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Antibody-Mediated Immobilization of *Cryptococcus neoformans* Promotes Biofilm Formation▽†

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Most microbes, including the fungal pathogen *Cryptococcus neoformans*, can grow as biofilms. Biofilms confer upon microbes a range of characteristics, including an ability to colonize materials such as shunts and catheters and increased resistance to antibiotics. Here, we provide evidence that coating surfaces with a monoclonal antibody to glucuronoxylomannan, the major component of the fungal capsular polysaccharide, immobilizes cryptococcal cells to a surface support and, subsequently, promotes biofilm formation. We used time-lapse microscopy to visualize the growth of cryptococcal biofilms, generating the first movies of fungal biofilm growth. We show that when fungal cells are immobilized using surface-attached specific antibody to the capsule, the initial stages of biofilm formation are significantly faster than those on surfaces with no antibody coating or surfaces coated with unspecific monoclonal antibody. Time-lapse microscopy revealed that biofilm growth was a dynamic process in which cells shuffled position during budding and was accompanied by emergence of planktonic variant cells that left the attached biofilm community. The planktonic variant cells exhibited mobility, presumably by Brownian motion. Our results indicate that microbial immobilization by antibody capture hastens biofilm formation and suggest that antibody coating of medical devices with immunoglobulins must exclude binding to common pathogenic microbes and the possibility that this effect could be exploited in industrial microbiology.

Cryptococcus neoformans is a fungal pathogen that is ubiquitous in the environment and enters the body via the inhalation of airborne particles. The *C. neoformans* cell is surrounded by a layer of polysaccharide that consists predominantly of glucuronoxylomannan (GXM), which forms a protective capsule around the microbe. The capsule has been shown to be essential for virulence in murine models of infection (5–7) and, thus, is considered a key virulence factor. *C. neoformans* is the causative agent of cryptococcosis, a disease that primarily affects individuals with impaired immune systems, and is a significant problem in AIDS patients (21, 31). The most common manifestation of cryptococcosis is meningoencephalitis.

Biofilms are communities of microbes that are attached to surfaces and held together by an extracellular matrix, often consisting predominantly of polysaccharides (8, 10). A great deal is known about bacterial biofilms (3, 9, 24, 30), but fungal biofilm formation is much less studied. *Candida albicans* is known to synthesize biofilms (11, 28, 29), as is *C. neoformans*. Biofilm-like structures consisting of innumerable cryptococcal cells encased in a polysaccharide matrix have been reported in human cases of cryptococcosis (32). Biofilm formation confers upon the microbe the capacity for drug resistance, and microbial cells in biofilms are less susceptible to host defense mechanisms (2, 4, 9, 12). In this regard, cells within *C. neoformans* biofilms are significantly less susceptible to caspofungin and amphotericin B than are planktonic cells (19). The cells within the biofilm are also resistant to the actions of fluconazole and voriconazole and various microbial oxidants and peptides (17, 19).

Bacterial and fungal biofilms form readily on prosthetic materials, which poses a tremendous risk of chronic infection (10, 13, 15, 27). *C. neoformans* biofilms can form on a range of surfaces, including glass, polystyrene, and polyvinyl, and material devices, such as catheters (16). *C. neoformans* can form biofilms on the ventriculoatrial shunts used to decompress intracerebral pressure in patients with cryptococcal meningoencephalitis (32).

The polysaccharide capsule of *C. neoformans* is essential for biofilm formation (18), and biofilm formation involves the shedding and accumulation of large amounts of GXM into the biofilm extracellular matrix (16). Previously, we reported that antibody to GXM in solution could inhibit biofilm formation through a process that presumably involves interference with polysaccharide shedding (18, 20). However, the effect of antibody-mediated immobilization of *C. neoformans* cells on cryptococcal biofilm formation has not been explored. In this paper, we report that the monoclonal antibody (MAb) 18B7, which is specific for the capsular polysaccharide GXM, can capture and immobilize *C. neoformans* to surfaces, a process that promotes biofilm formation. Interestingly, we identified planktonic variant *C. neoformans* cells that appeared to escape from the biofilm, but whose functions are not known. The results provide new insights on biofilm formation.

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MATERIALS AND METHODS

Yeast strains and culture conditions. *C. neoformans* var. *grubii* strain H99 was obtained from Mauricio del Poeta (Charleston, NC). Strains were grown in Sabouraud dextrose broth at 30°C with agitation (150 to 180 rpm). *C. neoformans* was killed by heating in a 65°C water bath for 30 min.

Time-lapse microscopy. Poly-D-lysine glass bottom culture dishes (Ashland, MA) were coated with 10 μg/ml MAb 18B7 to *C. neoformans* capsule component GXM or MOPC-21, an irrelevant isotype-matched control MAb that does not bind *C. neoformans*, and incubated at room temperature for 1 h. After incubation, the plates were washed three times with phosphate-buffered saline (PBS). Control plates were not coated with MAb and contained only *C. neoformans*. *C. neoformans* cells were collected by centrifugation at 10,600 × *g* for 30 s, washed three times with PBS, and resuspended in media used to induce biofilm formation, termed inducing media (10% Sabouraud dextrose broth diluted in 50 mM MOPS [morpholinepropanesulfonic acid] [pH 7.5]), and a total of 2×10^5 cells were added to the culture dish. Live imaging was performed using an Axiovert 200 M inverted microscope and photographed with an AxioCam MRm camera controlled by the Axio Vision 4.6 software (Carl Zeiss Micro Imaging, New York, NY). Imaging was performed at 4-min intervals, using a 10× or 20× (numerical optovar of 1.6) objective.

Immunofluorescence microscopy. *C. neoformans* biofilms were incubated for 10 h at room temperature with Alexa Fluor 488-labeled MAb 18B7 (10 μg/ml) and then washed with PBS. Fluorescence microscopy was performed using an Axiovert 200 M inverted microscope (10× objective, numerical optovar of 1.6) using green fluorescent light.

Measurement of biofilm growth by XTT reduction assay. To induce biofilm formation, sterile 96-well polystyrene enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 μl (10 μg/ml) of either MAb 18B7 or MOPC-21 and incubated at room temperature for 2 h. Microtiter wells containing heat-killed *C. neoformans* were included as negative controls. Assays were carried out in six wells, thus yielding six repetitions. Wells were washed three times with 0.05% Tween 20 in PBS (PBS-T). *C. neoformans* cells were harvested as described above and resuspended in inducing media, and 1×10^6 cells were added to the wells. Plates were incubated at 37°C for 2, 4, 6, 8, or 12 h to induce biofilm formation. Following incubation, wells were washed in triplicate with PBS-T, to remove any planktonic cells. A semiquantitative measurement of *C. neoformans* biofilm formation was obtained from the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT) reduction assay. For each well, 50 μl of XTT salt solution (1 mg/ml in PBS) and 4 μl of menadione solution (1 mM in acetone) were added. The colorimetric change was measured using a microtiter reader at 492 nm. In prior studies we have demonstrated that the XTT reduction assay measurements correlate with biofilm fungal cell number (16).

Spot ELISA. Sterile 96-well polystyrene ELISA plates were coated with 50 μl of MAb 18B7 or MOPC-21 (10 μg/ml) and incubated for 1 h at room temperature. Control wells containing no MAb were also included. Measurements were performed in triplicate. Wells were washed three times with PBS-T. *C. neoformans* cells were harvested as described above and resuspended in inducing media, and 500 cells were added to each well. Plates were incubated at 37°C for 0.5, 1, 2, 4, or 6 h. Wells were washed in triplicate with PBS-T and blocked using 100 μl of 1% bovine serum albumin in PBS (PBS-B). Incubation steps were performed for 1 h at 37°C or overnight at 4°C. After washing, 50 μl of 10 μg/ml secondary antibody 2D10 (GXM-specific immunoglobulin M [IgM]) in PBS-B was added to each well and incubated, and the wells were subsequently washed. Next, 50 μl biotin-labeled goat anti-mouse IgM (10 μg/ml) in PBS-B was added to each well and incubated for 1 h, followed by 50 μl Vectastain ABC mix (Vector Laboratories, CA), and plates were subsequently incubated at room temperature for 30 min. To each well, 50 μl of 1 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Amresco, Solon, OH) per ml diluted in AMP buffer (0.2 g $MgCl_2 \cdot 6H_2O$, 0.1 ml Triton X-405, 95.8 ml 2-amino-2-methyl-1-propanol in 800 ml distilled H_2O [pH 9.8]) was added and incubated at room temperature for 3 h. Plates were washed three times with PBS-T, followed by one time with distilled water. Spots were viewed using an inverted microscope, with a 10× objective, or an ELISPOT plate reader (AID GmbH).

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 5.0a). XY or column graphs were generated, and standard deviations of multiple values were determined. *P* values were determined using *t* tests.

RESULTS

Surface-bound MAb 18B7 immobilizes *C. neoformans* to glass surfaces. We observed that when *C. neoformans* cells suspended in PBS were added to poly-D-lysine glass bottom culture dishes coated with MAb 18B7, they readily attached to the surface and became immobile, such that they were no longer susceptible to movement, which presumably is driven by Brownian motion (Fig. 1; also see Movie S1 in the supplemental material). In contrast, when cells were added to plates not coated with MAb, a smaller number of cells adhered, and the unattached cells could be seen moving, even when placed on surfaces coated with poly-D-lysine (Fig. 1). When MAb MOPC-21, which does not bind GXM, was used to coat the plates, some adhesion of *C. neoformans* to the slide was observed, as suggested by reduced movement of cryptococcal cells (Fig. 1), but the effect was much less than when MAb 18B7 was used. These data suggest that coating with MAb 18B7 is an effective method to capture *C. neoformans* cells to glass surfaces, since in the absence of this antibody, the *C. neoformans* cells do not attach as effectively.

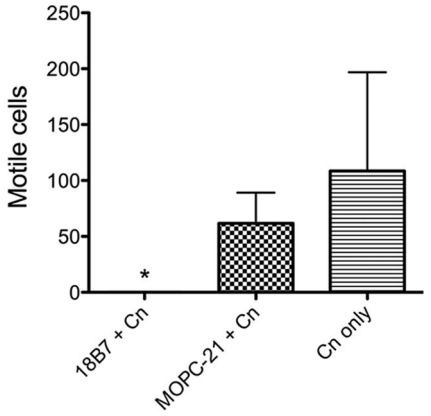

FIG. 1. *C. neoformans* motility in presence and absence of specific MAb. Glass bottom petri dishes were uncoated or coated with either MAb 18B7 or MOPC-21, and *C. neoformans* (Cn) cells in PBS were added. The number of motile cells entering the view of the microscope was counted over 10 h. * denotes that the number of motile cells in the 18B7 + Cn group was zero.

Antibody-mediated immobilization of *C. neoformans* promotes biofilm formation. Time-lapse microscopy indicated that *C. neoformans* cells began to bud very soon after immobilization by placement of glass slides coated with MAb 18B7 (Fig. 2; also see Movie S1 in the supplemental material). Each budding cell in the microscope view continued to divide and eventually formed a surface-attached community that evolved into a biofilm (Fig. 2). Biofilm growth was a dynamic process, and cells were visualized moving within the growing biofilm. In contrast, when *C. neoformans* cells were placed in wells without antibody coating or in wells coated with the irrelevant control MAb MOPC-21, numerous motile cells that had not been immobilized were apparent, presumably drifting by Brownian motion. Cells that had bound to the surface, however, rapidly formed microcolonies that grew into biofilms (data not shown).

Biofilm formation was measured using an XTT reduction assay to determine metabolic activity. When *C. neoformans* cells were added to the bottom of a polystyrene well coated with MAb 18B7, biofilm formation occurred at a significantly higher rate than in wells coated with MAb MOPC-21 or no antibody (Fig. 3). An initial increase in biofilm growth was observed as early as 2 h after placement of *C. neoformans* cells into the 18B7-coated wells. Over a period of 12 h, the cells that were captured by MAb 18B7 exhibited a continually larger biofilm than the other samples described above. A small in-

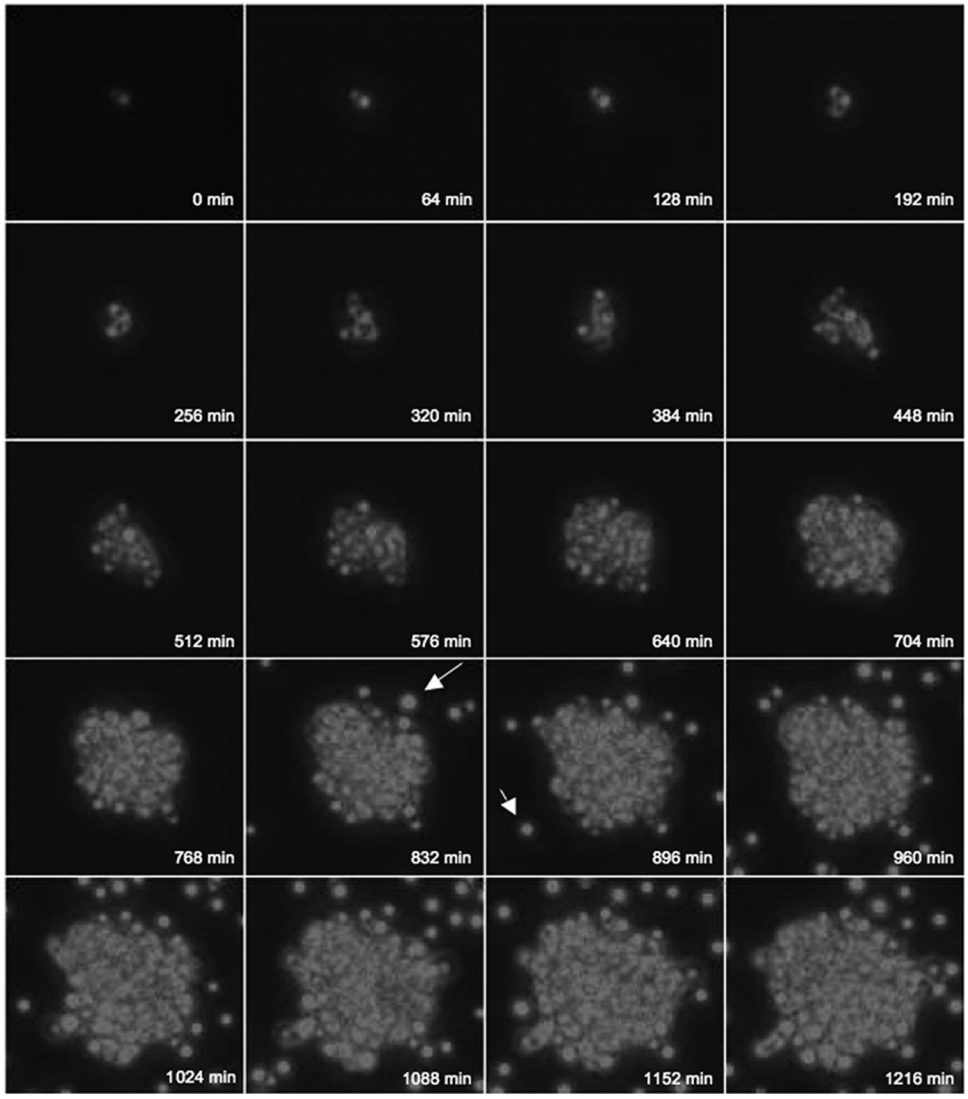

FIG. 2. Time-lapse microscopic study of *C. neoformans* biofilm formation. *C. neoformans* cells were anchored to a glass bottom petri dish, using MAb 18B7, and biofilm formation was induced. Photographs of biofilm formation were taken every 4 min using a 20× (numerical optovar of 1.6) objective. Variant cells (indicated by white arrows) were released from the biofilm community and exhibited motility.

crease in biofilm growth was seen when MAb MOPC-21 was used to coat the wells, compared to that with untreated wells.

Polysaccharide is deposited faster when *C. neoformans* is immobilized by GXM-specific antibody. Spot ELISA was used to determine the amount of polysaccharide shed during the early stages of biofilm synthesis (Fig. 4). We measured the area of 9 to 30 spots, and the amount of shed polysaccharide was inferred from the area of the spot (Fig. 4b). When *C. neoformans* was placed in polystyrene wells coated with MAb 18B7, the spot areas were significantly larger than those for cells growing in wells coated with MOPC-21 or no antibody. When MAb 18B7 was present, polysaccharide shedding was observed after only 30 min and consistently increased over 6 h. Alternatively, on plates coated with MAb MOPC-21, a significant increase in shedding occurred after only 2 h. Spot ELISAs were also used to determine the number of cryptococcal cells on the surface of the culture dish, over time (Fig. 4c). An approximately equal number of cells were present during the initial 2 hours when MAb 18B7 coated the surface, and this number was greater than when the wells were left untreated. After 4 h, there was an apparent reduction in the number of spots under the MAb 18B7-coated conditions, when live *C. neoformans* cells were present, because the spots were so large that they became confluent. Over time, the number of fungal cells steadily increased when surfaces were not coated, suggesting that time was required for the cells to settle.

Planktonic cells are released from biofilm matrix. Approximately 7 h after addition of *C. neoformans* to MAb 18B7-coated plates, planktonic cells were observed drifting away from the biofilm (Fig. 2). These cells were untethered and motile, despite the fact that they were presumably rolling on a surface that was coated with MAb 18B7. Release of variant planktonic cells was not dependent on the presence of MAb 18B7, as cells were also released from biofilms grown on un-

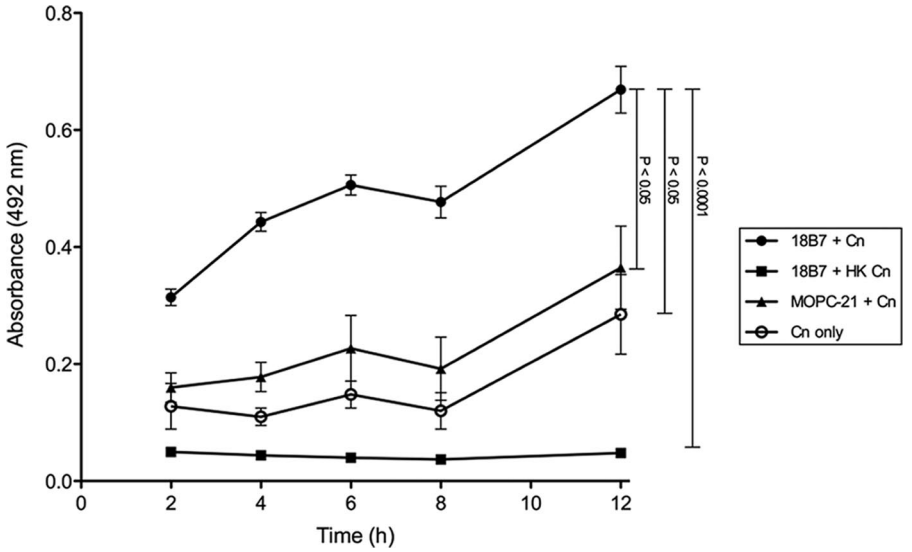

FIG. 3. Biofilm formation as measured by XTT reduction assay. *Cryptococcus neoformans* (Cn) cells were grown in 96-well plates that were coated with MAb 18B7 or MAb MOPC-21 or left untreated. As a control, MAb 18B7-coated wells were inoculated with heat-killed *C. neoformans* (HK Cn). Cell viability was measured at intervals over 12 h. Conditions were measured for six wells, and the average was plotted. Error bars represent standard deviations, and *P* values were calculated using unpaired *t* tests. Assays were performed three times, and they generated similar results.

treated plates. When these cells were harvested and plated onto agar, there was no discernible difference in colony morphology from that of cells obtained from the original culture. To determine any changes in capsule composition, planktonic cells were carefully isolated from the biofilm and studied by capture ELISA. There was no significant difference in binding affinity to MAb 18B7 when cultures derived from these planktonic cells were compared to cells derived from regular planktonic cultures in a capture ELISA (data not shown). The variant planktonic cells had the capacity to form biofilms when removed from the original plates and added to new MAb 18B7-coated plates (data not shown).

Fluorescence microscopy using Alexa Fluor 488-labeled MAb 18B7 was performed on mature biofilms to visualize any GXM release around the biofilm and within the extracellular matrix (Fig. 5). It was possible to see halos of GXM around the biofilm community of cells, and this matrix could join a number of biofilm communities together. XTT assays were used to determine whether the addition of purified GXM to MAb 18B7 would inhibit the subsequent binding of *C. neoformans* to the antibody. No reduction in biofilm growth was observed in the presence of GXM (data not shown).

DISCUSSION

Cryptococcal biofilm formation is influenced by conditioning surfaces with compounds such as bovine serum albumin and proteins contained within nutrient-rich media (19, 22). Indeed, it has been described that a range of host fluids, such as blood, urine, and saliva, can coat biomaterials and aid the attachment of microbes to them (22). In this study, we coated glass and polystyrene surfaces with MAb to the *C. neoformans* major capsule component GXM and an isotype-matched control and studied biofilm formation. Time-lapse microscopy indicated that MAb 18B7-coated glass surfaces were more effective at immobilizing *C. neoformans* than MAb MOPC-21-coated or uncoated glass surfaces. Given that the capsular polysaccharide is highly negatively charged, we evaluated the ability of poly-D-lysine, an adhesive substrate, to capture *C. neoformans* cells, but this coating did not effectively immobilize cryptococcal cells.

Antibody-mediated immobilization of *C. neoformans* cells translated into a significantly faster onset of biofilm formation, as measured by XTT reduction assays, which presumably resulted from more efficient capture of cryptococcal cells on polystyrene surfaces. Spot ELISAs showed both more and larger spots when *C. neoformans* was plated onto surfaces coated with MAb 18B7, the latter result reflecting increased shedding of polysaccharide by cryptococcal cells immobilized onto the support surface. This observation, combined with the earlier observation that solution antibody binding to the polysaccharide capsule inhibits biofilm formation (18), indicates that capsular polysaccharide shedding and its binding to the support matrix are early and necessary steps in cryptococcal biofilm formation. Using immunofluorescence microscopy, we observed GXM halos around fungal microcolonies, which could also be seen forming as one large matrix around a number of biofilm colonies. It is possible that these matrices permit a form of communication, such as quorum sensing between biofilm communities.

Time-lapse microscopy allowed us to visualize fungal biofilm formation. After several hours of biofilm formation imaging, we observed that certain cells broke away from the mass of cells in the biofilm and drifted from the biofilm and, thus, reverted back to a planktonic state. We thought that the mobility of these planktonic variants, presumably by Brownian motion, was remarkable, given that they were moving over surfaces coated with MAb 18B7, and we initially hypothesized that they expressed a variant type of polysaccharide that re-

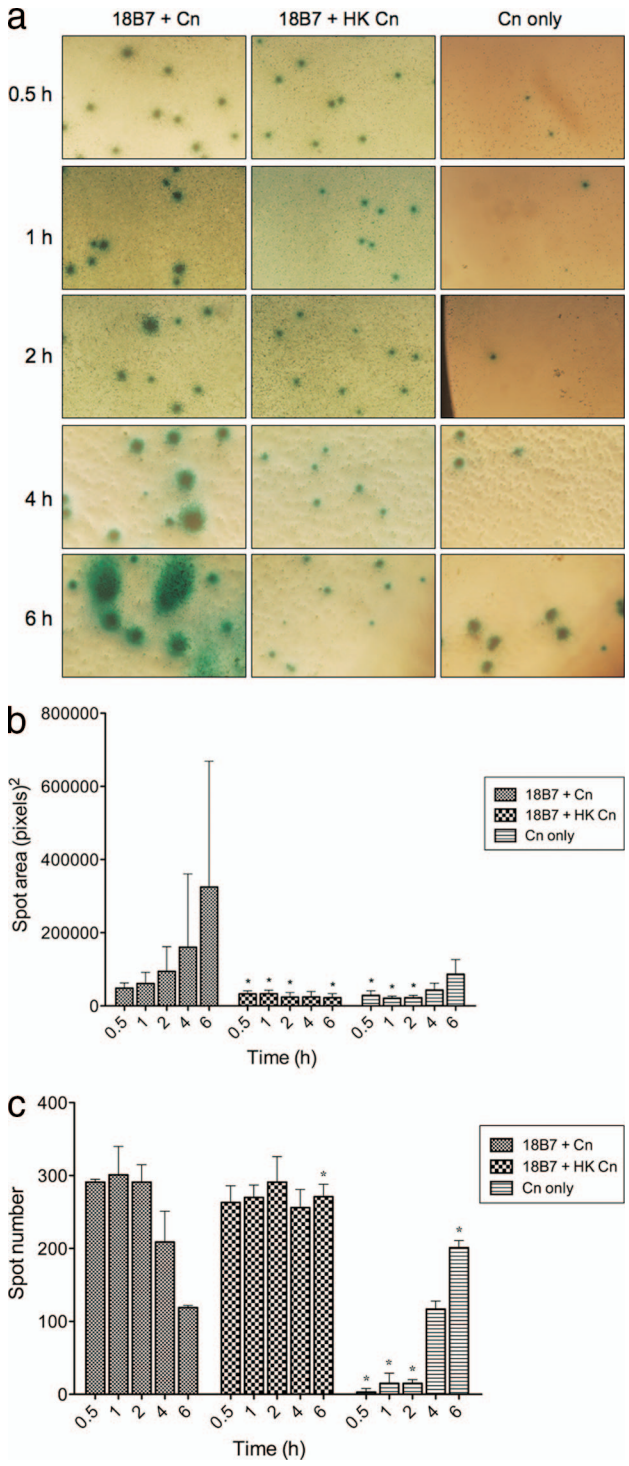

FIG. 4. Biofilm formation as determined by spot ELISA. (a) Spot ELISA was used to determine polysaccharide shedding during the initial stages of biofilm formation. (b) Area of polysaccharide shedding, as determined by spot ELISAs. (c) *C. neoformans* cell deposition onto plastic surfaces was determined using spot ELISAs. *C. neoformans* (Cn) cells were added to 96-well plates coated with MAb 18B7 or to plates left untreated. Heat-killed (HK) cells were also added to wells containing MAb 18B7. Spot area and number were measured over 6 h. Bars represent the averages of 9 to 30 spots (Fig. 5b) and the average number of spots from three wells (Fig. 5c). Error bars indicate standard deviation, and *P* values were calculated using Student's *t* tests with the Bonferroni correction for multiple comparisons. * denotes *P* values of <0.0001, corresponding to the time points in 18B7-treated wells.

leases them from the biofilm and precludes them from binding to the antibody. However, comparison of these biofilm-derived planktonic cells to culture-derived planktonic cells by capture ELISA revealed no significant difference in binding affinity to 18B7. When the plates were stained for GXM, however, it was evident that a significant amount of the area around the growing biofilm was coated with shed polysaccharide. Hence, the ability of these cells to move over an antibody surface may reflect the fact that the antibody becomes saturated with shed polysaccharide and is not able to capture these emergent planktonic cells. Although the biological purpose of planktonic cell release from the biofilm is not understood, we hypothesize that these cells represent planktonic variants that are released from the attached mass of cells so that they could colonize new locations. If such cells are generated during infection, they could promote dissemination of this pathogen throughout the body. Alternatively, it has been described that when nutrient levels within a biofilm are low, cells have the capacity to release from them, by breaking down the extracellular matrix, and revert back to a planktonic state (1, 26). Thus, the observation of planktonic variant cells drifting away from the cryptococcal biofilm raises the possibility that similar mechanisms occur with fungal biofilms in nutritional stress.

The results of this study showing that immobilized specific antibody promotes attachment and biofilm formation are in contrast to the fact that soluble specific MAb prevents polysaccharide release and biofilm formation (18, 20). Although these results may appear contradictory at first glance, we note that they represent the outcome of very different experimental conditions. Prevention of biofilm synthesis, by the addition of soluble antibody to cryptococcal cells, is a result of the antibody binding to the capsule, which prevents release of GXM. Since GXM is a constituent of the cryptococcal biofilm matrix, the ability of antibody binding to the capsule to prevent biofilm formation probably reflects an absolute requirement for GXM in *C. neoformans* biofilm formation. In contrast, in the current study, MAb 18B7 was itself immobilized first by absorption to glass surfaces and, in this absorbed state, does not appear to interfere with polysaccharide shedding, as demonstrated by spot ELISA, and presumably promotes biofilm formation by anchoring the cell to the surface.

Recently, there is great interest in protecting medical devices from clot formation by coating surfaces with MAbs (14). Although antibodies to mammalian antigens generally do not bind microbial antigens, there is always the concern that such antibodies may bind to a microbe through the phenomenon of molecular mimicry or antibody cross-reactivity. If our observations are applicable to other microbes, it may be a worthwhile precaution to establish that antibodies used for coating medical devices do not immobilize common bloodstream pathogens.

It should be noted that since specific antibody is not immobilized in tissues, we do not believe that this effect would occur in vivo. However, a range of commercial manufacturing processes relies on the formation of microbial biofilms (23, 25, 26). Hence, it may be possible to adapt antibody-mediated immobilization to promote biofilm formation to exploit the industrial use of these microbes.

Although we did not investigate the molecular mechanism responsible for this effect, one can posit explanations for the

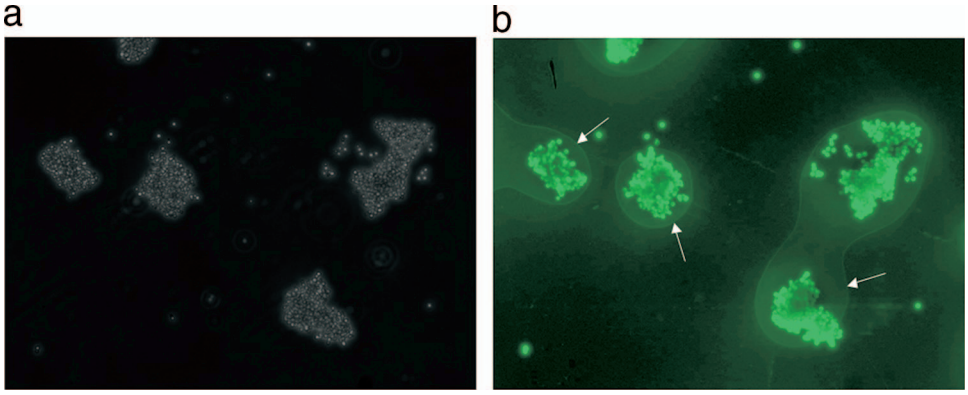

FIG. 5. Fluorescence microscopy of GXM within biofilm matrix. (a) Bright-field microscopy results of mature biofilms. (b) Biofilms were stained with Alexa Fluor 488-labeled 18B7 and viewed using green fluorescent light. Arrows indicate halos of GXM surrounding biofilm microcolonies. A 10× objective (numerical optovar of 1.6) was used.

observed phenomenon. Given that biofilm formation involves the secretion of an extracellular polysaccharide matrix (16), yeast cell immobilization could enhance the efficiency of this process by confining matrix production to a small area. Alternatively, or possibly concomitantly, it is conceivable that immobilization triggers changes in gene expression that promote biofilm formation. Our results highlight the importance of attachment in biofilm formation and suggest the need for additional studies to identify the mechanism(s) by which surface contact translates into changes in microbial physiology and metabolism.

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