<td>3.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Fab 439 + serotype A GXM (strain 288)</td>
<td>3.7</td>
<td>0.6</td>
</tr>
<tr>
<td>mAb 439</td>
<td>4.7</td>
<td>1.8</td>
</tr>
<tr>
<td>mAb 439 + serotype A GXM (strain 288)</td>
<td>4.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**DISCUSSION**

A molecular model of Fab 439 was constructed using molecular mechanics, molecular dynamics, and the atomic coordinates of Fab 4-4-20 as a template. Fab 4-4-20 was chosen as a template because its x-ray coordinates were known and it has a strong sequence similarity to Fab 439. The model predicted several properties of the antibody that were supported by experimental methods.

The sequence of Fab 439 in good agreement with the sequence of other antibodies specific for GXM of *C. neoformans* (48). The light chain sequence of the variable region differed by only 4–10 amino acids from the sequences of other reactive antibodies. All of the light chains contained a tryptophan at position 101. Our studies showed that this tryptophan can be an important reporter group in fluorescence binding and quenching assays. The presence of the tryptophan in other anti-GXM antibodies suggests that this reporter group may be of general value in studying the interaction of GXM with monoclonal antibodies. The heavy chain sequence differed by 10–17 amino acids from the sequences of several published anti-GXM monoclonal antibodies (48).

Our approach to the construction of a molecular model is based on the principles outlined by Chothia et al. (42, 43). The homology modeling techniques used are similar to those used to construct models of the binding sites of two antibodies reactive with α-1,6-dextran (49, 50). It is of particular interest that the model of one of the anti-dextran antibodies revealed the presence of a single tryptophan residue in the wall of a deep cavity near the heavy and light chain interface (49). The model of the second anti-dextran antibody showed that 2 tryptophan residues were located at the heavy and light chain interface (49).
Both anti-dextran antibodies exhibited a ligand-induced tryptophanyl fluorescence change, which allowed a calculation of the $K_D$ for interaction of the antibodies with a series of ligands. Previous studies have employed fluorescence perturbation to measure affinity and to determine binding constants for antibodies to several carbohydrates (51, 52). This is consistent with the presence of a tryptophan in the binding cavity of many antibodies directed against polysaccharides as demonstrated here and by others (49, 50).

The total number of tryptophan and tyrosine residues was determined because only the variable region of the Fab fragment was sequenced. This was important for two reasons. First, it supported the accuracy of our molecular model. Second, a determination of the total number of tryptophan and tyrosine residues was required for interpretation of the binding and quenching data obtained from the fluorophores.

Fluorescence enhancement and acrylamide quenching studies provided information regarding the interactions that occur within the microenvironment of the binding site between the cryptococcal polysaccharide and Fab 439. The blue shift in the emission maximum of bound Fab is consistent with antigen binding, which causes the environment of 1 or more tryptophan residues to change from a polar to a less polar environment. Polysaccharide binding could cause the displacement of water from the antigen-binding site. A tryptophan located at the binding site would therefore experience a decrease in the polarity of its environment when associated with the antigen. Exclusion of most or all water molecules at the antigen-antibody interface has been demonstrated by x-ray crystallography of several antigen-antibody complexes (53–56).

Acrylamide quenching showed that 3.5 tryptophan residues were exposed to solvent in unbound Fab 439, and 2.5 residues were exposed in bound Fab. Tryptophan 101 is located in a pocket between heavy chain CDR3 and light chain CDR3 and is probably positioned in the binding site. Binding of the polysaccharide to the Fab fragment could cover this tryptophan residue, making it inaccessible to solvent. Thus, the experimental data are consistent with the model.

Stern-Volmer plots of the quenching data established that both dynamic and static quenching occurred. The $K_{sv}$ (dynamic quenching constant) and $K_{q}$ (static quenching constant) values were calculated. A $K_{q}$ of 2.4 M$^{-1}$ was obtained from unbound Fab, and a much lower $K_{q}$ of 0.6 M$^{-1}$ was calculated for Fab bound to serotype A GXM. Both the static and dynamic quenching constants were lower in the antibody than in the model compound, N-acetyl-L-tryptophanamide. Quenching constants obtained from the antibody and the model compound are not directly comparable because fluorescence at 285 nm in the antibody is the result of both tryptophan and tyrosine residues, whereas fluorescence in N-acetyl-L-tryptophanamide is due to tryptophan alone. However, the calculated constants for Fab 439 are comparable to the $K_{sv}$ and $K_{q}$ values determined in other proteins (57–59).

Binding of antigen has no significant effect on dynamic quenching ($K_{sv}$), but does have a large effect on static quenching ($K_{q}$), which decreased from 2.4 to 0.6 M$^{-1}$. Analyzing the constants by the ground-state complex static quenching model (60) indicates that the decrease is the result of a reduced binding of the acrylamide to the Fab fragment when complexed with GXM. This indicates that 1 or more tryptophans in bound Fab exhibits limited accessibility to the acrylamide when compared to unbound Fab. Thus, results from Stern-Volmer analysis are consistent with acrylamide quenching data showing that binding of the polysaccharide excludes a tryptophan from the quencher.

Binding titrations with Fab 439 demonstrated that the apparent $K_D$ values for serotypes A–C are almost identical, with an apparent $K_D$ of $\sim 1.7 \times 10^{-6}$ M. Serotype D exhibited weaker binding, with a $K_D$ of $\sim 1.9 \times 10^{-5}$ M. The $K_D$ for Fab 439 and GXM is comparable to that obtained for other antibodies reactive with polysaccharides (49). For example, an IgG1 reactive with the Salmonella O antigen had a $K_D$ of $5 \times 10^{-6}$ M (61). It should be noted that $K_P$ values in our studies were calculated on the basis of the molecular weight of one repeating unit of polysaccharide. It is unlikely that antibodies would bind to contiguous repeating units on an intact polysaccharide because of the steric bulk of the antibodies and the small size of the polysaccharide epitope. As a consequence, the actual $K_P$ values are probably smaller than our estimates.

The similarity in $K_{D}$ for binding of GXM of serotypes A–C suggests that similar epitopes are recognized on each polysaccharide. Despite a similarity in $K_D$, GXM of serotypes A–C exhibited different $\Delta F_{max}$ values, indicating differences within the microenvironment of the binding site near the tryptophan residue. This is particularly evident in the case of serotype C GXM, which binds with the same apparent affinity as polysac-
mAb Binding to Cryptococcal GXM

The binding of mAb to the cryptococcal polysaccharide GXM is crucial for the interaction between the antibody and the complex polysaccharide antigens. The binding sites for the antibodies are often shared epitopes that are common among different serotypes. The presence of these epitopes is essential for the strong binding of the mAb to the carbohydrate antigen. The study of the mAb binding is important for understanding the molecular basis of the antibody-antigen interaction.

The binding affinity of mAb to GXM is affected by various factors such as the glycosylation pattern, the presence of acetyl groups, and the length of the carbohydrate chains. The de-O-acetylation of the polysaccharide reduces the binding affinity of the mAb, indicating that the acetyl groups are important for the mAb binding.

The studies also showed that the binding of mAb to GXM is influenced by the presence of other molecules, such as the host proteins. The binding of mAb to the polysaccharide is also affected by the presence of other polysaccharides in the sample, indicating that the binding is specific to the GXM.

The results of these studies are important for the development of diagnostic and therapeutic strategies for the treatment of cryptococcosis. The understanding of the molecular basis of the binding is crucial for the development of new strategies for the treatment of the disease.