Monoclonal Antibody to Fungal Glucosylceramide Protects Mice against Lethal Cryptococcus neoformans Infection

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Glucosylceramides (GlcCer) are involved in the regulation of Cryptococcus neoformans virulence. In the present study, we demonstrate that passive immunization with a monoclonal antibody to GlcCer significantly reduces host inflammation and prolongs the survival of mice lethally infected with C. neoformans, revealing a potential therapeutic strategy to control cryptococcosis.

Cryptococcosis, a fungal infection caused by the yeast-like pathogen Cryptococcus neoformans, is an important cause of morbidity and mortality in immunosuppressed individuals, affecting especially the central nervous system (3, 33). Treatment failures occur and, despite high doses of antifungal drugs, the fungus is frequently not eradicated, resulting in recurrent disease in individuals coinfected with human immunodeficiency virus in the absence of continued antifungal therapy (3, 8).

Passive immunization with monoclonal antibodies (MAbs) to cryptococcal antigens can protect mice against lethal C. neoformans infections (6, 11, 13, 36). Targets of protective MAbs include glucuronoxylomannan (GXM), the major capsular polysaccharide of C. neoformans (6, 11, 13), the pigment melanin (36), and the cell wall polysaccharide glucan (30). Antibodies to GXM have been characterized as potential therapeutic agents (6) and are in clinical trials for individuals with cryptococcosis (3, 8).

In the present study, we demonstrate that passive immunization with a monoclonal antibody to GlcCer significantly reduces host inflammation and prolongs the survival of mice lethally infected with C. neoformans, revealing a potential therapeutic strategy to control cryptococcosis.
pronounced when a 500-µg antibody dose was used, passive immunization with 250 µg of purified antibody also prolonged mice survival. In other experiments, 100 µg of the MAb to GlcCer was not protective (data not shown).

For CFU, cytokine, and histological analyses, mice were given 500 µg of a MAb (commercial IgG2b or the MAb to GlcCer) or of PBS i.p. Twenty-four hours later, mice were infected i.t. with C. neoformans (10^6 cells/mouse). Age-matched, noninfected mice were used to provide baseline histology and cytokine values. Antibody or PBS-treated (n = 5 per group) and noninfected mice (n = 1 per group) were sacrificed on days 1 and 7 after infection. In all experimental groups, the right upper lobe of the lung was removed and placed into formalin for histology. Formalin-fixed, paraffin-embedded tissues were examined by hematoxylin-eosin staining for evaluation of histopathology and fungal distribution.

The remaining tissue of each lung was homogenized in 4 ml of PBS, and aliquots were plated on brain heart infusion agar supplemented with penicillin-streptomycin, followed by incubation at 30°C for CFU determination. The remaining part of the suspension was centrifuged, and the supernatant was stored at -20°C. When all samples had been collected, the supernatants from each group at each time point were pooled and cytokine/chemokine analyses were performed with a RayBio mouse cytokine antibody array II kit (RayBiotech, Inc., Norcross, GA) according to the manufacturer’s instructions. In this assay, cytokines and chemokines are detected by interaction with antibodies specific to each of the molecules measured. Cytokine samples were analyzed in duplicate, and the experiment was repeated with similar results. The membranes were developed radiographically, and ImageJ (http://rsb.info.nih.gov/ij/) was used to analyze the results. Statistical analyses and comparisons between the cytokine/chemokine values of the control group and mice given anti-GlcCer were performed using the Student t test.

A histological analysis of lung sections revealed that administration of antibody to GlcCer significantly reduced inflammation (Fig. 2). On day 1, although increased cellularity, especially peribronchial inflammation, occurred in the lungs of mice treated with antibody to GlcCer, there was greater preservation of alveolar airspace in those mice than in the controls. C. neoformans yeast cells were clearly visible in the alveolae of mice in all experimental groups. On day 7, increased numbers of yeasts were seen in the air spaces, but there was a dramatic reduction in inflammation in the lungs of mice treated with the MAb to GlcCer, whereas dense inflammation persisted in the control mice, and numerous multinucleated giant cells were present. A comparison of the total number of cells in the lungs of mice given PBS, the control MAb, or the MAb to GlcCer revealed, for the last group, a significantly smaller number of cells at both day 1 (P < 0.05) and day 7 (P < 0.01) after infection, as statistically analyzed using analysis of variance. In this early time range (1 to 7 days) of infection, the administration of MAb to GlcCer did not reduce the numbers of CFU in the lungs of infected mice (data not shown).

Lung cytokine levels of mice treated with PBS, control MAb, or MAb to GlcCer were measured on days 1 and 7 after infection with C. neoformans (Fig. 3). On day 1 after infection, interleukin-4 (IL-4), IL-12, IL-12p70 and RANTES (regulated upon activation, normal T-cell expressed, and secreted protein) were detected in significantly higher levels (P < 0.05) in mice given GlcCer antibody, whereas the levels of other tested cytokines were very similar between groups. On day 7 postinfection, mice given GlcCer antibody had significantly higher pulmonary concentrations of IL-4, IL-6, IL-12, monocyte chemoattractant protein 5 (MCP-5), and stem cell factor (P < 0.05).

GlcCer in pathogenic fungi was first described two decades...
These molecules have been fully characterized at the structural level and found to be extremely conserved in a number of fungal species (reviewed in reference 1). Recently, there have been several investigations of the roles of GlcCer in fungal physiology and pathogenesis. For example, GlcCer was characterized by different groups as the target of antimicrobial antibodies (9, 23, 29, 35) and plant defensins (39). In addition, the inhibition or inactivation of GlcCer synthase, the enzyme responsible for the last step in GlcCer biosynthesis, resulted in aberrant morphogenesis in different fungal models (19). In *C. neoformans*, a direct relationship between GlcCer expression and yeast replication was established by molecular and antibody-based approaches (31, 35). GlcCer also regulates fungal virulence in animal models of cryptococcosis (31). Finally, this lipid is a key component of secretory vesicles transporting capsular polysaccharide (34). Given the clear relevance of GlcCer in the biology and pathogenesis of *C. neoformans*, we speculated that passive immunization of mice with GlcCer-binding antibodies could have the potential to control cryptococcosis.

In the present study, we demonstrate that a MAb to GlcCer protects mice against lethal *C. neoformans* infection. Although protective antibodies to glycolipids in other microbes have been described (16), this is, to our knowledge, the first demonstration of protection using a MAb targeting a fungal glycolipid. Preliminary analyses in our laboratory indicate that the MAb to GlcCer, which binds to the cryptococcal cell wall, is cytotoxic in vitro (25, 35) but does not opsonize *C. neoformans* cells in vitro (unpublished data), possibly because of a masking of the Fc domains by capsular polysaccharides. Our in vivo data showed that CFU levels were not different between mice receiving MAb to GlcCer and controls, suggesting that direct fungal cytotoxicity by the MAb was minimal during early infection. Nevertheless, in comparison to controls, mice given MAb to GlcCer had dramatically reduced lung inflammatory responses. These observations suggest that the MAb-mediated protection may be a consequence of downregulation of the inflammatory response. We therefore believe that in the presence of the antibodies to GlcCer, inflammatory responses are better controlled by the host, resulting in reduced damage to host tissues and more effective killing of the invading organism by host effector cells (4). Similar results were described for mice passively immunized with a protective IgG1 to GXM prior to infection with *C. neoformans* (14, 32). The finding of reduction of inflammation by nonopsonic antibody has also been shown with other pathogenic fungi, such as *Histoplasma capsulatum* (26).

The reduced inflammation in mice treated with the MAb to GlcCer corresponds to the increased lung concentrations of IL-4 on day 1 postinfection and anti-inflammatory cytokines, such as IL-4 and IL-6 (28), on day 7. It has been shown that IL-4 is necessary for MAb-mediated protection and that mice genetically deficient in IL-6 and IL-12 production have increased susceptibility to *C. neoformans* infections (2). In addi-
tion, an association between high levels of IL-6 and resistance to C. neoformans infection has been recently described (37). Administration of MAb to GlcCer in our cryptococcal model also increased lung expression of IL-12 and IL12p70 on days 1 and 7 postinfection. IL-12 has in fact been demonstrated to have a protective effect against pulmonary infections with C. neoformans in mice (17). The elevated levels of RANTES in the lungs of mice given MAb to GlcCer were in accordance with results from a study by Uicker et al., which demonstrated that this chemokine is associated to a protective cell-mediated immunity against C. neoformans (40). On day 7, pulmonary levels of MCP-5 and stem cell factor were also elevated, a finding which may be correlated with the recruitment of monocytic cells. These results reinforce the complexity of the mechanisms through which antibodies modulate infection by C. neoformans.

Cerebroside expression is associated with virulence in pulmonary cryptococcosis (31), which suggested that antibodies to GlcCer could be efficacious in modifying disease. In summary, as recently theorized by Mitchell (21), GlcCer represents a valuable target for new therapeutic agents against cryptococcosis. Considering the fact that fungal cerebrosides are extremely conserved and expressed in virtually all pathogenic species studied so far (1), GlcCer-binding antibodies may also be useful tools in the control of other mycoses.

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**FIG. 3.** Lung cytokine/chemokine levels of mice given the anti-GlcCer MAb, the control MAb, or PBS prior to lethal C. neoformans infection. The relative increase in cytokine/chemokine levels in animals immunized with the MAb to GlcCer was defined by dividing densitometric values obtained for mice immunized with the control MAb by those for mice given PBS (control) or mice given the anti-GlcCer MAb by those for mice given the isotype control MAb (effective variation in cytokine/chemokine expression). Cytokine/chemokine levels were determined on days 1 (A) and 7 (B) postinfection. *P* values are shown for levels in which statistically significant differences were observed. GCSF, granulocyte colony stimulating factor; GMCSF, granulocyte-macrophage colony stimulating factor; IFN-γ, gamma interferon; SCF, stem cell factor; sTNFR1, soluble TNF receptor p55; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.
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