Passive Immunization against *Cryptococcus neoformans* with an Isotype-Switch Family of Monoclonal Antibodies Reactive with Cryptococcal Polysaccharide

JOAN E. SANFORD, DAVID M. LUPAN, ANNETTE M. SCHLAGETER, AND THOMAS R. KOZEL*

Department of Microbiology and Cell and Molecular Biology Program, School of Medicine,
University of Nevada, Reno, Nevada, 89557

Received 30 January 1990/Accepted 27 March 1990

The *in vivo* properties of an immunoglobulin isotype-switch family of monoclonal antibodies specific for the polysaccharide capsule of *Cryptococcus neoformans* were examined in a murine model of cryptococcosis. Subclass-switch variants were isolated by sequential sublining of an immunoglobulin G subclass 1 (IgG1)-secreting cell line. Antibodies of the IgG1, IgG2a, and IgG2b isotypes with identical reactivities with cryptococcal polysaccharide were prepared. The antibodies had the distinct biological properties associated with the heavy chains of each respective isotype. The antibodies were used prophylactically or therapeutically in an attempt to alter the course of cryptococcal infection in mice. Survival of mice and a tissue census of the numbers of viable cryptococci in the lung, spleen, and brain were used as indicators of efficacy. Passive immunization with the IgG2a and IgG2b antibodies effected a reduction in the numbers of cryptococci in lung and spleen. Passive immunization with the IgG1 antibody was markedly less effective. Passive immunization had little or no effect on the numbers of cryptococci in brain tissue, regardless of the immunoglobulin isotype. Despite apparent efficacy with regard to reduction in the numbers of yeast cells in the lung and spleen, the results showed no improvement in survival from murine cryptococcosis. Our results indicate that passive immunization produces a modest effect on the course of murine cryptococcosis in tissues other than brain. However, under the experimental conditions used, such treatment does not have a measurable impact on the ultimate outcome of the infection.

Cryptococcosis is a serious threat to the immunocompromised patient. Current approaches to therapy present numerous problems for the clinician. Available antibiotics frequently have a high toxicity, and failure rates can be high, particularly in the patient with acquired immunodeficiency syndrome. One possible alternative or adjunct to chemotherapy is the use of passive immunization with immune globulin. Passive immunization with monoclonal antibodies (MAbs) has proven successful in animal models of infection by several encapsulated bacteria, including *Haemophilus influenzae* type b (6), *Streptococcus pneumoniae* (1), group B *Streptococcus* spp. (24), and *Neisseria meningitidis* (2).

The use of antibody for passive immunization against experimental cryptococcosis has been an elusive goal. Gadebusch (5) and Graybill et al. (10) reported that mice could be protected against experimental cryptococcosis by passive immunization with rabbit anticryptococcal antibody. A key feature of both reports was the need for antibody to be present at the time and site of challenge. In contrast, Louria and Kaminski found that passive immunization had no effect on survival time or the numbers of cryptococci in brain tissue if the mice were treated 24 h after challenge at a site that differed from the challenge route (15). Without doubt, the most impressive results with passive immunization have been obtained by using MAbs specific for the capsular polysaccharide (3, 4). Dromer et al. (3) found that a MAb of immunoglobulin G subclass 1 (IgG1) could extend the mean survival time of heavily infected DBA/2 mice from 3 days in untreated mice to 18 days for mice treated with MAb.

The biological activity of an immunoglobulin, and its potential efficacy in passive immunization, is influenced by

the immunoglobulin subclass. For example, the subclasses of mouse IgG differ with regard to serum half-life (26), ability to fix complement (19), and ability to direct antibody-dependent cell-mediated cytotoxicity (13). *In vivo* studies have established the importance of antibody isotype in immunotherapy by using systems as diverse as (i) reduction in the number of schistosomes of *Schistosoma mansoni* in the lungs of infected rats (11), (ii) inhibition of tumor growth in nude mice (25), (iii) treatment of a murine B-cell lymphoma (12), (iv) use of monoclonal anti-CD-4 antibodies to treat autoimmune disease mediated by T lymphocytes (27), and (v) treatment of *Escherichia coli* K1 infection (20).

In the accompanying paper, we describe the production and characterization of a subclass-switch family of MAbs reactive with an epitope shared by all four serotypes of cryptococcal polysaccharide (23). The relative opsonic activity of these antibodies for phagocytosis of encapsulated cryptococci by murine macrophages and cultured human monocytes was IgG2a > IgG1 > IgG2b. The subclass switch involves only the heavy-chain constant region, leaving the light chains and the heavy-chain variable region unaltered. The role of the heavy-chain isotype in passive immunization against cryptococcosis can be determined, because any differences in the protective activities of the antibodies would be due to differences in the biological activities attributable to the heavy chain rather than possible differences in the ability of the antibodies to bind to the capsule. The specific objective of our study was to investigate the prophylactic and therapeutic efficacy of this subclass-switch family of MAbs. Our results showed that passive immunization with MAbs of the IgG2a and IgG2b subclasses significantly and reproducibly reduced the numbers of yeast cells in lung and spleen but not brain tissue. Despite lowered

* Corresponding author.

TABLE 1. Effect of prophylactic injection of MAbs 24 h before challenge with cryptococci on the numbers of cryptococci in the lung, spleen, and brain 7 days after challenge^a

Tissue	Isotype of MAb used for passive immunization ^b			
	None (control)	IgG1	IgG2	IgG2b
Experiment 1				
Lung	78,000 ± 80,000	20,000 ± 18,000 (<i>P</i> = 0.08)	5,800 ± 7,400 (<i>P</i> = 0.03)	11,000 ± 9,200 (<i>P</i> = 0.03)
Spleen	5,600 ± 7,400	3,000 ± 4,400 (<i>P</i> = 0.26)	3,200 ± 4,500 (<i>P</i> = 0.27)	500 ± 300 (<i>P</i> = 0.08)
Brain	26,000 ± 11,000	19,000 ± 9,800 (<i>P</i> = 0.16)	22,000 ± 14,000 (<i>P</i> = 0.28)	22,000 ± 7,900 (<i>P</i> = 0.28)
Experiment 2				
Lung	77,000 ± 95,000	24,000 ± 16,000 (<i>P</i> = 0.17)	18,000 ± 15,000 (<i>P</i> = 0.11)	800 ± 600 (<i>P</i> = 0.06)
Spleen	129,000 ± 124,000	9,700 ± 7,600 (<i>P</i> = 0.14)	12,000 ± 7,000 (<i>P</i> = 0.08)	450 ± 300 (<i>P</i> = 0.07)
Brain	180,000 ± 110,000	100,000 ± 48,000 (<i>P</i> = 0.16)	78,000 ± 55,000 (<i>P</i> = 0.10)	51,000 ± 27,000 (<i>P</i> = 0.03)

^a Data reported as the mean CFU per 100 mg of tissue ± standard deviation for four replications.

^b For each tissue, *P* was calculated for each treatment group compared with the control group. Significant differences are those values for which *P* < 0.05.

numbers of yeast cells in spleen and lung, passive immunization had no effect on the survival of mice challenged with *Cryptococcus neoformans*.

MATERIALS AND METHODS

MAbs and passive immunization. An isotype-switch family of MAbs was used for all experiments. The lineage of the complete family of isotype-switch variants was IgG1 → IgG2b → IgG2a. Procedures for the production of the class-switch variants and the in vitro properties of the antibodies are described in an accompanying paper (23). All antibodies were isolated from ascites fluid by immunoaffinity purification with a column in which cryptococcal polysaccharide had been coupled to AH-Sepharose (14). The antibodies of each isotype were further purified on protein A-Sepharose to assure that there was no contamination by antibodies of other isotypes (23).

Mice were injected intraperitoneally with MAbs of the IgG1, IgG2a, and IgG2b subclasses. Two treatment protocols were used. In a prophylaxis protocol, mice were given an injection of 1.0 mg of antibody 24 h prior to challenge with viable cryptococci. In the therapeutic protocol, mice were given 0.5 mg of antibody 48 h and again 96 h after infection with viable cryptococci. Control mice received a similar injection of sterile saline.

Murine cryptococcosis. *C. neoformans* 104 is an encapsulated serotype A isolate that was used in all studies. Cultures were maintained on slants of Sabouraud agar at room temperature. Seventy-two-hour cultures were used for challenge experiments. Yeast cell concentrations were determined by counting in a hemacytometer and confirmed by quantitative pour plates. Yeast viability was always greater than 85%. Female Swiss Webster mice, 6 to 10 weeks old (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), were challenged intravenously in the lateral tail vein with 1×10^5 yeast cells. This was considered a lethal inoculum, because preliminary experiments established that this dose typically killed 50% of a group of Swiss Webster mice in 20 to 25 days and 90% of the group after 35 days.

Evaluation of treatment efficacy. Survival of mice and a tissue census of yeast cells in lungs, spleen, and brain were used to assess the extent of cryptococcal infection. Tissue census studies were done by using six mice per group. Mice were killed by CO₂ asphyxiation. Tissues were removed, weighed, and then homogenized in sterile saline with a glass tissue grinder (model 1975-00015; Bellco Glass, Inc., Vineland, N.J.). Serial 10-fold dilutions of the homogenates were prepared in sterile saline and plated on Sabouraud agar.

Colony counts were determined after incubation for 72 h at room temperature. Results are expressed as means ± standard error. Differences between means were determined by the Student's *t* test. Significance was determined at the *P* < 0.05 level. In survival studies, both prophylactic and treatment protocols were used with groups of 12 mice.

RESULTS

Prophylactic use of MAb. A series of experiments was done to assess the effects of MAb administered prior to challenge on the course of an experimental cryptococcal infection. Mice were given intraperitoneal injections of 1 mg of antibodies of the IgG1, IgG2a, or IgG2b isotype. Mice were challenged intravenously 24 h later with viable cryptococci. Results from preliminary experiments indicated that 1 mg of antibody is approximately 4,000 times more than needed to effectively opsonize the challenge inoculum of 1×10^5 cryptococci (23). The efficacy of prophylaxis was assessed by survival time as well as by a tissue census of lung, brain, and spleen 7 days after challenge.

Table 1 shows the results of tissue census from two separate experiments. There was considerable variation within each experimental group. As a consequence, standard deviations were large and statistical comparison was difficult. IgG1 antibodies were the least effective in reducing numbers of viable cryptococci in all the tissues sampled. Despite numerical reductions in lung and spleen, in no instance did IgG1 antibodies have a statistically significant effect. Regardless of immunoglobulin isotype, antibody was least effective in preventing cryptococcal infection of brain tissue. However, there was one instance (experiment 2, with IgG2b) in which a significant reduction (*P* < 0.05) in the number of yeast cells in brain tissue was observed. A substantial decrease in the number of cryptococci was observed in lung and spleen tissue of mice treated with IgG2a and IgG2b antibodies. However, the effect on spleen was not statistically significant in either experiment, and a significant reduction in the number of cryptococci in lung tissue was observed in only one of the two experiments.

Survival after challenge with a lethal inoculum was used as an indicator of the effect of each MAb on the ultimate outcome of a cryptococcal infection. A comparison of mean survival time between groups of 12 mice indicated that no significant protection was conferred by any of the MAbs (Fig. 1). Mice that died had the weight loss and hydrocephalus characteristic of cryptococcal meningoencephalitis (4).

Therapeutic use of MAb. Experiments were done to examine the ability of MAbs of the three isotypes to treat a

FIG. 1. Survival of mice after challenge with a lethal dose of viable *C. neoformans*. Mice were treated with saline or 1.0 mg of anticryptococcal antibody of the IgG1, IgG2a, or IgG2b isotype 24 h before challenge. SD, Standard deviation.

FIG. 2. Survival of mice after challenge with a lethal dose of viable *C. neoformans*. Mice were treated with saline or 0.5 mg of anticryptococcal antibody of the IgG1, IgG2a, or IgG2b isotype 48 and 96 h after challenge. SD, Standard deviation.

cryptococcal infection. Mice were injected intraperitoneally with 0.5 mg of antibody at intervals of 48 and 96 h after intravenous challenge with 1×10^5 cryptococcal cells. Increased survival time and a tissue census done on brain, lung, and spleen tissues 7 days after infection were used as indicators of treatment efficacy.

The results of tissue census of two separate experiments are shown in Table 2. As with the prophylaxis protocol, a wide variation within all experimental groups contributed to large standard deviation. Treatment of infected mice with IgG1, IgG2a, or IgG2b had no effect on the number of yeast cells in brain tissue. In contrast to the lack of effect on brain tissue, the IgG2a and IgG2b antibodies significantly reduced the number of cryptococci in lung and spleen in both experiments. The IgG1 antibody had no significant effect on the number of cryptococci in the lung but reduced the number of viable yeast cells in the spleen. The effect of IgG1 on the spleen was significant in one of the two experiments.

The studies described above showed that the treatment of infected mice with antibodies of the IgG2a and IgG2b isotypes altered the course of experimental cryptococcosis in the lung and spleen. Despite this apparent efficacy, survival studies (Fig. 2) showed that none of the MAb influenced mean survival time.

DISCUSSION

Our studies were designed to assess the ability of a class-switch family of MAbs to alter the course of experi-

mental cryptococcosis. In the accompanying paper, we describe the production and characteristics of a class-switch family of MAbs reactive with an epitope on cryptococcal polysaccharide (23). The antibodies were opsonic for phagocytosis by murine macrophages. Moreover, the antibodies had an identical reactivity with cryptococcal polysaccharide. Thus, any variability in ability to passively immunize would be due to differences in the immunoglobulin subclass rather than differences in reactivity with antigen.

Antibodies of the IgG2a and IgG2b subclasses were more effective in clearing yeast cells from tissue than antibody of the IgG1 subclass. This difference was most apparent when mice were treated with antibody 48 and 96 h after challenge. The IgG2a and IgG2b antibodies effected a significant reduction in the number of cryptococci in the lung and spleen in each of two separate experiments. In contrast, the IgG1 antibody had little effect on the number of cryptococci in the lung and spleen.

The relative inability of the IgG1 antibody to reduce the number of cryptococci in the lung and spleen was somewhat unexpected. Studies of the opsonic activity of this isotype-switch family had shown that the IgG1 antibody was more opsonic for encapsulated cryptococci than the IgG2b antibody (23). There are at least two possible explanations. First, the distribution of IgG1 in vivo may be such that appreciable amounts of the antibody fail to reach either the yeast or the appropriate target organs. Alternatively, opsonization for phagocytosis may not be the critical effector

TABLE 2. Effect of intraperitoneal injection of MAbs 48 and 96 h after challenge with cryptococci on the numbers of cryptococci in lung, spleen, and brain 7 days after challenge^a

Tissue	Isotype of MAb used for passive immunization ^b			
	None (control)	IgG1	IgG2a	IgG2b
Experiment 1				
Lung	49,000 $\pm$ 14,000	48,000 $\pm$ 30,000 ($P = 0.47$)	1,400 $\pm$ 3,000 ($P < 0.001$)	6,400 $\pm$ 6,900 ($P < 0.001$)
Spleen	21,000 $\pm$ 22,000	7,100 $\pm$ 6,600 ($P = 0.12$)	1,100 $\pm$ 2,700 ($P = 0.03$)	2,000 $\pm$ 3,600 ($P = 0.03$)
Brain	43,000 $\pm$ 26,000	43,000 $\pm$ 32,000 ($P = 0.48$)	27,000 $\pm$ 7,500 ($P = 0.11$)	42,000 $\pm$ 15,000 ($P = 0.47$)
Experiment 2				
Lung	2,300,000 $\pm$ 1,500,000	1,800,000 $\pm$ 2,400,000 ($P = 0.37$)	190,000 $\pm$ 280,000 ($P = 0.004$)	320,000 $\pm$ 70,000 ($P = 0.004$)
Spleen	100,000 $\pm$ 78,000	14,000 $\pm$ 9,800 ($P = 0.01$)	4,400 $\pm$ 1,900 ($P = 0.01$)	7,600 $\pm$ 3,200 ($P = 0.006$)
Brain	600,000 $\pm$ 320,000	650,000 $\pm$ 610,000 ($P = 0.44$)	500,000 $\pm$ 500,000 ($P = 0.38$)	710,000 $\pm$ 540,000 ($P = 0.37$)

^a Data reported as the mean CFU per 100 mg of tissue $\pm$ standard deviation for four replications.

^b For each tissue, P was calculated for each treatment group compared with the control group. Significant differences are those values for which $P < 0.05$.

mechanism for passive immunization with antibody. Nabavi and Murphy demonstrated that the anticryptococcal activity of natural killer cells was appreciably enhanced by anticryptococcal antibody (17). Kipps et al. (13) have found that murine IgG1 is unable to direct antibody-directed cell-mediated cytotoxicity by human K cells. Thus, an alternative effector function, such as direction of antibody-directed cell-mediated cytotoxicity in the lung and spleen, might be the relevant action of the IgG2a and IgG2b antibodies. Previous studies of the biological properties of subclass-switch antibodies have also noted discrepancies between in vitro assays and in vivo efficacy (16, 25). For example, Mujoo et al. (16) found that IgG1 antibodies would not direct complement-mediated lysis or antibody-directed cell-mediated cytotoxicity against neuroblastoma tumor cells, but the IgG1 antibody suppressed the growth of the tumor cells in nude mice.

Passive immunization with MABs had little effect on the number of cryptococci in brain tissue regardless of the treatment protocol or the antibody subclass. A significant reduction was noted in one group of mice treated with IgG2b in a prophylaxis protocol. This was only a threefold reduction, and the effect was not observed in a duplicate experiment. Our inability to appreciably influence the number of cryptococci in brain tissue is similar to an observation by Perfect et al. (21) that rabbits with high levels of serum anticryptococcal antibody do not have an increased ability to clear cryptococci from cerebrospinal fluid. The inability of MABs to influence the number of cryptococci in brain tissue was reported recently by Dromer et al. (4).

There are several possible reasons for the failure of passively administered antibody to reduce the number of cryptococci in brain tissue. One explanation is that passively administered antibody may not cross the blood-brain barrier. This explanation is consistent with the observation of Goren that encapsulated cryptococci in the liver and spleen of mice with high levels of circulating anticapsular antibody are coated with antibody (9). In contrast, cryptococci in the brain tissue of hyperimmunized mice were not coated with antibody, suggesting that serum antibody is unable to reach cryptococci in brain tissue. More recently, Gigliotti et al. (7) examined a rabbit model of *S. pneumoniae* meningitis and reported that there was poor penetration of serum IgG into cerebrospinal fluid, even under conditions of acute inflammation. Additional factors that may contribute to the failure of passive immunization may be the relative absence of phagocytic cells or low complement levels in central nervous system tissue.

Despite a significant effect of passive immunization on the number of cryptococci in the lung and spleen, none of the antibodies influenced survival time. Thus, there was a close parallel between the inability to reduce the number of cryptococci in brain tissue and the inability to prolong survival time. Results from the prophylaxis protocols emphasize the ability of cryptococci to avoid the opsonizing action of antibody in establishing a central nervous system infection. Further, once established, a central nervous system infection is refractory to the action of antibody.

Our inability to prolong the lives of infected mice differs from results reported originally by Dromer et al. (3). Dromer et al. found that untreated mice had a mean survival time of 1.5 to 3 days, whereas mice treated with 100 µg of MAB had a mean survival time of 12 to 18 days. The critical difference between the two studies that undoubtedly accounts for the disparate results was the strain of mouse used in each study (4). We used the Swiss Webster mouse; Dromer et al. used

the DBA/2 mouse in their studies. The DBA/2 mouse has an inherited deficiency of complement component 5 (C5) (18). Rhodes demonstrated that cryptococcosis takes a markedly different course in C5-deficient mice than in C5-sufficient mice (22). C5-deficient mice develop an acute pneumonia that is rapidly fatal, whereas C5-sufficient mice do not. Our results showed that passive immunization reduced the burden of cryptococci in the lung approximately 10-fold. Thus, it is not surprising that the previous paper by Dromer et al. (3) reported significant prolongation of life in C5-deficient mice. It is important to note that all mice in the Dromer study eventually died of cryptococcosis, presumably because of the meningoencephalitis characteristic of C5-sufficient mice.

The importance of complement in the experimental model was recently confirmed in a report that compared the ability of anticapsular MAB to protect complement-sufficient BALB/c mice and C5-deficient DBA/2 mice from experimental cryptococcosis (4). Passive immunization protected C5-deficient mice from the early acute pneumonia that followed intravenous injection of a large number of cryptococci. In contrast, passive immunization did not protect against the slow progressive meningoencephalitis that occurs in either BALB/c mice or C5-deficient mice that are given a lower dose of cryptococci that does not cause an acute pneumonia. Our results are consistent with the observation in this recent study that MABs reduce the tissue burden in the spleen and lung but not brain. Further, treatment with MABs did not prolong the life of BALB/c mice given a lethal intravenous inoculum of cryptococci. Taken together, these studies provide a consistent pattern in which MABs are very effective in protecting C5-deficient mice from a fatal pneumonia, but MABs are minimally effective against the fatal meningoencephalitis that occurs in either C5-sufficient mice or C5-deficient mice given low doses of cryptococci.

The potential value of MABs in the treatment of cryptococcosis is uncertain. Given the ability of the antibodies to reduce the fungal load in the lungs, it is possible that passive immunization might prove more effective in protection against an intranasal challenge. The inability of passive immunization to prolong life and to effect clearance of the yeast from the brains of Swiss Webster mice suggests that it may be more difficult to treat a central nervous system infection by passive immunization alone. However, our results also indicate that MABs of the IgG2a and IgG2b isotypes will reduce the fungal burden in the lung and spleen. There is a possibility that passive immunization could be used in conjunction with more conventional antibiotic therapy to either increase the efficacy of a treatment protocol or reduce the levels of antibiotic needed for the treatment of cryptococcosis. The viability of this approach is suggested by an earlier study which reported that passive immunization with anticryptococcal antibody markedly improved the ability of amphotericin B to prolong the survival of mice with experimental cryptococcosis (8). Future studies of passive immunization should examine the ability of antibodies of the IgG2a and IgG2b isotypes to act synergistically with more conventional antifungal therapy (3).

Immunoglobulin isotype may be of importance in avoiding potential side effects of passive immunization. Cryptococcosis frequently produces high levels of antigenemia, particularly in patients with acquired immunodeficiency syndrome. Passive immunization has the potential for producing in vivo immune complexes or inducing widespread complement activation with serious complications. We have found that the IgG2b antibody has a markedly decreased ability to

precipitate cryptococcal polysaccharide (23). As a consequence, this antibody may be less active in producing large-lattice complexes in vivo. Other studies have shown that antibodies of the IgG2a isotype have a diminished capacity for complement activation (19). Taken together, these studies indicate that immunoglobulin isotype is a significant factor in any strategy that might use passive immunization for the treatment of cryptococcosis.

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