

## Monoclonal Antibodies to *Cryptococcus neoformans* Capsular Polysaccharide Modify the Course of Intravenous Infection in Mice

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**Immunoglobulin G1 (IgG1) monoclonal antibodies (MAbs) to the capsular glucuronoxylomannan (GXM) were studied for their ability to modify the course of intravenous *Cryptococcus neoformans* infection in mice. A/J mice were given intraperitoneal injection of 1.0 mg of either a GXM-binding IgG1 MAb (2H1 or 2D10γ1) or the irrelevant isotype-matched control MAb 36-65 prior to intravenous infection. Parameters used to study antibody efficacy were lung and brain tissue fungal burden, lung and brain weights, serum GXM levels, and histopathological examination of lung, brain, heart, kidney, and spleen tissues. Mice given GXM-binding MAb had significantly reduced lung tissue fungal burden as measured by CFU. In contrast to the reduction in lung tissue burden, the reduction in brain tissue burden was small and did not achieve statistical significance. Serum GXM levels were reduced in mice receiving GXM-binding MAb. Histopathological examination revealed reduced numbers of granulomas and *C. neoformans* organisms in the lungs, brains, and kidneys of MAb 2H1-treated mice relative to control mice. The lungs and brains of mice receiving GXM-binding MAb weighed significantly less than those of control animals, consistent with the reduced inflammation noted histologically. Subendocardial inflammation and kidney cortical infarctions were present in control infected mice but not in MAb 2H1-treated mice. Immunocytochemical staining for polysaccharide antigen revealed a marked reduction in the amount of tissue polysaccharide in mice treated with MAb 2H1 relative to control mice. The results support an useful role for passive antibody administration in *C. neoformans* infections.**

*Cryptococcus neoformans* causes life-threatening meningoencephalitis in approximately 10% of patients with AIDS (77). In the setting of AIDS, cryptococcal infections are usually incurable because antifungal therapy does not eradicate the infection (73) despite in vitro susceptibility of the organism to antifungal drugs (6). This has renewed interest in the potential of vaccines (9) and passive antibody therapy (15, 45, 58, 59, 67) to prevent infection. Passive antibody is an attractive option because it could enhance residual immunity in immunosuppressed patients (2). Precedent for the use of antibody in therapy of human cryptococcosis exists: in 1925, Shapiro and Neal used rabbit immune sera in an unsuccessful attempt to treat a boy with meningitis (71); in 1959, Littman used human gamma globulin and amphotericin B in a small number of patients with encouraging results (48); and in 1963, Gordon and Vedder used rabbit immune globulin and amphotericin B to successfully treat two patients with cryptococcosis (23, 25). Specific antibody can potentiate the effect of amphotericin B against *C. neoformans* in mouse models (14, 24, 59a), suggesting a rationale for combined therapy.

It is generally accepted that cellular immunity is the primary line of defense against *C. neoformans* (1, 29, 31, 42, 45, 47, 54, 60) and that complement provides opsonins for phagocytosis (1, 13, 43). In contrast, the importance of natural antibody immunity has remained uncertain. Passive immunization with polyclonal sera has produced evidence for (22, 28) and against (49) a role for antibody in protection. Casting doubt on the importance of antibody immunity are the observations that an

immunogenic vaccine failed to protect mice (26), B-cell deficient mice are not at increased risk (55), and antibody is not an absolute requirement for phagocytosis (1, 13) or killing of *C. neoformans* (27, 52). However, the observation that antibodies are potent opsonins (40, 41, 69) which are required for NK cell (53, 61) and leukocyte (10, 11, 51) antifungal activity in vitro suggests a role for antibody immunity. Other observations supporting an important role for antibody are as follows: the appearance of antibody in cerebrospinal fluid of rabbits is temporally correlated with decreased colony counts in brain tissue (36); mice succumb to infection when their serum antibody titers decline (70); serum antibody is a good prognostic indicator in humans (12); recovery from cryptococcal meningitis is accompanied by the production of specific antibody in cerebrospinal fluid (44); and cryptococcosis in the setting of hypogammaglobulinemia is occasionally reported (30, 68). Three groups have shown that administration of monoclonal antibody (MAb) to the capsular polysaccharide can mediate protection in murine models of *C. neoformans* infection (14–17, 57–59, 67). Antibody efficacy is dependent on the quantity (15, 16), the isotype (58, 67), and the fine specificity (58) of the MAb used, findings that could explain the discordant results obtained in earlier experiments with polyclonal sera.

In 1981, Graybill showed that the choice of animal model was crucial for demonstrating efficacy with polyclonal sera (28). The best animal model for identifying potential antibody reagents against *C. neoformans* for human use is not known and will not be known until human efficacy data are correlated with data from animal studies. We have previously evaluated the efficacy of some of our MAbs in intraperitoneal (i.p.) (57, 58) and intracerebral (i.c.) (59) murine models of *C. neoformans*

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*mans* infection. Here we extend our studies to an intravenous (i.v.) model of murine cryptococcosis.

## MATERIALS AND METHODS

**Abbreviations.** The following abbreviations are used in this paper: GXM, glucuronoxylomannan; MAb, monoclonal antibody; Ig, immunoglobulin; i.p., intraperitoneal; i.v., intravenous; i.c., intracerebral; PBS, 0.02 M phosphate-buffered saline. ELISA, enzyme-linked immunosorbent assay; DAB, diaminobenzidine.

**MAbs.** *C. neoformans* GXM-binding MAbs 2H1 (IgG1) and 2D10 (IgM) were generated in BALB/c mice immunized with a serotype A GXM-tetanus toxoid vaccine (3). The serological properties (3, 4), molecular analysis (57), and efficacy against *C. neoformans* (58, 59) of MAbs 2H1 and 2D10 have been described. MAbs 2H1 and 2D10 were derived from the same pre-B cell, share the same variable gene usage, have the same fine specificity, and differ by only four amino acid residues in the variable region resulting from somatic mutations (57). MAb 2D10 $\gamma$ 1 is an isotype switch variant of MAb 2D10 (IgM) which arose spontaneously in cell culture and which was isolated by the technique of sib selection with the ELISASpot modifications recently described (72). MAb 2D10 $\gamma$ 1 hybridoma cells were cloned in soft agar and characterized by Southern blot analysis of V<sub>H</sub> and V<sub>L</sub> gene rearrangements using the J<sub>11</sub> and J<sub>k</sub>1-5 DNA probes as described previously (5). The 2D10 $\gamma$ 1 hybridoma has the same productive variable gene rearrangements as the parent 2D10 hybridoma (57). MAb 36-65 (IgG1) (66) has specificity for the hapten *p*-azophenylarsenate, does not bind to cryptococcal polysaccharide, and was used as an isotype-matched irrelevant antibody control in the experiments described here and in previous studies (59). Ascites fluid was prepared in pristane primed BALB/c mice, and the concentration of MAb was determined by ELISA relative to IgG1 standards of known concentration.

***C. neoformans.*** *C. neoformans* 24067 (serotype D) was obtained from the American Type Culture Collection (Rockville, Md.). Although the MAbs used here were generated in response to a serotype A GXM-tetanus toxoid vaccine, they bind to *C. neoformans* capsular polysaccharides from all four isotypes (3). Strain ATCC 24067 was used because of prior experience with it in i.p. and i.c. murine models of cryptococcosis (57-59). *C. neoformans* was grown to a cell density of 10<sup>6</sup> cells per ml in Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.) at 37°C (overnight growth). Yeast cells were collected by centrifugation and washed three times in sterile PBS (pH 7.2) prior to infection. Inocula were determined in a hemocytometer and confirmed by plating efficiency in Sabouraud's agar (Difco). ATCC 24067 strain cells grown in Sabouraud dextrose broth have small capsules, but in mouse tissues the yeast forms have large capsules.

**Mice.** A/J female mice, 6 to 8 weeks of age, were obtained from the Jackson Laboratories. Mice were rested for 1 to 2 weeks prior to use in experiments.

**MAb administration and infection.** MAb was administered by i.p. injection as ascites 1 day (experiment I) or 4 h (experiment II) prior to infection. i.p. administration results in rapid distribution to the intravascular compartment such that the serum MAb levels resulting from i.v. and i.p. administration are approximately the same after 24 h (59a). Two experiments were done 6 months apart. In experiment I, mice were infected with *C. neoformans* by i.v. injection of 4 × 10<sup>6</sup> yeast cells suspended in 100  $\mu$ l of PBS into the lateral tail vein; 10 mice were given MAb 36-65 (irrelevant isotype matched control group), 10 mice were given MAb 2D10 $\gamma$ 1, and 5 mice

were given MAb 2H1. Each mouse was given 1.0 mg of MAb. One mouse in the 36-65 group died within 24 h of infection and was removed from the experiment. In experiment II, mice were infected with 10<sup>6</sup> cryptococci 4 h after the administration of 1.0 mg of MAb i.p. In experiment II there were 10 mice per group and each group received either MAb 2H1, 2D10 $\gamma$ 1, or 36-65.

**Organ *C. neoformans* counts and histopathological studies.** Mice were sacrificed by cervical dislocation at either day 7 or 9 of infection. Day 7 was chosen because Sanford et al. (67) had demonstrated reduced lung CFU in antibody-treated mice at that day of infection. Brain and lung tissue were homogenized by mechanical grinding in 3 ml of PBS containing 1,000 U of penicillin G per ml and 1,000  $\mu$ g of streptomycin sulfate per ml (Gibco Laboratories, Grand Island, N.Y.). Tissue homogenates were plated in Sabouraud dextrose agar (Difco) to determine the number of *C. neoformans* CFU. Microscopic examination of the agar surface after plating the tissue homogenates revealed that the overwhelming majority of cryptococci were single, well-encapsulated yeast forms and there was no evidence of clumping. Thus the number of CFU reflects the number of viable yeast cells deposited in the agar.

Brain, lung, heart, kidney, and spleen from mice in the MAb 36-65 (control) and MAb 2H1 groups sacrificed at day 9 of infection were fixed in 10% buffered formalin and imbedded in paraffin for histopathological examination. In the case of brain and lung tissues in experiment I, half of the organ was used for CFU determination and half was used for histopathology studies. The brain hemisphere used for histopathology was bisected in the parasagittal plane. Tissue sections of 4  $\mu$ m thickness were examined after hematoxylin-eosin, mucicarmine, and Gomori's methenamine silver staining (PolyScientific, Bay Shore, N.Y.). In experiment II, the lung and brain were weighed in an analytical balance prior to whole-organ homogenization for CFU determination.

**Immunocytochemical and lectin histochemistry.** Paraffin sections were examined for tissue polysaccharide antigen by using MAb 3E5 (IgG3) (3). Briefly, sections were deparaffinized and hydrated, washed in PBS, and incubated in 0.3% H<sub>2</sub>O<sub>2</sub> in PBS for 30 min to block endogenous peroxidase activity. Sections were then incubated in 10% normal goat serum in PBS for 1 h at room temperature to block nonspecific Fc receptor binding. Primary antibody 3E5 was then used at a concentration of 2.6  $\mu$ g/ml in 10% normal goat serum in PBS and applied for 16 h at 4°C. Peroxidase-conjugated goat anti-mouse IgG3 (Fisher Scientific, Orangeburg, N.Y.) was diluted at 1:250 in PBS and applied for 1 h at room temperature. Color was developed with DAB. Sections were examined after counterstaining with hematoxylin. For detection of mouse tissue IgG1 (to determine whether MAb 2H1-treated animals had any detectable tissue IgG1), sections were immunostained in the same manner as above with the exception that the primary antibody was omitted and the secondary antibody was substituted with peroxidase-conjugated goat anti-mouse IgG1 (Fisher Scientific).

To identify tissue macrophages, lectin histochemistry was performed using *Griffonia simplicifolia* B<sub>4</sub> isolectin (GSA I-B<sub>4</sub>) that specifically binds to alpha-D-galactose (50, 74). Deparaffinized tissue sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min and then incubated with peroxidase-conjugated GSA-IB<sub>4</sub> (Sigma, St. Louis, Mo.) at 10  $\mu$ g/ml in cation buffer (PBS containing CaCl<sub>2</sub>, MgCl<sub>2</sub>, and MnCl<sub>2</sub> at a concentration of 0.1 mM each) for 16 h at 4°C, and DAB was added as a chromogen.

**Serum GXM level determination.** Levels of GXM in serum were measured by using an antigen capture ELISA which has been described (4). Briefly, sera were diluted 1:50 in PBS, proteinase K (Boehringer Mannheim, Mannheim, Germany)

TABLE 1. Lung and brain CFU

Expt, organ, and day <sup>a</sup>	GXM-binding MAb (2H1 and 2D10γ1)		Control MAb (36-65)		<i>P</i> <sup>b</sup>
	Mean log <sub>10</sub> CFU ± SD	<i>n</i>	Mean log <sub>10</sub> CFU ± SD	<i>n</i>	
<b>I</b>					
Lung					
7	3.48 ± 2.12	5	6.40 ± 0.43	4	0.1099
9	3.08 ± 1.78	10	5.71 ± 0.92	5	0.0150*
Combined	3.21 ± 1.83	15	6.01 ± 0.86	9	0.0018*
Brain					
7	3.51 ± 1.88	5	4.97 ± 1.38	4	0.2703
9	3.34 ± 1.93	10	4.21 ± 2.04	5	0.7469
Combined	3.40 ± 1.85	15	4.55 ± 1.85	9	0.3047
<b>II</b>					
Lung, 7	5.99 ± 0.38	20	6.92 ± 0.17	10	0.0001*
Brain, 7	6.25 ± 0.15	19 <sup>c</sup>	6.33 ± 0.25	10	0.2328

<sup>a</sup> Combined refers to the combination of day 7 and day 9 data for statistical analysis.

<sup>b</sup> \*, *P* values significant at the 0.05 level.

<sup>c</sup> Because of a dilution error, the CFU value for one of the antibody-treated mice was lost and hence the number of 19.

was added to a concentration of 200 μg/ml, and the solution was incubated at 37°C for 1 h and then at 100°C for 5 min. Proteinase-heat treatment was done to eliminate the possibility that preexisting antibody bound to GXM would block the ELISA. Control experiments showed that proteinase-heat treatment of purified GXM did not affect the epitope density in the polysaccharide. The ELISA configuration used MAb 2D10 for GXM capture and MAb 2H1 for detection (4). Serum GXM levels were determined relative to a GXM standard from strain 24067 purified by cetylmethylammonium bromide (Aldrich, Milwaukee, Wis.) complexation (7).

**Statistical analysis.** Wilcoxon signed-rank analysis of CFU in brain and lung tissues in antibody-treated and control animals was done with the SAS statistical package by Chee Jen Chang of the Department of Epidemiology and Social Medicine of the Albert Einstein College of Medicine. For the statistical analysis, the data obtained with MAbs 2H1 and 2D10γ1 were pooled since these antibodies are both of the IgG1 isotype, use the same variable genes (57), display identical fine specificities (3), and were derived from the same pre-B cell in vivo (57). In experiment I, the data obtained at days 7 and 9 for the control and GXM-binding MAb groups were analyzed individually before and after pooling (Table 1). Prior to pooling, the data sets for the control and GXM groups were compared at day 7 and 9 and no significant difference was found within groups at the two time points, thus justifying their combination. Student's *t* test analysis was done with Primer of Biostatistics: The Program, Version 3.01 (McGraw-Hill, New York, N.Y.).

## RESULTS

**Brain and lung fungal burden.** In experiment I, the lung and brain CFU of 15 mice receiving either of the IgG1 GXM-binding MAbs 2H1 and 2D10γ1 and 9 mice receiving the isotype-matched control MAb 36-65 were determined at either day 7 or 9 of infection (Fig. 1). For lung tissue, mice receiving GXM-binding MAb had reduced CFU at days 7 and 9 (*P* = 0.1099 and 0.0150, respectively; Table 1), and combined analysis of the day 7 and 9 data revealed a significant reduction relative to control mice (*P* = 0.0018) (Table 1). In contrast to the reduction in lung tissue CFU, the reduction in brain tissue

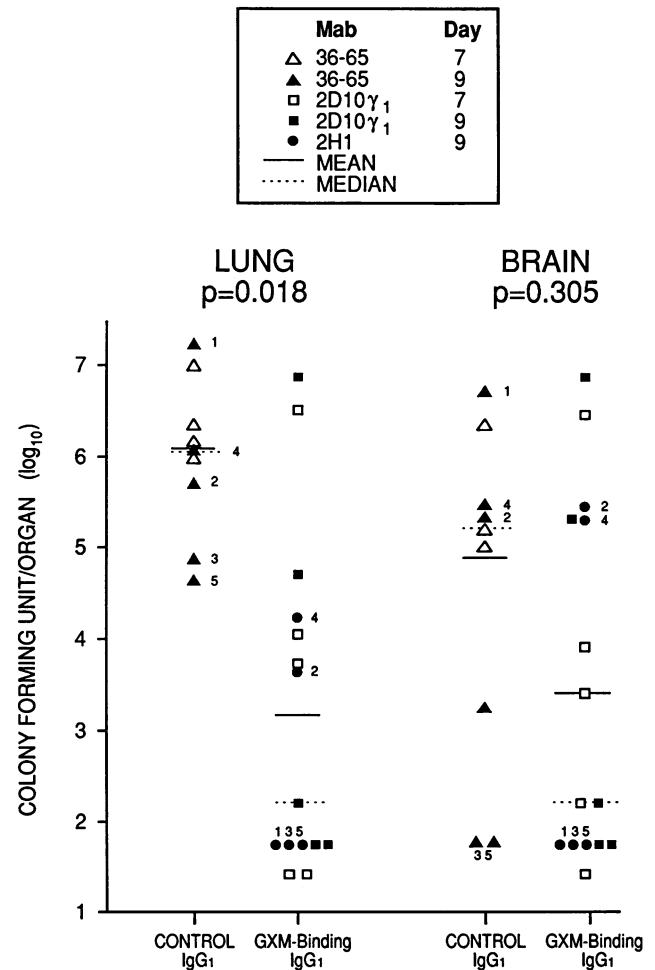


FIG. 1. Colony counts in the lung and brain of mice given the GXM-binding MAbs 2H1 and 2D10γ1 and the control MAb 36-65. Points corresponding to the MAb 2H1 and 36-65 mice studied for serum GXM level and histopathology are numbered for cross-referencing with the other data. Solid bars correspond to average values for each group. Dashed bars correspond to the median values for each group. Statistical *P* values were obtained by Wilcoxon nonparametric analysis of the data set (Table 1).

CFU in mice receiving GXM-binding MAb was smaller and not statistically significant at the 0.05 level at either day 7 or 9 or when combined (Table 1).

In experiment II, three groups of 10 mice were given either control MAb 36-65 or one of the GXM-binding MAbs 2D10γ1 and 2H1. Experiment II differed from experiment I in that the infecting inocula was reduced to  $1 \times 10^6$  per mouse ( $4 \times 10^6$  were used in experiment I), antibody was given 4 h prior to infection instead of 24 h earlier, and the entire lung and brain were homogenized for CFU determination. Average log<sub>10</sub> CFU for the control, 2D10γ1, and 2H1 groups were  $6.92 \pm 0.17$ ,  $6.26 \pm 0.21$ , and  $5.73 \pm 0.33$ , respectively. For both 2D10γ1- and 2H1-treated mice the reduction in lung CFU was highly statistically significant (*P* = 0.0002). Combined 2D10γ1 plus 2H1 average lung log<sub>10</sub> CFU were  $5.99 \pm 0.38$  with *P* = 0.0001 relative to control mice (Table 1). Average brain log<sub>10</sub> CFU for the control, 2D10γ1, and 2H1 groups were  $6.25 \pm 0.48$ ,  $6.22 \pm 0.22$ , and  $6.45 \pm 0.19$ , respectively, and there was no statistically significant difference at the *P* = 0.05 level

TABLE 2. Lung and brain weights of mice

Group	n	Lung wt (g) <sup>a</sup>	P <sup>b</sup>	Brain wt (g) <sup>a</sup>	P <sup>b</sup>
Control (36-65)	10	0.262 ± 0.034		0.356 ± 0.030	
2D10γ1	10	0.173 ± 0.045	<0.001*	0.304 ± 0.042	0.007*
2H1	10	0.178 ± 0.026	<0.001*	0.317 ± 0.048	0.043*
2H1 and 2D10γ1	20	0.176 ± 0.036	<0.001*	0.310 ± 0.044	0.007*

<sup>a</sup> Lung and brain weights are averages ± standard deviation.

<sup>b</sup> P values are relative to the control group; \*, values significant at the 0.05 level (Student's *t* test).

between the control and antibody-treated mice when the groups were considered either individually or combined (Table 1). In experiment II the effect of GXM-binding MAb on lung fungal burden was smaller than in experiment I, though statistically more significant. Since the level of i.p. administered MAb in serum peaks at 24 h (56a), we attribute the smaller effect in experiment II to lower serum MAb levels at the time of infection.

Measurement of lung and brain weights of control and GXM-binding MAb-treated mice in experiment II revealed significant differences between the two groups. The lungs and brains of control mice weighed more than those of GXM-binding MAb-treated mice, and the effect was greater for lung than for brain (Table 2). The higher weight of control lung and brain tissue presumably reflects increased inflammation in the absence of specific antibody (see below).

**Histopathological studies.** Organ histopathology from the five MAb 36-65-treated mice and the five MAb 2H1-treated mice sacrificed at day 9 of infection was evaluated with regard to the type and degree of tissue inflammation, number of fungi, and degree of polysaccharide infiltration in the tissue. The organs studied were lung, brain, heart, kidney, and spleen. Paired lung and brain tissues of the individual mice were studied separately, and the relative severity of the histological findings was given an arbitrary scale of – (none) to +++++ (most severe), and the findings are summarized in Table 3. Each organ will be considered separately.

(i) **Lung.** In control mice (MAb 36-65 group) the lung was the organ with the most severe inflammatory response. Numerous granulomas were scattered (average, 10 to 20 per cross-sectional area), each containing a few intact yeast forms

(Fig. 2a). Foci of *C. neoformans* organisms without cellular reaction (Fig. 2c) were present in the lungs of control animals. There was significant mouse-to-mouse variation in the degree of tissue inflammation and the number of organisms observed (Table 3), but for a given mouse there was good correlation between the three parameters studied. In contrast, the lungs of MAb 2H1-treated mice had much less inflammation than control mice (Table 3 and Fig. 2e). In two MAb-2H1 treated mice, only two granulomas containing 0 to 3 yeast forms per granuloma were observed. In the remaining three MAb 2H1-treated mice no granulomas or yeast forms were observed.

(ii) **Brain.** For control mice the pathology in brain tissue was less severe than in lung tissue (Table 3). Control mouse 1 had multiple areas of *C. neoformans* accumulations in the parenchyma and meninges without an inflammatory reaction (Fig. 2f). The brains of the four other control mice had one or two foci of *C. neoformans* accumulations, and only mouse 2 had a lymphohistiocytic cellular infiltrate. In contrast, only mouse 4 in the MAb 2H1-treated group showed brain pathology, a well-formed granuloma in the cerebellum containing many *C. neoformans* forms.

(iii) **Heart.** Three of the five control mice had lymphocytic infiltration in the subendocardial space, and one mouse had a severe myocarditis consisting of multiple collections of *C. neoformans* surrounded by mononuclear inflammatory cells. Three control mice showed extensive epicardial calcification over the right ventricle. In contrast, none of the MAb 2H1-treated mice had subendocardial infiltrate and only one mouse showed epicardial calcification.

(iv) **Kidney.** The kidneys of all control mice had areas of *C. neoformans* accumulation associated with granulomatous inflammation and cortical infarcts. In contrast, only one MAb 2H1-treated mouse had inflammation and none had infarcts.

(v) **Spleen.** No significant pathology was observed in either the control of MAb 2H1-treated mice. Similar absence of spleen histopathology in the setting of disseminated infection was reported in a model of dermal infection (56).

**Serum GXM levels and tissue immunocytochemistry.** The serum GXM concentrations at day 9 of infection for the five mice in the MAb 36-65 and MAb 2H1 groups were determined by using an antigen capture ELISA which we developed (4). Figure 3 shows that the mice in the MAb 2H1 group had markedly reduced serum GXM concentrations relative to the

TABLE 3. Reactions of mouse organs

Mouse (MAb)	Result <sup>a</sup> in organ					
	Lung			Brain		
	No. of <i>C. neoformans</i> cells	Cellular reaction <sup>b</sup>	Immunoreactivity for polysaccharide	No. of <i>C. neoformans</i> cells	Cellular reaction <sup>b</sup>	Immunoreactivity for polysaccharide
1 (36-65)	+++++	+++++	+++++	+++	–	+++
2 (36-65)	+++	+++	+++	+	+	+
3 (36-65) <sup>c</sup>	NA	NA	NA	+	–	+
4 (36-65)	+++	+++	+++	+	–	+
5 (36-65)	+	+	+	+	–	+
1 (2H1)	–	–	–	–	–	–
2 (2H1)	±	+	+	–	–	±
3 (2H1)	–	–	–	–	–	–
4 (2H1)	±	+	+	+	+	+
5 (2H1)	–	–	–	–	–	–

<sup>a</sup> NA, not available. For details on scale, see Results.

<sup>b</sup> Degree of granulomatous inflammatory response.

<sup>c</sup> Lung tissue for mouse 3 was lost.



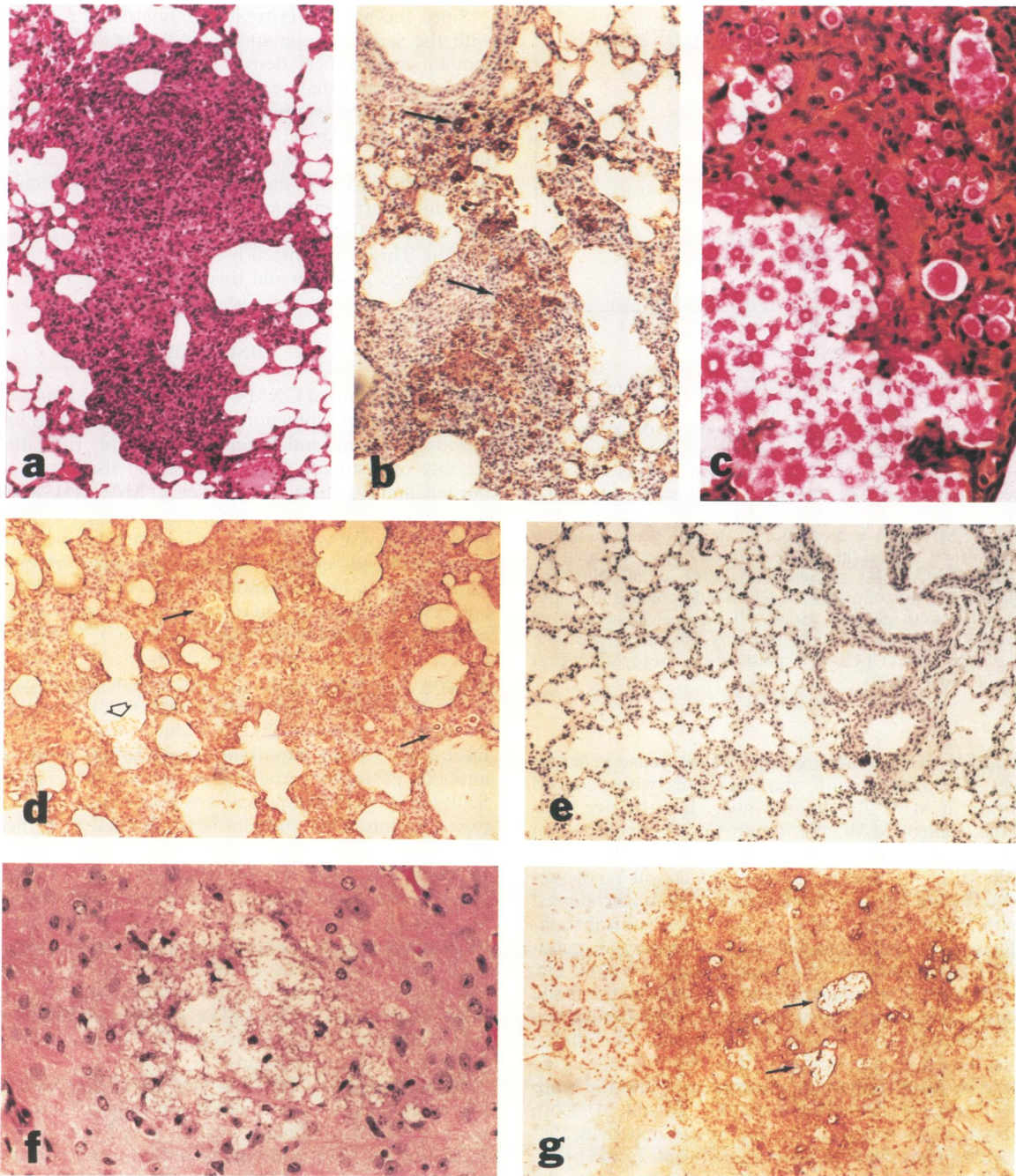


FIG. 2. Histopathology of lung and brain of i.v. murine cryptococcosis. (a) The lung of control mouse 2 with severe granulomatous inflammation and loss of alveolar space (hematoxylin-eosin stain; magnification,  $\times 80$ ); (b) an adjacent area in the lung of control mouse 2 immunostained for polysaccharide antigen displays positive cells (arrows) within granulomas (hematoxylin counterstaining; magnification,  $\times 80$ ); (c) staining of lung tissue from a control mouse shows an accumulation of cryptococci adjacent to an area of granulomatous inflammation (mucicarmine staining; magnification,  $\times 200$ ); (d) diffuse consolidation of lung tissue by granulomatous inflammation and widespread immunoreactivity for polysaccharide antigen in control mouse 1; note yeast forms with various degrees of immunoreactivity (thin arrows) and a focal accumulation of *C. neoformans* within an alveolar space (open arrow) (magnification,  $\times 80$ ). (e) Lung tissue of MAb 2H1 mouse 1 immunostained for cryptococcal polysaccharide antigen. Note the lack of inflammation, fungal forms, and immunoreactivity for polysaccharide (hematoxylin counterstain; magnification,  $\times 80$ ). (f) High-power view of brain tissue from control mouse 1 displaying an accumulation of *C. neoformans* in the parenchyma with apparent lack of tissue inflammatory response (hematoxylin-eosin stain; magnification,  $\times 200$ ). (g) Low-power view of a similar area immunostained for polysaccharide antigen showing intense staining of the tissue around *C. neoformans* accumulations (arrows) that diffuses in a streaky pattern resembling cell processes (magnification,  $\times 80$ ).

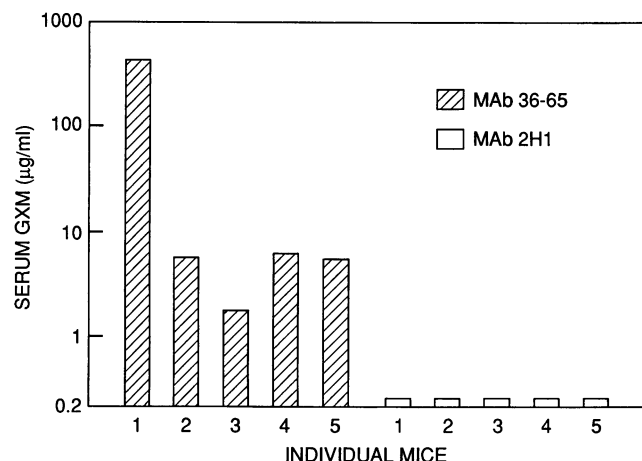


FIG. 3. Serum GXM levels at day 9 of infection for mice given MABs 2H1 and 36-65 (control). GXM levels were measured by using an ELISA capture assay (4). Prior to measurement the serum was digested with proteinase K and subsequently boiled to destroy any residual 2H1 antibody which could have affected the measurement. Protease-heat treatment of polysaccharide control had no effect relative to untreated polysaccharide. Polysaccharide was not detected in the serum of the MAb 2H1-treated mice by an assay designed with a detection limit of  $<0.2$   $\mu\text{g/ml}$  (hence the serum GXM levels for all the mice treated with MAb 2H1 are plotted as  $<0.2$   $\mu\text{g/ml}$ ). Note the marked differences in the control group, which reflect mouse-to-mouse variability in response to infection. Mouse 1 had a much higher level of GXM in serum, consistent with the fact that this animal had the highest lung and brain CFUs and most severe histopathological findings (Fig. 1 and Table 3).

mice in the MAb 36-65 control group. The decrease in serum GXM level is consistent with and correlates with reduced fungal burden in MAb 2H1-treated mice. For the five mice receiving the control MAB, the serum GXM concentration showed significant animal-to-animal variation (Fig. 3).

The tissue distribution of *C. neoformans* capsular polysaccharide in brain and lung was studied by immunocytochemical staining with MABs to the GXM. Immunocytochemical staining revealed a good correlation between the degree of immunoreactivity and the severity of histopathology (Table 3; Fig. 2). MAb 2H1-treated mice had marked reduction in the tissue polysaccharide immunoreactivity relative to control mice, and staining was limited to the few granulomas present. In contrast, control mice had diffuse immunoreactivity for polysaccharide involving the alveolar epithelium, macrophages, and the luminal border of the bronchial epithelium (Fig. 2b and 2d). Both lung and brain showed polysaccharide immunoreactivity that was more widespread than the intact yeast forms. In the brain, the most intense staining occurred around the accumulations of *C. neoformans*, but the staining diffused to the adjacent tissues (Fig. 2g). Lung granulomas showed immunoreactivity for polysaccharide (Fig. 2b and 2d), but within a granuloma certain cells stained while others remained negative (Fig. 2b). The cells containing polysaccharide reactivity were stained in adjacent parallel sections by lectin histochemistry with GSA-IB<sub>4</sub>, indicating that they were macrophages (Fig. 4). The theoretical possibility that colocalization of GSA-IB<sub>4</sub> and GXM staining was due to cross-reactivity of GSA-IB<sub>4</sub> with *C. neoformans* capsular polysaccharide was considered and ruled out given that (i) GSA-IB<sub>4</sub> does not stain encapsulated *C. neoformans* in tissue (data not shown) and (ii) GSA-IB<sub>4</sub> staining was localized to macrophage membranes (as expected

[50, 74]) whereas GXM staining was diffuse and involved both cell cytoplasm and surrounding tissues (Fig. 2 and 4).

Since the MAb 2H1-treated animals had received a MAB with the same epitope specificity as the MAb 3E5 used for immunocytochemical detection of tissue polysaccharide, we were concerned that residual MAb 2H1 could have masked immunochemical staining. To address this concern, tissue sections from MAb 2H1-treated mice were immunostained for mouse IgG1. No tissue staining was detected. Thus, the marked differences in immunochemical staining between MAb 2H1 and control mice are not the result of antibody masking but rather reflect large differences in tissue polysaccharide burden. This is supported by the overall correlation between GXM levels in serum and tissue immunoreactivity.

## DISCUSSION

Administration of GXM-binding MAB in a murine model of i.v. *C. neoformans* infection resulted in reduced (i) lung fungal burden, (ii) lung and brain tissue weight, (iii) number and severity of histopathologic lesions, (iv) tissue polysaccharide burden, and (v) serum GXM levels in MAb 2H1- and 2D10 $\gamma$ 1-treated mice relative to control mice. There was considerable mouse-to-mouse variation in each parameter used to evaluate passive antibody efficacy. We have previously observed significant variation in the response of individual mice to i.p. (58) and i.c. (59) infection, and variation is also apparent in the data reported by other investigators (18, 56, 67). The variation may reflect the fact that some mice limit the spread of infection, possibly by developing an effective cell-mediated response (18, 37, 56) or escaping from immunologic paralysis and producing an antibody response (5). Individual variation within lots of inbred mice is an important consideration in the design of antibody protection experiments because it increases the difficulty of demonstrating statistically significant differences between groups. This problem is not limited to *C. neoformans* infections and was observed in the mouse protection test used to standardize horse sera for human therapy (19). Interexperimental variation in the overall severity of infection is apparent in the CFU for experiments I and II, which were done 6 months apart. Mice in experiment II had higher lung and brain CFU than those in experiment I despite receiving a lower infecting inoculum. Significant interexperimental variation has been observed in i.p. (58) and i.c. (59) models and could reflect subtle differences in the susceptibility of mouse lots or virulence in the infecting strain.

In control mice, the lung was the organ with the most histopathological lesions, which probably reflects trapping of yeast cells in the pulmonary capillary bed (39). GXM-binding MAB-treated mice had significantly lower numbers of lung CFU than control animals, and histopathologic examination revealed a marked reduction in the number of yeast forms, granulomas, and cellular reaction in lung tissue. Lungs of control mice were 34% heavier than those of GXM-treated mice, consistent with the loss of air spaces and diffuse inflammation noted in the histopathology study. The decrease in lung CFU in mice given MAB before infection may reflect enhanced clearance of *C. neoformans* cells by cell-mediated and nonspecific immune mechanisms (see below), resulting in a less severe initial infection.

In contrast to lung tissue, administration of GXM-binding antibody resulted in a small reduction in brain CFU which did not achieve statistical significance at the  $P = 0.05$  level. Statistical significance may be achieved for this parameter with



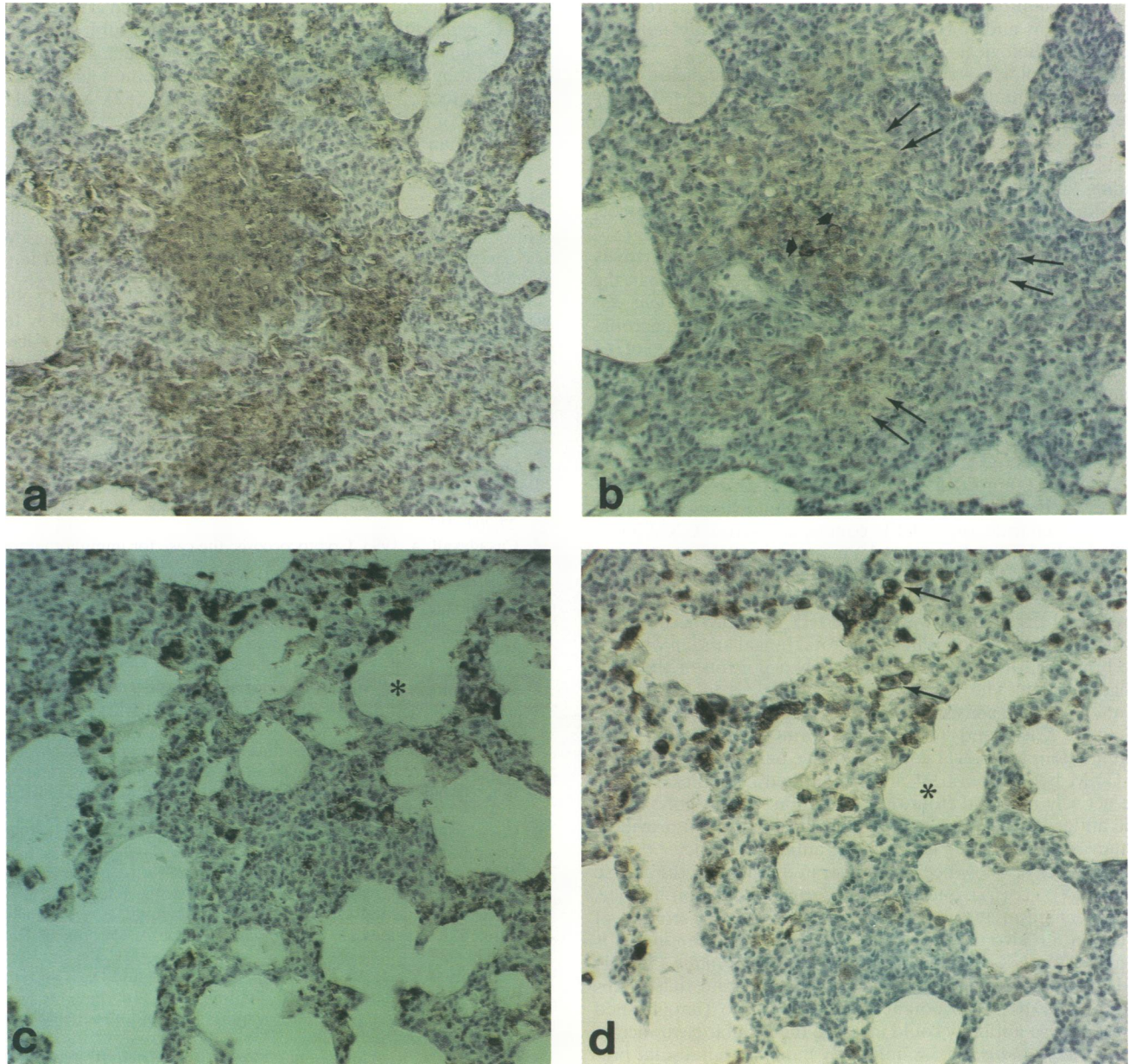


FIG. 4. Colocalization of GXM immunoreactivity and GSA-IB<sub>4</sub> binding in mouse cryptococcal lung lesions. Serial paraffin sections of lung tissue from control mouse 2 were immunostained for GXM antigen (panels a and c) and GSA-IB<sub>4</sub> (panels b and d). The same granuloma is shown to contain GXM-positive epithelioid cells (panel a) and various degrees of GSA-IB<sub>4</sub> lectin binding (panel b). Double arrows indicate faint GSA-IB<sub>4</sub> reactivity, and short thick arrows indicate macrophages with intense membrane staining (panel b). (c and d) Another area of thickened interstitium with cells exhibiting identical staining for GXM antigen (panel c) and GSA-IB<sub>4</sub> lectin binding (panel d). Single arrows indicate dark membrane staining of macrophages with GSA-IB<sub>4</sub> (panel d), which contrasts with the faint staining of epithelioid histiocytes (double arrows, panel b) in the granuloma. Asterisks denote the same alveolar air space in two serial sections.

larger samples. Similar results have been obtained with IgG1 GXM-binding MAbs by others (16, 67). The ineffectiveness of IgG1 GXM-binding antibody in significantly reducing brain CFU in the i.v. infection model differs from the result obtained with the i.c. model, in which MAb 2H1 reduced brain CFU (59). This difference in antibody effectiveness could reflect increased antibody penetration into the brain in i.c. infection due to more severe initial meningoencephalitis or trauma resulting from i.c. injection (59). Alternatively antibody could have been preferentially depleted in the clearance of the lung

infection such that less antibody was available for diffusion into the brain compartment. Nevertheless, a beneficial effect of antibody on brain infection is suggested by the reduction in histopathological lesions and polysaccharide immunoreactivity and the lower brain weights observed in GXM-binding MAb-treated mice relative to control mice. Cryptococcal polysaccharide can induce accumulation of fluid in brain tissue (32–35), and brain edema contributes to mortality in human cryptococcosis (8). The lower brain weights measured for GXM-binding MAb-treated mice presumably reflect reduced brain edema

relative to control mice, a finding consistent with the reduced inflammation and polysaccharide immunoreactivity noted in the histopathological study (Table 3).

Histopathological examination of kidney and heart tissue provided additional evidence of the GXM-binding MAb effectiveness in modifying the course of systemic infection. MAb 2H1-treated mice had no myocarditis or cortical kidney infarcts, whereas these lesions were found in control mice. The finding of myocarditis in control mice is interesting given occasional reports of cardiac involvement in human cryptococcosis (38, 46).

Immunohistochemical staining revealed GXM reactivity some distance away from yeast forms, suggesting that the capsular antigen diffused from areas where *C. neoformans* was present. Intracellular polysaccharide was found in macrophages, where its presence could reflect the remains of *C. neoformans* destroyed after phagocytosis or possibly cellular uptake of shed polysaccharide antigen. In animals given MAb 2H1, tissue polysaccharide was limited to granulomas, whereas in control mice there was more diffuse infiltration of polysaccharide antigen. Control mice had more tissue polysaccharide and higher serum GXM levels than MAb 2H1-treated mice. There was overall consistency in the immunochemical staining for polysaccharide, organ CFU counts, and serum GXM levels for individual mice. For example, mouse 1 in the control group had the highest lung and brain CFU and, by far, the highest serum GXM level. However, the correlation between GXM in serum and organ CFU was not perfect, possibly because the level of GXM in serum reflects the total fungal burden whereas the brain and lung CFU measure only a portion of the total fungal burden. The lower serum GXM levels in MAb 2H1-treated mice are consistent with reduced fungal burden shown by lower CFU and fewer yeast forms in histopathologic examination. MAb 2H1 could also have enhanced elimination of GXM by formation of antigen-antibody complexes which are actively removed from the circulation. Serum polysaccharide antigen levels are useful in clinical practice and provide a noninvasive parameter for studying antibody efficacy.

MAb 2H1 has now been demonstrated to modify the course of i.p. (58), i.c. (59), and i.v. *C. neoformans* infection in mice. The mechanism by which antibody modifies the course of infection *in vivo* is uncertain, but given that antibody is required for NK-cell activity against *C. neoformans* (53, 61) and for fungicidal activity by leukocytes (10) and that MAbs 2H1 and 2D10y1 are potent opsonins *in vitro* (unpublished data), passive antibody could increase the cellular clearance of the *C. neoformans*. In i.v. infection models, the results presented here and by other investigators (16, 67) show that IgG1 GXM-binding antibody is effective at reducing fungal burden outside the brain compartment. Although the clinical manifestations of *C. neoformans* infection in humans are primarily the result of meningoencephalitis, it is noteworthy that human cryptococcosis is a systemic infection often accompanied by fungemia and diffuse organ involvement (63). Passive i.v. antibody administration could have a role in facilitating the clearance of systemic infection. In the brains of patients with AIDS the blood-brain barrier is leaky (64, 65) and antibody penetration may be greater than in normal brains. However, if serum antibody penetration into human brain is poor, there are two potential alternatives to circumvent this problem. First, antibody can be administered directly into the subarachnoid space, bypassing the blood-brain barrier. In the preantibiotic era, meningococcal meningitis was successfully treated with antibody alone given intraspinally (20). Second, antibody derivatives for improved blood-brain-barrier penetration can be designed (21, 75), and the murine i.v. infection model could

provide a useful system for the testing of such compounds. Anti-*C. neoformans* antibodies may also be useful for prophylaxis of high-risk groups, and the feasibility of such a strategy is supported by the successful use of passive immunoglobulin to prevent infection in pediatric AIDS patients (62). MAbs 2H1 and 2D10, or their derivatives (76), are potential candidates for use in human treatment and prevention trials.

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