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Antibody to *Cryptococcus neoformans* Glucuronoxylomannan Inhibits the Release of Capsular Antigen

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Cryptococcus neoformans **releases capsular polysaccharide in the supernatant of liquid cultures and in tissues. Significantly less glucuronoxylomannan (GXM) was released by *C. neoformans* in the presence of capsule-binding monoclonal antibody (MAb). MAb-mediated inhibition of GXM release may be another mechanism by which humoral immunity can mediate protection against this pathogen.**

Immunoglobulins (Ig) are the effector molecules of the adaptive humoral immune response. The classical mechanisms by which antibody mediates protection against microbes include opsonization, complement activation, toxin neutralization, antibody-dependent cellular cytotoxicity, and viral neutralization (1). Specific antibody is also a potent immunomodulator whose presence can alter cytokine expression and leukocyte recruitment and modulate the cellular response (1).

Studies of antibody-mediated protection against the human pathogenic fungus *Cryptococcus neoformans* have provided insight into the complexity of antibody-mediated protective mechanisms (1). *C. neoformans* is an encapsulated opportunistic yeast-like fungus responsible for life-threatening meningoencephalitis in immunocompromised and previously healthy individuals. Approximately 6 to 8% of patients with AIDS develop cryptococcal infection, although the proportion in Africa may be significantly higher. *C. neoformans* infections present formidable problems for the host immune response, including the presence of giant cells in tissue, a lack of antibody responsiveness to capsular polysaccharide, and extensive accumulation of polysaccharide in tissue (2). The presence of glucuronoxylomannan (GXM), the major capsular polysaccharide of *C. neoformans*, in tissue is believed to be a major contributor to *C. neoformans* pathogenesis since this compound has been associated with a variety of immunosuppressive effects (20). For instance, GXM can interfere with phagocytosis, antigen presentation, leukocyte migration and proliferation, and specific antibody responses, and the capsular polysaccharide can enhance HIV replication (20). *C. neoformans* releases abundant capsular polysaccharide in the supernatant of liquid cultures and in tissues (7, 10). In individuals with impaired immunity affected by cryptococcosis, high levels of *C. neoformans* capsular polysaccharide are often detected in serum and cerebrospinal fluid. Recent investigations suggest that the accumulation of GXM in the cytoplasmic vacuoles of phagocytic cells might contribute to cell destruction (10).

Administration of monoclonal antibody (MAb) directed against GXM significantly reduces serum GXM levels in rodents infected with *C. neoformans* through the formation of antigen-antibody complexes that are taken up by reticuloendothelial cells (13). MAb to *C. neoformans* capsular polysaccharide promotes opsonization, phagocytosis, and growth inhibition of encapsulated *C. neoformans* by phagocytic cells (15). However, we hypothesized that antibody may also reduce GXM levels by interfering with the release of polysaccharide from the capsule.

C. neoformans strains H99 (serotype A) and 24067 (serotype D) were acquired from John Perfect (Durham, N.C.) and the American Type Culture Collection (Rockville, Md.), respectively. These strains have been well characterized. MAbs 18B7 (IgG1), 12A1 (IgM), 13F1 (IgM), and 21D2 (IgM) each bind to GXM and have been described previously (3, 4, 6, 15). The murine IgG1 MAbs 3671 and 3665 and the murine IgM MAb 4F11 were used as isotype-matched controls, having specificity for phenylarsonate and arabinomannan, respectively (9, 19). Neither MAb 3665, 3671, nor 4F11 binds to *C. neoformans* polysaccharide. MAbs 18B7, 3665, and 3671 were purified by protein G affinity chromatography (Pierce, Rockford, Ill.). IgM MAbs 12A1, 13F1, 21D2, and 4F11 were used as ascites or after mannan-binding protein affinity chromatography (Pierce). Antibody concentration was determined by enzyme-linked immunosorbent assay (ELISA) relative to isotype-matched standards. *C. neoformans* capsular GXM was measured by capture ELISA as described previously (4). Briefly, samples of *C. neoformans* supernatant to be assayed for GXM were treated with 20 μg of proteinase K/ml overnight at 37°C to digest MAb before evaluation by capture ELISA. After proteolytic digestion, the samples were heated at 100°C for 15 min to inactivate the enzyme. Microtiter polystyrene plates were coated with goat anti-mouse IgM (1 μg/ml) and blocked with 1% bovine serum albumin in phosphate-buffered saline. Next, the IgM GXM binding MAb 2D10 (2 μg/ml) was added as a capture antibody, and the plate was incubated for 1 h. The solution to be tested for GXM was then added, serially diluted on the plate, and incubated for 1 h. The ELISA was completed by adding, in successive steps, MAb 18B7 (2 μg/ml) in phosphate-buffered saline (1% bovine serum albumin), 1 μg of alkaline phosphatase-labeled goat anti-mouse IgG1/ml, and 50 μl of *p*-nitrophenyl phosphate (5 mg/ml) in substrate buffer. Between every step, the wells were washed with 0.05% Tween 20 in Tris-buffered saline. All incubations were done at 37 or

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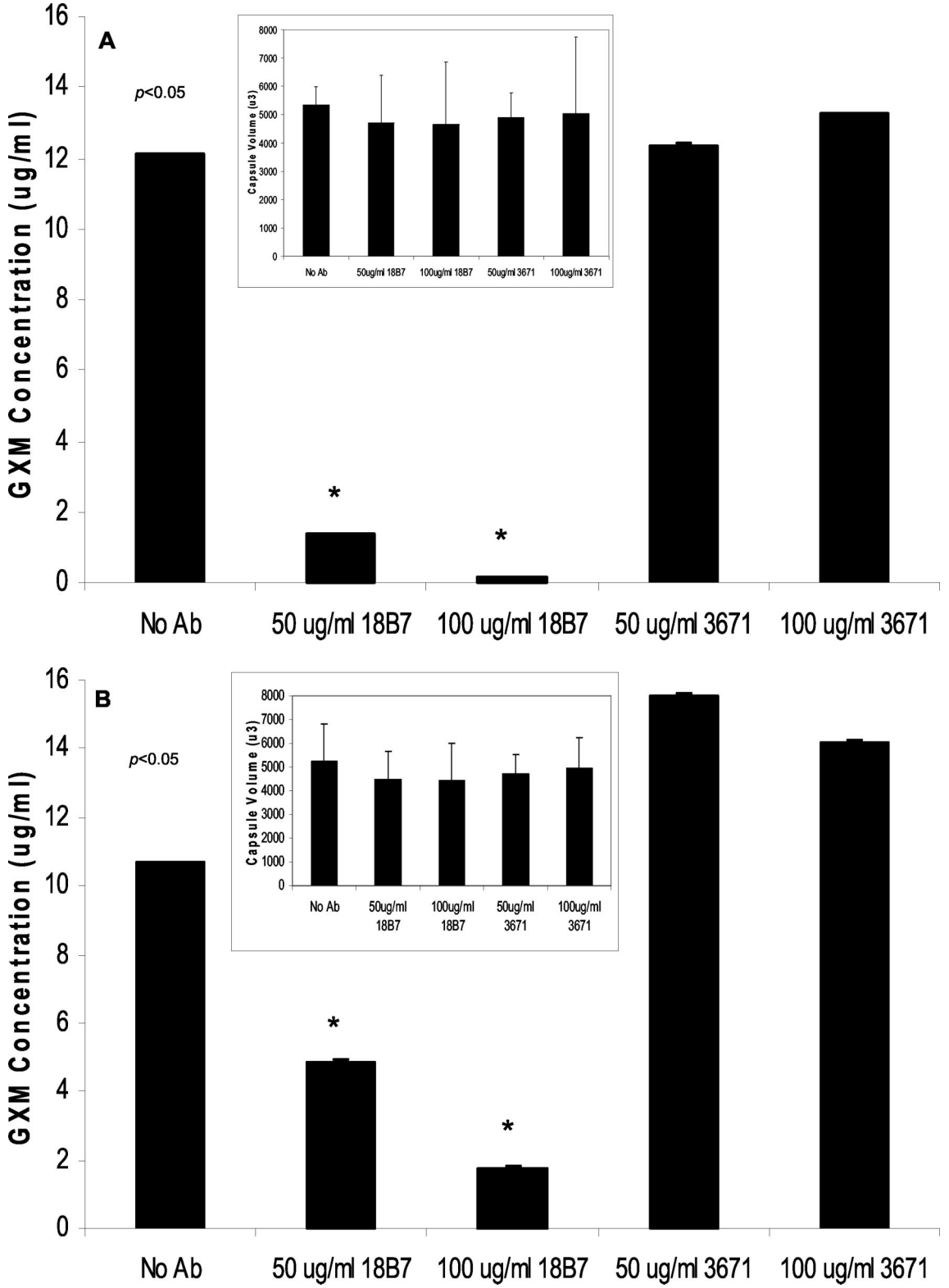
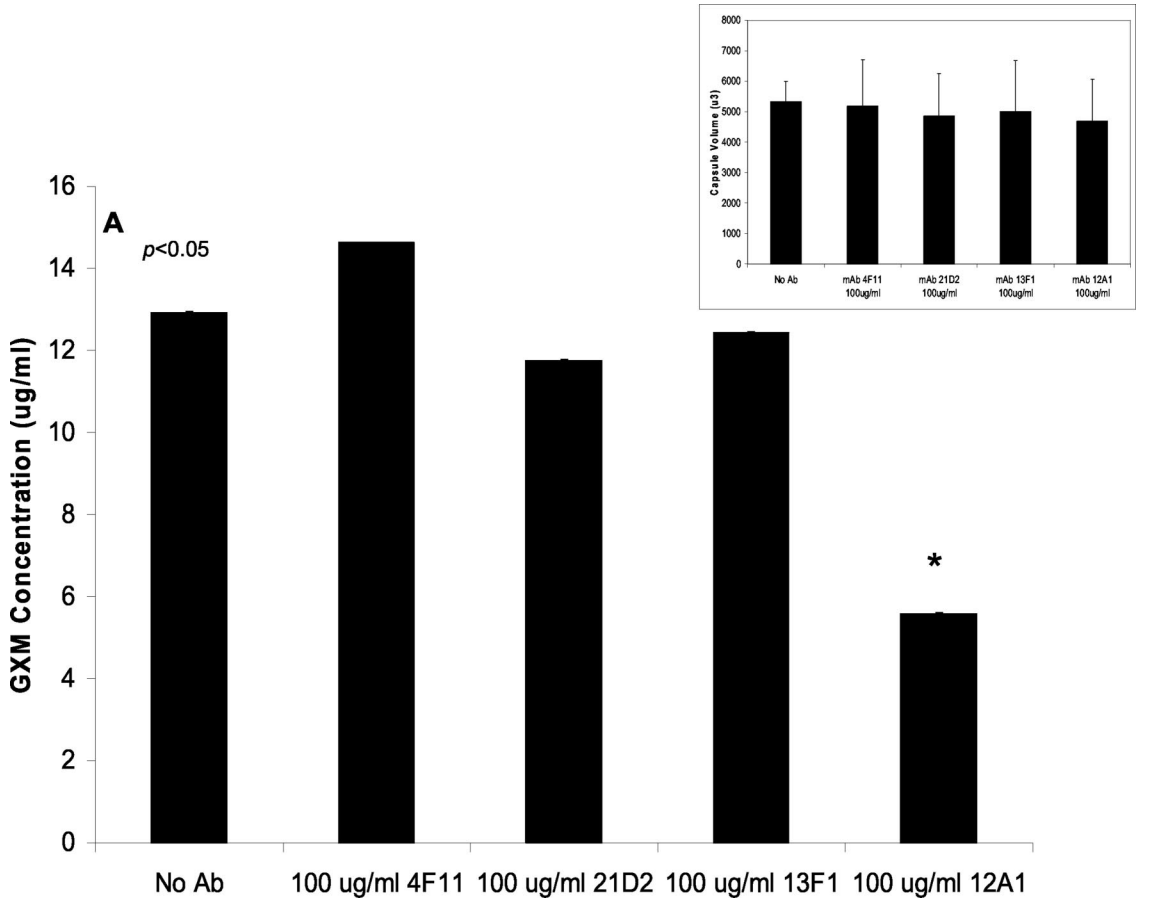
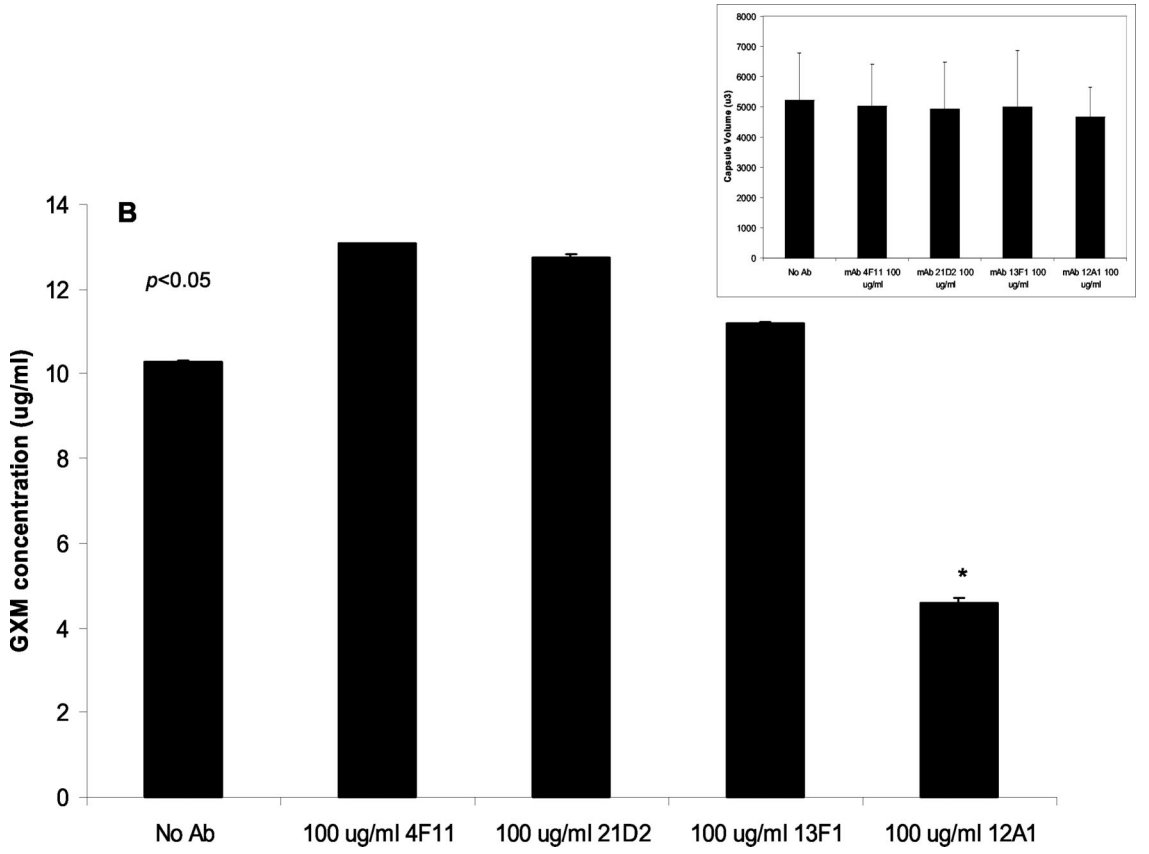


FIG. 1. GXM concentration in the supernatant of *C. neoformans* H99 and 24067 cultures (panels A and B, respectively) in the presence and absence of IgG1-specific MAbs. The inset graphs correspond to capsule size measurements of *C. neoformans* H99 and 24067 cells. Bars are the average of three GXM concentration measurements, and brackets denote standard deviations. Asterisks denote *P* value significance calculated by analysis of variance and adjusted by the Bonferroni correction. Ab, antibody.


A
p<0.05
GXM Concentration (ug/ml)
16
14
12
10
8
6
4
2
0
*
No Ab
100 ug/ml 4F11
100 ug/ml 21D2
100 ug/ml 13F1
100 ug/ml 12A1
Capsule Volume (u3)
8000
7000
6000
5000
4000
3000
2000
1000
0
No Ab
mAb 4F11 100ug/ml
mAb 21D2 100ug/ml
mAb 13F1 100ug/ml
mAb 12A1 100ug/ml


B
p<0.05
GXM concentration (ug/ml)
14
12
10
8
6
4
2
0
*
No Ab
100 ug/ml 4F11
100 ug/ml 21D2
100 ug/ml 13F1
100 ug/ml 12A1
Capsule Volume (u3)
8000
7000
6000
5000
4000
3000
2000
1000
0
No Ab
mAb 4F11 100 ug/ml
mAb 21D2 100 ug/ml
mAb 13F1 100 ug/ml
mAb 12A1 100 ug/ml

4°C overnight. All data were subjected to statistical analysis by using Primer of Statistics-The Program software (McGraw-Hill Co., New York, N.Y.). *P* values of <0.05 were considered significant.

Capsule size measurements of *C. neoformans* cells were done for cells grown in the presence and absence of MAb 18B7, as described previously (21). *C. neoformans* H99 and 24067 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) with MAbs 18B7, 12A1, 13F1, and 21D2 or irrelevant control MAbs 3671, 3665, and 4F11 (100 μg/ml) for 48 h at 37°C without shaking to prevent antibody-mediated clumping of cells. Capsule size of *C. neoformans* cells grown in DMEM (10% FCS) culture was measured at 0, 24, and 48 h after incubation. Three microliters of culture of cells grown without MAb and in the presence of MAbs 18B7, 12A1, 13F1, and 21D2 or MAbs 3671, 3665, and 4F11 was placed on a slide, and a small drop of India ink was added. The slides were viewed under a microscope. The thickness of the *C. neoformans* capsule and cell body was measured by tracing the circumference of the whole organism and cell body at the equatorial plane (18).

To evaluate the effect of antibody on polysaccharide release by *C. neoformans* H99 or 24067, cells were grown in DMEM (10% FCS) in presence or absence of MAbs 18B7, 12A1, 13F1, and 21D2 or irrelevant control MAbs 3671, 3665, and 4F11 (100 μg/ml) for 48 h at 37°C in 10% CO_2 without shaking to prevent antibody-mediated clumping of cells. CO_2 induces *C. neoformans* polysaccharide capsule growth (11). To study GXM release, 1 ml of supernatant from each condition was collected after 48 h. *C. neoformans* cells were then separated by centrifugation, and the supernatant was utilized fresh or frozen at −20°C until analyzed, which usually occurred within a day or two. GXM concentrations in supernatant were then measured by capture ELISA as described above.

C. neoformans cells are known to release GXM during growth in culture. *C. neoformans* H99 or 24067 cells were grown in the absence of MAb or with 25, 50, 75, and 100 μg of MAb 18B7 or irrelevant MAbs 3671 and 3665 per ml and 100 μg of MAb 12A1, 13F1, and 21D2 or irrelevant MAb 4F11 per ml under capsular induction conditions at 37°C without shaking. After 48 h, supernatants of *C. neoformans* cultures grown in the presence of MAb 18B7 or 12A1 had significantly lower levels of GXM than cells grown in absence of MAb or grown in the presence of MAb 13F1 or 21D2 and control MAb 3671 or 4F11 (Fig. 1 and 2). A reduction in supernatant soluble polysaccharide was observed for antibody concentrations greater than 25 μg/ml. We observed considerable interexperimental variation in the magnitude of reduction in GXM concentration in the presence of MAb 18B7, but this effect was statistically significant in 19 of 27 independent experiments done over 2 years. The GXM concentration in the presence of MAb 3665 was highly variable and in some experiments, it was higher than the condition of no antibody (data not shown), suggesting that this "irrelevant" IgG1 may have may have some uncontrolled effect on capsule release. In contrast, MAb 18B7 was not associated with an increased GXM level relative to the no-antibody control in any experiment. Consequently, we used 3671 as another IgG1 control and found no difference between the irrelevant IgG control and the no-antibody-added condition. There was no difference in capsule size among cryptococcal cells grown in the presence or absence of MAbs 18B7, 12A1, 13F1, and 21D2 or irrelevant MAbs 3671, 3665, and 4F11. The reduction in solution GXM measured in the presence of antibody was not a consequence of differences in growth rates since capsule-binding MAbs have no effect on *C. neoformans* replication as measured by cell counts with a hemocytometer.

Furthermore, the low concentration of GXM in the supernatant of cultures containing MAb 18B7 was not a false-negative result arising from the masking of GXM by specific antibody or precipitation since equal amounts of GXM were measured by ELISA in control experiments where 7-day-old supernatant from *C. neoformans* cultures was mixed with MAb 18B7 or 3671 and then subjected to proteinase K digestion and our ELISA protocols (data not shown). The ability of MAbs 18B7 and 12A1 to reduce the concentration of GXM in the supernatant was confirmed by a secondary method using the CALAS (cryptococcal antigen latex agglutination system) kit (Meridian Bioscience, Cincinnati, Ohio) (data not shown).

In addition, we investigated the effect of specific antibody on capsule size and the phenomenon of serum-induced capsular enlargement (21). The capsule size of *C. neoformans* H99 cells was measured with India ink in cells grown in the presence or absence of MAb 18B7 to determine whether or not CO_2 induces capsule growth and its effect in GXM released in culture. After 24 h, the capsule size of *C. neoformans* H99 cells was similarly highly induced under all conditions. There was no significant difference in *C. neoformans* polysaccharide capsule size in the presence or absence of capsular-binding MAb 18B7. However, growth of *C. neoformans* in concentrations of 100 μg/ml to 1 mg/ml produced aberrant changes to the capsule (Fig. 3), suggesting interference with capsular remodeling during growth. Although the biological consequences of this phenomenon are uncertain, it is noteworthy that concentrations of >100 μg/ml in serum are achievable with the dose of 1 mg/mouse used in many passive protection experiments (15, 16).

Previous studies have shown that antibodies to *C. neoformans* GXM significantly reduced serum GXM levels in rodents. The generally accepted explanation for this effect is that antibody promotes ingestion of soluble GXM by reticuloendothelial cells (10, 12). In fact, this mechanism has been validated by studies showing that the injection of GXM into mice and rats followed by the administration of specific antibody results in polysaccharide uptake by macrophages. In this study, we demonstrate a second potential mechanism by which the ad-

FIG. 2. GXM concentration in the supernatant of *C. neoformans* H99 and 24067 cultures (panels A and B, respectively) in the presence and absence of IgM-specific MAbs. The inset graphs correspond to capsule size measurements of *C. neoformans* H99 and 24067 cells. Bars are the average of three GXM concentration measurements, and brackets denote standard deviations. Asterisks denote *P* value significance calculated by analysis of variance and adjusted by the Bonferroni correction. The experiment was done four times, with similar results. Ab, antibody.

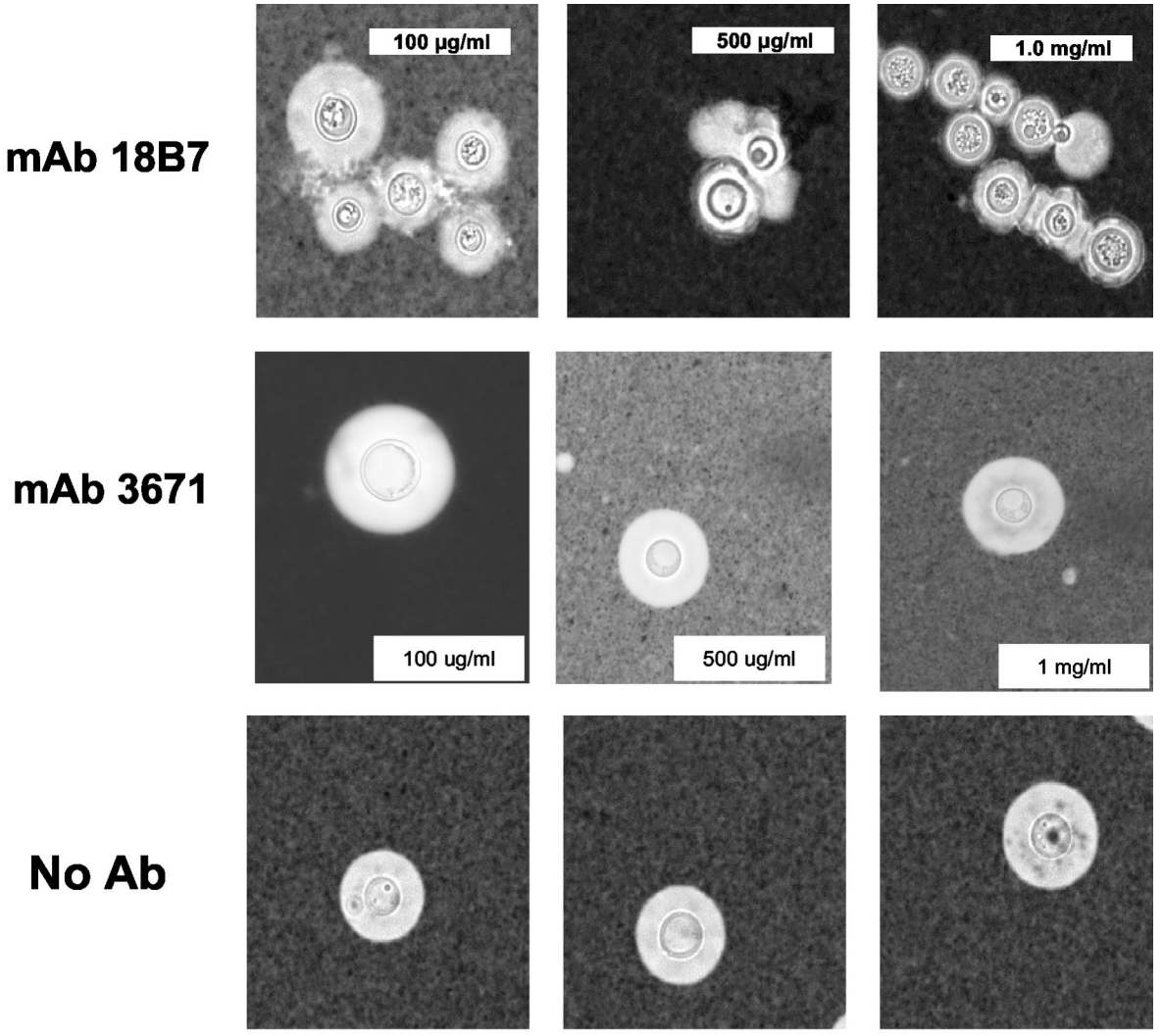


FIG. 3. The effect of MAb (mAb) on capsule size of *C. neoformans* H99 and the phenomenon of serum-induced capsular enlargement were measured with India ink. Ab, antibody. Magnification, ×400.

ministration of specific antibody can result in lower GXM levels in serum that involves inhibiting GXM release from encapsulated cells. Although the mechanism by which GXM is released from cells is not understood, we propose that antibody interferes with this process by cross-linking GXM molecules in the capsule and thus preventing their release. In this regard, antibody cross-linking of GXM fibrils in the polysaccharide capsule has been implicated in differences in capsule complement deposition (13). In addition, we report differences in the ability of two IgMs differing in protective efficacy to interfere with GXM release from *C. neoformans* cells. IgM MAbs 12A1 and 13F1 originated from the same B-cell precursor but differed in epitope specificity and protective efficacy, apparently due to somatic mutations in their variable region (5, 14). MAb 12A1 is protective and produces an annular immunofluorescence pattern on *C. neoformans* serotype D, whereas MAbs 13F1 and 21D2 are nonprotective and produce a punctate appearance upon binding to the capsule (8, 17). MAb 12A1 was more effective than either MAb 21D2 or MAb 13F1 in preventing *C. neoformans* GXM shedding. The observation that antibody can inhibit the release of GXM suggests another mechanism by which specific antibody can contribute to host defense against *C. neoformans*. Our results establish a new effect of antibody that may be operative in the defense against pathogens that release immunologically active microbicidal products into tissue.

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