Published in final edited form as:

Mol Microbiol. 2009 October ; 74(1): 126–138. doi:10.1111/j.1365-2958.2009.06855.x.

Phenotypic heterogeneity in expression of epitopes in the *Cryptococcus neoformans* capsule

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Summary

The opportunistic yeast *Cryptococcus neoformans* is surrounded by a polysaccharide capsule comprised primarily of glucuronoxylomannan (GXM). GXM is a key component of the antigenic character of the capsule. Expression of the epitope that allows for binding of mAbs that require *O*-acetylation of GXM for mAb recognition was greatly influenced by cell age, growth conditions and serotype. Yeast cells of serotype A grown in vitro under capsule induction conditions showed considerable cell-to-cell variability in binding of two *O*-acetyl-dependent mAbs, and such mAbs uniformly failed to bind to GXM that covers yeast buds. Expression of the *O*-acetyl-dependent epitope increased with cell age. In contrast, all serotype A cells harvested from brain tissue bound the same *O*-acetyl-dependent mAbs. The ability of the cryptococcal capsule to activate the complement cascade and bind C3 occurred uniformly over the surface of all yeast cells, including the bud. Finally, the cell-to-cell variability in binding of *O*-acetyl-dependent mAbs with strains of serotype A was not found with strains of serotype D; almost all cells of serotype D showed homogeneous binding of *O*-acetyl-dependent mAbs. These results indicate that variability in expression of antigenic epitopes by GXM should be considered in selection of mAbs used for immunodiagnosis or immunotherapy.

Keywords

Cryptococcus neoformans; monoclonal antibody; epitope; capsule

Introduction

Cryptococcus neoformans is a fungal pathogen that is surrounded by an antiphagocytic capsule. *C. neoformans* produces a life-threatening meningitis, particularly in individuals with impaired cell-mediated immunity, including those with AIDS. *C. neoformans* has multiple virulence factors that contribute to yeast pathogenesis; one such virulence factor is the capsular polysaccharide, glucuronoxylomannan (GXM), a linear polysaccharide with a (1-3)- α -D-mannopyranan backbone with single β -D-xylopyranosyl and β -D-glucopyranosyl-uronic acid substituents (Bhattacharjee *et al.*, 1984; Cherniak *et al.*, 1998; Casadevall *et al.*, 2003). There are four major serotypes of GXM that are distributed among three distinct varieties. *C. neoformans* var. *grubii* produces serotype A GXM; *C. neoformans* var. *neoformans* produces serotype D, and *C. neoformans* var. *gatii* produces serotypes B and C (Bennett *et al.*, 1977; Franzot *et al.*, 1999). GXM serotypes are distinguished primarily by the extent of *O*-acetylation and xylose substitution on the mannose backbone (Brandt *et al.*, 2003; Turner and Cherniak, 1991). *O*-acetyl groups are of particular interest because they

are immunodominant epitopes recognized by monoclonal and polyclonal antibodies to GXM (Cherniak *et al.*, 1980; Belay and Cherniak, 1995).

Several groups have made monoclonal antibodies that bind to distinct epitopes within the *C. neoformans* capsule (Eckert and Kozel, 1987; Dromer *et al.*, 1987; Mukherjee *et al.*, 1992). Previously, we examined the interaction between GXM-specific mAbs and the cryptococcal capsular matrix by a variation in the classical quellung reaction (Neufeld, 1902) in which we utilized differential interference contrast (DIC) microscopy to evaluate mAb binding. We found three different reactions. MAb with different epitope specificities may i) fail to react with GXM and thus cannot visualize the capsule; ii) produce an annular or “rim” pattern that is characterized by a sharp increase in the optical gradient at the edge of the capsule followed by a decrease in the optical gradient; or iii) produce a diffuse pattern termed “puffy” where there is an increase in the optical gradient at the capsular edge marked by the absence of the immediate decrease in the optical gradient that is observed with the rim pattern (MacGill *et al.*, 2000). Importantly, mAbs that produce a rim pattern are also protective in murine models of cryptococcosis, whereas mAbs that give a puffy pattern fail to protect in the same model (MacGill *et al.*, 2000).

During mammalian infection, an increase in capsule size occurs, resulting in encapsulated yeast equipped to evade phagocytosis and modulate immune responses through the shedding of capsular material into the host (Bergman, 1965; Casadevall *et al.*, 1998; Feldmesser *et al.*, 2001). Additionally, acapsular strains of *C. neoformans* are avirulent (Bulmer *et al.*, 1967; Kozel and Cazin, Jr., 1971; Kwon-Chung and Rhodes, 1986). Despite the importance of encapsulation to virulence, assembly of the capsular matrix surrounding each cell is a complex process that is still poorly understood. The goal of our study was to examine expression of capsular epitopes by use of a library of mAbs that are reactive with GXM. The experimental design included examination of mAb reactivity with parent vs. budding cells, cells of different ages, and cells cultured in different conditions, including the use of cells isolated from infected murine brain tissue. The results showed that expression of epitopes reactive with two mAbs that are dependent on GXM *O*-acetylation for binding occurs independent of capsule formation and is greatly influenced by growth conditions.

Results

Variability in mAb binding to parent and budding cells

A library of mAbs reactive with GXM has been developed in our laboratory. These mAbs differ in their binding to cells of different serotypes and their requirements for *O*-acetylation of GXM for binding to cells of serotype D (Kozel *et al.*, 2003) (Table 1). These mAbs exhibited a striking variability in binding to the capsule that surrounded serotype A CN6 cells that had been grown in synthetic medium. mAbs 3C2, 471, 1255 and 339 showed a uniform bright staining of the entire population of cells (Fig. 1A and 1C). In contrast, there was considerable variability in the binding of mAbs 302 and 1326 to these same cells. Some cells showed a pattern of bright binding to the capsule that surrounded the parent cell; other cells showed a fine punctate binding pattern, but most cells failed to bind the antibodies. The variability in mAb binding patterns by mAbs 302 and 1326 is illustrated in Fig. 1B. An examination of yeast cells that exhibited the bright pattern of binding showed an additional difference between the mAbs. mAbs 3C2, 471 and 1255 bound to capsular material that surrounded both buds and parent cells; mAbs 339, 302 and 1326 did not bind to capsule surrounding buds of cells that exhibited the bright, uniform binding pattern (Fig. 1A).

Cell to cell variability in binding of mAbs 302 and 1326 could be due to the presence of a mixed cell population or to phenotypic variability within a population. As a consequence, CN6 was streaked for isolation, and binding of mAbs was assessed for 10 individual

colonies. The results showed that each of the clones displayed the same variability in binding of mAbs 302 and 1326 that was observed with the parent isolate (data not shown).

Variability in expression of the epitope recognized by mAbs 302 and 1326 within a population of clonal cells as well as the absence of mAb binding to capsule surrounding buds suggested that cell age might be a factor influencing epitope expression. This question was examined further by evaluation of binding of mAb 1326 to cells of different ages. For this and several subsequent experiments, mAb 1326 was chosen to evaluate the expression of the epitope recognized by mAbs 302 and 1326. We refer to this as the 1326 epitope. In order to study changes that may occur with cell aging, a system was designed to identify original “parent” cells that would, with increasing time in culture, be surrounded by an increasing number of daughter cells. This was achieved by fluorescently labeling the cell wall of live *C. neoformans* cells with Alexa Fluor 488. After labeling, parent cells were returned to culture conditions in fresh synthetic medium and allowed to continue growth. Samples were taken daily and examined for mAb 1326 binding patterns to parent and daughter cells (Fig. 2A). As parent cells aged, there was a significant increase in the frequency of cells with bright binding patterns after 4 or 6 days of continued growth ($P < 0.001$ vs time 0). In contrast, there was no change in the ratio of binding patterns among the growing population of daughter cells. Images captured at days 0 and 6 illustrate the impact of cell age on the pattern of mAb 1326 binding to parent and daughter cells (Fig. 2B and 2C). At day 0 (Fig. 2B), most parent cells (shown by labeling of the cell wall with the green Alexa Fluor 488) are negative. At day 6 (Fig. 2C), the Alexa Fluor 488-labeled parent cells show a much higher percentage of bright staining with mAb 1326.

Activation of the alternative complement pathway is an important biological activity of the cryptococcal capsule that leads to deposition of iC3b within the capsular matrix. Given the variability in epitope expression between capsule that surrounds the parent and buds (Fig. 1A), we examined deposition of C3 fragments over parent and buds in relation to the binding sites of the various mAbs. Yeast cells were incubated with normal human serum to deposit C3 in the capsular matrix. Alexa 488-labeled C3 was added to the human serum in trace amounts to allow for recognition of the sites for binding of C3 fragments (Gates and Kozel, 2006). This was followed by incubation with Alexa 555-labeled mAbs. The results (Fig. 3) showed deposition of C3 in the matrix over both the parent and bud. mAbs 3C2, 471 and 1255 bound to the capsule surrounding both parent and bud, whereas mAbs 339, 302 and 1326 only bound to capsule over the parent, confirming results shown in Fig. 1A.

Dependence of mAb binding on GXM-acetylation

Our previous studies reported the binding of this set of mAbs to *CAS1* (*O*-acetyl positive [NE167 and NE 169]) and *cas1* Δ mutants (isogenic *O*-acetyl negative strains [NE168 and NE 170]) of a serotype D strain of *C. neoformans* (JEC156) (Kozel *et al.*, 2003). mAbs 3C2, 471 and 1255 bound to both the parent and mutant strains; mAbs 339, 302 and 1326 bound to the parent cells but not the *O*-acetyl negative mutant strain. These prior studies suggested that variability in binding of mAbs 302 and 1326 to cells within the population of CN6 cells could be due to differences in *O*-acetylation. JEC156 is derived from a serotype D strain, whereas CN6 is a serotype A strain. Since cells of serotype A and D differ in the extent of *O*-acetylation, mAb binding was assessed for untreated and chemically de-*O*-acetylated cells of CN6. The results (Fig. 4) showed that the pattern of binding of mAbs 3C2, 471 and 1255 shifted from a rim-type pattern to a puffy pattern with de-*O*-acetylated cells, but all cells continued to bind mAbs. mAb binding was evident by both the capsule reaction (Fig. 4A) and by use of directly labeled mAbs (Fig. 4B). In contrast, mAbs 339, 302 and 1326 failed to bind to the de-*O*-acetylated cells. These results indicate that binding of mAbs 339, 302 and 1326 to cells of serotype A strain CN6 is dependent on an intact *O*-acetyl group. Note

the speckled pattern of binding of mAb 1255 to the de-*O*-acetylated cells, suggesting a partial contribution of *O*-acetylation to binding by mAb 1255.

Influence of growth conditions on mAb binding

Variable expression of an *O*-acetyl-dependent epitope within clonal cells grown in synthetic medium prompted additional experiments to explore the influence of growth conditions on presentation of the 1326 epitope. In previous studies, we examined CN6 cells isolated from brain tissue of infected mice (Gates *et al.*, 2004). Yeast cells isolated from brain tissue have an increased concentration of GXM within the capsular matrix compared to the original in vitro cultured cells used to infect the mice. To investigate expression of the 1326 epitope further, yeast cells isolated from brain tissue were incubated with Alexa 555-labeled mAbs (Fig. 5A). In contrast to the cell to cell variability in binding of mAbs 302 and 1326 to yeast cells grown in synthetic medium (Fig. 1), the mAbs showed homogenous bright binding to all cells isolated from brain. To confirm the importance of *O*-acetylation in binding of mAbs 339, 302 and 1326, the yeast cells derived from brain tissue were de-*O*-acetylated by treatment at pH 11 with NH₄OH and mAb binding was evaluated. The results showed bright uniform binding of the *O*-acetyl-independent mAbs 3C2 and 471, a speckled binding of mAb 1255 and an absence of binding by the *O*-acetyl-dependent mAbs 339, 302 and 1326 (Fig. 5B).

The comparison of binding by the *O*-acetyl-dependent mAbs 302 and 1326 to yeast cells grown in synthetic medium and cells isolated from brain tissue suggested that yeast culture conditions influence expression of the 1326 epitope. Previous studies found induction of large capsule synthesis by growth in PBS + 10% fetal bovine serum (FBS) (Zaragoza *et al.*, 2003). An evaluation of binding by mAbs 302 and 1326 to yeast cells grown for 48 h at 37°C in 10% FBS with 5% CO₂ found that >80% of the cells bound the mAbs (not shown). Growth conditions that did not produce a change in the heterogenous binding of mAbs 302 and 1326 included i) growth on Sabouraud's dextrose broth, ii) incubation in PBS, iii) growth in synthetic medium at different temperatures (21°C vs. 30°C vs. 37°C), or iv) growth in synthetic medium in the absence of shaking (not shown).

The shift from limited binding of *O*-acetyl-dependent mAbs 302 and 1326 to cells grown in synthetic medium to extensive binding of these same mAbs to cells shifted to 10% FBS could be due to new expression of the *O*-acetyl epitope by GXM in the capsule of existing cells or to the growth of new cells that expressed the epitope. In an effort to better understand the nature of the shift from limited *O*-acetyl epitope expression to extensive epitope expression, yeast cells were grown for 48 h in synthetic medium, directly labeled with Alexa Fluor 488 which stained the cell wall, washed with PBS to remove excess Alexa 488, inoculated into 10% FBS in PBS and cultured for an additional 18 hours. In this manner, the original parent cells carried a green label from the Alexa 488 on the cell wall, whereas the progeny cells lacked a labeled cell wall. Examples of binding of the *O*-acetyl-dependent mAb 1326 to parent and daughter cells are shown in Fig. 6A.

Examination of the binding of mAb 1326 to parent cells at various times after the shift from synthetic medium to 10% FBS showed only 11% of the cells with bright homogeneous binding at the time of the shift (Fig. 6B). The percentage of cells displaying bright homogeneous binding increased to 44% after 18 h incubation in FBS ($P < 0.001$ vs. time 0). Conversely, the percentage of parent cells that did not bind mAb 1326 dropped from 60% at the time of the shift to 24% after 18 h in FBS ($P < 0.001$ vs. time 0). These results indicated that the parent cells either synthesized new GXM that bound the *O*-acetyl-dependent mAb after the shift to growth in FBS or that the 1326 epitope was newly expressed on pre-existing GXM. This result is consistent with results shown in Fig. 2 that the percentage of parent cells showing bright staining increases with cell age. An

examination of daughter cells found that the progeny cells were apparent 6 h after the shift to FBS. All of the cells bound mAb 1326 with either the bright or punctate patterns at 6 h, and almost all cells showed bright patterns after 18 h; no daughter cells were negative at any time point. This result contrasts with the high percentage of daughter cells that showed no binding of mAb 1326 after shift to synthetic medium (Fig. 2).

Strain and species variability in expression of the mAb epitope

Studies described above showing variability in expression of the 302/1326 epitope were done with a single strain of serotype A (CN6). As a consequence, an experiment was done with two additional serotype A strains to determine if the observed variability in expression of the *O*-acetyl-dependent epitope recognized by mAb 1326 was generalizable to other strains. In addition, we examined three isolates of serotype D (*C. neoformans* var. *neoformans*). Cells from three strains of serotype A and three strains of serotype D were incubated with Alexa 555-labeled mAb 1326 (*O*-acetyl-dependent), and the patterns of mAb binding were assessed. The results (Fig. 7) showed that the cell-to-cell variability in expression of the mAb 1326 epitope by strain CN6 was also observed with serotype A strains MU-1 and H99. In contrast, almost 100% of cells of serotype D strains 3501, 24067 and EJ127 produced a bright homogeneous binding pattern.

Variability in epitope expression with soluble GXM

Studies shown in Figs. 1–7 describe the binding of *O*-acetyl-dependent and *O*-acetyl-independent mAbs to GXM as it is presented in an intact capsule. GXM is also shed into body fluids in large amounts during infection and exerts a number of biological effects that may impact pathogenesis. As a consequence, we also examined variability in binding of mAbs to soluble GXM.

An antigen capture ELISA was constructed using mAb F12D2 in the solid phase to capture GXM from the samples (Brandt *et al.*, 2003). mAb F12D2 was raised from mice immunized with de-*O*-acetylated serotype A GXM. As a consequence, it shows uniform binding to cells of all serotypes and to de-*O*-acetylated GXM. HRPO-labeled mAb 1326 (*O*-acetyl-dependent) was used in the fluid phase to assess expression of the 1326 epitope by the GXM preparations. Serotype A GXM (strain CN6) was isolated from supernatant fluids of i) yeast cells grown in synthetic medium, ii) cells grown in 10% FBS, and iii) homogenized brain tissue from *C. neoformans*-infected mice. Although several purification steps were performed on the supernatant from infected brain tissue, the possibility of non-GXM polysaccharide contamination still existed. To ensure accuracy of comparisons made between GXM preparations, F12D2, an *O*-acetyl-independent GXM mAb, was used to normalize GXM concentrations. The results (Fig. 8) showed identical binding of the *O*-acetyl-dependent mAb 1326 to GXM captured by ELISA from brain tissue or growth on 10% FBS (ED₅₀ of 0.0049 µg/ml and 0.0056 µg/ml, respectively). In contrast, there was a significant reduction in binding of the mAb to GXM from cultures grown in synthetic medium (ED₅₀ of 0.066 µg/ml).

Discussion

Unicellular microbes may exhibit marked variation in their surface components, a property that facilitates adaptation to a changing environment, the sequential colonization of various tissues during infection, and the avoidance of immune surveillance (Saunders, 1989; Beale and Wilkinson, 1961). In an early review, Beale and Wilkinson noted that the environment plays an important role in determining which out of an array of alternative antigens is formed by a given cell (Beale and Wilkinson, 1961). Given the fact that *C. neoformans* is a microbe that has an environmental niche and that humans are likely accidental hosts,

expression of *O*-acetyl-dependent epitopes may be a response to the need to adapt to a changing nutritional environment or the need to adapt to changing hosts or predators such as amoebae.

Variable expression of *O*-acetyl-dependent epitopes might influence the pathogenesis of cryptococcosis. Strains that have a *CAS1* deletion are *O*-acetyl negative and are hypervirulent in mice (Janbon *et al.*, 2001). However, the *cas1* Δ parent strain is serotype D; serotype D does not show the variability in expression of the *O*-acetyl-dependent epitope that we found with serotype A (Fig. 7). The *O*-acetyl group is an immunodominant epitope for GXM (Brandt *et al.*, 2003), and many protective antibodies are *O*-acetyl dependent (Casadevall *et al.*, 1994). Variable expression of this critical antibody target as cells age or in response to the host environment may influence the ability of an antibody to protect or tissue-specific adaption during infection. Notably, cryptococcal cells of advanced generational age have increased resistance to phagocytosis and killing by antifungals (Jain *et al.*, 2009). These adaptive properties, accompanied by variability in epitope expression on GXM likely contribute to pathogenesis during chronic infection.

Sumner and Avery define phenotypic heterogeneity as “non-genetic variation that exists between individual cells within isogenic populations” (Sumner and Avery, 2002). The authors further note that “some of the key parameters that drive non-genetic heterogeneity include cell cycle progression, cell aging, mitochondrial activity, epigenetic regulation and potentially also stochastic variation.” Our results clearly implicate cell aging as an operative variable in cell-to-cell variability in expression of the epitope recognized by the *O*-acetyl-dependent 1326 mAb.

Cell-to-cell variability in expression of capsular epitopes within a clonal population of cryptococcal cells has been described previously. For example, Janbon reported phenotypic variation in expression of an epitope recognized by mAb E1 (Janbon, 2004; Garcia-Hermoso *et al.*, 2004). Variability in structure of GXM over buds vs. parental capsule has also been reported (Pierini and Doering, 2001). Our results suggest a link between the two reports. If expression of the 1326 epitope increases with cell age, beginning with no expression of the epitope over budding cells (Fig. 1) to the bright staining pattern observed with aged yeast cells (Fig. 2), this result suggests that an examination of an unsynchronized cell population would show the variability that we and others have observed. The absence of expression of the 1326 epitope in GXM during bud formation also supports the report by Pierini and Doering that GXM produced over the bud is newly synthesized polysaccharide (Pierini and Doering, 2001). Finally, a change in capsule structure as a function of cell age was reported by Pierini and Doering who found an increase in matrix density as a function of culture time (Pierini and Doering, 2001).

Interpretation of results from our study should be limited to what is known about the epitopes that are recognized by the available mAbs. Reactivity of mAbs 339, 302 and 1326 is lost if GXM is chemically de-*O*-acetylated or if there is a mutation on the pathway to *O*-acetylation (Kozel *et al.*, 2003). Detailed genetic studies of *O*-acetylation have been reported for serotype D (Janbon *et al.*, 2001). Our studies with chemically de-*O*-acetylated encapsulated cryptococci suggest a similar result with cells of serotype A. These studies do not identify a specific structure that is phenotypically varied. It is possible that the *O*-acetyl group itself is the relevant structure. Alternatively, variability in *O*-acetylation could mask or unmask structures other than the *O*-acetyl group, or the presence or absence of an *O*-acetyl group could have a secondary effect on epitopes that are not by themselves *O*-acetylated.

A shift in expression of GXM epitopes has been reported previously. For example, Cherniak *et al.* noted variation in the structure and antigenic makeup of GXM in isolates from patients with recurrent cryptococcal meningitis, despite the fact that the isolates were indistinguishable by DNA RFLP analysis (Cherniak *et al.*, 1995). Garcia-Hermoso *et al.* reported structural evolution of GXM epitope expression during mouse passage and prolonged in vitro culture (Garcia-Hermoso *et al.*, 2004). Such changes in epitope structure were relatively stable traits that were evident on yeast subculture. Our report of phenotypic variability in epitope expression in response to environmental cues most closely resembles the report by Charlier *et al.* that the cryptococcal capsule undergoes a rapid change in epitope expression on passage across the blood brain barrier (Charlier *et al.*, 2005). Invasion of the brain after intravenous infection was associated with the acquisition of reactivity with mAb CRND-8 and loss of reactivity with mAb E1. CRND-8 is an *O*-acetyl-dependent mAb raised against serotype D that does not react with cells of serotype A that have been grown in vitro (Ikeda *et al.*, 1996); mAb E1 produces serotype-specific binding patterns to cells of serotypes A, B or D and no binding to cells of serotype C (Dromer *et al.*, 1993). Notably, Charlier *et al.* used a serotype A strain (H99), the serotype that our studies found to undergo phenotypic variability.

In contrast to expression of the *O*-acetyl-dependent epitope that is dependent on cell age, the capsular structure that is responsible for activation of the complement cascade and deposition of C3 into the capsular matrix is expressed on all cells in a non-synchronized cell culture. C3 was also deposited in the capsular matrix that surrounded yeast buds. This independence of complement activation from expression of an *O*-acetyl-dependent epitope is in agreement with our previous reports that de-*O*-acetylated cryptococci are fully able to activate the complement system and bind C3 fragments (Young and Kozel, 1993; Kozel *et al.*, 2003).

C. neoformans serotypes A and D were previously designated as *C. neoformans* var. *neoformans*, whereas serotypes B and C were designated as *C. neoformans* var. *gattii* (Kwon-Chung *et al.*, 1982; Vanbreuseghem and Takashio, 1970). More recently, designation as *C. neoformans* var. *neoformans* has been limited to isolates of serotype D, and isolates of serotype A are designated *C. neoformans* var. *grubii* (Franzot *et al.*, 1999). Despite considerable genetic differences, *C. neoformans* var. *grubii* and var. *neoformans* are distinguished by only a few phenotypic characteristics (Franzot *et al.*, 1999). Our results suggest an additional difference; isolates of serotype A show considerable phenotypic variability in expression of the 1326 epitope, whereas isolates of serotype D show high constitutive expression of this same epitope. It is likely that the cryptococcal capsule evolved as a means to survive in a hostile environment such as soil amoebae (Ruiz *et al.*, 1982) and desiccation (Ophir and Gutnick, 1994). If this is the case, differences in variability of epitope expression by strains of serotypes A and D raise the possibility of different environmental niches for the two varieties.

mAbs 339, 302 and 1326 all required an intact *O*-acetyl group for binding. However, the epitope recognized by mAb 339 differed from that recognized by mAb 302 and 1326. The *O*-acetyl-dependent epitope bound by mAb 339 was found on all parent cells within the population, whereas expression of the epitope recognized by mAbs 302 and 1326 was found only after cells aged. This result suggests two distinct modes by which *O*-acetyl-dependent epitopes can be regulated and/or expressed. Despite the differences in patterns of expression of epitopes recognized by mAb 339 vs. mAbs 302 and 1326 on parent cells, all three failed to bind to capsule overlying buds.

Our results provide further information as to GXM production and modification. GXM is synthesized intracellularly and is secreted from the cell via exocytosis (Yoneda and Doering,

2006). The impact of cell age on binding of mAb 1326 suggests that expression of the 1326 epitope is a late event in capsule formation. These results support the argument that GXM acetylation is the last step in GXM biosynthesis (Janbon *et al.*, 2001). Capsular polysaccharide is clearly evident over the surface of yeast buds (Fig. 1A). Moreover, capsule surrounding yeast buds bind GXM mAbs 3C2, 471 and 1255, activates the complement cascade, and binds C3 in a manner similar to capsule surrounding the parent cells. However, these same buds fail to bind mAbs that require *O*-acetylation for recognition.

Finally, our results have implications for efforts using mAbs to target GXM for immunodiagnosis of infection or treatment. It is clear from our studies that expression of capsular epitopes recognized by GXM mAbs are influenced by both cell age and growth conditions. These results suggest that efforts to target the capsule for immunodiagnosis or immunotherapy must consider the influence of *in vivo* growth conditions on expression of epitopes that might be recognized by such mAbs.

Experimental procedures

C. neoformans cells and GXM

C. neoformans serotype A strains CN6 (ATCC 62066) and H99 (ATCC 208821) were obtained from the American Type Culture Collection. Serotype A strain MU-1 and serotype D strains 3501, 24067 and EJ127 were originally provided by Dr. Robert Cherniak. Unless indicated otherwise, cells were induced for large capsule production by incubation for 4 days at 37°C with 5% CO₂ in synthetic medium (Cherniak *et al.*, 1982) supplemented with 24 mM sodium bicarbonate and 25 mM Hepes (Granger *et al.*, 1985) and killed by overnight treatment with formaldehyde. Some experiments used yeast cells harvested from infected tissue. In this instance, BALB/c mice were injected by the intravenous route with 5000 cells of strain CN6. Once hydrocephalus was observed, mice were sacrificed and yeast cells were isolated from brain tissue as previously described (Gates *et al.*, 2004).

GXM was isolated from culture supernatant fluids and infected brain tissue by differential precipitation with ethanol and hexadecyltrimethylammonium bromide (Cherniak *et al.*, 1991). GXM shed *in vivo* was isolated from supernatant fluid after brain tissue from CN6-infected mice was minced, passed through cheesecloth to remove cellular debris and then centrifuged to remove yeast cells. De-*O*-acetylation of cells and soluble GXM was done by alkaline hydrolysis. The pH was adjusted to 11 with ammonium hydroxide followed by an overnight incubation at room temperature. De-*O*-acetylated cells were washed and resuspended in phosphate-buffered saline (PBS), whereas soluble GXM was dialyzed overnight in PBS.

Reagents, sera, and mAbs

mAbs 3C2, 471, 1255, 339, 1326, and 302 are IgG1 antibodies that are reactive with GXM, the major capsular polysaccharide of *C. neoformans*. These mAbs are described in further detail in Table 1. mAb F12D2 was raised from mice immunized with de-*O*-acetylated serotype A GXM (Brandt *et al.*, 2003). Human complement protein C3 was purified from pooled human plasma by differential precipitation with polyethylene glycol, ion exchange chromatography on Mono Q and Mono S and molecular sieve chromatography on Superdex 200 (Alsenz *et al.*, 1992; Hammer *et al.*, 1981; Tack and Prahl, 1976). Purified human C3 and various mAbs were conjugated to Alexa Fluor dyes (488 or 555) according to manufacturer's instructions (Molecular Probes). Complement deposition studies were done with pooled human serum obtained from Innovative Research (Novi, MI).

mAb and C3 binding to the capsule

C. neoformans cells (1.2×10^5) were incubated with mAbs (50 $\mu\text{g/ml}$) in a 50 μl reaction volume for 10 min at 37°C to assess mAb binding to the capsular matrix by DIC microscopy. Direct immunofluorescence examining antibody binding was done similarly, except the concentration of mAbs conjugated to Alexa Fluor 555 was 10 $\mu\text{g/ml}$. Statistical comparisons of antibody binding patterns in populations of cells were done by Chi-square analysis. Direct labeling of the cell wall was accomplished by incubating live yeast at 0°C with Alexa Fluor 488. To remove unbound fluorophore, cells were washed 3 times with cold PBS before transferring to fresh synthetic medium or 10% FBS in PBS. In control experiments, the Alexa Fluor dye was found to co-localize with calcofluor white, a dye that binds to chitin in fungal cell walls (Pringle, 1991). Additional controls found that cells labeled with Alexa Fluor retained the dye for several days without loss of fluorescence and that labeling with the fluorophore had no effect on yeast viability as shown by quantitative culture of labeled and sham labeled cells (not shown). Similar results for labeling of the cryptococcal cell wall were reported by Pierini and Doering with the exception that labeling was done with amino-fluorescein via a carbodiimide reaction (Pierini and Doering, 2001).

Binding of C3 to the capsular matrix was determined by incubating *C. neoformans* cells (1.2×10^5) in 40% pooled normal human serum supplemented with 50 $\mu\text{g/ml}$ of Alexa Fluor 555- conjugated human C3 for 32 min at 37°C. Cells were treated with 10 mM EDTA in PBS to stop complement deposition, then washed with PBS and incubated with fluorescent GXM mAbs as described above. All cells were immediately transferred to poly-L-lysine-coated slides and examined with a Nikon C1 confocal equipped with differential interference contrast (DIC) optics attached to a Nikon Eclipse E800 microscope (100 $\times$ objective). Confocal images were processed using Nikon EZC1 software, version 1.70. Images were merged and cropped using Simple PCI software (Compix, Cranberry Township, PA).

Assays for antibody activity

An ELISA was used to assess expression of the *O*-acetyl-dependent epitopes recognized by mAb 1326 in GXM isolated from culture supernatant fluids or from homogenates of brain tissue. In this immunoassay, microtiter plates were coated overnight with the *O*-acetyl-independent mAb F12D2 (1 $\mu\text{g/ml}$ in PBS) (Brandt *et al.*, 2003). Plates were washed with PBS-Tween (PBS containing 0.05% Tween 20, pH 7.4) and incubated for 2 h with a blocking solution (PBS containing 0.5% Tween 20 and 5% skim milk). GXM in blocking solution was added in serial two-fold dilutions and incubated for 90 min. After washing plates 3 times with blocking solution, horseradish peroxidase conjugated GXM mAbs 1326 (*O*-acetyl-dependent; 16 $\mu\text{g/ml}$) was added and incubated for 90 min. Finally, plates were washed 3 times with PBS-Tween and incubated for 30 min with TMB substrate (KPL, Gaithersburg, MD). Stop solution (H_3PO_4) was added and the absorbance was read at 450 nm. Immunoassay reactivity with mAb F12D2 in the indicator phase was used to normalize the GXM content in the various samples. The concentration of GXM that produced half maximal OD_{450} (ED_{50}) was calculated with the assistance of SigmaPlot (Systat Software, Inc., San Jose, CA).

Acknowledgments

This study was supported by Public Health Service grant AI014209 from the National Institute for Allergy and Infectious Diseases.

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Fig. 1.

Binding of a library of GXM mAbs to the capsular matrix of serotype A cells. Panel A – Binding of Alexa 555-labeled mAbs to serotype A CN6 cells grown for 4 days in synthetic medium under capsule induction conditions. Results are representative of cells showing the “bright” binding pattern (as illustrated in Panel B). Panel B – Examples of three different capsular binding patterns observed with mAbs 302 and 1326. Fluorescence images in Panel A are equatorial slices; images in Panel B are projections of slices collected through the entire cell. Panel C - Percentage of cells showing bright, punctate and negative binding (n = 200 cells).

Fig. 2.

Binding of *O*-acetyl-dependent mAb 1326 to serotype A cells of different ages. Cells were grown in synthetic medium for 3 days, directly stained with Alexa Fluor 488 (green) to label cell walls of parent cells, washed and added to fresh medium. Cells were collected at subsequent time points and analyzed for changes in binding of Alexa 555-labeled (red) mAb 1326. Panel A – Patterns of mAb binding to parent and daughter cells as a function of incubation time. Panel B – Example of mAb binding patterns to Alexa Fluor 488-labeled parent cells used to inoculate fresh medium. Panel C – After 6 days in culture, example of mAb binding to parent cells identified by green staining of the cell wall and daughter cells distinguished by their lack of cell wall staining. Parent with bright pattern (PB), parent with punctate pattern (PP), parent with negative binding (PN), daughter with bright pattern (DB), daughter with punctate pattern (DP), and daughter with negative binding (DN).

Fig. 3. Relative sites for C3 deposition and mAb binding in the capsular matrix surrounding parent and buds on serotype A cells. Yeast cells were incubated in 40% normal human serum containing trace amounts of Alexa 488-labeled human C3 (green), washed and incubated with Alexa 555-labeled mAbs (red).

Fig. 4. Capsule reactions of mAbs with untreated serotype A cells [O-Ac(+)] and cells that have been de-*O*-acetylated by treatment at pH 11 with NH_4OH [O-Ac(-)]. mAb binding was evaluated by capsule reaction via DIC microscopy (Panel A) and by use of Alexa 555-labeled mAbs (Panel B).

Fig. 5.
Binding of Alexa 555-labeled mAbs to serotype A cells isolated from infected brain tissue.
Panel A – Untreated yeast cells. Panel B – Yeast cells from infected brain that have been de-
O-acetylated by treatment at pH 11 with NH_4OH .

Fig. 6. Binding of *O*-acetyl-dependent Alexa 555-labeled mAb 1326 to serotype A cells that were grown in synthetic medium, washed and shifted to growth in 10% FBS in PBS. Cells were grown in synthetic medium for 2 days, directly stained with Alexa Fluor 488 to label cell walls of parent cells, washed and added to fresh medium containing serum. Cells were collected at subsequent time points and analyzed for changes in binding of mAb 1326. Panel A – Examples of mAb binding patterns to parent cells showing green staining of the cell wall and daughter cells which do not retain the Alexa 488-stained cell wall. Panel B – Patterns of mAb binding to parent and daughter cells as a function of time after shift to medium containing serum. Daughter cells did not appear until 6 h after the shift.

Fig. 7.

Impact of strain and species differences on variability in expression of the epitope recognized by mAb 1326. Cells from three strains of serotype A (*C. neoformans* var. *grubii*) and serotype D (*C. neoformans* var. *neoformans*) were incubated with Alexa 555-labeled mAb 1326, and the patterns of mAb binding were assessed. Panel A – Image showing heterogeneity of binding to cells of serotype A strain CN6 and uniformity of binding to cells of serotype D strain 3501. Top panels show fluorescence images alone; bottom panels show merge of fluorescence and DIC images. Panel B – Percentage of cells of different strains showing bright, punctate and negative binding with mAb 1326. Patterns of binding were assessed as described for Fig. 1 (n = 200 cells).

Fig. 8.

Reactivity of *O*-acetyl-dependent mAb 1326 in sandwich ELISA with GXM isolated from infected brain tissue or supernatant fluid from *C. neoformans* serotype A strain CN6 grown in synthetic medium or 10% FBS. Microtiter wells were first coated with the *O*-acetyl-independent mAb F12D2 to allow for capture of GXM, incubated with various concentrations of GXM, and then incubated with HRPO-labeled mAb 1326.

Table 1

Characteristics of mAbs used for this study.

mAb	GXM used for immunization	Isotype	Serotype reactivity	Reactivity with de-O-acetylated serotype D cells ^a	References
3C2	Serotype C	IgG1	A, B, C, D	Yes	(Belay <i>et al.</i> , 1997; Spiropulu <i>et al.</i> , 1989; Kozel <i>et al.</i> , 2003)
471	Serotype A	IgG1	A, B, C, D	Yes	(Belay <i>et al.</i> , 1997; Spiropulu <i>et al.</i> , 1989; Kozel <i>et al.</i> , 2003)
1255	Serotype A	IgG1	A, B, D	Yes	(Belay <i>et al.</i> , 1997; Eckert and Kozel, 1987; Kozel <i>et al.</i> , 2003)
339	Serotype B	IgG1	A, B, D	No	(Belay <i>et al.</i> , 1997; Spiropulu <i>et al.</i> , 1989; Kozel <i>et al.</i> , 2003)
302	Serotype D	IgG1	A, D	No	(Belay <i>et al.</i> , 1997; Eckert and Kozel, 1987; Kozel <i>et al.</i> , 2003)
1326	Serotype A	IgG1	A, D	No	(Netski and Kozel, 2002; Kozel <i>et al.</i> , 2003)

^aReactivity determined by Quellung-type reaction with cells of strains NE167 or NE169 (*O*-acetyl positive) vs. strains NE168 or NE170 (*O*-acetyl negative) (Kozel *et al.*, 2003).