Characterization of Anticapsular Monoclonal Antibodies That Regulate Activation of the Complement System by the Cryptococcus neoformans Capsule

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Incubation of the encapsulated yeast Cryptococcus neoformans in human serum leads to alternative pathway-mediated deposition of C3 fragments in the capsule. We examined the ability of monoclonal antibodies (MAbs) specific for different epitopes of the major capsular polysaccharide to alter the kinetics for classical and alternative pathway-mediated deposition of C3 onto a serotype A strain. We studied MAbs reactive with capsular serotypes A, B, C, and D (MAB group II); serotypes A, B, and D (MAB group III); and serotypes A and D (MAB group IV). The MAB groupings are based on antibody variable region usage which determines the antibody molecular structure. When both the classical and alternative pathways were operative, group II MAbs induced early classical pathway-mediated binding of C3 but reduced the overall rate of C3 accumulation and the amount of bound C3. Group III MAbs closely mimicked the effects of group II MAbs but exhibited reduced support of early classical pathway-facilitated accumulation of C3. Depending on the antibody isotype, group IV MAbs slightly or markedly enhanced early binding of C3 but had no effect on either the rate of C3 accumulation or the amount of bound C3. When the classical pathway was blocked, group II and III MAbs markedly suppressed C3 binding that normally would have occurred via the alternative pathway. In contrast, MAbs of group IV had no effect on alternative pathway-mediated C3 binding. These results indicate that anticapsular antibodies with different epitope specificities may have distinct regulatory effects on activation and binding of C3.

Cryptococcus neoformans is the etiological agent of cryptococcal meningitis, a life-threatening infection of particular importance in patients with deficiencies in cellular immunity, most notably patients with the AIDS. The yeast is surrounded by a polysaccharide capsule that is composed primarily of glucuronoxylomannan (GXM), which has a linear (1→3)-α-D-mannopyranan backbone bearing β-D-xylopyranosyl, β-D-glucopyranosyluronic acid, and O-acetyl substituents (3, 9, 54). The cryptococcal capsule occurs as four major serotypes (A, B, C, and D) and is an essential virulence factor for the yeast. One of the most striking features of the cryptococcal capsule is its ability to activate the alternative complement pathway. Incubation of encapsulated cryptococci in normal human serum (NHS) leads to the deposition of $10^7$ to $10^8$ C3 fragments on the yeast (28, 56). The C3 is deposited at the surface and throughout the capsule (30). Available evidence indicates that the amount of anti-GXM antibodies found in NHS is not sufficient to initiate the classical pathway (24); consequently, activation and binding of C3 to the cryptococcal capsule are mediated entirely by the alternative complement pathway (29, 30, 55). One of the hallmark features of alternative pathway deposition of C3 onto encapsulated cryptococci is a delay of 5 to 8 min before readily detectable amounts of C3 are found on yeast cells incubated in NHS (29, 55). Once past the initial lag, C3 fragments rapidly accumulate on the yeast cells as incubation proceeds for an additional 10 min.

Recently, there has been interest in antibody-mediated resistance to cryptococcosis. Monoclonal antibodies (MAbs) have been proposed for treatment of cryptococcosis (7), and immunization with GXM-protein conjugates has been suggested for prevention of cryptococcosis (6, 12, 13). However, it is becoming increasingly clear that anti-GXM MAbs may have distinct specificities and biological activities. Anti-GXM MAbs which differ in (i) reactivities with GXM of the four major serotypes (2), (ii) apparent binding sites in the cryptococcal capsule (32, 37), and (iii) abilities to provide protection in a murine model of cryptococcosis (32, 37) have been described. Some differences in biological activity are related to differences in the epitope specificities of the various MAbs (32, 37).

One means by which antibodies could enhance resistance to cryptococcosis is through accelerated deposition of opsonic C3 fragments via the action of the classical pathway. Such an acceleration would reduce or eliminate the 5- to 8-min lag that occurs during alternative pathway-mediated deposition of C3 fragments. The objectives of our study were to evaluate the effects of anti-GXM MAbs on the kinetics and sites for deposition of C3 fragments into the cryptococcal capsule. We examined several well-characterized antibodies that differed in the epitope specificity of the MAbs. The results showed that MAbs with different isotypes and epitope specificities had distinctly different effects on activation and binding of C3 via the classical and alternative pathways; many antibodies markedly suppressed C3 binding; some antibodies accelerated C3 binding, and other antibodies had little or no effect.

MATERIALS AND METHODS

Yeast cells. C. neoformans 388 is an encapsulated isolate of serotype A that was used throughout the study. The yeast cells were grown at 30°C on a synthetic

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TABLE 1. Serotype specificity and molecular characteristics of MAbs reactive with C. neoformans GXM

<table>
<thead>
<tr>
<th>MAb group</th>
<th>MAb</th>
<th>Isotype</th>
<th>Serotype specificity</th>
<th>Molecular structure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>439</td>
<td>IgG1</td>
<td>+ + + +</td>
<td>V_{H} family: 7183 2 7 k5.1 1</td>
<td>2, 5, 15, 50</td>
</tr>
<tr>
<td></td>
<td>471</td>
<td>IgG1</td>
<td>+ + + +</td>
<td>J_{H}: 7183 2 7 k5.1 1</td>
<td>2, 5, 50</td>
</tr>
<tr>
<td>III</td>
<td>3C2</td>
<td>IgG1</td>
<td>+ + + +</td>
<td>D: size 7183 2 7 k5.1 1</td>
<td>2, 5, 50</td>
</tr>
<tr>
<td></td>
<td>339</td>
<td>IgG1</td>
<td>+ + + +</td>
<td>V_{L}: 10 4 6 k21 5</td>
<td>2, 5, 50</td>
</tr>
<tr>
<td></td>
<td>1255</td>
<td>IgG1</td>
<td>+ + + +</td>
<td>J_{L}: 10 4 6 k21 5</td>
<td>2, 5, 15, 50</td>
</tr>
<tr>
<td>IV</td>
<td>302</td>
<td>IgG1</td>
<td>+ + + +</td>
<td>V_{G}am: 3 4 k4.5 1</td>
<td>2, 5, 50</td>
</tr>
<tr>
<td></td>
<td>36b</td>
<td>IgM</td>
<td>+ + + +</td>
<td>V_{G}am: 3 4 k4.5 1</td>
<td>2, 5, 50</td>
</tr>
</tbody>
</table>

*a Based on differences in antibody variable region usage, which determines antibody molecular structure (5).

medium (8), killed by treatment overnight with 10% formaldehyde, washed with sterile 0.01 M phosphate-buffered 127 mM saline, pH 7.3 (PBS), and stored at 4°C until use.

Serum and serum proteins. Peripheral blood samples were collected from at least 10 adult volunteers. The sera were pooled and stored at ~85°C until use. This pool was used as the source of NHS for all studies. C3 was isolated from frozen human plasma by a modification (23) of a procedure originally described by Tack et al. (52). C3 was labeled with 125I by the iodogen (Pierce Chemical Co., Rockford, Ill.) procedure (20). Typically, 2 mg of C3 was labeled to a specific activity of 4 × 10^5 cpm/μg.

Antibody production and purification. Seven MAbs reactive with cryptococcal GXM (MAbs 439, 471, 3C2, 339, 1255, 302, and 386) were used in this study. These antibodies fall into three groups on the basis of molecular structure and antibody variable region usage, which determines antibody molecular structure (5). Subclass switch families (immunoglobulin G1 [IgG1]—IgG2b—IgG2a—IgM) were produced from MAbs 471 and 439. Production and characterization of the MAb 471 subclass switch family have been reported (45); the MAb 439 subclass switch family have been reported (47).

The number of molecules of each MAb binding to yeast cells was determined by binding experiments in which cells (4 × 10^7) were incubated with various amounts of 125I-labeled MAb (50,000 cpm/μg) for 30 min at 37°C in a 200-μl reaction volume. All MAbs were labeled with 125I by the iodogen procedure. Samples (50 μl) were taken in triplicate, layered over 250 μl of 30% (wt/wt) sucrose in 400-μl microcentrifuge tubes, and centrifuged for 1 min at 12,500 g. The tubes were frozen cut above the sucrose layer, and the amounts of radiolabeled MAb in the upper portion (free MAb) and bottom portion (bound MAb) were determined with a Packard 5650 AUTO-GAMMA gamma counter. The numbers of MAb molecules bound per cell were calculated by the method of equation (47).

Analysis of MAb binding to yeast cells. The number of molecules of each MAb binding to yeast cells was determined by binding experiments in which cells (4 × 10^7) were incubated with 125I-labeled MAb (50,000 cpm/μg) for 30 min at 37°C in a 200-μl reaction volume. All MAbs were labeled with 125I by the iodogen procedure. Samples (50 μl) were taken in triplicate, layered over 250 μl of 30% (wt/wt) sucrose in 400-μl microcentrifuge tubes, and centrifuged for 1 min at 12,500 g. The tubes were frozen cut above the sucrose layer, and the amounts of radiolabeled MAb in the upper portion (free MAb) and bottom portion (bound MAb) were determined with a Packard 5650 AUTO-GAMMA gamma counter. The numbers of MAb molecules bound per cell were calculated by the method of equation (47).

Effect of anti-GXM MAbs on the kinetics of classical pathway-mediated C3 binding. Incubation of encapsulated cryptococci in NHS leads to activation and binding of C3 to the yeast via the alternative complement pathway (29, 30). Alternative pathway initiation is characterized by a lag of 4 to 8 min before readily measurable amounts of C3 are found on the yeast cell surface. An initial experiment evaluated the relative abilities of MAbs from different molecular groups with differing epitope specificities to initiate the classical pathway leading to accelerated activation and binding of C3.

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presence or absence of each MAb. The results (Fig. 1) showed that the MAbs had distinctly different effects on the kinetics for accumulation of C3 on the yeast cells; moreover, the effects segregated largely according to the molecular group and epitope specificity of the MAb. At the highest concentration, those MAbs with strong reactivity for an epitope shared by cryptococcal serotypes A, B, C, and D (MAbs 439 and 3C2; group II) altered the kinetics for accumulation of C3 in two ways. First, there was limited, but readily detectable, C3 on the yeasts at early time intervals such as 4 and 6 min, indicating that the lag had been greatly reduced. Second, the rate of accumulation in the presence of the MAbs (33 μg/ml) was much slower than the rate observed in the absence of the group II MAbs. Identical results (not shown) were also obtained with a third antibody of group II (MAb 471). The overall accumulation of C3 at the end of the 25-min incubation in the presence of the group II MAbs (33 μg/ml) was markedly less than the accumulation in the absence of MAb.

MAbs reactive with an epitope shared by cryptococcal serotypes A, B, and D (MAbs 1255 and 339; group III) altered the kinetics for activation and binding of C3 in a manner that differed slightly from the effects produced by MAbs of group II. Unlike the increased early binding in the presence of group II MAbs, MAbs 1255 and 339 had little or no effect on early binding of C3 to the cells; the lag was quite similar to the lag found when cryptococcal cells were incubated with NHS alone. However, in a result similar to the effect of the group II MAbs, at high MAb concentrations (33 μg/ml), the rate of accumulation was lower in the presence of group III MAbs than the rate observed in the absence of added antibody.

MAbs with reactivity for epitopes shared only by GXMs of serotypes A and D (MAbs 386 and 302; group IV) influenced the kinetics of accumulation of C3 in a manner that was distinct from that of MAbs of either group II or group III. MAb 386 markedly reduced, in a dose-dependent fashion, the lag observed in the absence of added MAb but had little or no effect on the rapid rate of accumulation once past the lag. MAb 302 slightly reduced the lag but had no apparent effect on the rate of accumulation. The different effects of MAbs 386 and 302 on the lag are likely due to the fact that MAb 386 is an IgM whereas MAb 302 is an IgG1. The maximum levels of bound C3 observed in the presence of either MAb 302 or 386 were similar to the level observed in the absence of MAb.

Effect of anti-GXM MAbs on the cellular sites of classical pathway-mediated C3 binding. Kinetics experiments such as those shown in Fig. 1 do not provide information as to the cellular sites and patterns of C3 deposition. The pattern of C3 deposition on encapsulated cryptococci incubated in NHS is characterized by a delayed appearance of focal initiation sites that are asynchronous in their occurrence and appear to expand with time to eventually fill the cryptococcal capsule (29). Given the ability of some MAbs to accelerate deposition of C3 onto the yeast, we considered the possibilities that the early binding of C3 was simply an acceleration of the focal initiation pattern or was a fundamental qualitative change from the pattern observed in the absence of MAb.

Incubation of encapsulated cryptococci in NHS containing group II MAb 439 or 3C2 produced a pattern of C3 deposition characterized by early binding of C3 at sites diffusely spread over the capsule (Fig. 2). MAb 471 produced identical results (not shown). This contrasts with the delayed, focal pattern of C3 binding in the absence of MAb. In addition, the intensity of the observed fluorescence with cells incubated for 8 and 16 min was substantially lower than the fluorescence observed when encapsulated cryptococci are incubated in NHS alone. Overall, the immunofluorescence analysis of C3 binding in the presence of the group II MAbs largely reflects results of the kinetics experiments; there is an early deposition of C3 on the capsule followed by a very gradual rate of amplification.

Incubation of encapsulated cryptococci in NHS containing
group III MAbs 1255 and 339 produced patterns of C3 deposition consistent with the quantitative results from the kinetics assays (Fig. 1). Very little bound C3 was seen after 2 min of incubation, a pattern that distinguishes antibodies of group III from those of group II. Bound C3 observed after 8 min of incubation showed patterns that were a mixture of limited focal staining, e.g., MAb 339 at 8 min, and less intense diffuse binding similar to that observed in the presence of the group II antibodies.

The patterns of C3 binding in the presence of group IV MAbs 302 and 386 differed considerably from one another. Binding in the presence of MAb 302 was similar to, or slightly more rapid than, the typical alternative pathway pattern of focal initiation observed when cryptococcal cells were incubated with NHS alone. This result suggests that MAb 302 had little effect on or slightly accelerated the process of C3 deposition that would have occurred in the absence of MAb. In contrast, binding in the presence of MAb 386 showed an immediate binding of C3 in a largely diffuse pattern, indicating that the IgM antibody had initiated the classical pathway.

**Effect of anti-GXM antibodies on activation and binding of C3 via the alternative pathway.** Suppression of the rate of accumulation of C3 in the presence of the group II and group III MAbs raised the possibility that the MAbs were altering the microenvironment of the cryptococcal capsule which is necessary for C3 activation and/or deposition. Experiments shown in Fig. 1 to 3 were done in the presence of NHS, and the results likely represented both antibody-mediated initiation of the classical pathway and normal activation of the alternative pathway which occurs in the absence of the MAb. Treatment of serum with Mg-EGTA chelates the Ca\(^{2+}\) needed for activation via the classical pathway while leaving activation of the alternative pathway intact (19, 40). Previous studies from our laboratory found that the kinetics of activation and binding of C3 to encapsulated cryptococci are not markedly altered if the classical pathway is blocked by incorporation of Mg-EGTA into the reaction mixture (29). As a consequence, we examined the effect of each MAb on activation and binding of C3 from NHS in the presence of Mg-EGTA.

An analysis of the kinetics of activation and binding of C3 to encapsulated cryptococci in the presence of 50 \(\mu\)g of MAb per ml and 10 mM Mg-EGTA (Fig. 4) showed almost complete suppression of C3 binding in the presence of group II antibodies (MAbs 439, 471, and 3C2). Unlike the results observed with NHS in the absence of Mg-EGTA (Fig. 1 to 3), there was no readily detectable early deposition of C3 when only the alternative pathway was operative. Suppression of C3 binding was also observed in the presence of group III antibodies (MAbs 1255 and 339) and Mg-EGTA. In contrast, the group IV antibodies had no effect on the kinetics of alternative pathway mediated activation and binding of C3 (Fig. 4).

An evaluation by immunofluorescence of the sites for C3 deposition onto cryptococci incubated with NHS containing...
Mg-EGTA showed that initiation foci were formed in the presence of MAbs of all three groups (Fig. 5). However, there were subtle differences in the patterns of formation and growth of the foci. Focal initiation sites were not visible on cells incubated for 2 min in NHS-Mg-EGTA alone or on cells incubated with both NHS-Mg-EGTA and MAbs of group IV (MAbs 302 and 386). However, after 4 min of incubation in the absence of MAb or in the presence of MAb 302 or 386, limited numbers of foci of a relatively large size were readily apparent, and there was dense accumulation of C3 after 8 min, suggesting rapid expansion of the initiation foci. In contrast, very small initiation foci were observed on cells incubated for 2 min with NHS-Mg-EGTA in the presence group II (MAbs 439 and 3C2) or group III MAbs (MAbs 1255 and 339). These foci were small and faint, requiring 50 integrations during image acquisition to produce figures which illustrated the sites of C3 deposition. A similar attempt at image acquisition in the case of cells incubated for 2 min with NHS-Mg-EGTA alone or NHS-Mg-EGTA plus MAbs of group IV failed to demonstrate C3 binding. An examination of alternative pathway-mediated C3 binding after 4 and sometimes 8 min in the presence of group II and III MAbs showed the presence of initiation foci that were much smaller than foci found on cells incubated for similar times with NHS-Mg-EGTA alone or on cells incubated with NHS-Mg-EGTA plus group IV MAbs.

The numerous minute foci of C3 formed on cells incubated with NHS-Mg-EGTA and the group II antibodies were not readily detected and imaged under the conditions used to visualize denser deposits of C3 formed on cells incubated for 4 min with NHS-Mg-EGTA in the absence of the MAb (Fig. 2 and 5). Sites formed in the presence of the MAb were faint and very small and faded rapidly under UV illumination. Moreover, only center sections are shown in Fig. 5, and the figures do not illustrate the pattern of C3 binding over the entire cell.

FIG. 3. Kinetics of activation and binding of C3 fragments to C. neoformans cells incubated with 40% NHS in the absence of anti-GXM MAbs or in the presence of an isotype switch (IgG1→IgG2b→IgG2a) family of MAbs derived from MAb 439 (top row) and MAb 471 (bottom row). The indicated antibody concentrations are micrograms of MAb per milliliter of reaction mixture volume. Binding of C3 fragments was determined by incorporation of trace amounts of 125I-labeled C3 into the reaction mixture.

FIG. 4. Kinetics of alternative pathway-mediated activation and binding of C3 fragments to C. neoformans cells incubated with 40% NHS containing 10 mM Mg-EGTA in the presence (50 μg/ml) or absence of anti-GXM MAbs. Binding of C3 fragments was determined by incorporation of trace amounts of 125I-labeled C3 into the reaction mixture.
In an effort to generate an image of the entire cell, the conditions for image collection were modified to optimally visualize and photograph this early pattern of C3 deposition on cells incubated with NHS-Mg-EGTA plus MAb 3C2 (see Materials and Methods). The patterns of C3 deposition were determined by use of FITC-labeled antiserum to C3. Unless otherwise indicated, images were collected under identical conditions of image acquisition, including the number of image integrations (five) and camera gain (−3 db). *, 20 image integrations; **, 50 image integrations. As in the case with images shown in Fig. 2, the fluorescence found with some samples was so intense that digital deconvolution of the images could not completely remove haze found in the center of the cell.

Taken together, these results indicate that alternative pathway mediated initiation foci readily form in the presence of the suppressive group II and III MAbs. The critical difference between initiation sites formed in the presence of the suppressive antibodies and initiation sites produced either in the absence of MAbs or in the presence of nonsuppressive MAbs (group IV) appears to be the extent to which and the rate at which the sites are able to expand to eventually fill the capsule with C3 fragments.

MAbs of groups II, III, and IV readily bind to C. neoformans and do not block binding of metastable C3b to the cells. The results presented above suggest that MAb-mediated suppression of C3 deposition occurs via a mechanism involving regulation or restriction of C3 amplification from focal initiation sites. Two experiments were done to eliminate alternative explanations for the ability of anti-GXM MAbs from different groups to exert different effects on alternative pathway-mediated binding of C3 to the cryptococcal capsule. First, we considered the possibility that antibodies of the three groups differed in the number of MAb molecules binding to the yeast cells. Radiolabeled MAbs were prepared, and binding was assessed according to the method of Scatchard. The results (Table 2) showed that saturation of binding sites occurred with similar numbers of the IgG1 antibodies regardless of the MAb group. Modest differences were noted between some antibodies, but these differences did not readily distinguish antibodies of one molecular group from those of another. A calculation of the number of antibodies expected to bind to cells under experimental conditions similar to those used in Fig. 1 to 4, e.g., 50 μg of MAb per 4 × 10⁷ yeast cells, also showed no differences in predicted binding that would suggest an association between numbers of MAb molecules bound and suppressive versus nonsuppressive activity. Fewer numbers of MAb 386 molecules

![FIG. 5. Immunofluorescence analysis of the sites for binding of C3 to C. neoformans cells incubated for 2, 4, 8, and 16 min with 40% NHS containing 10 mM Mg-EGTA in the presence (50 μg/ml) or absence of anti-GXM MAb 3C2. Sites of C3 deposition were determined by use of FITC-labeled antiserum to C3. Unless otherwise indicated, images were collected under identical conditions of image acquisition, including the number of image integrations (five) and camera gain (−3 db). *, 20 image integrations; **, 50 image integrations. As in the case with images shown in Fig. 2, the fluorescence found with some samples was so intense that digital deconvolution of the images could not completely remove haze found in the center of the cell.](http://iai.asm.org/)

![FIG. 6. Stereoscopic images of sites for binding of C3 to C. neoformans cells incubated for 4 min with 40% NHS containing 10 mM Mg-EGTA in the presence (50 μg/ml) or absence of anti-GXM MAb 3C2. Sites of C3 deposition were determined by use of Oregon Green 514-labeled antiserum to C3. Conditions for collection of the immunofluorescence image were optimized for the intensity of fluorescence exhibited by each cell type (NHS-Mg-EGTA, 10 image integrations and camera gain of 0 db; NHS-Mg-EGTA plus MAb 3C2, 30 image integrations and camera gain of 9 db).](http://iai.asm.org/)

![TABLE 2. Binding of MAbs to encapsulated cryptococci as determined by Scatchard analysis](http://iai.asm.org/)
bound to the cryptococcal cells, a result that likely reflects the fact that MAb 386 is an IgM antibody.

An alternative explanation for the ability of MAbs with different epitope specificities to have suppressive or nonsuppressive effects is the possibility that metastable C3b has preferred binding sites on GXM which are blocked by suppressive MAbs. This explanation is supported by a report which shows increased binding of metastable C3b to serotypes of *C. neoformans* that are rich in xylose (44). As a consequence, an experiment was done in which the binding of trypsin-generated metastable C3b was evaluated in the presence or absence of MAbs that did or did not have the ability to suppress activation and binding of C3 via the alternative pathway. Cryptococcal cells were preincubated with each MAb, radiolabeled C3 was added, metastable C3 was formed by the addition of trypsin, and the amount of bound C3b was assessed. The results (Table 3) showed that similar (P = 0.25) numbers of C3b molecules bound to cells that were untreated or were coated with each of the MAbs.

**DISCUSSION**

Activation and binding of C3 to encapsulated cryptococci incubated in NHS are characterized by a prominent lag of approximately 4 to 8 min before appreciable amounts of C3 fragments accumulate on the yeast (29). This process is mediated entirely via the alternative pathway (29, 30). The biological significance of this lag is not known, but this delay could be an important factor in the pathogenesis of cryptococcal meningitis because production of meningitis most likely follows dissemination of the yeast from the lung via the bloodstream or lymphatics to eventually cross the blood-brain barrier. Thus, there would be a brief moment of opportunity for clearance of the encapsulated cryptococci before the yeast reaches relatively protected sites. One means to accelerate activation and binding of C3 to the capsule is initiation of the classical pathway by antcapsular antibodies. Anti-GXM antibodies could facilitate clearance of cryptococci from the lungs, bloodstream, or other tissues through a combination of opsonization via classical pathway deposition of C3 and opsonization via the Fc fragment of the antibody. Accordingly, studies from several laboratories have convincingly demonstrated that antcapsular IgG and IgM antibodies can alter the course of experimental cryptococcosis (14, 33, 34, 45). The most striking result of our study was the observation that some MAbs suppress rather than enhance binding of C3 to the cryptococcal capsule; moreover, this suppressive activity is dependent on the antibody isotype and epitope specificity.

An examination of an isotype switch family of anti-GXM MAbs belonging to MAb group II produced the expected result that anticapsular MAbs are able to initiate the classical pathway and mediate early deposition of C3 fragments onto the capsule. The effect was dose and IgG subclass dependent. Antibodies of the IgG1 subclass showed limited but readily detectable acceleration of early activation and binding of C3; however, there was an overall suppression in both the rate of C3 accumulation and the amount of bound C3 that would have occurred via the alternative pathway in the absence of the antibody. In contrast, IgG2a and IgG2b subclass switch variants of the MAb 439 and 471 lines produced marked early deposition of C3 when used at 5.0 μg/ml. These results are consistent with previous reports that murine IgG1 antibodies are less effective activators of the classical pathway than IgG2a or IgG2b antibodies (10, 35, 38). Both IgG2a antibodies displayed a prozone-like phenomenon in which an increase in the antibody concentration from 5.0 to 50 μg/ml effected a reduction in both the rate of accumulation and the total amount of C3 bound to the yeast cells. The available data do not suggest a mechanism for this prozone effect.

Readily observable classical pathway initiation at 5.0 μg/ml as was seen with the IgG2a and IgG2b subclasses is consistent with the amount of antibody needed to provide protective immunity against a variety of encapsulated bacteria. For example, efficient complement-dependent opsonization, phagocytosis, and killing of type III group B streptococci required ≥2 μg of antibody per ml of serum (1, 17). Adults immunized with group A and C meningococcal polysaccharides produce serum antibody levels of approximately 15 μg/ml (21). Protective levels of anti-*Haemophilus influenzae* type b antibodies have been estimated to be 0.04 to 0.1 μg/ml of serum in nonvaccinated normal adults (42) and 1.0 μg/ml among immunized children (25). Finally, antipolysaccharide antibodies produced in response to the various protein conjugate *H. influenzae* type b vaccines ranged from 0.28 to 3.64 μg/ml (11).

The abilities of anti-GXM antibodies of the IgG2a and IgG2b subclasses to accelerate deposition of C3 into the capsule is not surprising given the known complement activating potential of these subclasses. However, blocking of alternative pathway-mediated activation and binding of C3 fragments by the IgG1 antibodies in a manner that is related to the epitope specificity of the antibody was unexpected. Numerous studies have found that antibodies can enhance activation of the alternative pathway by particulate activators (4, 18, 31, 41, 48, 53). Explanations proposed for the facilitating action of antibody include a blockade of surface sites that would normally favor the action of the regulatory protein factor H (18, 41), enhanced deposition of C3b or factor B (31, 48), and increased efficiency of the cell-bound C3 convertase (C3b, Bb) in the presence of antibody (36). The literature also identifies situations where antibodies directed against microbial surfaces can suppress activation of the complement system. These suppressive antibodies are typically IgA antibodies that block the ability of IgG antibodies to initiate the classical pathway (16, 22, 23, 43, 57). Mechanisms proposed for this antibody-mediated suppression of antibody-dependent complement activation include competition between nonactivating immunoglobulin isotypes and activating isotypes for antigenic sites on the target cell (23, 43) and sterile interference with C1 binding to IgG (43). The ability of anti-GXM antibodies to suppress activation of the alternative pathway clearly differs from these forms of antibody-mediated regulation of complement activation and, to our knowledge, is without a parallel in the literature.

There are several possible mechanisms for the ability of anti-GXM antibodies to suppress, in an epitope-specific manner, alternative pathway mediated activation and binding of C3 fragments to encapsulated cryptococci. First, the antibody
could bind to a preferred acceptor for metastable C3b. Sahu et al. reported data which suggest that the xylose side chains of GXM may be preferred binding sites for metastable C3b (44). If a MAb is specific for such residues, epitope-specific suppression of C3 binding might occur. None of the group II, III, or IV antibodies had a measurable effect on binding of trypsin-generated metastable C3b to encapsulated cryptococci (Table 3). Further, an examination of binding of alternative pathway-generated C3 to encapsulated cryptococci in the presence of the suppressive group II antibodies showed the presence of numerous minute sites of C3 binding, suggesting the initial foci of C3b readily form in the presence of the suppressive antibodies. These results suggest that the suppressive group II antibodies block the amplification phase of the alternative pathway.

There are at least two mechanisms by which the suppressive group II antibodies could inhibit alternative pathway amplification in an epitope-specific manner. First, the capsule could contain specific regulatory domains that influence the rate of alternative pathway amplification. For example, in the absence of the suppressive antibody, specific capular structures could favor the action of complement factor B which would support continued amplification. Blockade of such a regulatory domain would have the indirect effect of restricting amplification.

An alternative mechanism for decreased amplification is inhibition of the amplification process through mechanical or physical means. Amplification of the alternative pathway by particulate activators requires formation of a solid-phase C3 convertase followed by generation of metastable C3b, which, in turn, binds to the particle, forming a new C3 convertase with Bb (39). A bivalent IgG that cross-links the capsule could interfere with this amplification process by reducing diffusion of complement components through the capsule or by physically blocking expansion of the initial sites of C3b deposition. Given the short half-life of metastable C3b, estimated at 60 μs (49), a reduction in the ability of metastable C3b to diffuse to new binding sites would “cage” the initial reactive site and prevent expansion of the bound C3b to fill the capsule. In contrast, an antibody with a different epitope specificity could cross-link the capsule in a manner that does not interfere with this process. This explanation is consistent with the observed formation of numerous sites of C3 deposition without the expansion and coalescence of these sites which occur in the absence of the suppressive antibodies (Fig. 6). This explanation is also consistent with the results of another study, in which we demonstrated that the ability of a MAb to suppress activation of the alternative pathway is critically dependent on bivalency (27). Intact MABs of group II and their F(\(\text{ab}\)\(_2\)) fragments readily suppress activation and binding of C3, whereas Fab fragments of the antibodies have little or no suppressive activity.

Previous studies of the biological activities of anti-GXM MABs identified two properties of the MABs that were related to epitope specificity. MAbs 12A1 provided significant protection in a murine model of cryptococcosis, whereas MAb 13F1 failed to protect (32, 37). In a second assay, examination of the sites of binding in the cryptococcal capsule by indirect immunofluorescence showed that MAb 12A1 was diffusely distributed throughout the capsule, whereas MAb 13F1 was distributed throughout the capsule in a punctate pattern (32, 37). Our studies of the distinctly different effects of MABs with different epitope specificities on activation of the classical and alternative pathways by the cryptococcal capsule add a third biological activity to the manner that epitope specificity influences the biological activity of anticyptococcal antibodies. Such differences may be important in selection of antibodies for use in passive immunotherapy (7, 14, 33, 34, 45) and in design of antigens to be used in a cryptococcal vaccine (6, 12, 13).

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