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Therapeutic Efficacy of Monoclonal Antibodies to *Cryptococcus neoformans* Glucuronoxylomannan Alone and in Combination with Amphotericin B

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The therapeutic efficacy of the immunoglobulin G1 (IgG1) monoclonal antibody (MAb) 2H1 to the *Cryptococcus neoformans* capsular polysaccharide was studied with and without amphotericin B (AmB) in a murine model of intravenous (i.v.) infection. MAb and AmB were administered by intraperitoneal (i.p.) injection after i.v. infection with a *C. neoformans* serotype D strain. Intraperitoneal administration of MAb 2H1 resulted in rapid distribution to the intravascular compartment, and the half-lives of i.p. and i.v. administered MAb were similar. Administration of MAb 2H1 alone resulted in increased survival, decreased lung fungal burden, and reduced serum glucuronoxylomannan antigen levels when given 2 to 6 h but not 24 h after infection. In vivo, the combination of MAb 2H1 and AmB was more effective at prolonging survival than either agent alone. MAbs of IgM, IgG1, IgG3, and IgA isotypes given 1 day after infection were effective in reducing serum GXM-D levels, with their relative efficacy being IgG1 > IgG3 > IgM > IgA. In vitro, MAb 2H1 was a potent opsonin of *C. neoformans* and the combination of MAb 2H1 and AmB was more effective than either agent alone in decreasing *C. neoformans* colony counts in the presence of the murine macrophage cell line J774.16. The results confirm that capsule-binding MAbs can enhance the effect of AmB against *C. neoformans* and provide support for considering combined therapy in humans.

Cryptococcus neoformans infections in patients with AIDS are often incurable because antifungal therapy does not eradicate the infection (49), despite in vitro susceptibility of the fungus to the antifungal drugs used (5). The difficulty in treating *C. neoformans* infections in patients with severe immunodeficiencies has renewed interest in the potential of specific antibody as a therapeutic adjunct to antifungal therapy (3, 11, 47). Passive antibody is an attractive option for augmenting host defenses by promoting fungal clearance through effector cells. Capsule-specific antibody potentiates phagocytosis by macrophages (31, 48), fungal killing by leukocytes (9, 10), and fungistasis by NK cells (35, 41). The experience with a small number of patients treated with a combination of polyclonal antibody and amphotericin B (AmB) was positive (18, 20, 32). In addition, passively administered antibody could be effective in binding to and possibly enhancing elimination of *C. neoformans* capsular exopolysaccharide, which accumulates in tissues of infected individuals. The polysaccharide capsule is required for virulence (27), and soluble polysaccharide has been associated with such host deleterious effects as immune paralysis (30, 40), inhibition of phagocytosis (29), and enhancement of human immunodeficiency virus infection (44).

Three independent groups have shown that administration of monoclonal antibodies (MAbs) can modify the course of infection in mice, resulting in prolongation of survival and/or reduction in tissue fungal burden (11–13, 36–38, 47). Earlier, Graybill et al. (25) and Gadebusch (17) reported modest benefits with passive administration of polyclonal immune sera. Gordon and Lapa (19) showed that polyclonal rabbit sera enhanced the antifungal effect of AmB against *C. neoformans*, and recently Dromer and Charreire (11) reported similar results with a MAb primarily reactive with serotype A strains. These studies, together with the in vitro data showing antibody enhancement of effector cell activity against *C. neoformans* (see above), suggest a role for antibody in therapy.

We have identified several mouse MAbs to the *C. neoformans* capsular polysaccharide which bind to the four serotypes and which modify the course of lethal intraperitoneal (i.p.) (36, 37) and intracerebral (38) infection in mice. The therapeutic efficacy of one of these murine MAbs (2H1) was studied with and without AmB in a murine model of intravenous (i.v.) infection. Passive administration of MAb after infection prolonged survival and decreased lung fungal burden and serum antigen polysaccharide levels. The combination of MAb and AmB was better than either agent alone in both in vitro and in vivo studies.

MATERIALS AND METHODS

***C. neoformans* and capsular polysaccharide.** ATCC 24067 (serotype D) was obtained from the American Type Culture Collection (ATCC) (Rockville, Md.). Strain 24067 was used because of prior experience with it (36–38). Cultures were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) slants at 4°C and grown in Sabouraud dextrose broth (Difco Laboratories) at 37°C overnight. Serotype D glucuronoxylomannan (GXM-D) from ATCC 24067 was purified by using cetyltrimethylammonium bromide as described elsewhere (7).

Pharmacokinetics of i.p. administered MAb. To determine the pharmacokinetics of MAb 2H1 (immunoglobulin G1 [IgG1]) administered i.p. and compare them with those of MAb 2H1 administered by i.v. route, we examined the rate of entry of i.p. administered MAb 2H1 into the vascular compartment and measured the intravascular and whole-body rates of antibody disappearance. For these experiments, MAb 2H1

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ascites was produced in pristane-primed SCID mice, purified over a protein G column (Pharmacia, Piscataway, N.J.), and radiolabeled with either ^{125}I or ^{131}I by the Iodogen technique (Pierce, Rockford, Ill.) (16) to a specific activity of approximately 1 μCi/μg. SCID mice were used because they produce little if any endogenous IgG1, with the result that the only antibody in ascitic fluid is hybridoma MAb. Free iodine was removed by passage over a size exclusion column (Sephadex G-25; Pharmacia). Mice were prepared by supplementing their drinking water with 1% Strong Iodine (Lugol's) Solution (USP, Regional Service Center, Inc., Woburn, Mass.) for a 1-week period to block thyroid uptake.

Presence of intravascular 2H1 was assessed in groups of A/JCr mice (five mice per group) by assay of radioactivity in the blood after i.v. or i.p. injection of 1 μg of ^{125}I-labeled MAb 2H1 in 100 μl of sterile phosphate-buffered saline (PBS). Whole-body counts were obtained immediately after injection to measure the amount of activity administered. Blood samples were then obtained at 1, 29.5, 144.5, and 243 h. More frequent sampling was not performed to minimize the artifactual depletion of the blood volume and intravascular immunoglobulin pool. At these times, approximately 50 μl of blood was collected from the tail vein into preweighed bleeding tubes (Sarstedt, Newton, N.C.) which were reweighed on an analytical balance to enable precise determination of the sample volume. All blood samples were counted in a gamma scintillation counter at the conclusion of the study, and by comparison with the baseline whole-body counts, antibody levels were expressed as a percentage of the injected activity per milliliter of blood.

The rate of whole-body antibody catabolism was determined in other groups of five adult female A/JCr mice after i.v. and i.p. injection of ^{131}I-labeled 2H1. Because these mice are not bled or otherwise manipulated after antibody administration, half-life values ($t_{1/2}$s) obtained by this method are highly reliable (51). Approximately 1 μg of labeled antibody in 100 μl of PBS was administered i.v. via the lateral tail vein or i.p. by direct injection. Whole-body activity was assayed at 1 and 4.5 h and on a daily or alternate-day basis by using an external sodium iodide scintillation probe (Picker Corporation, Cleveland, Ohio). After correction for background and isotopic decay, radioactive counts were expressed as a percentage of the initial 1-h count rate for each mouse. MAb whole-body $t_{1/2}$ was calculated from clearance curves by nonlinear regression by using a pharmacokinetics software package (PCNONLIN; SCI Software, Inc., Lexington, Ky.). Although no obvious alpha phase of rapid initial clearance was apparent (52), data prior to 24 h were omitted to minimize potential contribution from this component.

In vivo studies. The ability of MAb 2H1 to reduce lung CFU burden when administered after infection was studied in female A/JCr infected i.v. via the lateral tail vein with 2.5×10^5 ATCC 24067 cells. The inocula were determined by counting yeast cells in a hemocytometer and confirmed by plating efficiency on Sabouraud dextrose agar. Six hours following infection, mice were given either 250 μl of sterile PBS or 1 mg of MAb 2H1 (in ascitic fluid) i.p. Four days following infection, mice were bled from the orbital sinus to isolate serum and sacrificed by cervical dislocation to harvest lung tissue. Lung tissue was homogenized, and a dilution of the homogenate was plated on Sabouraud dextrose agar to determine CFU burden. Microscopic examination of the agar surface immediately following plating revealed that the overwhelming majority of organisms were single well-encapsulated yeast forms. Thus the CFU reflect the numbers of viable yeast cells in lung tissue. Serum GXM-D concentrations were determined as described for the MAb-mediated GXM-D clearance study (see below).

The efficacy of MAb 2H1 in a therapeutic role (i.e., when given after infection) was studied in a murine model of cryptococcosis in which the infection was given i.v. and the MAb was given i.p. This model has been used by other investigators to study antibody-mediated protection against *C. neoformans* (11, 12, 47). Our model consisted of i.v. administration via the lateral tail vein of 0.5 to 1×10^6 cells of ATCC 24067 to female A/JCr mice. The inocula were determined by counting yeast cells in a hemocytometer and confirmed by plating efficiency on Sabouraud dextrose agar. At various times after infection each of 10 mice was given, i.p., either 250 μl of sterile PBS, 1 mg of MAb 2H1 (in ascitic fluid), 0.5 mg of AmB (GIBCO/BRL, Grand Island, N.Y.) per kg, or 1 mg of MAb 2H1 (in ascitic fluid) and 0.5 mg of AmB per kg (one to three separate injections) (details of each experiment are shown in Table 1). Mice were observed at least twice daily, and survival was recorded every 12 h.

MAb-mediated serum GXM clearance. The effect of MAb administration on serum GXM-D levels in mice infected with *C. neoformans* was studied by using a previously described antigen capture enzyme-linked immunosorbent assay (ELISA) (4). Forty female A/JCr were infected with 1.7×10^5 ATCC 24067 cells i.v. One day after infection, each mouse received 0.25 ml of sterile PBS (control) or 1 mg of either MAb 2D10, 2H1, 3E5, or 18G9. There were eight mice in each group. Serum was isolated by bleeding the mice via the orbital sinus just prior to and 2 and 6 days following MAb administration. To dissociate antibody-antigen immune complexes which may mask the GXM-D, a 1:25 dilution of serum was incubated overnight at 37°C with 0.2 mg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml in PBS and then heated for 20 min at 100°C. Previous work has shown no interfering substances in mouse sera (4), and this ELISA has been used to measure polysaccharide antigen levels in infected mice (37, 39). Control experiments have shown that addition of MAb to GXM-D solutions at MAb/GXM-D weight ratios of up to 20:1 does not inhibit detection of GXM-D by this ELISA (presumably because GXM-D is a polyvalent antigen). Thus the proteinase-heating step may or may not be necessary but is used here since clinical detection of polysaccharide antigen in serum includes a proteinase step (24). Ninety-six-well microtiter plates (no. 25801; Corning Glass Works, Corning, N.Y.) were prepared by coating them with a goat anti-mouse IgM (Fisher Scientific, Orangeburg, N.Y.) solution in TBS (10 mM Tris-HCl, 150 mM NaCl, 1 mM NaN_3), blocking them with 1% bovine serum albumin (ICN Biomedicals, Inc., Costa Mesa, Calif.)–0.5% horse serum (Hyclone Laboratories, Sterile Systems, Inc., Logan, Utah) in TBS, and incubating them with a 1-μg/ml solution of an IgM anticryptococcal MAb. Proteinase- and heat-treated serum was serially diluted 1:3 in duplicate across prepared microtiter plates (4) against a standard containing a known amount of GXM-D. The plates were then coated and incubated successively with a 1-μg/ml solution of an IgG_1 anticryptococcal MAb and alkaline phosphatase-labeled goat anti-mouse IgG_1 (Fisher Scientific), and then the alkaline phosphatase substrate *p*-nitrophenyl (Sigma, St. Louis, Mo.) was added.

In vitro murine macrophage assay. The ability of MAb 2H1 to potentiate in vitro fungistatic or fungicidal activity of AmB was examined by using the J774.16 murine macrophage-like cell line. These experiments were modelled on previous work which used J774.16 cells to study macrophage killing of *Mycobacterium tuberculosis* (6). J774.16 cells were grown in DME (Mediatech Inc., Washington, D.C.) containing 10% fetal calf

TABLE 1.

Expt. no.	Inoculum[a]	Treatment (group)	Avg survival (no. of days ± SD) [P][b] of treatment group: A	B	C	D	Comment
I	5×10^5 (94%)	PBS (A), 1 mg of 2H1 at 24 h (B), 0.5 mg of AmB per kg at 24, 48, and 72 h (C), B + C (D)[c]	5.5 ± 2.58[d]	5.8 ± 1.23[d] [P_1 = 0.5106]	23.2 ± 7.84[d] [P_1 = 0.0001]*	22.60 ± 5.32[d] [P_1 = 0.0001, P_2 = 0.1012, P_3 = 0.0001*]	AmB effect predominant; expt terminated on day 26 when all mice were sick and several agonal
II	1×10^6 (82%)	PBS (A), 1 mg of 2H1 at 2 h (B), 0.5 mg of AmB per kg at 24 h[e] (C), B + C (D)[c]	1.55 ± 0.16[d]	4.85 ± 0.65[d] [P_1 = 0.0001]*	1.86 ± 0.60[f,g] [P_1 = 0.1094]	5.77 ± 1.40[g] [P_1 = 0.0001,* P_2 = 0.0205,* P_3 = 0.0759]	MAb 2H1 effect predominant; antibody alone prolongs survival when given 2 h after infection
III	1×10^6 (94%)	PBS (A), 1 mg of 2H1 at 6 h (B), 0.5 mg of AmB per kg at 6 h (C), B + C (D)[c]	1.5 ± 0.0[d]	2.3 ± 1.86[d] [P_1 = 0.1896]	2.25 ± 1.38[d] [P_1 = 0.0118]	4.25 ± 0.68[d] [P_1 = 0.0001,* P_2 = 0.0182,* P_3 = 0.0313*]	Combination of AmB and MAb 2H1 better than either agent alone

[a] Inocula were determined by counting yeast cells in a hemocytometer. Plating efficiency is shown in parentheses.
[b] P_1 applies to the comparison between the treatment group and group A; P_2 applies to the comparison between groups C and D; P_3 applies to the comparison between groups B and D; and * indicates a P value significant at 0.05 confidence level or better.
[c] Group D received a combination of the treatments received by groups B and C.
[d] n = 10.
[e] All mice were alive at 24 h when the AmB was administered to treatment groups C and D.
[f] One mouse in treatment group C was still alive at the termination of the experiment on day 15, and this datum point was censored in the calculation of the mean.
[g] n = 11.

serum (Bioproducts for Science, Indianapolis, Ind.), 10% NCTC-109 (GIBCO Laboratories, Life Technologies Inc., Grand Island, N.Y.), and 1% nonessential amino acids; plated at 10^5 cells per well on a 96-well tissue culture plate (Falcon no. 3072; Becton Dickinson and Co., Lincoln Park, N.Y.); and stimulated with 500 U of murine recombinant gamma interferon (IFN-γ) (Genzyme, Cambridge, Mass.). Cells were incubated at 37°C overnight. The medium in each well was replaced with medium containing a final concentration of 500 U of IFN-γ per ml, 3 μg of lipopolysaccharide (LPS) (Sigma) per ml, 1×10^5 or 2×10^4 ATCC 24067 cells, and 0 or 5 μg of MAb 2H1 purified by a protein G column (Pierce). AmB at a final concentration of 0 or 0.12 μg/ml was added in the presence and absence of MAb 2H1. Assuming one J774 cell division per 24 h, the addition of 1×10^5 ATCC 24067 cells per well results in an initial effector/target (E/T) ratio of 2:1, whereas addition of 2×10^4 ATCC 24067 cells per well results in an initial E/T ratio of 10:1. Following a 20-h incubation at 37°C, the supernatants from each well were collected and 0.1 ml of sterile distilled H_2O was added to each well, after which the cells were lysed by incubation at room temperature for 30 min. The lysate was vigorously aspirated and ejected with a pipette several times and added to the supernatant from the same well. The supernatant and the lysate were diluted and plated on Sabouraud dextrose agar. This protocol overcomes the problem of yeast cell aggregation by a combination of antibody dilution and mechanical disruption.

Phagocytic index was determined in a chamber slide (Lab-tek no. 178599; Nunc Inc.). Briefly, 10^5 J774.16 cells were incubated overnight with 500 U of IFN-γ per ml. The medium was then replaced with a suspension of 10^5 *C. neoformans* cells in fresh medium with 3 μg of LPS (Sigma) and 500 U of IFN-γ per ml with and without 5 μg of protein G-purified MAb 2H1 per ml. After 2 h of incubation at 37°C, the wells were washed several times and the cells were methanol fixed and stained with Giemsa. The phagocytic index is the number of attached plus ingested cryptococci per the number of macrophages per field.

Statistical analysis. Survival data were analyzed by using a log-rank analysis written by C. J. Chang, a statistician at our institution, with the SAS statistical package. Comparisons between two groups were analyzed by Student's t test using version 3.01 of Primer of Biostatistics program (McGraw-Hill Inc.).

RESULTS

Pharmacokinetics of MAb IgG1 administered i.p. Since all mouse experiments used i.p. MAb administration, we examined the biodistribution and intravascular metabolism of radiolabeled IgG1 MAb injected into the peritoneum. The advantage of the i.p. route relative to i.v. administration is that it is technically easier and larger volumes of antibody solution can be given. Intravascular MAb 2H1 levels following i.p. and i.v. administration are illustrated in Fig. 1A. Serum MAb levels reflect the entry of antibody into the intravascular compartment, the rate of metabolism, and the rate of equilibration with levels in other body compartments. Intravenous administration results in high initial levels of antibody in serum which equilibrated with levels in the extravascular space by 29.5 h of measurement. In contrast, i.p. administered antibody is initially present at low levels but rapidly increases as antibody is transferred to the serum compartment. By 29.5 h, the levels of MAb 2H1 administered by the two routes and their rates of intravascular clearance are similar. After 2 days, the i.p. route results in slightly higher MAb concentrations than the i.v. route, presumably because some antibody continues to be slowly released from the peritoneal cavity. In addition, the variation among individual animals was greater when the MAb was given i.p. than when it was given i.v. In one mouse, i.p. injection did not result in significant blood radioactive counts, and this presumably reflects inadvertent injection of the anti-

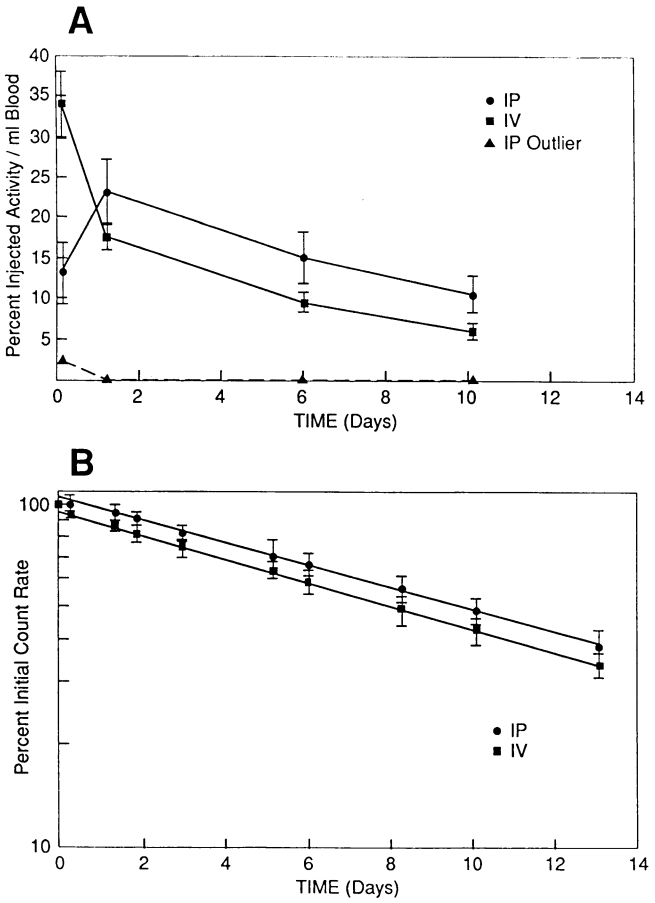

FIG. 1. (A) Intravascular levels of i.v. and i.p. administered ^{125}I-labeled 2H1. For each time point, the mean value for five mice and standard deviation (error bar) are displayed. One of the mice receiving i.p. injection was noted to differ in having markedly diminished blood activity from the initial time point onwards and is graphed separately; the phenomenon of absent intravascular antibody after i.p. injection is attributed to inadvertent entry and injection into the bowel lumen. (B) Whole-body clearances of i.v. and i.p. administered ^{131}I-labeled 2H1. Means and standard deviations are as described in the legend to panel A, with the exception that data are portrayed on a semi-log plot to illustrate the exponential nature of the antibody disappearance. Straight-line fit through the points represents a nonlinear regression of the data from 24 h onwards, yielding $t_{1/2}$ s of 8.68 and 9.12 days for antibody administered via i.v. and i.p. routes of injection, respectively.

body solution into the lumen of the intestine (Fig. 1A). Whole-body disappearance of MAb 2H1 after i.v. and i.p. routes of administration is shown in Fig. 1B. Rates of whole-body clearance of the antibody were similar for the two methods of administration, although slightly more of the i.p. administered MAb was retained in the body. Whole-body $t_{1/2}$s of 8.68 and 9.12 days were calculated for the i.v. and i.p. routes of injection, respectively. These values are consistent with the reported $t_{1/2}$ of IgG1 (45).

Therapeutic efficacy of MAb with and without AmB. The ability of MAb 2H1 to modify the course of lethal i.v. *C. neoformans* infection was studied by using A/JCr mice. A/JCr mice are very susceptible to *C. neoformans* infection, presumably because of partial complement deficiency (46). Mice with partial complement deficiency succumb rapidly with fulminant pneumonitis when infected i.v. (13). Complement-deficient mice provide a logical model for the testing of antibody reagents against *C. neoformans* since human cryptococcosis is accompanied by complement depletion (33).

Table 1 shows three experiments done to determine the effect of MAb, and the AmB dose used was suggested by the studies of Dromer and Charreire (11), who demonstrated a benefit with combined antibody and AmB therapy. In experiment 1, administration of MAb 24 h after infection did not prolong survival but three injections of AmB at 24, 48, and 72 h significantly enhanced survival. MAb alone administered at 24 h also had no effect on lung fungal burden in mice given 10^6 cryptococci i.v., with $\log_{10}$ mean CFU values for control and MAb-treated mice of 6.93 and 7.10, respectively ($P = 0.45$). Thus administration of MAb 2H1 24 h after infection resulted in no prolongation of survival or decrease in lung fungal burden.

The protocol for experiment 2 was modified by shortening the timing of MAb administration to 2 h postinfection and reducing the AmB dose to one 0.5-mg/kg dose 24 h after infection. The aim was to decrease the efficacy of the AmB regimen while increasing the likelihood of a MAb-mediated therapeutic effect. Shortening the timing of MAb administration significantly prolonged survival for the MAb-treated mice, but the reduced AmB dose used did not significantly prolong survival of AmB-treated mice (Table 1). Although the combination of MAb 2H1 and AmB was more effective than AmB alone, the benefit was primarily an antibody effect. Experiment 2 shows that antibody therapeutic effects can be demonstrated if the antibody is given shortly after infection, even by the i.p. route, which requires some time to achieve maximum intravascular concentrations.

In experiment 3, MAb 2H1 administration was delayed to 6 h after infection to reduce the therapeutic effect noted in experiment 2 and one dose of AmB (0.5 mg/kg) was administered at the same time. This protocol resulted in no statistically significant prolongation of the average survival of mice receiving either MAb 2H1 or AmB alone, but those receiving combined therapy lived almost three times longer than the controls ($P = 0.0018$). Thus the combination of AmB and MAb 2H1 was more effective than either agent alone, confirming similar observations by Dromer and Charreire (11). To determine whether antibody alone could decrease lung CFU after infection, the inoculum was reduced to 2.5×10^5 cryptococci and MAb 2H1 was administered i.p. 6 h later. Four days after infection, MAb-treated mice had significantly reduced average lung CFU burden and a lower serum GXM-D level relative to those of the control mice, indicating a potential mechanism for therapeutic efficacy of MAb (Fig. 2).

Passive antibody reduces serum GXM-D levels. In addition to enhancing cellular immunity by serving as an opsonin, passive antibody could theoretically be useful for removing GXM-D antigen. To determine the ability of antibody to decrease serum GXM-D, we measured the effect of i.p. IgM, IgG3, IgG1, and IgA MAb administration on serum GXM-D concentration. These antibodies are all derived from a single B cell, have very similar if not identical fine specificities, and differ only by isotype and a few amino acid residues in their heavy and light chains as a result of somatic mutation (36). For this experiment, we reduced the i.v. inocula to 1.7×10^5 to prolong survival so that the successive measurements could be done, and five groups of eight mice were infected. On the second day of infection, all mice had serum GXM-D concentrations in the range of 10 to 20 μg/ml and there was no difference between the five groups (Fig. 3). MAb was administered i.p. 1 day after infection, and serum GXM-D was measured on the third and seventh days of infection. Control mice showed a progressive increase in serum GXM-D concen-

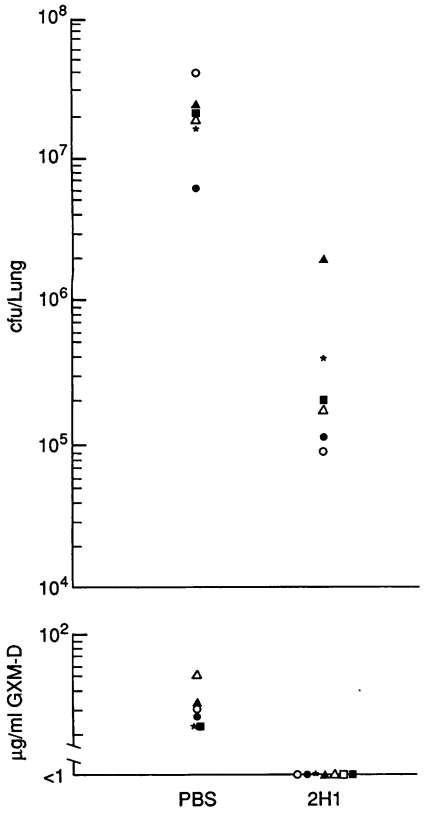

FIG. 2. Lung CFU and serum GXM-D levels for mice infected with 2.5×10^5 *C. neoformans* cells and treated with MAb 2H1 alone 6 h after infection. The PBS (control) and MAb 2H1 groups had six and seven mice, respectively. MAb 2H1 administration resulted in ~100-fold reduction in average CFU ($P < 0.001$). The serum GXM values for the seven MAb 2H1-treated mice are shown as <1.0 µg/ml because this was the limit of detection for the assay used. Each symbol represents a different mouse.

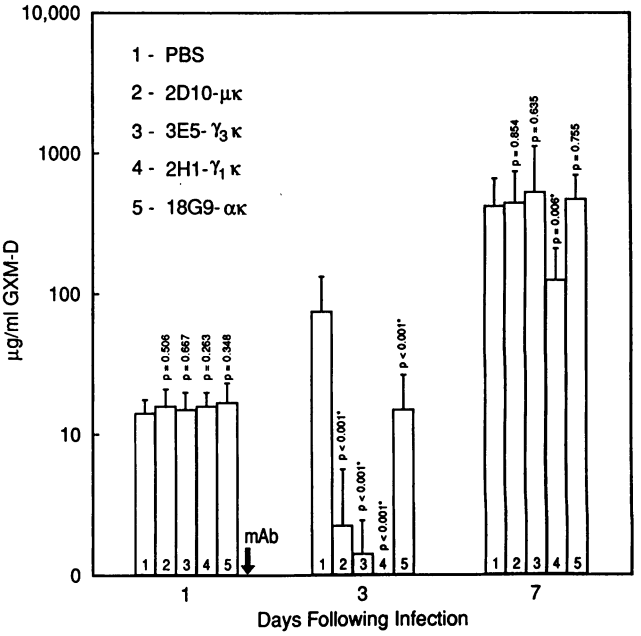

FIG. 3. Serum GXM-D levels in mice infected i.v. with 1.7×10^5 *C. neoformans* cells and treated with MAbs 2D10 (IgM), 3E5 (IgG3), 2H1 (IgG1), and 18G9 (IgA). Each of the five groups had eight mice. GXM-D levels are the averages for the eight mice, and the error bars denote the standard deviations. *P* values are calculated relative to the PBS control, and asterisks denote *P* values significant at the 0.05 level or better.

tration. However, there was a marked decrease in serum GXM-D for each of the four groups receiving i.p. MAb. The reduction in GXM-D was greatest for mice given MAb 2H1 (IgG1) and lowest for mice given MAb 18G9 (IgA), with the relative efficacy being IgG1 > IgG3 > IgM > IgA. The decrease in GXM-D resulting from IgM, IgG3, and IgA administration was transient, and by day 7 the serum GXM-D levels in mice receiving these isotypes were not different from those in control mice. In contrast, the mice receiving MAb 2H1 continued to have significantly decreased serum GXM-D levels at day 7.

Antibody enhances the fungistatic effect of macrophages and AmB in vitro. To determine whether MAb 2H1 potentiated the effect of AmB in vitro, we studied the interaction of J774.16 murine macrophage cells with *C. neoformans* in the presence and absence of MAb 2H1 and AmB (Fig. 4). These experiments require specific E/T (macrophage/*C. neoformans*) ratios and optimal concentrations of antibody and AmB to demonstrate an effect. Titration of AmB with *C. neoformans* ATCC 24067 to determine its effect on fungal viability indicated a narrow working concentration for AmB of > 0.1 to ≤ 0.14 µg/ml. AmB concentrations of >0.14 µg/ml killed all *C. neoformans* cells, whereas concentrations of ≤0.1 µg/ml had no effect on CFU. The phagocytic indexes for *C. neoformans*, obtained by using J774.16 cells in the presence and absence of 5 µg of MAb 2H1 per ml, were 1.55 ± 0.50 and 0.06 ± 0.02, respectively ($P < 0.001$). Thus the concentrations of 5 µg/ml for MAb 2H1 and 0.12 µg/ml for AmB were chosen to study their effects on *C. neoformans* viability in the presence of J774.16 cells. Figure 4 shows the results of two experiments utilizing different initial E/T ratios. At an initial E/T ratio of 2:1, addition of AmB ($P < 0.001$) but not MAb 2H1 ($P = 0.948$) significantly enhanced fungicidal or fungistatic activity of IFN-γ–LPS-activated J774.16 cells. However, the combination of AmB and MAb 2H1 was more effective than AmB alone ($P = 0.002$). At an initial E/T ratio of 10:1, both AmB ($P = 0.012$) and MAb 2H1 ($P = 0.023$) significantly reduced the CFU compared with the CFU measured with activated J774.16 cells alone, and their combination was again more effective at reducing CFU than either agent alone.

DISCUSSION

Intraperitoneal injection is a commonly used route of MAb administration in passive antibody protection experiments (12, 25, 37, 47). MAb delivered into the peritoneum appeared rapidly in the serum compartment, and by 24 h i.p. and i.v. administered MAbs had achieved similar concentrations in serum. This result indicates that the i.p. route is very efficient for delivering MAb systemically and provides strong support for its continued use. In the present study, a single mouse did not have significant serum radioactive counts after i.p. injection, which most likely reflects injection of antibody into the intestinal lumen. The combined findings from the data reported here and earlier preliminary studies (unpublished data) suggest that the frequency of intestinal injection is less than 10%. This result illustrates one drawback of the i.p. approach which could contribute to some of the animal-to-animal vari-

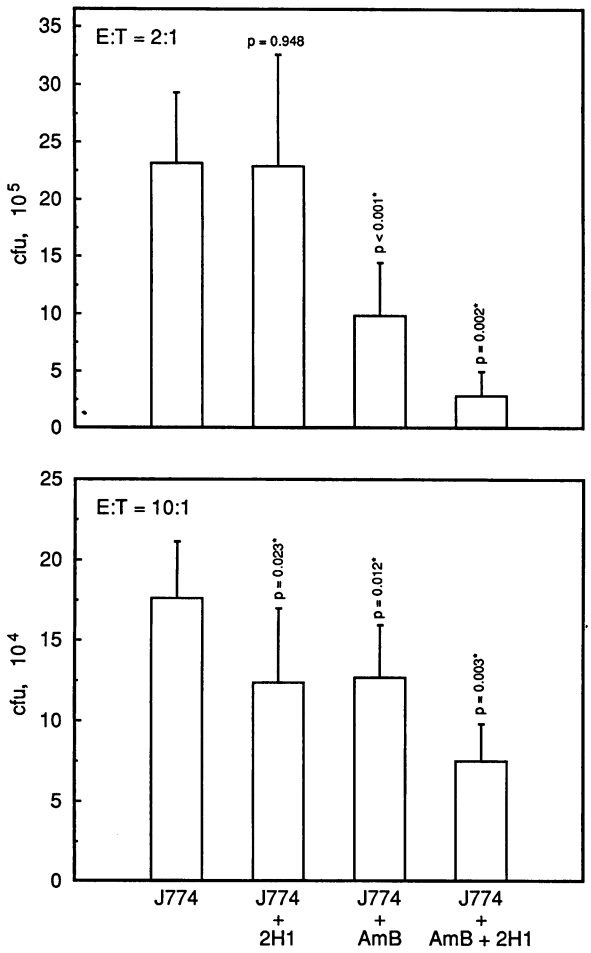

FIG. 4. Interaction of J774.16 cells in vitro with *C. neoformans* in the presence and absence of MAb 2H1 and AmB. The bars denote the *C. neoformans* CFU after 20 h of incubation with J774.16 cells with and without MAb 2H1 and AmB. The MAb and AmB concentrations used were 5 and 0.12 μg/ml, respectively. The top and bottom panels show data obtained at initial J774.16 cells/*C. neoformans* (E:T) ratios of 2:1 and 10:1, respectively. Values shown are the averages and standard deviations (error bars) of seven to eight replications. *P* values are calculated relative to the J774.16 cells alone, and asterisks denote values significant at the 0.05 level or better.

ation observed in survival studies (37, 38). The advantages of the i.p. route, namely, ease of injection, ability to deliver large volumes, and rapid intravascular dissemination, are mitigated somewhat by uncertainty as to the exact delivery site.

MAb administered after infection was able to decrease lung tissue fungal burden, reduce serum GXM-D, and prolong survival of lethally infected mice relative to that of untreated controls. The therapeutic efficacy of MAb 2H1 was dependent on the time elapsed between infection and antibody administration. Reduction in lung tissue CFU burden and prolongation of survival were observed for MAb 2H1 administered 6 h but not 24 h after infection. The lung was chosen to study fungal burden because yeast cells injected into the lateral tail vein drain into the pulmonary vasculature (26) and because the i.v. infection model in complement-deficient mice produces a fulminant pneumonitis (13). The inability of MAb to modify the course of infection if given after a certain time is consistent with earlier observations made for other pathogens such as *Neisseria meningitidis* and *Streptococcus pneumoniae*, animals infected with which could not be saved if antibody treatment was delayed for more than a few hours (8, 15). However, it is noteworthy that in the preantibiotic era, clinically useful sera were often ineffective in the therapy of established infection in animal models (8, 15, 34). Thus, antibody therapeutic ineffectiveness in animal models of established infection does not preclude therapeutic usefulness in human infection. In the case of *C. neoformans* infections, one explanation for the ineffectiveness of passive antibody in mediating protection if given 24 h after infection is suggested by the fact that mice infected with 1.7×10^5 organisms had serum GXM-D levels of 10 to 20 μg/ml 1 day after infection (Fig. 3). Presumably, serum GXM-D sequesters antibody in complexes such that little or no MAb is available to enhance cellular effector function against *C. neoformans*. However, the finding that MAb 2H1 given after infection was able to prolong survival, decrease lung CFU burden, and reduce serum GXM-D indicates a therapeutic effect for antibody alone. The mechanism by which MAb 2H1 mediates protection in vivo (37, 38) is uncertain but is likely to involve the promotion of phagocytosis (28, 48) by host effector cells and the potentiation of antifungal effects by NK cells (35, 41) and leukocytes (9).

In vivo, the combination of AmB and MAb 2H1 was more effective than either agent alone in prolonging the survival of lethally infected mice. This result had been reported previously for polyclonal rabbit sera (19) and for another capsule-binding MAb (11) and is confirmed by our findings. IFN-γ–LPS-activated J774.16 cells inhibit *C. neoformans* growth in the absence of antibody (38a). Inhibition of *C. neoformans* by activated murine macrophages has been reported by others (1, 14, 21–23, 43) and has been attributed to nitric oxide production (1, 21) or secreted antifungal proteins (14). MAb 2H1 alone promoted phagocytosis and enhanced the fungistatic or fungicidal activity of J774.16 cells, consistent with the observation that fungistasis is more efficient when cryptococci are phagocytosed by murine macrophages (22). In vitro, the combination of AmB and MAb 2H1 was more effective than either agent alone at potentiating the antifungal effects of IFN-γ–LPS-activated murine macrophages. AmB has direct fungicidal effects on *C. neoformans* (2) and indirect antifungal effects by activating macrophage function (50). The finding that MAb 2H1 potentiates *C. neoformans* fungistatic or fungicidal effects by murine macrophages supports previous observations that antibody enhances killing by leukocytes (9, 10) and provides an in vitro correlate for the in vivo effects.

Mice treated with MAb 2H1 had significantly reduced levels of serum GXM-D, which could reflect the lower fungal burden observed in the antibody-treated mice and/or antibody-mediated clearance of polysaccharide. Administration of IgM, IgG3, IgG1, and IgA MAbs 24 h after infection markedly reduced serum GXM-D. The IgG isotypes were more effective than the polyvalent IgA and IgM in decreasing serum GXM-D levels, possibly by promoting removal of antigen-antibody complex through Fc receptors. The effect of IgM and IgA on serum GXM-D levels was transient, and their long-term ineffectiveness could be related to their very short intravascular $t_{1/2}$ (45). IgG3, however, has an intravascular $t_{1/2}$ similar to that of IgG1, and its effect was also transient, so that at day 7 of infection only mice given IgG1 showed reduced serum GXM-D levels relative to those observed for the control and other isotypes. This is consistent with our earlier finding that IgG1 but not IgG3 mediates protection against *C. neoformans* (37) and could reflect reduced tissue fungal burden with IgG1, similar to that shown in Fig. 2.

In human cryptococcosis, the high concentrations of GXM

antigen in body fluids provide both an obstacle and an opportunity for passive antibody therapy. Free GXM antigen is an obstacle because it could, in principle, inhibit opsonization of *C. neoformans* by competing with and sequestering anticapsular antibody. However, the finding that antibody can reduce serum GXM-D in mice suggests a potential role for passive antibody therapy in the removal of polysaccharide antigen. *C. neoformans* polysaccharide has been associated with a variety of deleterious effects on immune function, including immune paralysis (30, 40), inhibition of phagocytosis (29), and enhancement of human immunodeficiency virus infection in vitro (44). Thus, polysaccharide removal could be beneficial to the host. Limited human experience with polyclonal rabbit antisera suggests that passive antibody therapy reduces antigenemia (18, 20). Repeated dosing with antibody could result in clearance of antigen such that subsequent doses are more likely to bind to and opsonize the fungus. Alternatively, patients could be treated initially with AmB to reduce antigen load, with the subsequent addition of antibody therapy.

Our results provide additional support for the importance of antibody-mediated immunity against *C. neoformans* infections. More importantly, the results confirm previous observations (11, 19) which indicate a potential role for passive antibody in treatment of established infection. In human infection, passive antibody could be useful clinically as an adjunct to antifungal therapy if it decreases the incidence of relapse, shortens hospitalization, and/or reduces mortality or the dose of AmB necessary for treatment. In addition, passive antibody could have a role in the prevention of *C. neoformans* infections in high-risk groups. In this regard, passive polyclonal antibody is already used prophylactically with considerable success in the prevention of infection in children with AIDS, indicating that prophylactic approaches are feasible in targeted populations (42). Passive antibody offers a means of enhancing existing antifungal chemotherapy in the setting of severe immunosuppression.

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