ARTÍCULO ORIGINAL

# Anti-glucuronoxylomannan IgG1 specific antibodies production in *Cryptococcus neoformans* resistant mice

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**Background.** *Cryptococcus neoformans* is a widely disseminated fungus shown to be responsible for infections in individuals with impaired cell mediated immunity, such as patients with human immunodeficiency virus (HIV). *Cryptococcus neoformans* has a polysaccharide capsule composed of glucuronoxylomannan (GXM), which acts as a major virulence factor and is considered to be a thymus independent type-2 antigen (TI-2).

**Objective.** In the current study, the production kinetics were evaluated for IgG subclasses specific for GXM, and assessed with the cross reactive antibodies to *Streptococcus pneumoniae* polysaccharide. In addition, spleen B cell subpopulations were quantified in murine models of cryptococcosis with different susceptibilities to the infection.

**Material and methods.** Antibodies were detected by ELISA at different time intervals after *C. neoformans* infection in moderately resistant (Balb/c), highly resistant (CBA/j) and susceptible (C57BL/6) mouse strains. B cells subpopulations were determined by flow cytometry analysis.

**Results.** Early production of $IgG_1$, described as protector antibodies, coincided with a decrease of the number of *C. neoformans* colony forming units in the lungs. Polysaccharide cross-reactive antibodies were detected in each of the three mouse strains. Antibody titers were highest in the susceptible strain (C57BL/6), a strain which also showed the highest proportion of splenic CD5+ B lymphocytes. In contrast, CBA/J mice showed the highest levels of CD43+ B.

**Conclusions.** These findings suggest that $IgG_1$ antibodies specific for GXM, are implicated in host protection against *C. neoformans* infection and may be regulated by CD43+ cells. They also suggest that cross reactivity antibodies are not important in the protection against *C. neoformans* infection.

**Keywords:** *Cryptococcus neoformans*, glucuronoxylomannan, cross-reactive antibodies, IgG subclasses.

**Producción de anticuerpos IgG1 anti-glucuronoxilomanana en ratones resistentes a la criptococosis**

**Introducción.** *Cryptococcus neoformans* es un hongo ubicuo que está relacionado con infecciones oportunistas en pacientes inmunocomprometidos, especialmente los pacientes infectados con el virus de la inmunodeficiencia humana. *C. neoformans* posee una cápsula que contiene principalmente glucuronoxilomanana que actúa como factor de virulencia y es considerado como antígeno timo independiente tipo-2.

**Objetivos.** Determinar la cinética de producción de las subclases de IgG específicas para la glucuronoxilomanana, medir los anticuerpos de reactividad cruzada contra el polisacárido de *Streptococcus pneumoniae* y determinar las subpoblaciones esplénicas de linfocitos en tres modelos de criptococosis en ratones con diferentes susceptibilidades a la infección.

**Materiales y métodos.** Los anticuerpos fueron detectados por la técnica inmunoenzimática ELISA después de la infección con *C. neoformans* en ratones moderadamente resistentes (Balb/c), resistentes (CBA/j) y susceptibles (C57BL/6). Las subpoblaciones de linfocitos B se determinaron por citometría de flujo.

**Resultados.** Se observó la producción temprana de anticuerpos $IgG_1$, que coincidía con una

disminución en el número de unidades formadoras de colonias de *C. neoformans* en pulmón. Los anticuerpos de reactividad cruzada se detectaron en las tres cepas de ratones después de la infección; fueron mayores en la cepa susceptible C57BL/6, la cual mostró el porcentaje más alto de linfocitos B, CD5$^+$ en el bazo. En contraste, los ratones CBA/J presentaron los mayores niveles de linfocitos B, CD43$^+$.

**Conclusiones.** Estos hallazgos sugieren que los anticuerpos IgG$_1$ específicos para la glucuronoxilomanana están implicados en la protección del hospedero contra la infección por *C. neoformans* y podrían estar regulados por células CD43+. También sugiere que los anticuerpos de reactividad cruzada no poseen un papel relevante en el control de la infección.

**Palabras claves:** *Cryptococcus neoformans*, glucuronoxilomanana, anticuerpos de reactividad cruzada, subclases de IgG.

*Cryptococcus neoformans* is an ubiquitous fungus which has been shown to be responsible for infections in individuals with impaired cell mediated immunity, such as patients infected with human immunodeficiency virus (HIV) (1). *C. neoformans* has a polysaccharide capsule composed of glucuronoxylomannan (GXM), which acts as a major virulence factor (2) and is considered to be a thymus independent type-2 antigen (TI-2). A murine model in which *C. neoformans* is inoculated *via* intratracheal route has allowed the definition of different levels of susceptibility among the various mouse strains (3,4). It has been proposed that these differences are attributed mainly to the presence of a Th1 cellular immune response (3).

The role of the humoral response in protection against cryptococcal infection has been well demonstrated. Passive serotherapy with monoclonal IgG$_1$ antibodies directed against GXM offers a possible alternative therapy for cryptococcal infection in immunosupressed patients (5). Studies in lethally infected mice have shown that passive administration of monoclonal antibodies reduced serum polysaccharide levels and lung fungal burden and prolonged survival. In addition, these studies suggest that the protective efficacy depends on the isotype of antibodies used; the conclusion was drawn that anti-GXM specific IgG$_1$ antibodies were more effective than IgM and IgG$_3$ antibodies (5-8).

Isotype switching of a non-protective IgG$_3$ antibody to a protective IgG$_1$ antibody confirmed the isotype importance for antibody efficacy against *C. neoformans* (9,10). Interestingly, the immune response to purified cryptococcal capsular polysaccharide is influenced by the genetic background of the animal (11-13). In spite of the relevant role of this anti-polysaccharide antibody in the control of *in vivo* infection, to date, only the kinetics of the humoral response to cryptococcal proteins has been studied (14). The relationship between the isotype of the antibody produced after cryptococcal infection and the antigen specificity *in vivo* is thus far only partially understood. In other infections with microorganisms such as *Streptococcus pneumoniae* that express antigenic polysaccharide moieties, the presence of cross-reactive anti-polysaccharide antibodies has been demonstrated (15). However, its protective or deleterious role in host defense is incompletely understood.

Cells implicated in the proliferative responses to TI-2 antigens, including polysaccharides, are CD5$^+$ lymphocytes (16-18) that are characterized phenotypically as B220$^{low}$IgM$^{low}$ cells normally found in the spleen (18). These cells are able to undergo self-renewal upon stimulation with TI-2 antigens. The paucity of CD5$^+$ B cells in mice is correlated with an inability to respond to TI-2 antigens (19). However, *in vivo* activation of CD5$^+$ lymphocytes seems to be deleterious and is strongly implicated in the generation of non-protective humoral responses (20). CD5$^+$ lymphocyte expansion is controlled by the presence of CD43$^+$ cells, suggesting that deleterious responses might be naturally regulated (21).

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In this study, we wanted to analyze IgG subclass production after intratracheal infection with *C. neoformans* of three mouse strains, and observed differential production of $IgG_1$ depending on the strain of mouse. Our results provide additional evidence for the efficacy of the antibody response to *C. neoformans*, which is dependent on the subclass of antibody elicited and on the specificity of recognition.

## Materials and methods

### *Animals*

Six 8 week-old male CBA/J ($H2^k$), BALB/c ($H2^d$) and C57BL/6 ($H2^b$) mice were purchased from Jackson Laboratories and maintained in the animal facilities of the Instituto Nacional de Salud (INS). Animals were maintained on food and water *ad libitum*, and treated in accordance with the international guidelines at the INS in Bogotá, Colombia.

### *Maintenance of C. neoformans, and purification of capsular polysaccharide and GXM*

*C. neoformans* var. *grubii*, serotype A (ATCC 90113), was used in all experiments. The fungus was cultured in Sabouraud dextrose agar medium at 27°C and passaged weekly *in vitro*. To maintain virulence, *C. neoformans* was passed *in vivo* in C57BL/6 mice, recovered from spleen and cultured in Sabouraud agar; capsular polysaccharide was obtained as previously described (22). Briefly, purified polysaccharide was stored at 4°C, and the carbohydrate content was determined by Dubois technique (22). To purify the GXM, polysaccharide was diluted in 0.2M NaCl and sonicated for 15 minutes, after which 3.0 mg of cetyltrimethyl-ammonium (CTAB) per mg of polysaccharide was added. Precipitation was carried out using two volumes of 0.05% CTAB and centrifuged at 12,000*g* at 23°C for 1 hour. The precipitate was washed with 10% ethanol and diluted in 1M NaCl. The GXM was dialyzed, dried and stored at 4°C. The presence of LPS in GXM preparation was determined by the *Lymulus amebocyte* lysate assay (LAL). Only samples in which LPS was less than 0.25 µg/ml were used. Protein content was determined by Bradford technique (Biorad) and revealed to be consistently less than 10 µg/mL.

### *Mice infection and blood sampling*

Mice were infected by intratracheal inoculation. Briefly, aseptic exposure of the trachea was done under anesthesia with ketamine (150 mg/kg) and xylazine (10 mg/kg), followed by inoculation with 50 µL of PBS containing $10^6$ colony-forming units (CFU) of *C. neoformans*. Uninfected mice were used as negative controls. Animals were bled *via* cardiac puncture on day 0, 4, 7, 11, 17, 23 and 45 post-infection. Blood was allowed to coagulate at room temperature and serum was obtained after centrifugation. Lungs and spleens were extracted aseptically and weighed, after which the lungs and spleens were minced in 1 mL 0.9% sterile saline solution. The number of CFU in each organ was determined after culturing in Sabouraud and cafeic acid agar by incubation at 27°C for 72 h.

### *Kinetics of the antibody anti-GXM response in C. neoformans-infected mice*

Sera were evaluated for the presence of specific antibodies to GXM by ELISA technique. Briefly, a total of 2.5 µg GXM in 100 µl of PBS (pH 7.2) was used to coat 96-well flat-bottom plates (NUNC, MaxiSorp, Naperville, IL, U.S.A), which were incubated overnight at 4°C. Plates were washed 5 times with PBS, 0.1% of BSA and 0.05% Tween 20 (ICN, Cappel, USA) and blocked with PBS and 1% BSA for 1 hour at 37°C. After blocking, plates were washed again and serum samples were added. Sera and controls were diluted 1/10 in PBS (pH 7.2) and 0.1% BSA. Samples were incubated for 1 hour at 37°C and washed again. Goat anti-mouse isotype antibodies (anti-$IgG_1$, anti-$IgG_2$a, anti-$IgG_2$b, anti-$IgG_3$) (ICN) were diluted in PBS (pH 7.2) and 0.1% BSA and used either at 2.5 µg/ml or ($IgG_2$b) 3.5 µg/ml concentration (ICN). After incubation for 1 hour at 37°C plates were washed and conjugated antibody rabbit anti-goat IgG labeled with alkaline phosphatase (100 µl of diluted 1/9000 in PBS and 0.25% BSA per well) was added (ICN) and incubated for an additional 1 hour at 37°C. After washing, 100 µl of substrate (1 mg of P-nitrophenyl phosphate (ICN) diluted in 100 ml of buffer 2-amino-2-methy-propaneidiol [pH 10.3]) was added to each well and incubated for 45 minutes at room temperature. Reaction was stopped using 100 µl of 3N NaOH per well. Plates were read at 405 nm using a spectrophotometer

(Multi-Scan 340, Biosystems). As positive controls anti-GXM mAb of isotype $IgG_1$ (provided by F. Drommer, Institute Pasteur, Paris) $IgG_3$, $IgG_{2a}$ and $IgG_{2b}$ (provided by A. Casadevall, Albert Einstein Medicine College, New York) were used.

### Cross-reactivity assay

The cross-reactivity of sera from mice infected with *C. neoformans* to the capsular polysaccharide from *Streptococcus pneumoniae* serotype 14 (ATCC, USA) was determined using the ELISA technique as described above with minor modifications. Briefly, a mouse polyclonal antibody was used as a positive control for anti-pneumococcal polysaccharide and antibody titres were obtained against polysaccharide from *S. pneumoniae* serotype 14 and cross reactivity against GXM. For the ELISA test, the cutoff was established by determining optical density plus 3 standard deviations, of serum from non-infected mice tested in plates covered with GXM.

In some experiments, cross reactivity was measured in soluble competition ELISA. Briefly, sera from *C. neoformans* infected mice were adsorbed with 0.5, 5.0 and 50 µg/mL of *S. pneumoniae* polysaccharide (serotype 14) for 2 hours at 37°C. Supernatants were withdrawn, centrifuged to remove debris and tested using GXM as antigen. Results are shown as percentage of inhibition: OD adsorbed serum x 100/OD total serum – 100.

### Flow cytometric analysis

Mononuclear cells (MNC) obtained from spleens of C57BL/6 or CBA/j mice were used to determine the relative percentage of B cells subpopulations. Fc receptors were blocked before staining with normal mice serum and then cells were washed twice in PBS (supplemented with FCS 2% and 0.01% sodium azide). MNC ($3x10^5$) were added with fluorescent antibodies anti-CD5-FITC, anti-CD43 FITC, anti-IgM-PE and anti-B220-Biotin (BD Pharmingen, San Diego, CA, U.S.A). After 30 min of incubation at 4°C, MNC were washed and streptavidin-APC (BD, Pharmingen) was added for 30 min at 4°C. Finally, MNC were washed and a three-color analysis was performed using a flow cytometer (Becton Dickinson, FACScan, San Jose, CA).

### Statistical analysis

Unpaired Student *t*-test was applied for statistical analysis of the results. Values were considered statistically significant when *p* value was £ 0.05.
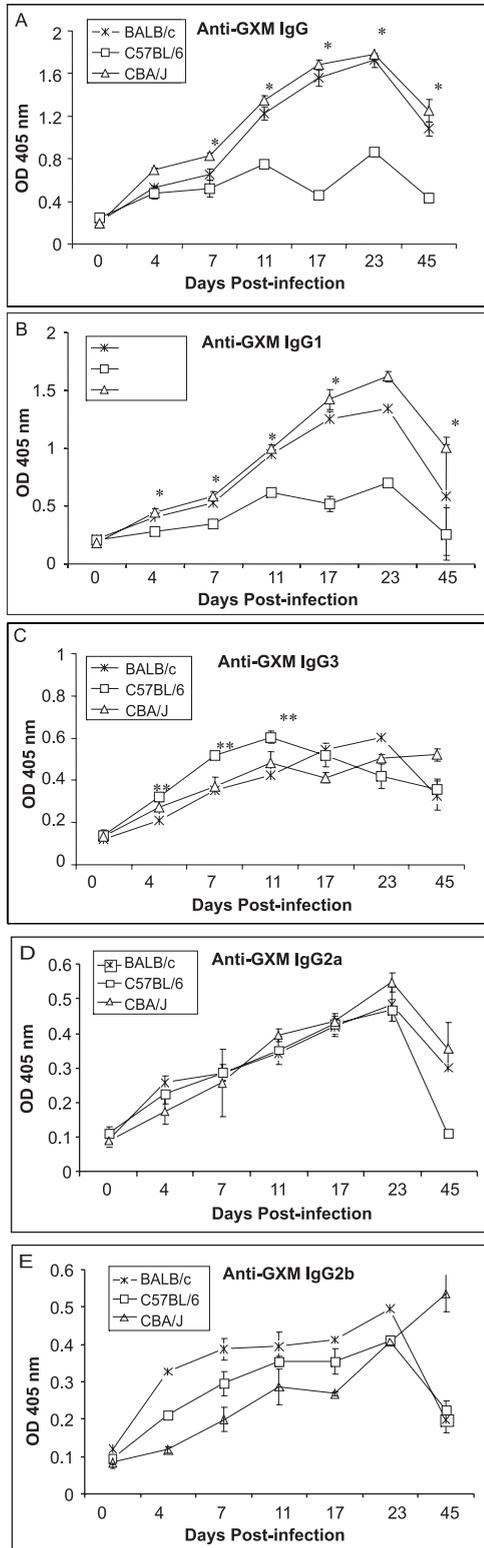
### Results

### Differential infection profile and humoral antibody response of mice to infection with C. neoformans

Intratracheal inoculation of *C. neoformans* had the highest rate of infectivity in susceptible C57BL/6 mice, with CFU values reaching $5.6x10^6$ and $4.6x10^6$ by day 14 and 23, respectively (figure 1; open squares). In moderately resistant BALB/c mice, a gradual decrease in the number of CFU from $1.7x10^6$ at day 14 to $5.1x10^5$ at day 23 was observed (figure 1; filled crosses), while in highly resistant CBA/J mice no CFU were detected after 7 days post-infection (figure 1; open triangles). These results demonstrate that there is indeed a differential pattern of infectivity that is dependent on the strain of mice infected.

Measurement of total anti-GXM IgG antibodies after infection with *C. neoformans* showed that the highest levels of anti-GXM antibodies could be found in CBA/J and BALB/c mice, which significantly increased from day 7 until day 23
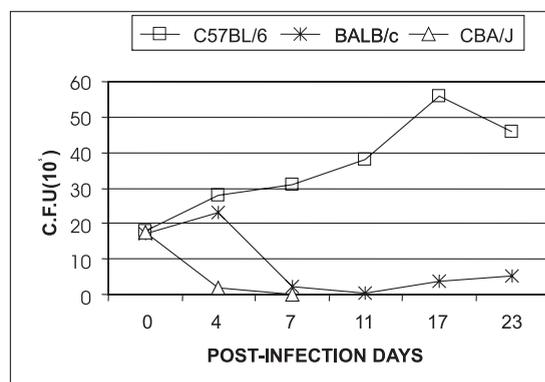


**Figure 1.** Differential infectivity of three strains of mice infected with *C. neoformans.* BALB/c (Ú), C57BL/6 (£) and CBA/J (r) three mice were infected with *C. neoformans* by intratracheal inoculation. At various times lungs were extracted, minced and the number of colony forming units was determined as described in materials and methods section. Results represent the mean colony forming units (CFU) ± SD.

(p<0.05) as compared to C57BL/6 mice (figure 2A). Infection of C57BL/6 mice with *C. neoformans* resulted in significantly lower levels of anti-GXM IgG from day 4 as compared to either BALB/c or CBA/J mice (figure 2A). We next determined the production of various anti-GXM IgG isotypes in the various mouse strains following intratracheal infection with *C. neoformans*. Measurement of $IgG_1$ antibodies revealed that this was the predominant isotype in both CBA/J and BALB/c mice, and was significantly higher than C57BL/6 mice from day 7 to day 45 (p<0.05) as compared to CBA/J mice (figure 2B). By day 45, $IgG_1$ antibody production diminished in both BALB/c and CBA/J mice, whereas, levels of IgG1 in C57BL/6 mice had returned to baseline levels (figure 2B). In contrast, the highest level of $IgG_3$ production was measured in C57BL/6, starting from day 4 to day 11, as compared to either CBA/J or BALB/c (**p<0.05, *p<0.01) (figure 2C). By day 45, levels of IgG3 in C57BL/6 mice had diminished. Measurement of specific anti-GXM $IgG_{2a}$ antibodies did not reveal any significant differences amongst the different species of mice, with the exception of day 45 post-infection with *C. neoformans,* when levels of anti-GXM IgG2a were significantly higher in CBA/J and BALB/c as compared to C57BL/6 mice (figure 2D). Measurement of anti-GXM $IgG_{2b}$ levels revealed that only CBA/J mice exhibited significantly higher anti-GXM IgG2b levels at day 45 post-infection with *C. neoformans* than either BALB/c or C57BL/6 (**p<0.05, *p<0.01) (figure 2E).

### Examination of cross-reactive IgG directed against pneumococcal polysaccharide after infection of mice with C. neoformans

We next examined whether cross-reactive anti-pneumococcal polysaccharide antibodies could be detected after infection of the various mouse strains with *C. neoformans*. By days 4 and 7 post-infection

**Figure 2.** Kinetics of *C. neoformans*-induced anti-GXM antibody concentration. BALB/c (Ú), C57BL/6 (£) and CBA/J (r) mice were infected with *C. neoformans* by intratracheal inoculation. At various times sera were measured for the level of (A) IgG anti-GXM, (B) IgG1 anti-GXM, (C) IgG3 anti-GXM, (D) IgG2a anti-GXM or (E) IgG2b anti-GXM as described in material and methods. Results represent the mean OD 405 nm ± SD of three animals per group.
**p<0.05 compared with lowest responding mouse strain
*p<0.01 compared with lowest responding mouse strain
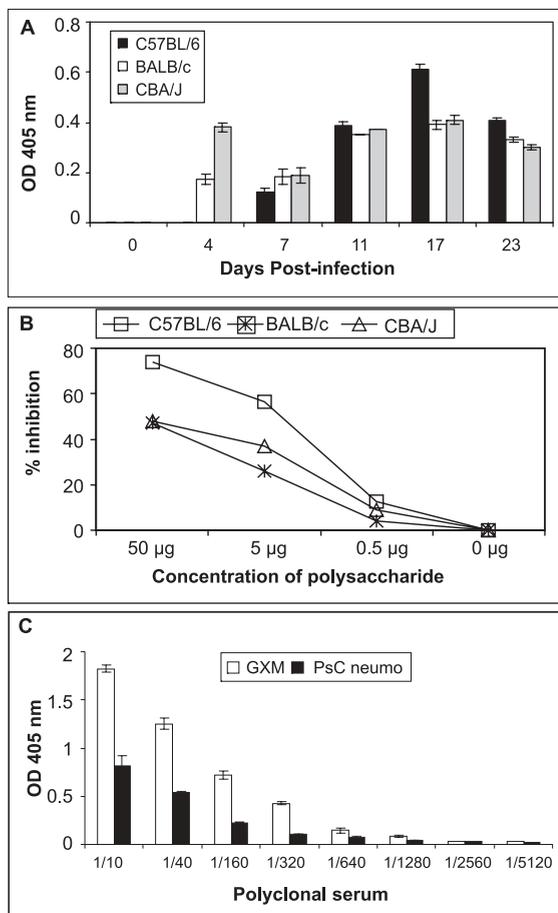
**Figure 3.** Effect of infection with C. *neoformans* on cross-reactive IgG directed against pneumococcal polysaccharide. (A) BALB/c mice (open bars), CBA/J (hatched bars) and C57BL/6 mice (filled bars) were infected with *C. neoformans* by intratracheal inoculation. On day 17 post-infection, cross-reacting antibodies specific for pneumococcal poly-saccharide were measured by ELISA as described in materials and methods section. Bars represent mean OD 405 nm ± SD, in three independently performed experiments in triplicate. (B) BALB/c (Ú), C57BL/6 (£) and CBA/J (r) were infected with *C. neoformans* by intratracheal inoculation. On day 17 post-infection, pneumococcal polysaccharide at 0.5, 5.0 and 50.0 mg/mL was adsorbed to serum as described in material and methods. Data represent the percent inhibition using the formula described in material and methods, and is the sum of three independently performed experiments in triplicates. (C) Normal serum drawn from non-infected BALB/c mice was inoculated with pneumococal polysaccharide and the amount of cross reactive antibodies recognizing GXM polysaccharide (GXM; open bars) was compared with specific antibodies to pneumococal polysaccharide (PsC neumo; filled bars), as described in material and methods. Bars represent mean OD 405 nm ± SD and are the sum of three independently performed experiments in triplicates.

with *C. neoformans,* detectable levels of anti-pneumococal antibodies could be measured in both BALB/c and CBA/J mice (figure 3A). However, from day 11 to day 23 post-infection, C57BL/6 mice produced more IgG directed against pneumococal polysaccharide than either CBA/J or BALB/c mice (figure 3A). To test whether these anti-pneumococcal polysaccharide antibodies were GXM cross-reactive antibodies, a competition assay was performed by mixing the serum from each mouse strain obtained after 17 days post-infection with various amounts of pneumococcal polysaccharide. A dose dependent inhibition of anti-GXM antibodies with pneumococcal polysaccharide was observed in all the mouse strains, although, inhibition was significantly higher in susceptible C57BL/6 mice as compared to DBA/J or BALB/c mice (figure 3B). Next, normal serum obtained from non-infected BALB/c mice and inoculated with pneumococal polysaccharide was tested. Cross reactive antibodies recognizing GXM polysaccharide were detected in a 1/40 dilution, as compared with specific antibodies to pneumococal polysaccharide that were detected at a 1/320 dilution (figure 3C).

### Characterization of B cell subpopulations after infection of mice with C. neoformans

To study whether resistant and susceptible mice present different B cell subpopulations in response to infection with *C. neoformans*, we analyzed the percentage of $CD5^+$ and $CD43^+$ B cells using flow cytometric analysis. B cells could be divided into $B220^{high}$ and $B220^{low}$ populations; C57BL/6 mice exhibited a higher percentage of $CD5^+$ B cells co-expressing the $B220^{low}$ phenotype (figure 4; upper panel). In contrast, CBA/J mice exhibited a higher percentage of $CD43^+$ cells with the $B220^{low}$ phenotype (figure 4; lower panel).

### Discussion

The present work constitutes the first study of the kinetics of the humoral response to GXM in susceptible and resistant mice infected with *C. neoformans.* We show that BALB/c and CBA/J mice, defined as moderately and highly resistant to *C. neoformans* infection (4), produce higher amounts of total IgG (mainly of the $IgG_1$ isotype) than the susceptible C57BL/6 mice. Protection
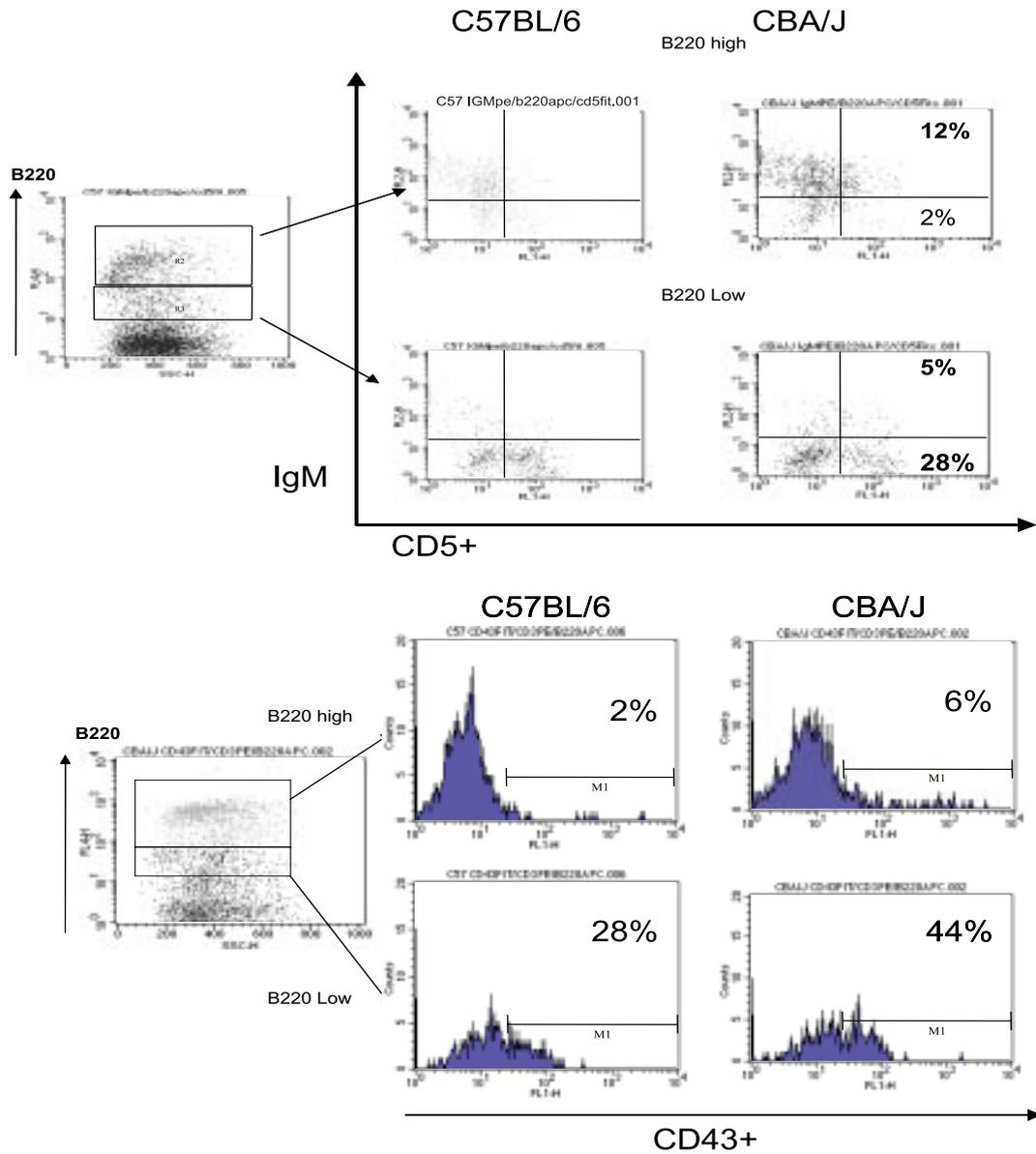
**Figure 4.** Phenotypic characterization of splenic B cells after infection with *C. neoformans.* C57BL/6 and CBA/J mice were infected with *C. neoformans* by intratracheal inoculation. Mononuclear cells (MNC) obtained from spleens of infected mice were used to determine the relative changes in B cells subpopulations after staining with anti-CD5, anti-CD43, anti-IgM-PE and anti-B220 antibodies. Analysis of the relative expression of B220^{+}/CD5^{+}/IgM^{+} cells (upper panel) and B220^{+}/CD43^{+}/IgM^{+} cells (lower panel) was performed by flow cytometry as described in materials and methods. Data are from a representative experiment of three independently performed analyses with similar results.

mediated by exogenous $IgG_1$ anti-GXM specific mAbs has been shown in several studies (6,10); in contrast to non-protective $IgG_3$ mAbs directed to the same epitope (10), IgG1 antibodies have been previously reported as protective in experiments of passive transfer in the mouse model (23-25). In these studies, it was observed that the three strains of infected animals produced both $IgG_{2a}$ and $IgG_{2b}$ but at relatively lower levels, and there were no significant differences between strains (23-25). Taken together, these results suggest that $IgG_{2a}$ and $IgG_{2b}$ do not play an important role in murine resistance or susceptibility to cryptococcosis *in vivo*.

The potent humoral response and resistance to cryptococcosis that is observed in the murine system remains to be established in humans. However, a close correlation has been observed between hypogammaglobulinemia (26), or HIV infected patients (11,27) and the risk of infection with *C. neoformans*. It has been suggested that a correlation exists between the decrease of certain IgG subtypes and *C. neoformans* meningitis in HIV patients (28). This is relevant, since HIV patients show deregulation in Ig rearrangement of heavy variable chains (VH) (28), and absence of V(H)3 segment is commonly associated with a low antibody response to polysaccharides (29). These patients have a deficient response to polysaccharide-based vaccines such as *S. pneumoniae* (29-31). It is important to emphasize that the specificity of the epitopes recognized by the protective antibodies is different from that of the non-protective antibodies (8,32).

These observations, together with our results, indicate that the level of susceptibility in humans and mice to cryptococcosis may be regulated by similar mechanisms, suggesting that the antibody response is as important as the cellular response. Classically, defects in cellular immunity, particularly those that require T lymphocytes, are considered as the main risk factor for the mycosis, both in humans and mice (3,4). In the present study, it was observed that the susceptible mouse strain produced the highest amount of cross-reactive antibodies. Polysaccharides, due to their biochemical characteristics, may contribute to the

kind of cross-reactivity observed with GXM (33), and cross-reactive antibodies are considered as critical elements for pre-immune resistance to other encapsulated pathogens (34).

The lack of an effective specific immune response might be intrinsically related to the type of B lymphocytes stimulated early during the infection process. In our present study, it was observed that C57BL/6 susceptible mice had a high percentage of $B220^{low}/CD5^+$ cells. In contrast, a high percentage of $B220^{low}/CD43^+$ cells were detected in CBA/J resistant mice. Although $CD5^+$ B lymphocytes or B1 subpopulations are classically implicated in the proliferative response to polysaccharides (15-17), antibodies produced by the B1 population are characterized by low affinity, and self-reactivity (35). Some of these responses are associated with a non-protective humoral response in humans (20). On the other hand, the $CD43^+$ population is generally implicated in the negative control of $CD5^+$ cells growth (21). The presence of a $B220^{low}/CD43^+$ population in CBA/J mice supports our hypothesis on the role of different B cell populations in the development of a protective or non-protective humoral response to *C. neoformans*.

In conclusion, this study demonstrates that production of anti-GXM antibodies of the $IgG_1$ isotype correlates with resistance to infection with *C. neoformans*. The presence of the lowest levels of specific anti-GXM antibodies in susceptible C57BL/6 mice together with the higher levels of cross-reactive anti-pneumococcal antibodies in these mice suggests that cross-reactivity antibodies are less protective. It is important to consider that this non-protective humoral response is developed in a non-immunosuppressed mouse strain, normally considered as a good Th1 cytokine producer (36). The fine specificity of these cross-reactive antibodies and the mechanisms which control their production should be further studied to better understand this anti-fungal immune response. Taken together, our findings suggest that the development of IgG1 antibodies specific for GXM might participate in the protection against *C. neoformans*, and that this response is dependent on the B lymphocyte subpopulation stimulated early during infection.

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