INFECTION AND IMMUNITY, Nov. 2007, p. 5085–5094 Vol. 75, No. 11
0019-9567/07/$08.00+0 doi:10.1128/IAI.00278-07

An Anti-β-Glucan Monoclonal Antibody Inhibits Growth and Capsule Formation of *Cryptococcus neoformans* In Vitro and Exerts Therapeutic, Anticryptococcal Activity In Vivo▽

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Received 20 February 2007/Returned for modification 17 March 2007/Accepted 18 June 2007

In this study we tested the in vitro and in vivo anti-*Cryptococcus neoformans* activity of an antilaminarin (anti-β-glucan) monoclonal antibody (MAb 2G8) (immunoglobulin G2b) which was previously shown to inhibit the growth of β-glucan-exposing *Candida albicans* cells. Here we show that MAb 2G8 binds to the cell wall of *C. neoformans* and inhibits its growth to an extent comparable to that observed for *C. albicans*. Binding and growth inhibition were detected almost equally for encapsulated and acapsular *C. neoformans* strains. In addition, at subinhibitory concentrations, MAb 2G8 reduced the capsule thickness without affecting protease or phospholipase production. Acapsular fungal cells, but not encapsulated fungal cells, were opsonized by the antibody and more efficiently phagocytosed and killed by human monocytes and by murine peritoneal macrophages. A single administration of MAb 2G8 resulted in a reduction in the fungal burden in the brains and livers of mice systemically infected with a highly virulent, encapsulated *C. neoformans* strain. This protective effect was also detected in neutropenic mice. Overall, these findings demonstrate that cell wall β-glucan of encapsulated *C. neoformans* is accessible to antibodies which can exert remarkable anticryptococcal activities in vitro and in vivo.

Deep-seated mycoses are a severe clinical problem because of well-known diagnostic difficulties and the partial inability of antifungal drugs to eradicate the infections in immunocompromised hosts, often resulting in toxicity, drug resistance, and associated high costs of supportive treatment. As a consequence, the mortality rate for invasive fungal infections remains high, particularly in severely immunocompromised patients (32). In this scenario, active and passive vaccinations must be considered valuable novel approaches which can be integrated with, if not replace, chemotherapy. Nonetheless, no vaccine against such infections exists, and the use of antibodies for immunotherapy is in the very early stages (23).

Together with *Candida albicans* and *Aspergillus* spp., *Cryptococcus neoformans* is one of the three leading causes of morbidity and mortality associated with fungal infections worldwide. The generation of immunologic tools to fight cryptococcosis has been pursued for a long time through a variety of approaches (15, 22). Considering the premises on which to build active and/or passive vaccination, Levitz and collaborators have pointed out the pivotal role of a cell-mediated immune response in fighting cryptococcosis (14, 24), while Casadevall and Pirofski have emphasized the importance of humoral responses in protection against cryptococcal disease (8, 10). First, a critical immunogenicity role has been ascribed to a heterogeneous family of cryptococcal mannoproteins, which are antigens responsible for stimulating T-cell responses necessary for effective host defense (21, 24). And second, the development of a vaccine for *C. neoformans* that induces antibody-mediated immunity was complicated by elicitation of protective, nonprotective, and disease-enhancing antibodies, depending on the isotypes (23). Nonetheless, it has been established that immunoglobulin G1 (IgG1) antibodies against glucuronoxylomannan (GXM), the principal constituent of capsular material of *C. neoformans*, are protective in different experimental models. Protection was achieved through active immunization with a GXM conjugate vaccine (15), as well as passive administration of antibodies (22). Furthermore, antibody therapy is being developed for treatment of human cryptococcosis. In this regard, an IgG1 monoclonal antibody (MAb) has been evaluated in a phase I/II trial in patients with AIDS-related cryptococcosis (22).

Prevention of and therapy for fungal infections could be more effectively implemented by exploitation of common antigens representative of the most important opportunistic fungi and capable of inducing a cross-protective response (11). In this regard, it has recently been reported that a novel polysaccharide-protein conjugate vaccine that includes the algal antigen laminarin (β-glucan) and a bacterial carrier protein is able to elicit antibodies to β-glucan, mediating protection against both experimental candidiasis and aspergillosis. Interestingly, these antibodies had direct antifungal effects. In fact, both vaccine-induced IgG and antilaminarin monoclonal IgG2b antibodies were able to inhibit fungal growth in the absence of immune effector cells (41).

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▽ Published ahead of print on 2 July 2007.

β-Glucan is an essential cell wall component in all pathogenic fungi and plays a critical role in cell viability (2, 17, 18, 29, 30). In fact, compounds that inhibit β(1,3)-glucan synthase show fungicidal activity (39, 40). A similar lethal event was observed following deletion of the gene(s) encoding the FKS subunit(s) of β(1,3)-glucan synthase (26).

The ectocellular structures of *C. neoformans* are composed primarily of polysaccharide polymers, which include capsular GXM, mannoproteins, and chitin. Electron microscopy studies using gold-labeled antibodies against (1,3)-linked β-glucan have confirmed the presence of these polysaccharides in the cell wall, localized beneath the large capsule (19). There is evidence that toxins or toxin-mimicking anti-idiotypic antibodies recognizing β-glucan receptors and/or inhibiting β(1,3)-glucan synthase are potent inhibitors of *C. neoformans* growth (37).

All these data make β-glucan a credible target for antibody therapy of cryptococcosis. On this basis, we examined whether the antilaminarin MAb 2G8 (41) was effective against *C. neoformans*. We also examined whether passive administration of this antibody could confer protection against a disseminated experimental infection by this fungus in normal or neutropenic mice.

MATERIALS AND METHODS

Microorganisms. Two encapsulated strains of *C. neoformans* (*C. neoformans* var. *neoformans* serotype D strain NIH B3501 [= ATCC 34873] and *C. neoformans* var. *grubii* serotype A strain H99 [= ATCC 208821]) and an acapsular mutant (CAP67 derived from strain NIH B3501) were obtained from the American Type Culture Collection (Manassas, VA). The CAP67 acapsular phenotype is the result of a single gene mutation; when the gene was complemented, the capsule and virulence of the strain were restored (20).

A virulent germ tube-forming strain of *C. albicans* (CA-6) isolated from a clinical specimen was used in this study. The origin of, characteristics of, and growth conditions for CA-6 have been described previously (3). The cultures were maintained by serial passage on Sabouraud agar (Fluka Biochemika, Steinheim, Switzerland). Log-phase yeast cells were harvested by suspending a single colony in saline, washed twice, and counted with a hemocytometer, and the concentration was adjusted to the desired level in the appropriate buffer.

Monocyte and macrophage isolation. Monocytes were purified from peripheral blood mononuclear cells from healthy donors as previously described (33). Heparinized venous blood was diluted with RPMI 1640 (Gibco, Paisley, Scotland, United Kingdom). Peripheral blood mononuclear cells were separated by density gradient centrifugation over Ficoll-Hypaque Plus (Amersham Biosciences AB, Uppsala, Sweden), recovered, washed twice and suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U penicillin/ml, and 100 μg streptomycin/ml, plated in a cell culture flask (BD Falcon, Bedford, MA), and incubated for 1 h at a density of 2×10^6 to 3×10^6 cells/ml. Adherent monocytes were recovered using a cell scraper (Falcon), washed twice, and counted, and the concentration was adjusted to the desired concentration.

Mouse peritoneal macrophages were obtained as previously described (34). Briefly, peritoneal macrophages were harvested by rinsing the exposed peritoneal cavity with RPMI 1640. Cells were washed three times and counted, and the concentration was adjusted to the desired level.

Phagocytosis assay. *C. neoformans* uptake and *C. albicans* uptake were performed by flow cytometry as previously described (12). Briefly, inactivated yeasts (60°C for 30 min) were suspended in phosphate-buffered saline (PBS) at a density of 10^8 yeast cells/ml. Cells were labeled with fluorescein isothiocyanate (FITC) (Sigma) at a concentration of 1 μg/ml in PBS at 22°C for 10 min. Labeled yeasts (10^7 cells) were incubated with monocytes (10^6 cells) at 37°C for 2 h. Phagocytosis was arrested by adding 1 ml of ice-cold PBS to the suspension. Trypan blue (200 μg/ml; Sigma) was added to each sample and incubated for 10 min to quench the fluorescence of noninternalized fungi. Unbound trypan blue was then removed by centrifugation, and the percentage of phagocytosis (i.e., the percentage of monocytes with ingested yeast cells) was determined by flow cytometry.

Killing assay. Killing activity was evaluated with a CFU inhibition assay. Briefly, human monocytes or mouse peritoneal macrophages (2×10^5 cells; 0.1 ml of suspension per well) were incubated in flat-bottom, 96-well microtiter tissue culture plates with 2×10^4 cells of *C. neoformans* B3501, CAP67, or H99 or *C. albicans* CA-6 in the presence of MAb 2G8 or an irrelevant MAb (20 μg/ml) for 4 h. After incubation at 37°C under 5% CO_2, plates were vigorously shaken, monolayers were lysed by adding 0.01% Triton X-100, and serial dilutions in distilled water were prepared from each well. Plates (triplicate samples) were made by spreading each sample on Sabouraud dextrose agar, and the CFU were visually evaluated after 48 h of incubation at 37°C. Killing activity was expressed as the percentage of CFU inhibition and was determined using the following formula: killing activity = 100 − [(number of CFU from experimental group/number of CFU from control culture) × 100].

Antibodies. MAb 18B7 is an IgG1 murine MAb that is specific for GXM and has been previously described (31). MAb 18B7 was obtained from Arturo Casadevall.

The anti-β-glucan MAb 2G8 and anti-CRM MAb (irrelevant MAb) have been described elsewhere (41). Briefly, a stable hybridoma secreting anti-β-glucan MAb 2G8 was generated after fusion of spleen cells of BALB/c mice immunized with soluble *C. albicans* β-glucan conjugated to diphtheria toxoid CRM 197 and myeloma cells of the murine line X63-Ag8653, using standard protocols (41). A stable hybridoma secreting anti-CRM MAb was generated after fusion of spleen cells of BALB/c mice immunized with CRM and myeloma cells of the murine line X63-Ag8653. Hybridomas were maintained in RPMI 1640 supplemented with 10% FCS, 100 U penicillin/ml, 100 μg streptomycin/ml, 1 mM sodium pyruvate, and 2 mM L-glutamine. MAb 2G8 and anti-CRM were precipitated from culture supernatants with ammonium sulfate and affinity purified on protein A-Sepharose resin (Amersham Biosciences) by following the manufacturer's instructions.

Fluorescence-activated cell sorting (FACS) analysis. MAb 2G8 binding to yeast cells was quantified by flow cytometry. Yeast suspensions (1×10^6 cells) in PBS containing 0.5% FCS and 0.1% sodium azide (FB) were incubated in the presence of 40 μg/ml of MAb 2G8 or anti-CRM for 30 min on ice. After incubation, cells were washed with FB and incubated with secondary antibody (anti-mouse IgG-FITC conjugate; Sigma) for 30 min. After labeling, yeast cells were washed and analyzed using flow cytometry (Becton Dickinson, San Jose, CA).

Confocal microscopy. Yeast cells were grown in suspension with constant agitation at 25°C for 18 h in Sabouraud medium. The cells were recovered and counted, and 10^5 cells were treated with MAb 2G8 or anti-CRM MAb, both at a concentration of 40 μg/ml, in the presence of anti-mouse IgG $F(ab')_2$ fragment-Cy3 conjugate (Sigma) for 30 min on ice. After incubation, cells were washed with FB and incubated with MAb 18B7 (40 μg/ml) in the presence of a secondary antibody (anti-mouse IgG-FITC conjugate; Sigma) for 30 min on ice. After labeling, yeasts were washed and placed on 12-well multitest slides (Flow Laboratories, United Kingdom). Then the cells were fixed with acetone (Carlo Erba, Italy), and 1 drop of the gel mount aqueous mounting medium (Sigma) was used to protect the fluorescence. Coverslips were mounted on the slides, which were analyzed with a confocal microscope. Images were collected with a Nikon C1 laser scanning confocal unit (Nikon D-Eclipse C1) attached to an inverted fluorescence microscope (Nikon Eclipse TE 2000-U) with a CFI plan fluor 60 × 0.50 to 1.25 (numerical aperture) oil iris objective (Nikon). Excitation on the Nikon C1 laser confocal microscope was performed with an argon laser (emission wavelength, 488 nm) and a helium/neon laser with 543-nm output. Appropriate filter sets were used to collect fluorophore emissions. Images were acquired digitally and processed using the operation software EZ-C1 for the Nikon C1 confocal microscope (Nikon).

Phospholipase assay. Extracellular phospholipase activity was determined by the method of Price et al. (35). Briefly, a few colonies from a 1- to 3-day-old agar plate were suspended in saline, and the concentration was adjusted to 2×10^5 cells/ml. Five microliters of the suspension together with 5 μl of MAb 2G8 at a concentration of 4 μg/ml were plated onto Sabouraud dextrose agar containing 1 M NaCl, 0.005 M $CaCl_2$, and 10% centrifuged egg yolk emulsion (Fluka Biochemika). The plates were incubated at 37°C for 21 days. Isolates that produced extracellular phospholipase had a distinct, white, opaque zone (precipitate) below and around each colony. The diameters of a colony (*a*) and of the colony plus its precipitation zone (*b*) were measured. Phospholipase activity (Pz) was determined as follows: $Pz = a/b$.

Protease assay. Protease production was determined as described by Aoki et al. (1). The test medium consisted of agar plates containing bovine serum albumin; 60 ml of a solution containing 0.04 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 1 g NaCl, 0.2 g dried yeast extract, 4 g glucose, and 0.5 g bovine serum albumin (fraction V; Acros Organics, New Jersey) was prepared, and the pH was adjusted

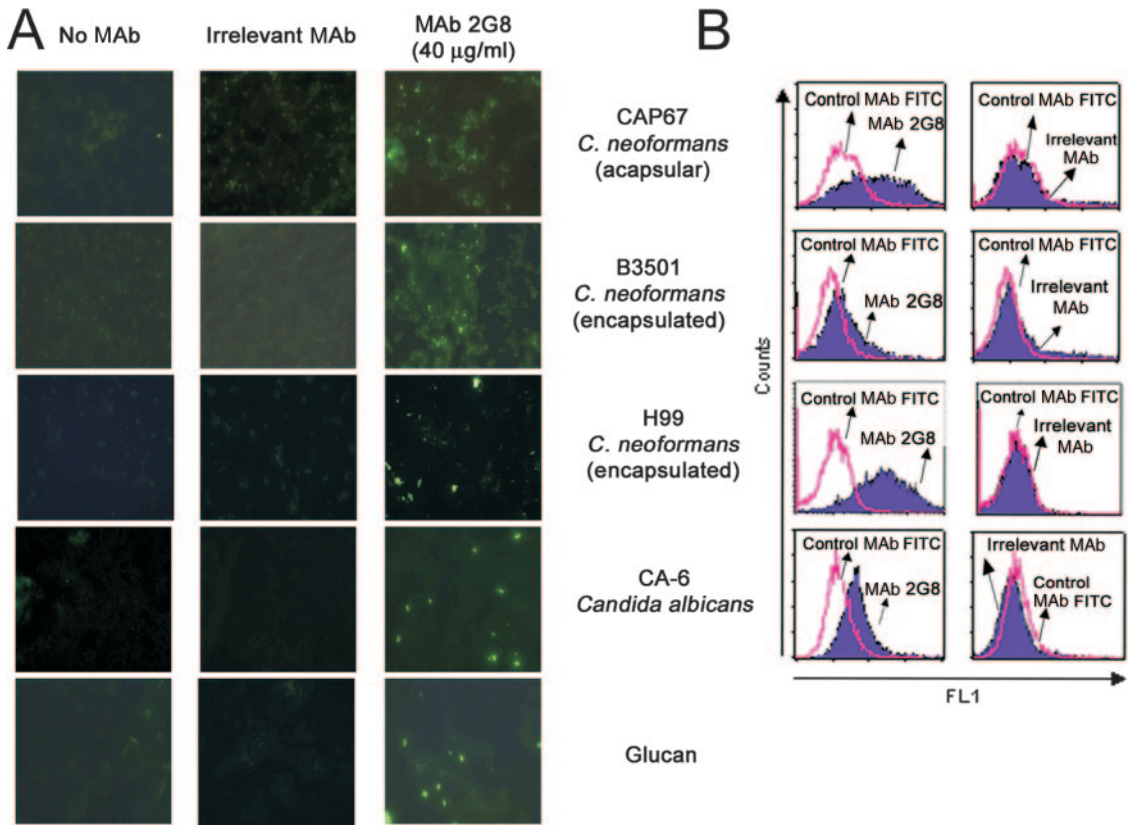

FIG. 1. MAb 2G8 binding to different strains of *C. neoformans.* (A) Indirect immunofluorescence staining of three strains of *C. neoformans* (CAP67, B3501, and H99), *C. albicans* CA-6, and β-glucan cell wall ghosts (Glucan) of *C. albicans* in the presence or absence of MAb 2G8 or the irrelevant MAb. Yeasts were incubated with 40 μg/ml of mouse MAb 2G8 or the irrelevant MAb for 30 min and subsequently with anti-mouse IgG-FITC for 30 min. (B) Cytofluorimetric analysis of *C. neoformans* CAP67, B3501, and H99 and *C. albicans* CA-6 performed in the presence or absence of MAb 2G8 or the irrelevant MAb. Yeasts were treated as described above. The data are representative of one of three independent experiments.

to 3.5 with 1 N HCl. The solution was sterilized by filtration and mixed with 140 ml of melted agar; 20 ml of this medium was poured into each petri dish, and 5 μl of yeast cells suspended in a saline solution (2×10^5 cells/ml) with 5 μl of MAb 2G8 at a concentration of 4 μg/ml was inoculated into each petri dish and incubated at 37°C for 21 days. The diameter of the area around the colonies was considered a measurement of protease production. Protease activity, determined by the method Price et al. (35), was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone.

Capsule thickness evaluation. Yeast strains B3501 and H99 at an initial concentration of 10^4 cells/ml, grown on Sabouraud dextrose broth (Sigma) for 7 days in the presence of 50 or 25 μg/ml MAb 2G8 at 37°C, were placed in India ink; for each sample 10 yeast cells in 10 different microscopic fields were observed to measure the capsules at a magnification of ×1,000. The capsule size of *C. neoformans* H99 in homogenized brains was evaluated by microscopy. Mice were sacrificed 7 days postinfection, brains were recovered and homogenized, and aliquots were stained with India ink.

Yeast growth inhibition assay. One hundred fifty *C. albicans* CA-6 or *C. neoformans* B3501, H99, or CAP67 yeast cells were incubated in 200 μl of RPMI containing FCS with 100 μg/ml of MAb 2G8. The cultures were incubated at 37°C for 18 h. *C. albicans* and *C. neoformans* growth was evaluated by classic CFU counting (25). To analyze the possibility that MAb 2G8 could induce yeast cell agglutination, experiments using flow cytometry were performed. The tests were carried out using forward light scattering, which correlates with cell size, and side light scattering, which correlates with cellular complexity.

Detection of GXM capsular polysaccharide of *C. neoformans*. *C. neoformans* B3501 and H99 yeast cells were incubated in 200 μl of RPMI containing FCS with 100 μg/ml of MAb 2G8 or anti-CRM MAb for 18 h at 37°C. After in vitro treatment supernatant fluids were recovered and stored at −20°C. Sera of mice infected with C. *neoformans* H99 and treated or not treated with MAb 2G8 were collected after 3 and 7 days and stored at −20°C. GXM detection was performed by using the cryptococcal antigen latex agglutination system test. The cryptococcal antigen latex agglutination system utilizes latex particles coated with anti-cryptococcal globulin which react with the GXM, causing visible agglutination. The agglutination score is expressed as follows: −, no visible clumping; +, fine granulation; ++, small but definite clumps; +++, large and small clumps; and ++++, large clumps.

Mice. Female BALB/c mice obtained from Harlan Italy Laboratories (Udine, Italy), were used at 4 to 6 weeks of age. Mice were allowed to rest for 1 week before the experiment; by that time the animals were roughly 6 to 7 weeks old. Animals were used under specific-pathogen-free conditions that included testing sentinels for unwanted infections; according to the Federation of European Laboratory Animal Science Association standards, no infections were detected. Immunosuppression was performed by treatment with cyclophosphamide 2 days before infection (150 mg/kg) and 3 days after infection (50 mg/kg).

***C. neoformans* infection.** *C. neoformans* H99 yeast cells from an overnight culture on Sabouraud liquid medium were washed with sterile endotoxin-free physiological saline and counted with a hemocytometer, and the concentration was adjusted to the desired level for intravenous injection. The number of viable yeast cells injected was confirmed by culturing dilutions of the inoculum on Sabouraud agar. Normal or immunosuppressed mice were treated with the irrelevant MAb or MAb 2G8 (200 μg/mouse given intraperitoneally) 2 h before and 1 day after infection with 10^4 CFU of *C. neoformans* in nonpyrogenic saline by intravenous inoculation.

Organ CFU assay. Three and 7 days after infection, mice were killed, sera were recovered, and the brains and livers were removed and homogenized. Serial 10-fold dilutions of each sample were plated on duplicate Sabouraud agar plates and incubated for 48 h. CFU counting was performed, and the results were multiplied by the dilution factor and then expressed as the mean CFU ± standard deviation per organ.

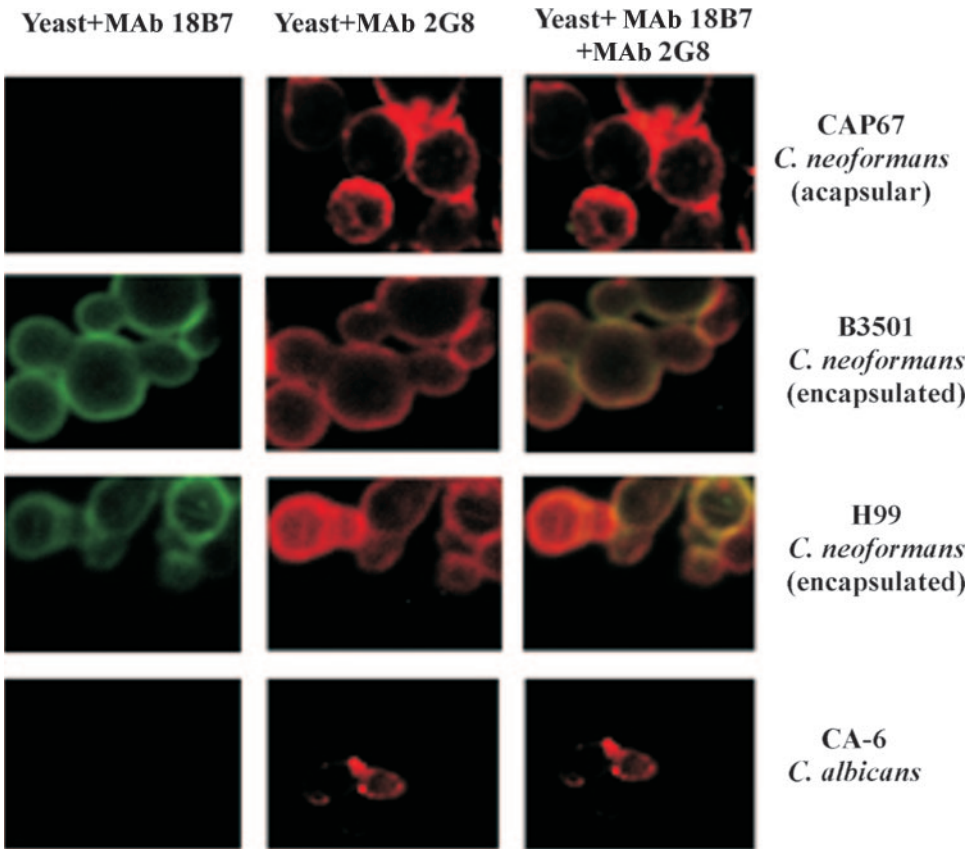

FIG. 2. Confocal microscope images of MAb 2G8 localization. Yeasts were treated with MAb to GXM and/or with MAb 2G8 or the irrelevant MAb. The images are from one of three experiments in which similar results were obtained.

Statistical analysis. The statistical significance of differences between groups was determined using analysis of variance or the Wilcoxon test. Results are presented below as means ± standard errors of the means.

RESULTS

MAb 2G8 binds to the cell wall of both acapsular and encapsulated *C. neoformans*. First we examined whether MAb 2G8 was able to bind to *C. neoformans* cells. To this end, we used several *C. neoformans* strains: acapsular avirulent strain CAP67, encapsulated virulent strain B3501, and encapsulated, highly virulent strain H99. The CAP67 strain was derived from strain B3501 by disruption of the CAP59 gene (13). All strains were treated for 30 min at 4°C with MAb 2G8 or the irrelevant MAb. *C. albicans* and β-glucan particles served as positive controls. The results of microscopic examination of cells of each fungus strain are shown in Fig. 1A, and the results obtained by FACS analysis are shown in Fig. 1B. As shown in Fig. 1A, cells of both encapsulated and acapsular *C. neoformans* strains were clearly fluorescent, and the fluorescence was not dissimilar from the fluorescence of the positive controls. No fluorescence was displayed by cells treated with the irrelevant MAb. The FACS analysis confirmed the binding of MAb 2G8 to *C. neoformans* and to *C. albicans* cells and quantified the positive reaction (Fig. 1B).

Since MAb 2G8 is specific for β-glucan (41) and this component is thought to be present in the cell wall and not in the capsule of *C. neoformans*, we attempted to obtain further insight into the binding of MAb 2G8 to the *C. neoformans* cell wall by using confocal microscopy. *C. albicans* and acapsular *C. neoformans* cells were also tested in our experimental system. *C neoformans* strains B3501, H99, and CAP67 and *C. albicans* were treated with a MAb to GXM that specifically binds the GXM of the *C. neoformans* capsule (6) alone or in combination with MAb 2G8. Figure 2 shows that, as expected, the anti-GXM MAb binds to the cells of both encapsulated *C. neoformans* strains, but not to the cells of the acapsular *C. neoformans* strain or to those of *C. albicans*. Conversely, the anti-β-glucan MAb binds to the cells of *C. albicans* and to all three strains of *C. neoformans*. In particular, fluorescence merging showed that the *C. neoformans* component recognized by MAb 2G8 is localized below the capsule, with a fluorescence intensity which was more prominent in younger growing cells, in both *C. neoformans* and *C. albicans* (Fig. 2).

MAb 2G8 inhibits the in vitro growth of *C. neoformans*. Having established that MAb 2G8 binds to the cell wall of *C. neoformans* cells, we analyzed whether this event influenced the growth of the fungus. To this end, we treated *C. neoformans* strains B3501 (encapsulated) and CAP67 (acapsular) with MAb 2G8 or the irrelevant MAb for 18 h at 37°C. Similarly, antibody-treated *C. albicans* was used as a control. Assuming that the growth of untreated yeast was 100%, treatment with MAb 2G8 resulted in approximately 50, 40, and 40%

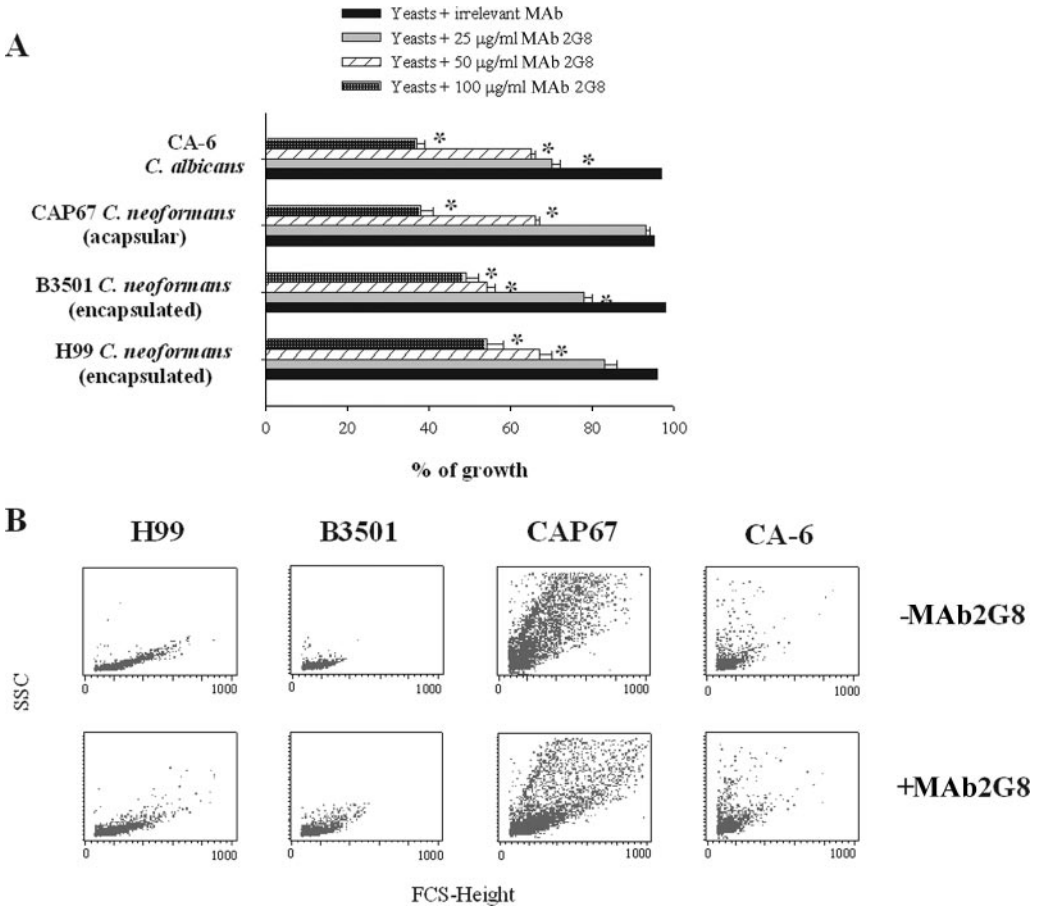

FIG. 3. Effect of MAb 2G8 on growth of *C. neoformans*. Yeasts were treated overnight with 100 μg/ml MAb 2G8 or the irrelevant MAb. The data are the means ± standard errors of the means of three separate experiments. The *P* value for a comparison of MAb 2G8-treated and irrelevant MAb-treated yeasts was <0.05. FCS, forward light scattering; SSC, side light scattering.

growth of *C. neoformans* strains B3501 and CAP67 and *C. albicans*, respectively (Fig. 3A). In a separate experiment, encapsulated strain H99 showed results comparable to those obtained with the B3501 strain (Fig. 3A). There was no statistically significant difference in the levels of growth inhibition caused by MAb 2G8 in *C. neoformans* and *C. albicans* strains, and treatment with the irrelevant antibody did not affect growth to any extent (Fig. 3A).

In these experiments, we also analyzed the cell population using forward light scattering, which correlates with cell size, and side light scattering, which correlates with cellular complexity. The results shown in Fig. 3B revealed a difference in the dimensions of different strains but no difference in cellular complexity in the presence or absence of MAb 2G8, indicating that no appreciable agglutination had occurred in the presence of MAb 2G8.

C. neoformans releases capsular material into the supernatants of in vitro cultures as well as in vivo cultures. Martinez et al. have demonstrated that the GXM level in supernatant fluid of a *Cryptococcus* culture is related to yeast growth (27). We therefore measured by using a semiquantitative method the GXM levels in the supernatants of untreated and MAb 2G8-treated *C. neoformans* cultures and noticed a dose-dependent decrease in GXM production in cultures of *C. neoformans* strains H99 and B3501 treated with the antibody compared to the quantity of GXM released in the absence of MAb 2G8. In particular, at a concentration of 25 μg/ml, MAb 2G8 was able to reduce the GXM level when the yeasts were cultured for 3 days but not when they were cultured for 7 days, while a dose of 50 μg/ml resulted in a decrease in the amount of GXM released after both 3 and 7 days of growth (GXM level, ++) compared with the amount released by cells not treated with the MAb (GXM level, +++).

MAb 2G8 treatment affects capsule size of *C. neoformans*. Next, we analyzed whether MAb 2G8 was able to affect the production of known virulence factors of *C. neoformans*. To this end, we tested the protease and phospholipase activities, as well as the capsule thickness, of encapsulated yeast strains B3501, 6995, and H99. For these experiments we used MAb 2G8 concentrations (25 to 50 μg/ml) lower than those that are optimal for fungus growth in order to minimize the likely confounding effects of retarded or inhibited growth. No appreciable effect on enzyme production by any of the *C. neoformans* strains was observed. Conversely, a remarkable effect of the antibody treatment on the capsule size of the highly virulent H99 strain was observed even at the lower concentration (25 μg/ml) (Fig. 4A and B). A reduction was also observed for

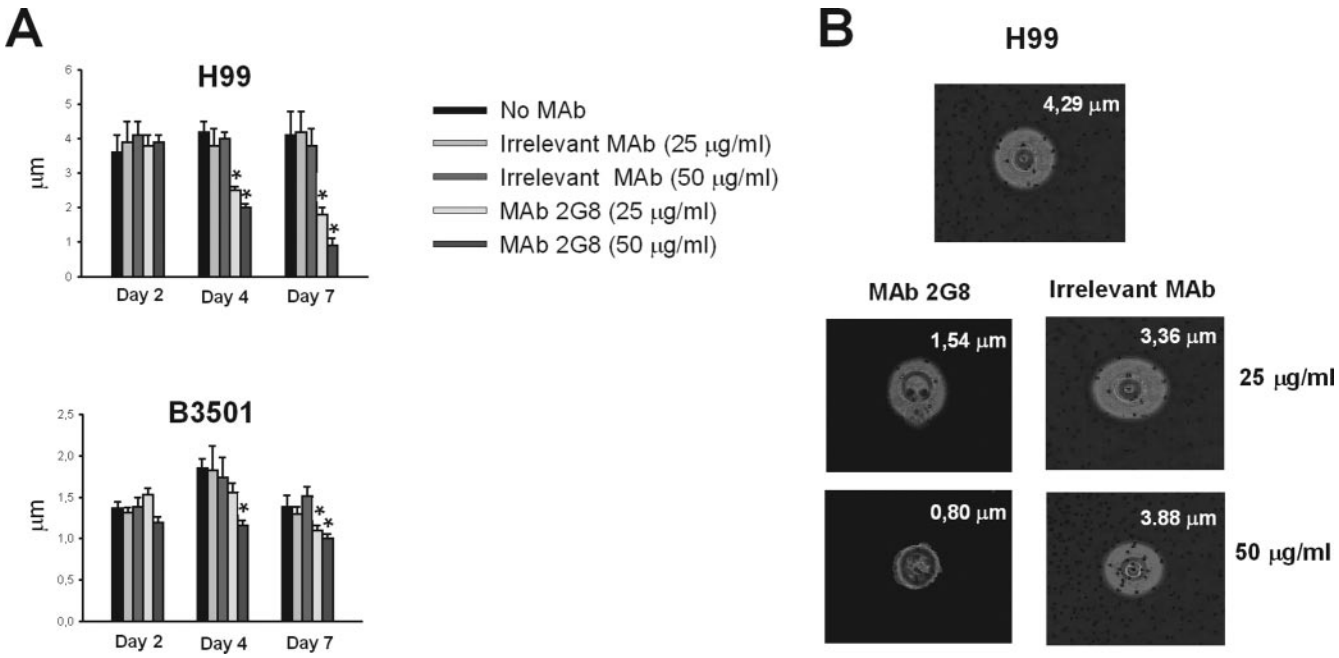

FIG. 4. Effect of MAb 2G8 treatment on capsule formation in different strains of *C. neoformans.* (A) *C. neoformans* H99 and B3501 cells were treated with MAb 2G8 (25 or 50 µg/ml) or with the irrelevant MAb (50 µg/ml) for 2, 4, and 7 days at 37°C. The results are expressed in capsule size (µm), and the data are the means ± standard errors of the means of three separate experiments. The *P* value for a comparison of MAb 2G8-treated and irrelevant MAb-treated yeasts was <0.05. (B) Micrographs of *C. neoformans* H99cells cultured for 7 days. Original magnification, ×100. The results are representative of one of three independent experiments.

B3501 (Fig. 4A), and the size of its capsule appeared to be significantly reduced within 4 to 7 days.

MAb 2G8 increases phagocytosis and killing of acapsular but not encapsulated strains of *C. neoformans*. The capsule of *C. neoformans* is a key virulence factor primarily because it inhibits phagocytosis. Thus, we wondered whether MAb 2G8 could facilitate the internalization of encapsulated *C. neoformans*. To this end, we used two isogenic strains, acapsular strain CAP67 and encapsulated strain B3501, and the highly pathogenic strain H99 of *C. neoformans*. *C. albicans* was used as a control. Figure 5 shows that the internalization of *C. albicans*, as well as *C. neoformans* acapsular strain CAP67, by human monocytes was significantly and specifically increased by treatment with MAb 2G8. As expected, the degree of phagocytosis of the two encapsulated strains of *C. neoformans*, strains B3501 and H99, was much lower than that of acapsular strain CAP67. In these strains neither the irrelevant MAb nor the antiglucan MAb was able to significantly increase phagocytosis (Fig. 5A). Similarly, MAb 2G8 did not increase killing by human monocytes of the encapsulated strains *C. neoformans* H99 and B3501, while it did increase killing in the case of *C. albicans* and acapsular *C. neoformans* strain CAP67 (Fig. 5B). Given that MAb 2G8 is a murine antibody, we also tested the phagocytic and killing activities of mouse peritoneal macrophages by using the same experimental conditions that were used for human monocytes. The results obtained were similar to those obtained with human monocytes. In particular, killing activity against encapsulated *C. neoformans* strains H99 and B3501 did not change in the presence or absence of MAb 2G8 (data not shown).

MAb 2G8 exerts anti-*C. neoformans* protective effects in vivo. To verify that the growth-inhibitory and anticapsular effects of the anti-β-glucan antibody observed in vitro could indeed be reproduced in vivo, normal mice or mice immunosuppressed with cyclophosphamide were inoculated with MAb 2G8 2 days before intravenous challenge with the *C. neoformans* H99 strain. The immunosuppression was maintained during the course of infection by administering repeated doses of cyclophosphamide. The level of immunosuppression was monitored by counting the white blood cells, which always resulted in values ranging from 200 to 400 cells/µl. After 3 and 7 days, mice were sacrificed, and brains and livers were analyzed to enumerate fungal cells. Figure 6 shows that in immunocompetent mice, there was a highly significant decrease in the fungus burden in MAb 2G8-treated animals compared with the irrelevant antibody-treated controls. In particular, the *C. neoformans* burden in the brain (Fig. 6A) was reduced by about 47% 3 days after infection, and the decrease was even greater (68%) 7 days after infection. A rather similar pattern was observed for the *C. neoformans* burden in the liver (Fig. 6B). In neutropenic mice, significant reductions in the fungus burdens in the brain and in the liver were observed on day 3 but not on day 7. It is worth noting that cyclophosphamide-treated mice had a higher *C. neoformans* burden than the normal mice in the brain on day 3 and in the liver on day 7.

Moreover, the GXM level in sera of mice treated or not treated with MAb 2G8 was evaluated. The results showed that there was a consistent decrease in the level of circulating GXM in animals treated with MAb 2G8 7 days postinfection (GXM

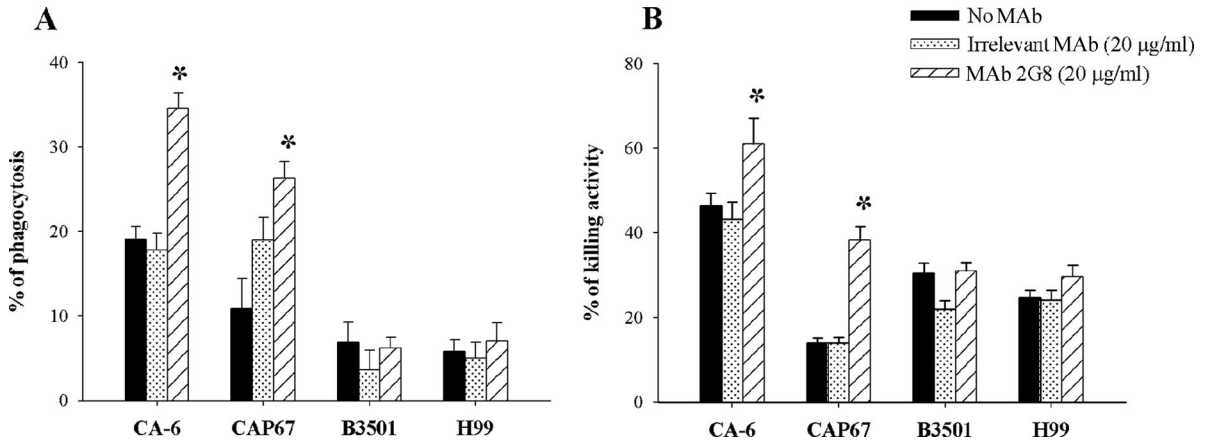

FIG. 5. Effect of MAb 2G8 on phagocytic and anticryptococcal activities of human monocytes. The phagocytic activity of monocytes was evaluated by cytofluorimetric analysis and was expressed as a percentage of the phagocytic cells. Monocytes were incubated with FITC-labeled *C neoformans* (CAP67, B3501, or H99) or FITC-labeled *C. albicans* (CA-6) at a ratio of 1:10 alone or in the presence of 20 μg/ml of the irrelevant MAb or MAb 2G8 for 2 h at 37°C in 5% CO_2. The data are the means ± standard errors of the means of three separate experiments performed with cells from three different donors. For determination of killing activity, monocytes were incubated with *C. neoformans* CAP67, B3501, or H99 or *C. albicans* CA-6 in the presence or absence of MAb 2G8. After incubation, monolayers were lysed. Plates were prepared by spreading each sample on Sabouraud dextrose agar, and CFU were visually evaluated after 48 h of incubation. The *P* value for a comparison of MAb 2G8-treated and irrelevant MAb-treated yeasts was <0.05.

level, +++) compared with animals not treated with MAb 2G8 (GXM level, ++).

To verify that the presence of MAb 2G8 influences the capsule size in vivo, on day 7 postinfection the brains of mice were recovered, homogenized, and stained by using India ink, and the capsule size was determined by microscopy. There was a significant reduction in capsule thickness (3.9 ± 1.5 μm versus 2.1 ± 1.1 μm; $P < 0.05$) in the brain lesions from MAb 2G8-treated immunocompetent mice compared with the brain lesions from control mice.

DISCUSSION

Because of the limited efficacy of the available antimicrobial armamentarium in immunocompromised subjects and the increasing resistance to antimicrobial drugs, there is a rather urgent need for new therapies against infections. Theory and history demonstrate that antibodies are ideal immunological tools to integrate or possibly even replace conventional antimicrobial drugs (5, 9). Fungal infections are primary targets for these new approaches, because treatment options are limited and often there is not cooperation by the immune system. Moreover, toxic effects have been observed for the currently available antifungal drugs (4). However, the perspectives of effective immunotherapy against fungal infections have so far been very limited (28) because of the prevailing assumption that cell-mediated immunoprotection is more effective than humoral immunity against fungal infections in humans (7). It is quite logical that the availability of protective antibodies targeting common, viability-critical components of fungal cells may greatly expand the potential and clinical perspectives for antifungal immunotherapy. β-Glucan is one such target that is present in all human pathogenic fungi, although it has distinctive molecular features in the various fungus genera (11, 16).

Following this strategic line, anti-β-glucan antibodies produced in mouse recipients of a glucan-conjugated vaccine have recently been shown to confer protection against *C. albicans* as well as *Aspergillus fumigatus* infections (11, 41). In particular, an IgG2b anti-β-glucan MAb has been generated and has shown in vitro and in vivo protective activity. This MAb was shown to efficiently bind hyphae of both fungi and germinating *Aspergillus* conidia, thus inhibiting hyphal growth in vitro (41). Therefore, it has been speculated that anti-β-glucan antibodies could be therapeutic against a variety of fungal organisms that have β-glucan in the cell wall (11, 41).

Within this conceptual framework, we addressed the effects of the anti-β-glucan MAb 2G8 on *C. neoformans* and show here that this antibody (i) binds to both acapsular and encapsulated *C. neoformans*; (ii) inhibits in vitro growth of acapsular and encapsulated *C. neoformans* strains at a rate similar to that observed for *C. albicans*; (iii) decreases the size of the capsule in both encapsulated strains used, H99 and B3501, in vitro and in vivo; (iv) opsonizes and favors the killing of the acapsular but not the encapsulated C. *neoformans* cells in human monocytes and in mouse peritoneal macrophages; and, importantly, (v) confers a certain degree of protection in an in vivo experimental model of systemic cryptococcosis in both normal and neutropenic mice.

Although *C. neoformans* has β-glucan in its cell wall, the effects of MAb 2G8 on this fungus were not foreseeable for reasons having to do with the likely differences in the β-glucan and the nature of the ectocellular structures in this fungus and *C. albicans*. In fact, *C. neoformans* is unique among human pathogenic fungi in possessing a thick capsule as the outermost cellular organelle, which is composed of GXM and does not contain β-glucan. Moreover, the capsule may hinder the access of even relatively low-molecular-weight compounds to the inner cell wall and cell membrane, which could explain, for instance, the inability of β-glucan synthase inhibitors, such as the echinocandin derivatives, to affect this critical enzyme in intact cells of *C. neoformans* (19). While the level of binding of MAb 2G8 to acapsular *C. neoformans*—which is shown here to

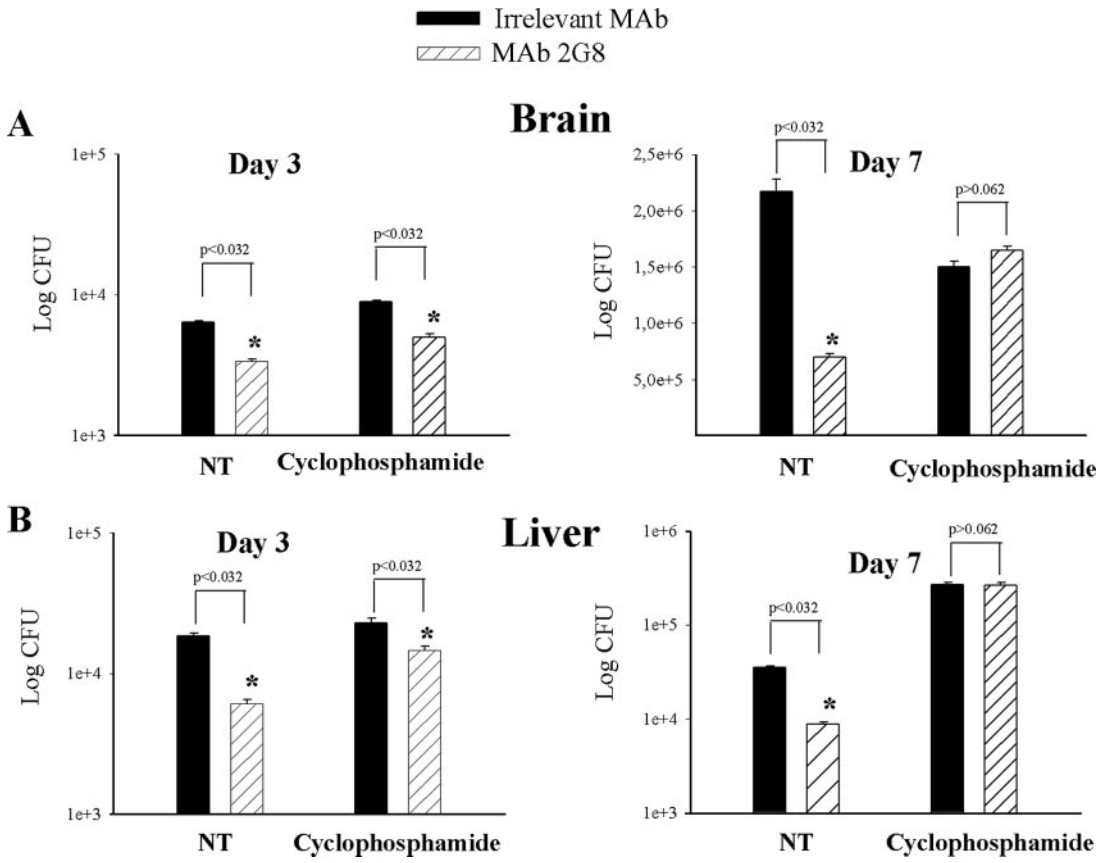

FIG. 6. Effect of MAb 2G8 on clearance of *C. neoformans* H99 cells by brains and livers of immunocompetent (NT) or immunosuppressed (Cyclophosphamide) mice. Immunocompetent or immunosuppressed mice were pretreated with MAb 2G8 or with the irrelevant MAb (200 μg/ml given intraperitoneally) 2 h before intravenous challenge with 10^4 *C. neoformans* cells. After 3 and 7 days, mice were killed, brains (A) and livers (B) were recovered, and viable yeast cells were counted by plating samples of homogenized tissue on Sabouraud agar. The results are expressed as the mean CFU ± standard error of the mean for three mice per time point. Data were analyzed by using the Wilcoxon test. The *P* value for a comparison of MAb 2G8-treated and irrelevant MAb-treated yeasts was <0.032.

be rather high and similar to that obtained by using *C. albicans* cells—and its ability to inhibit the in vitro growth of this strain were rather predictable, the fact that MAb 2G8 also effectively binds to, and inhibits the growth of, fully encapsulated strains of the fungus could not be anticipated.

Rather surprisingly, it appears not only that a large capsule does not prevent MAb 2G8 from binding to glucan but also that the capsule itself may be an indirect target of antibody action.

We did not specifically address the mechanism whereby MAb 2G8 decreases the capsule size, but the simplest explanation could be interference of the anti-β-glucan antibody, which binds to β(1,3)-linked glucan in the cell wall, with capsule formation or aggregation of capsular polymers during fungus replication. Of interest here is the report by Rodrigues et al. that binding of antibodies to the cell wall could interfere with the export of newly produced GXM across the cell wall (36). In addition, the decrease in capsule size may also be an indirect consequence of growth inhibition caused by the antibody, although in capsule size inhibition tests, we took great care to use concentrations of MAb 2G8 which had low or no activity against fungal growth. Moreover, we cannot totally exclude the possibility that some β-glucan moiety that is critical for capsule formation and stability is present in the capsule itself.

Under most experimental conditions, monocytes and macrophages avidly phagocytose microorganisms such as *C. albicans* but have limited capacity to ingest encapsulated *C. neoformans* (12). This was verified here when addition of MAb 2G8 to *C. albicans* yeast cells and acapsular *C. neoformans*, putatively exposing β-glucan antigens on cell surface, resulted in a significant enhancement of phagocytosis. However, MAb 2G8 was poorly effective, if it was effective at all, in increasing phagocytosis and the anticryptococcal activity of the encapsulated strains, suggesting that, despite its binding on fungal cells, it lacks opsonic capacity. This could be due to insufficient exposure on the surface of encapsulated *C. neoformans* of the MAb Fc portion, which is hindered by the presence of the capsule.

Notably, the anticryptococcal activity of MAb 2G8 was not limited to the in vitro systems. In fact, we show here that this antibody also restricts the in vivo growth of *C. neoformans* in both normal and (although to a less persistent degree) cyclophospamide-treated, heavily neutropenic mice. The effect is quite remarkable considering the fact that a single dose of MAb 2G8 was used. In leukopenic mice, the brain burden of *C.*

neoformans was significantly reduced on day 3 but not on day 7, suggesting that the antibody, at the dose and under the experimental conditions used, could not inhibit the heavy, late systemic fungus growth. Given that under the same conditions the nonleukopenic animals were capable of stably controlling fungus growth in vivo, it is clear that leukocytes, although not capable of phagocytosing the largely encapsulated cells, significantly contribute to maintaining restricted fungal growth in vivo. There are a multitude of supposed and demonstrated mechanisms by which host effectors of innate and adaptive immunity control *C. neoformans* growth in vivo (38).

In summary, the data reported here provide the first demonstration that an antibody directed against β-glucan confers protection against *C. neoformans*, possibly by interfering with cell wall and capsule formation during growth. Despite the lack of an opsonic capacity for encapsulated *C. neoformans* cells, the inhibition of capsule formation in vitro and in the brains of mice with systemic cryptococcosis supports the notion that, among other possible factors, the in vivo activity of MAb 2G8 may be directly or indirectly the result of an anticapsular effect. The initial demonstration of the anticryptococcal efficacy of this antibody in in vivo and in vitro systems is promising and may open new doors to further investigations addressing the full potential of anti-β-glucan antibodies as new therapeutic agents.

ACKNOWLEDGMENTS

This work was supported by the National Research Program on AIDS (contract 50G.38), by the Ministry of Education, University and Research (PRIN contract prot. 2005065913_003), and by a Marie Curie Network grant (Galar Fungail II project).

We are grateful to Gabriella F. Mansi for devoted editorial and secretarial assistance.

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Editor: A. Casadevall