

Specific Antibody to *Cryptococcus neoformans* Glucuronoxylomannan Antagonizes Antifungal Drug Action against Cryptococcal Biofilms In Vitro

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The fungus *Cryptococcus neoformans* possesses a polysaccharide capsule and can form biofilms on medical devices. We investigated the efficacy that the combination of a specific antibody to the capsular polysaccharide and antifungal therapy has against cryptococcal biofilms. The antibody enhanced the susceptibility of planktonic cells to antifungal agents, but an antagonistic effect was observed for combination therapy against *C. neoformans* biofilms. Our findings suggest that antibody therapies for infectious diseases that involve biofilms may antagonize certain antimicrobial therapies, and they also imply that products of the immune response may contribute to drug resistance of biofilms formed in vivo.

The encapsulated yeast *Cryptococcus neoformans* is a relatively frequent cause of life-threatening meningoencephalitis in immunosuppressed individuals. Glucuronoxylomannan (GXM) is the major component of the capsular polysaccharide, is shed abundantly during infection, and contributes to virulence by interfering with the immune response [1, 2]. GXM interferes with phagocytosis, antigen presentation, leukocyte migration and proliferation, and specific antibody responses; in addition, the capsular polysaccharide can enhance the replication of HIV [2].

C. neoformans forms biofilms on polystyrene plates [3] and medical devices, including ventriculoatrial-shunt catheters [4]. In addition, there have been several reports of *C. neoformans* infection of polytetrafluoroethylene peritoneal-dialysis fistula

and prosthetic cardiac valves, which highlights the ability of this organism to adhere to medical devices [5–7]. The increasing use of ventriculoperitoneal shunts to treat intracranial hypertension associated with cryptococcal meningoencephalitis highlights the importance of investigating the biofilm-forming properties of this organism. Cryptococcal biofilms are significantly less susceptible to antifungal drugs than are planktonic cells but are not less susceptible than biofilms caused by other organisms, which suggests an explanation for the difficulty in the eradication of infections of prosthetic devices [8]. *C. neoformans* biofilm formation begins with the adhesion of cryptococci to a solid surface, followed by the release of cryptococcal polysaccharides, including GXM [3]. Specific antibody to GXM has been shown to interfere with biofilm formation [3], but it is ineffective against established biofilms (L.R.M. and A.C., unpublished data).

The difficulties associated with the treatment of fungal diseases have stimulated interest in the development of immunotherapy. Passive antibody therapy is being developed for the treatment of both human cryptococcosis [9] and candidiasis [10]. Numerous studies of animal models of fungal infection [11–13] and in vitro [14] have established that specific antibody can enhance the efficacy of antifungal therapy. However, none of these models have involved the formation of fungal biofilms. Because biofilm formation results in the formation of a physical barrier against host immune mechanisms and antimicrobial therapy, it is important to investigate in this setting the interaction between immunoglobulins and antimicrobial drugs. In the present study, we investigated the efficacy the combination of GXM-binding monoclonal antibodies (MAbs) and antifungal drugs has against *C. neoformans* biofilms. The results indicate antagonism, which both suggests a new consideration in the use of combined therapy when biofilms are present and implies that immune molecules play a role in the phenomenon of acquired drug resistance.

Materials and methods. *C. neoformans* strain B3501 (serotype D) was used in the present study; it was acquired from the American Type Culture Collection. This strain was selected because it forms strong biofilms [3]. MAb 18B7 (IgG1) binds to GXM and has been described elsewhere [9]. The murine IgG1 MAb 3671 was used as an isotype-matched control, because it has specificity for phenylarsonate and does not bind to the *C. neoformans* polysaccharide. GXM-specific MAb 18B7 and control MAb 3671 were purified by protein G affinity chromatography (Pierce). Antibody concentrations relative to isotype-matched standards were determined by ELISA.

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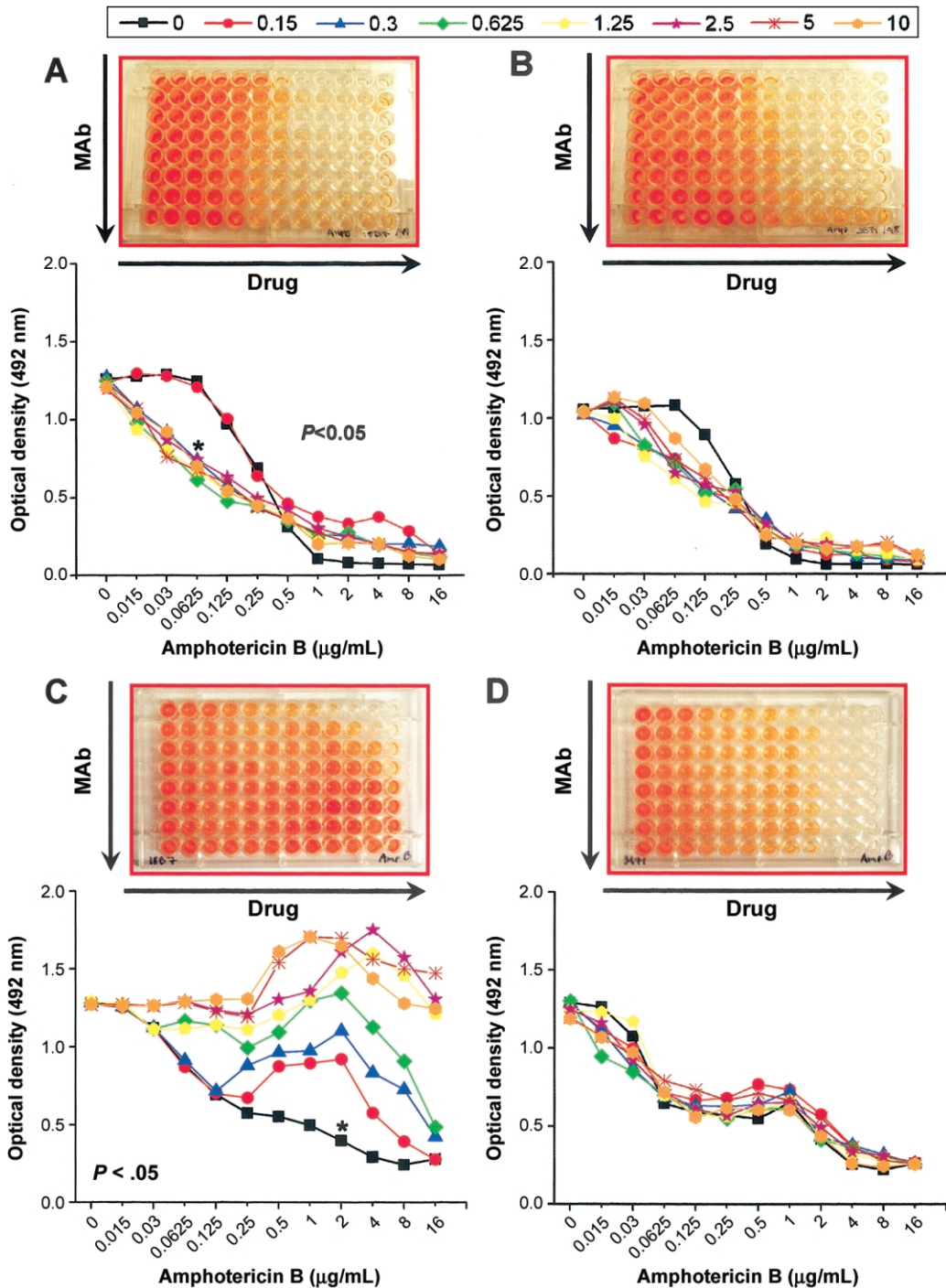


Figure 1. Susceptibility of *Cryptococcus neoformans* B3501 planktonic cells and biofilms to combination therapy, as measured by the tetrazolium hydroxide (XTT) reduction assay. Planktonic cells (A, B, E, and F) or biofilms (C, D, G, and H) were exposed to various combinations of glucuronoxylomannan (GXM)-specific monoclonal antibody (MAb) 18B7 and concentrations of amphotericin B (A and C), various combinations of control MAb 3671 and concentrations of amphotericin B (B and D), various combinations of GXM-specific MAb 18B7 and concentrations of caspofungin (E and G), and various combinations of control MAb 3671 and concentrations of caspofungin (F and H). Arrows denote increasing concentrations of either antibody or drug. For panels C and D, biofilms were similarly treated with amphotericin B and either MAb 18B7 (C) or MAb 3671 (D). Biofilms treated with MAb 18B7 and amphotericin B showed higher metabolic activity than biofilms treated with the drug but no antibody, which is consistent with an antibody-induced reduction in drug activity; in contrast, control MAb 3671 had no effect on the antifungal activity of amphotericin B. Asterisks denote significance ($P < .05$) for planktonic cells coincubated in the presence of GXM-specific MAb 18B7 and a selected concentration of amphotericin B (0.0625 $\mu\text{g}/\text{mL}$; A) and for biofilms coincubated in the presence of MAb 18B7 and a selected concentration of either amphotericin B (2 $\mu\text{g}/\text{mL}$; C) and caspofungin (32 $\mu\text{g}/\text{mL}$; G). P values were calculated by analysis of variance and adjusted by use of Bonferroni's correction. This experiment was done twice, with similar results each time.

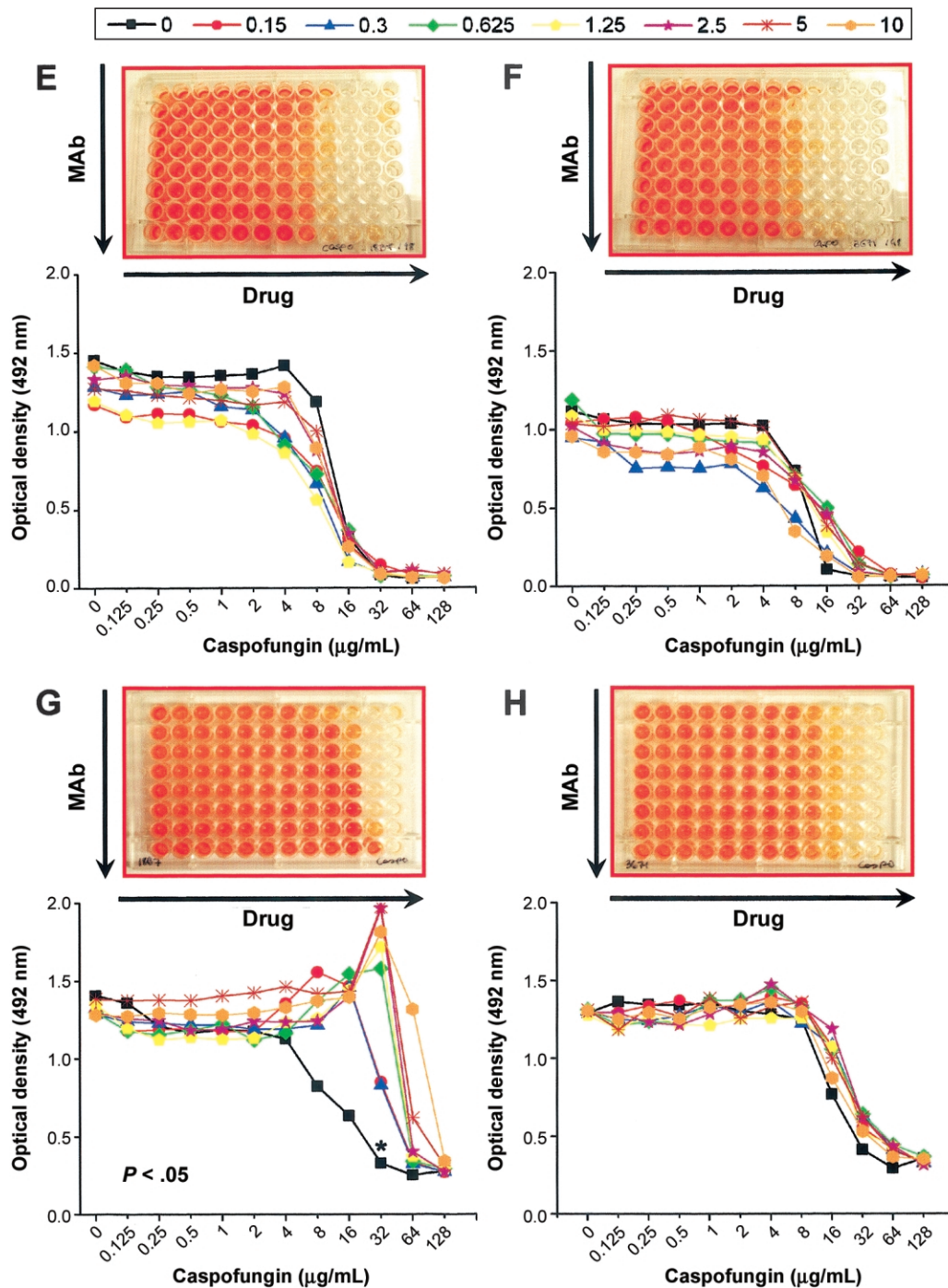


Figure 1. (Continued.)

A semiquantitative measurement of *C. neoformans* biofilm formation was obtained by use of the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay. For *C. neoformans* strains, 50 µL of XTT salt solution (1 mg/mL in PBS) and 4 µL of menadione solution (1 mmol/L in acetone) (Sigma) were

added to each well. Microtiter plates were incubated for 5 h at 37°C. Fungal mitochondrial dehydrogenase activity reduces XTT tetrazolium salt to XTT formazan, resulting in a colorimetric change that correlates with cell viability [15]. The colorimetric change was measured at 492 nm, by use of a microtiter reader (Labsystem Multiskan). The colorimetric signal

resulting from XTT reduction correlates with the number of colony-forming units of both planktonic and biofilm forms of *C. neoformans* [8].

To evaluate whether the GXM-binding MAb 18B7 enhances the efficacy that amphotericin B and caspofungin have against *C. neoformans* planktonic cells (free-swimming yeasts or yeast in suspension), fungal cells were grown in Sabouraud dextrose broth (Difco Laboratories), for 24 h at 30°C in a rotary shaker at 150 rpm (to the early stationary phase). *C. neoformans* cells were collected by centrifugation at 201.6 g, were washed twice with PBS, were counted using a hemacytometer, and were suspended at 5×10^7 cells/mL in minimal medium (20 mg/mL thiamine, 30 mmol/L glucose, 26 mmol/L glycine, 20 mmol/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 58.8 mmol/L KH_2PO_4). Combinations of either MAb 18B7 or the control MAb 3671 (0, 0.15, 0.3, 0.625, 1.25, 2.5, 5, or 10 $\mu\text{g}/\text{mL}$) and either amphotericin B (0, 0.015, 0.03, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, or 16 $\mu\text{g}/\text{mL}$) or caspofungin (0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, or 128 $\mu\text{g}/\text{mL}$) were serially diluted into individual wells of 96-well polystyrene plates (Fisher). Then, 100 μL of the suspension with cryptococci was added and incubated without shaking for 48 h at 37°C. The XTT reduction assay was used to assess the metabolic activity of planktonic cells.

To determine whether the GXM-binding MAb 18B7 enhances the efficacy of amphotericin B and caspofungin against *C. neoformans* biofilms, yeasts were grown as described above. *C. neoformans* cells were collected by centrifugation at 201.6 g, were washed twice with PBS, were counted using a hemacytometer, and were suspended at 1×10^7 cells/mL in minimal medium. Then, 100 μL of the suspension was added to individual wells of 96-well polystyrene plates and incubated without shaking at 37°C. Biofilms were allowed to form for 48 h. After the adhesion stage, the wells containing *C. neoformans* biofilms were washed 3 times with 0.05% Tween 20 in Tris-buffered saline, to remove nonadhered cryptococcal cells, using a microtiter plate washer (Skan Washer 400; Molecular Devices). Fungal cells that remained attached to the plastic surface were considered to be in biofilms. Then, 200 μL of a solution containing combinations of either MAb 18B7 or control MAb 3671 and either amphotericin B or caspofungin (each at the concentrations listed above) was added to each well. Biofilms treated with combination therapy were incubated for 24 h. The XTT reduction assay was used to determine the metabolic activity of the biofilms.

For confocal microscopy (CM), 96-well microtiter plates containing mature biofilms were washed 3 times with PBS and incubated with 100 $\mu\text{g}/\text{mL}$ MAb 18B7 for 2 h at 37°C. After treatment with MAb, the biofilms were incubated for 45 min at 37°C in 75 μL of PBS that contained the fluorescent stains FUN-1 (10 $\mu\text{mol}/\text{L}$), concanavalin A–Alexa Fluor 488 conjugate (ConA; 25 $\mu\text{mol}/\text{L}$), and goat anti–mouse IgG_1 –Alexa Fluor 350 conjugate (GAM- γ 1-AF; 50 $\mu\text{g}/\text{mL}$) (Molecular Probes).

FUN-1 (excitation wavelength, 470 nm; emission, 590 nm) is converted to orange-red cylindrical intravacuolar structures by metabolically active cells. ConA (excitation wavelength, 488 nm; emission, 505 nm) binds to glucose and mannose residues of cell-wall and capsule polysaccharides and fluoresces green. GAM- γ 1-AF (excitation wavelength, 346 nm; emission, 442 nm) reacts with the Fc portion of the heavy chain of mouse IgG_1 and fluoresces blue. Microscopic examinations of biofilms formed in microtiter plates were performed with CM using an Axiovert 200 M inverted microscope (magnification, $\times 40$; numerical aperture, 0.6). Depth was measured at regular intervals across the width of the device. To determine the structure of the biofilms, a series of horizontal (xy) optical sections with a thickness of 1.050 μm were measured throughout the full length of the biofilm. Confocal images of green (ConA), red (FUN-1), and blue (GAM- γ 1-AF) fluorescence were simultaneously recorded using a multichannel mode. Z-stack images and measurements were corrected using the Axio Vision software-deconvolution mode (version 4.4; Carl Zeiss MicroImaging).

All data were subjected to statistical analysis using Origin software (version 7.0; Origin Lab). *P* values were calculated by analysis of variance and were adjusted using Bonferroni's correction. *P* < .05 was considered to be significant.

Results. *C. neoformans* planktonic cells incubated with ≥ 0.3 $\mu\text{g}/\text{mL}$ MAb 18B7 showed greater susceptibility to amphotericin B, as measured by reductions in metabolic activity at drug concentrations of 0.015–0.25 $\mu\text{g}/\text{mL}$ (figure 1A). In contrast, there was no difference in planktonic-cell susceptibility when amphotericin B was combined with control MAb 3671 (figure 1B). When biofilms were treated with MAb 18B7 and exposed to amphotericin B, *C. neoformans* biofilms showed higher levels of metabolic activity (figure 1C). High metabolic activity displayed by biofilms was directly proportional to increases in the concentration of antibody in the medium. The protective effect of MAbs was observed at antibody concentrations > 1.25 $\mu\text{g}/\text{mL}$, even at higher concentrations of amphotericin B. The antifungal activity of amphotericin B was not inhibited when cryptococcal biofilms were incubated in the presence of control MAb 3671 (figure 1D).

C. neoformans planktonic cells showed no differences in susceptibility to caspofungin, in the presence of either MAb 18B7 or control MAb 3671 (figure 1E and 1F). Planktonic cells treated with either of these MAbs and caspofungin manifested a reduction in metabolic activity, at caspofungin concentrations of ≥ 8 $\mu\text{g}/\text{mL}$. Conversely, cryptococcal biofilms exposed to caspofungin manifested increases in cellular metabolism that were similar to those observed for biofilms treated with amphotericin B and MAb 18B7. Biofilms not treated with MAb 18B7 showed a reduction in metabolic activity when they were exposed to 4 $\mu\text{g}/\text{mL}$ of caspofungin (figure 1G). Nonetheless, a higher metabolic activity of biofilm was correlated with an increase in the

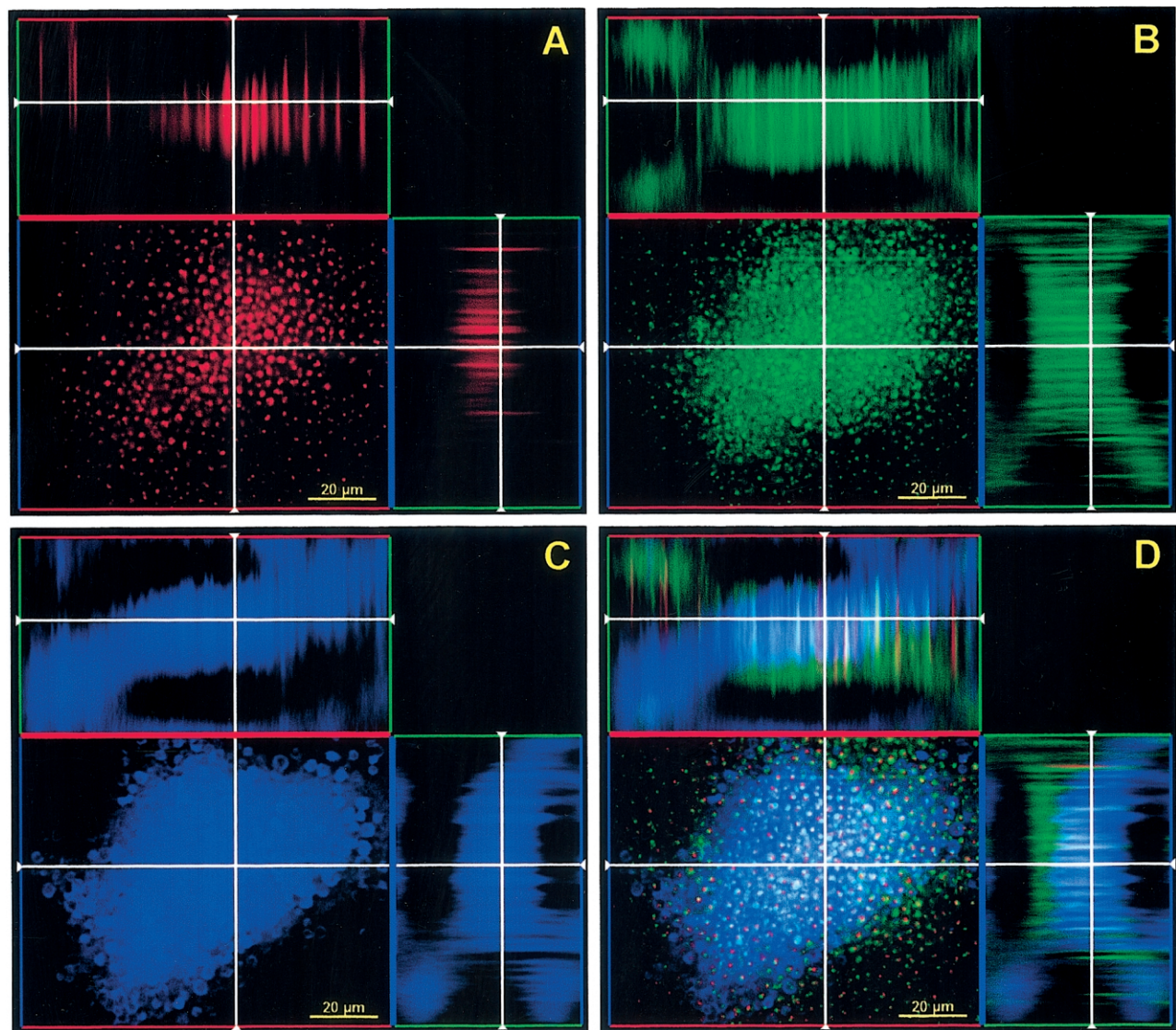


Figure 2. Confocal microscopic (CM) image of a mature *Cryptococcus neoformans* biofilm treated with the glucuronoxylomannan (GXM)-binding monoclonal antibody (MAb) 18B7. A–C, Orthogonal imaging of a mature *C. neoformans* biofilm, showing how metabolically active (red, FUN-1–stained) *C. neoformans* cells (A), extracellular polysaccharide material (green, concanavalin A–Alexa Fluor 488 conjugate–stained) (B), or GXM-binding MAb 18B7 (blue, goat anti–mouse IgG₁–Alexa Fluor 350 conjugate–stained) (C) binds throughout the exopolymeric matrix of a cryptococcal biofilm. D, Superimposition of panels A–C. Pictures were taken using a $\times 40$ -power field. Scale bar, 20 μm . The CM images reveal that glucuronoxylomannan-specific MAb bound throughout the polysaccharide surrounding the fungal biofilm, which suggests that its antagonistic effect may reflect impeded penetration of the drug molecules into the biofilm.

concentration of MAb 18B7. These effects were not observed when cryptococcal biofilms were incubated with caspofungin and control MAb 3671 (figure 1H).

Using a combination of 3 fluorescent stains—FUN-1, ConA, and GAM- γ 1-AF—we identified the relative location of metabolically active cells, polysaccharide matrix, and MAb 18B7. There was no difference in the gross appearance between biofilms treated with MAb 18B7 and those not treated with it. However, the results of the immunofluorescence study demonstrated that MAb 18B7 bound throughout the exopolymeric matrix of a biofilm (figure 2).

Discussion. Our results show that a combination of antibody and antifungal drugs has an antagonistic effect against established fungal biofilms; in contrast, the combination of antibody and amphotericin B is more active against planktonic cells. Studies of combination therapy in animal models of infection have shown that antibodies enhance the efficacy of antifungal drugs [11, 12]. This phenomenon has been attributed to enhanced *C. neoformans* killing by macrophages in the presence of a combination of opsonizing antibody and antifungal drug. Our results suggest that of novel antibody interactions with cells are present that enhance the susceptibility of plank-

tonic cells to drugs. In this regard, antibody binding to the cryptococcal capsule can have profound effects on capsular architecture, which may translate into increased drug penetration into the cell body.

For both amphotericin B and caspofungin, the combination of antibody and drug, at certain concentrations, resulted in a paradoxical increase in biofilm metabolic activity. This phenomenon reflects physical stress on the biofilm structure, which allowed increased penetration of small-molecule nutrients and/or changes in cellular metabolism via subtle cellular-tension effects. However, the most striking effect was that the presence of MAb 18B7 protected cryptococcal biofilms from either amphotericin B or caspofungin. This effect was not observed with control MAb 3671. CM revealed that MAb 18B7 binds through all of the exopolymeric matrix surrounding metabolically active yeast cells within *C. neoformans* biofilms. Given that amphotericin B and caspofungin are relatively large molecules (924 and 1213 Da, respectively), the antagonism observed may be a result of antibody-mediated interference with drug penetration.

The findings of the present study suggest the possibility of antagonistic effects when antibodies and drug therapy are combined for treatment of those clinical situations (e.g., infected prosthetic devices) where the presence of established biofilms can be expected. Furthermore, these results raise the possibility that products of the immune response contribute to the drug resistance of biofilms formed in vivo. One can anticipate that microbial biofilms formed on prosthetic devices in tissues contain antibodies and complement that may contribute to the acquisition of drug resistance in vivo.

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