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Role of Natural Killer Cells in Resistance to Systemic Cryptococcosis

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These studies demonstrate that *Cryptococccus neoformans* infection induced a dose-dependent augmentation of splenic natural killer (NK) cell activity by bg/+, but not bg/bg mice. To directly assess the role of NK cells in resistance to *C. neoformans*, bg/+ and bg/bg mice were treated with anti-NK-1.1 monoclonal antibody (mAb). Anti-NK-1.1-treatment abrogated the augmented NK cell activity observed during *C. neoformans* infection in bg/+ mice. Anti-NK-1.1-treated bg/+ mice had higher *C. neoformans* colony forming units (CFU) in their lungs on days 3 and 7 after intravenous (i.v.) challenge than control bg/+ mice. Moreover, the number of *C. neoformans* CFU in the lungs of anti-NK-1.1-treated bg/+ mice on days 3 and 7 were similar to those observed for infected bg/bg mice. By day 14, however, no differences in *C. neoformans* CFU were evident in the lungs of anti-NK-1.1-treated and control bg/+ mice. Anti-NK-1.1-treatment did not alter either the growth of *C. neoformans* in the spleens, livers, kidneys, or brain of bg/+ mice or the susceptibility of bg/bg mice to systemic cryptococcosis. These studies suggest that NK cells do not play a role in resistance to systemic cryptococcosis in the spleen, but do appear to play an early, but transient role in resistance to *C. neoformans* in the lungs. Overall, congenital defects in polymorphonuclear neutrophils (PMNs) and macrophages (Mϕs), in addition to defects in NK cells, contribute to the enhanced susceptibility of bg/bg mice to systemic cryptococcosis.

Key words: immunodeficient mice, bg/+ and bg/bg mice

INTRODUCTION

Natural killer (NK) cells comprise a discrete subset of leukocytes distinct from T, B, and myleomonocytic cells that mediate spontaneous cytotoxicity for a variety of normal, tumor, and virus-infected cells [8,21,37]. Morphologically, NK cells are characterized as large granular lymphocytes and by the expression of surface antigens, such as NK-1.1., asialo GM-1, and Ly5 [32]. Although the cytotoxic capacity of NK cells is spontaneous, their cytotoxic activity can be augmented by interferons (IFN), interleukin 2 (IL-2), and other biological response modifiers [1,11,36]. Moreover, NK cells can produce IFN and IL-2 and these factors may play an important role in autoregulation of NK cell activity [7,29]. NK cells, along with macrophages (Mϕs) and polymorphonuclear neutrophils (PMNs), are considered to be important components of innate immunity and studies have implicated NK cells in antimicrobial defense against bacteria, parasites, and fungi [9,10,15,17,27,30,31].

Both in vitro and in vivo evidence suggests that NK cells are important in host defense against the pathogenic yeast *Cryptococcus neoformans*. Murine NK cells bind to and inhibit the growth of *C. neoformans* in vitro [27,28]. In vivo studies also suggest that NK cells are involved in clearance of *C. neoformans* from infected tissues [6,12,13,20,22,24]. Some of the evidence supporting the role of NK cells in host defense to cryptococcal infection has come from beige mice [13,22,24], which have defects in NK cells, PMNs, and Mϕs. The NK cell defect is related to an inability to lyse NK-sensitive targets, while the Mϕ and PMN defects are characterized by delayed chemotaxis and phagolysosome fusion and reduced microbicidal capacity [33]. Beige mice are more susceptible to systemic cryptococcosis than immunocompetent bg/+ mice as measured by increased numbers of *C. neoformans* in NK rich organs like the lungs and spleen [13,34] and by reduced survival after challenge [22,34]. While these latter observations might suggest that NK cells are involved in resistance to cryptococcosis, it has been difficult to preclude a role for the PMN and Mϕ defect in enhancing the susceptibility of beige mice to *C. neoformans*. In a previous study, we reported that histopathology of *C. neoformans*-infected tissues indicated the recruitment of phagocytic cells and the shift from acute to chronic inflammation was delayed in bg/bg

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mice when compared with immunocompetent bg/+ mice [34]. While not precluding a role for NK cells, these observations suggested that the PMN and Mϕ defects resulted in altered in vivo inflammatory responses and contributed to the enhanced susceptibility of beige mice to cryptococcosis.

In this study, we used bg/bg and bg/+ mice to more fully examine the role of NK cells in resistance to systemic cryptococcosis. The immunomodulatory effects of *C. neoformans* infection are well documented and effect both innate and acquired immune responses [26]. In the following study, we assessed whether *C. neoformans* infection modulated NK cell activity of bg/bg and bg/+ mice. Additionally, we examined the relationship between levels of NK activity observed during systemic cryptococcosis and in vivo growth of *C. neoformans*. To more specifically evaluate the role of NK cells in in vivo resistance to *C. neoformans*, bg/bg and bg/+ mice were depleted of NK cells with anti-NK-1.1 monoclonal antibody (mAb), challenged with *C. neoformans*, and the growth of *C. neoformans* in infected tissues was monitored.

MATERIALS AND METHODS

Mice

Inbred germfree bg/bg and bg/+ C57BL/6N mice between 8–10 weeks of age were used in this study. Animals were obtained from the University of Wisconsin Gnotobiotic Laboratory (Madison, WI) and were maintained in accordance with the National Institutes of Health (NIH) guidelines. On the day each experiment was started, mice were removed from the germ free isolator and were maintained in sterile cages with filter bonnets in a laminar flow hood.

Organism

An encapsulated strain of *C. neoformans* (SLHA) serotype A was used in these studies. Yeast cells were maintained by passage on Sabouraud's dextrose agar (SDA). For inoculation, yeasts were grown in Sabouraud's dextrose broth and incubated at 37°C for 48 h. Cryptococci were harvested, washed three times by centrifugation, resuspended in non-pyrogenic saline, counted on a hemacytometer, and adjusted to the appropriate inoculum. To verify the number of viable cells, the inoculum was serially diluted in phosphate buffered saline (PBS), plated on SDA, incubated at 37°C for 48 h, and colony forming units (CFU) were determined. Mice were given viable *C. neoformans* by an intravenous (i.v.) route.

Antibody Depletion

The monoclonal mouse IgG2a antibody to NK-1.1 was produced as ascites in pristane-treated nu/nu BALB/c mice injected with hybridoma PK 136 (American Type and Culture Collection, Rockville, MD). The concentration of mouse IgG2a in ascites was quantified by enzyme-linked immunoassay (ELISA). Mice were given 200 μg of anti-NK-1.1 mAb (0.1 ml) or 200 μg of control ascites (0.1 ml) from hybridoma SP2/0 (Accurate Chemical and Scientific Corp, Westbury, NY) by the i.v. route 2 days prior to (day -2) i.v. challenge with 8×10^3 *C. neoformans* (day 0).

Target Cells and Medium

The tissue culture medium used in all experiments and cell preparations was RPMI-1640 supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and defined calf serum (10%). YAC-1 tumor targets were maintained in supplemented RMPI-1640.

NK Cell Assay

Splenic NK cell activity was assessed in 4-, 6-, or 18-h Cr^{51}-release assays as previously described [2]. Briefly, spleens were aseptically removed, placed in 10 ml of RPMI-1640, and single cell suspensions were prepared. Spleen cell suspensions from infected mice were divided into two aliquots; one aliquot (2.5 ml) was used to assess *C. neoformans* CFU as described below. The second aliquot (7.5 ml) was used in NK assays. In NK assays, spleen cells from saline-treated controls were run concomittantly with spleen cells from *C. neoformans*-challenged mice. Spleen cell suspensions for NK assays were treated with hemolytic Gey's solution to lyse erythrocytes, washed three times, and counted on a hemacytometer. Chromated YAC-1 targets (5×10^3, 0.1 ml) were mixed with splenic effector cells (0.1 ml) at effector: target ratios (E:T) of 100:1, 50:1, and 25:1 in round bottom plates. All E:T ratios were run in quadruplicate. The plates were centrifuged (60*g*, 5 min) to initiate cell contact and then incubated at 37°C in a humidified incubator (5% CO_2). Supernatants were collected using the Skatron supernatant collection system (Skatron, Inc., Sterling, VA) and counted in a gamma counter. Percent cytotoxicity was calculated as follows: (test cpm − spontaneous cpm)/maximum cpm − spontaneous cpm) × 100. Spontaneous release was obtained from labeled targets incubated alone, while maximum release was determined from labeled targets incubated with 2 N HCl. Spontaneous release never exceeded 10%, 12%, or 20% of maximum in 4-, 6-, and 18-h assays, respectively. Under the clean microisolator conditions used to house bg/bg and bg/+ mice, the splenic NK activity of saline-treated control mice remained stable over the 2-week experiment. To assess the number of viable *C. neoformans* in splenic effector cell preparations from infected mice, an aliquot of the effector cell preparation

(E:T = 100:1) was serially diluted, plated on SDA, and CFU were determined.

IL-2

rHu-IL-2 (111,184 U/mg of protein) was provided by E.I. DuPont De Nemaurs and Co., Inc. (Glenolden, PA) as a protein purified to homogeneity from transfected *Escherichia coli*. It was used at a concentration of 50 U/well in some NK assays.

Microbial Enumeration

The kidneys, liver, lungs, and brain were removed and homogenized in 5 ml PBS. Homogenates were serially diluted in PBS, plated in duplicate on SDA, and colonies were counted after incubation for 48 h at 37°C. Data are expressed as the mean Log_{10} number of *C. neoformans* per g (dry wt) of each tissue homogenate from three to six mice per group for each culture interval. Statistical differences in *C. neoformans* CFU between bg/bg mice and their bg/+ littermates were determined using the Student's t-test and analysis of variance.

Cold Target Inhibition

A standard 4-h Cr^{51}-release assay was set up as described above at an E:T of 100:1, with the following modifications: splenic effector cells were prepared from bg/+ mice and used at a concentration of 200,000 cells in 0.1 ml. Unlabeled ("cold") *C. neoformans* SLHA or YAC-1 tumor cells were added to wells (0.05 ml) so that the ratio of labeled ("hot") tumor targets to unlabeled ("cold") targets (hot:cold [H:C] ratio) was either 1:1 or 1:10 (2,000 or 20,000 competitors, respectively; 0.05 ml). Cytotoxicity was assessed as described above. Control wells contained effector cells, chromated YAC-1 tumor targets, and no competitor (H:C ratio = 1:0). Addition of unlabeled competitor to Cr^{51}-release assays did not alter spontaneous or maximum release of Cr^{51} from tumor targets.

RESULTS

Augmentation of Splenic NK Activity During *C. neoformans* Infection

Splenic NK activity was assessed on days 1, 7, 14, and 21 after i.v. challenge of bg/bg and bg/+ mice with 1×10^5 encapsulated *C. neoformans* (Fig. 1). In bg/+ mice, a 200% increase in splenic NK activity was observed on day 7 after i.v. challenge with 10^5 cryptococci. The NK activity of infected bg/+ mice declined to control levels on day 14 and was suppressed on day 21 (65% reduction) when compared with uninfected bg/+ controls. In contrast, infection with 1×10^5 *C. neoformans* did not alter splenic NK activity of bg/bg mice at any timepoint when compared with uninfected bg/bg

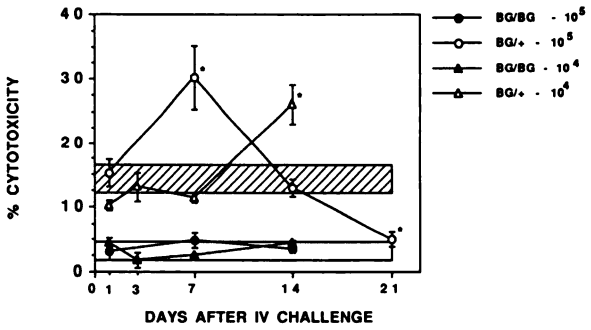

Fig. 1. Augmentation of splenic NK activity during *C. neoformans* infection. Bg/bg (●, ▲) and bg/+ (○, △) mice were i.v. challenged with 1×10^4 (▲, △; n = 3) or 1×10^5 (●, ○; n = 6) *C. neoformans*. Each data point represents the mean % cytotoxicity ± SEM assessed in a 4-h Cr^{51}-release assay. E:T ratio was 100:1. Splenic NK activity from control bg/+ mice is shown by hatched box (▨), while the stippled box (▩) represents control bg/bg mice. Asterisk (*) indicates that splenic NK activity was significantly different from the appropriate bg/bg or bg/+ control.

controls (Fig. 1). Beige (bg/bg) mice died 15–19 days after i.v. challenge with 1×10^5 *C. neoformans*.

When the challenge inoculum was reduced 10-fold to 1×10^4 *C. neoformans*, the kinetics of NK cell augmentation in bg/+ mice were altered. In bg/+ mice, augmented splenic NK activity (172% increase) was not observed until day 14 after i.v. challenge (Fig. 1). Cryptococcal infection did not alter the splenic NK activity of bg/bg mice i.v. challenged with 1×10^4 *C. neoformans* when compared with uninfected bg/bg controls (Fig. 1).

Pathogenesis of Encapsulated *C. neoformans* for Bg/bg and Bg/+ Mice

The spleen was cultured to assess whether modulated NK activity corresponded with altered growth of *C. neoformans* in infected tissues. We also cultured the lungs and brain, two important target organs, to further monitor the course of systemic cryptococcosis. On days 7 and 14 after i.v. challenge with 1×10^5 yeasts, bg/bg mice had significantly higher ($P < 0.05$) *C. neoformans* CFU in their spleens than bg/+ mice (Table 1). The course of cryptococcosis in the lungs and brain of bg/bg and bg/+ mice challenged with 10^5 cryptococci was progressive. With the exception of the lungs on day 14, no differences in the number of *C. neoformans* CFU in the lungs and brain of bg/bg and bg/+ mice were observed.

When the challenge inoculum was reduced 10-fold to 1×10^4 C neoformans, bg/bg mice had significantly higher ($P < .05$) CFU in their spleen and lungs on days 3, 7, and 14 and in their brains on day 14 after challenge than bg/+ mice (Table 1).

TABLE 1. Pathogenesis of Encapsulated *C. neoformans* in Bg/bg and Bg/+ Mice

Inoculum[a]	Days after i.v. challenge	Spleen		Lung		Brain	
		bg/bg	bg/+	bg/bg	bg/+	bg/bg	bg/+
10^5	1	4.1 ± 0.1[b]	4.1 ± 0.1	5.1 ± 0.2	5.3 ± 0.1	3.1 ± 0.1	2.6 ± 0.3
	7	5.1 ± 0.1[c]	3.9 ± 0.3	7.1 ± 0.1	6.8 ± 0.3	7.5 ± 0.1	7.5 ± 0.1
	14	6.4 ± 0.5[c]	4.4 ± 0.4	8.4 ± 0.1[c]	7.6 ± 0.2	8.0 ± 0.1	8.3 ± 0.2
	21	*[d]	6.5 ± 0.1	*	8.7 ± 0.2	*	8.3 ± 0.5
10^4	1	2.3 ± 0.9	0.8 ± 0.8	4.3 ± 0.3	2.9 ± 0.5	2.7 ± 0.1	2.4 ± 0.1
	3	2.4 ± 0.8[c]	0	4.5 ± 0.4[c]	3.2 ± 0.4	4.5 ± 0.2	4.2 ± 0.1
	7	5.5 ± 0.1[c]	2.4 ± 1.2	7.4 ± 0.1[c]	6.4 ± 0.5	7.0 ± 0.1	6.8 ± 0.4
	14	6.2 ± 0.3[c]	3.4 ± 0.5	7.6 ± 0.1[c]	6.4 ± 0.5	7.8 ± 0.1[c]	7.3 ± 0.1

[a] Mice were i.v. challenged with either 1×10^4 or 1×10^5 *C. neoformans* SLHA.
[b] Data are expressed as the Log_{10} CFU/g dry wt ± SEM. N = 3/group or 6/group for mice challenged with 1×10^4 or 1×10^5 cryptococci, respectively.
[c] Beige (bg/bg) mice had significantly higher CFU ($P < .05$) than similarly treated bg/+ mice at indicated time intervals.
[d] Bg/bg mice died 15-19 days after i.v. challenge with 1×10^5 *C. neoformans*.

Competitive Inhibition

Cold target competitive inhibition assays were carried out to assess whether the presence of viable *C. neoformans* in splenic effector cell preparations from infected mice would alter NK-mediated cytolysis of YAC-1 cells. Viable *C. neoformans* CFU in splenic effector cell preparations from bg/bg mice i.v. challenged with either 10^4 or 10^5 cryptococci were ≤2 cells/well on days 1, 3, and 7 after i.v. challenge and were ≤500 cells/well on day 14. In splenic effector cell preparations from bg/+ mice i.v. challenged with 10^4 or 10^5 *C. neoformans*, viable CFU were ≤2 cells/well on days 1, 3, and 7 and ≤100 CFU on days 14 and 21. Based on these data, H:C target ratios of 1:1 and 1:10 were chosen to assess the effect of *C. neoformans* on tumor cytotoxicity. A 1:1 H:C target ratio represented maximal competition by *C. neoformans* in cytotoxicity assays from infected mice, while 1:10 H:C target ratio represented at least 10-fold excess. Addition of unlabeled YAC-1 tumor cells to cytotoxicity assays significantly inhibited ($P < 0.01$) lysis of chromated YAC-1 tumor cells at H:C target ratios of 1:1 (36% reduction) and 1:10 (90% reduction; Table 2). In contrast, addition of unlabeled *C. neoformans* to cytotoxicity assays did not affect the lysis of chromated YAC-1 tumor targets (Table 2).

TABLE 2. Competitive Inhibition Assays

Unlabeled competitor	Hot: cold target ratios[a]		
	1:0	1:1	1:10
YAC-1	20.3 ± 0.2[b]	12.9 ± 1.6[c]	2.0 ± 0.3[c]
C. neoformans	17.6 ± 0.5	18.1 ± 1.9	18.8 ± 1.7

[a] Splenic effector cells from bg/+ mice were added to unlabeled "cold" competitor and chromated "hot" YAC-1 targets. The effector:chromated target ratio was 100:1.
[b] Data are expressed as mean % cytotoxicity ± SEM from six mice.
[c] Significant reduction ($P < 0.01$) in % cytotoxicity when compared to 1:0 hot:cold target ratio.

In Vivo Depletion of NK Cells

From the above studies, it appeared as if lower *C. neoformans* CFU in the spleen and lungs of bg/+ than in bg/bg mice corresponded with the presence of functional NK cells in vivo. To more specifically evaluate the role of NK cells in resistance to *C. neoformans* we used anti-NK-1.1 mAb to deplete NK cells in vivo. To evaluate the effectiveness of NK cell depletion, uninfected bg/+ mice were treated in vivo with anti-NK-1.1 mAb or control ascites and splenic NK activity was assessed 2 days later. After anti-NK-1.1-treatment, there was a significant ($P < 0.001$) reduction (85%) in splenic NK activity in 6-h Cr^{51}-release assays at all E:T ratios (Fig. 2). When the incubation time of the Cr^{51}-release assay was increased to 18-h, there was an increase in cytotoxic activity from ascites control-treated, but not anti-NK-1.1-treated, bg/+ mice. In 18-h Cr^{51}-release assays, the splenic NK activity of anti-NK-1.1-treated bg/+ mice remained significantly reduced (95%; $P < 0.001$) compared with ascites control-treated bg/+ mice (Fig. 2). Thus, in vivo treatment with anti-NK-1.1 mAb resulted in a drastic reduction in the in vitro cytotoxic activity of splenic NK cells.

To assess the duration of NK cell depletion, bg/+ mice were given 200 μg of anti-NK-1.1 mAb and splenic NK activity was monitored 2, 5, 9, and 16 days after treatment. Two days after treatment with anti-NK-1.1 mAb, splenic NK activity was again significantly ($P < 0.001$) reduced (85%; Fig. 3). This reduction (85–95%) of splenic NK activity was maintained through day 16 after anti-NK-1.1-treatment. When IL-2 was added to NK assays (in vitro), no recovery of splenic NK activity was observed when spleen cells from NK-1.1-treated mice were used (Fig. 3).

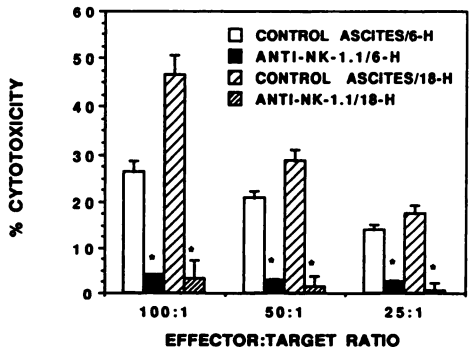

Fig. 2. In vivo depletion of splenic NK activity using mAb to NK-1.1. Bg/+ mice were injected (i.v.) with 200 μg of NK-1.1 mAb (■,▨) or 200 μg of control ascites (□,▨). Killing of YAC-1 tumor targets was assessed in a 6-h (□,■) or 18-h (▨,▨) Cr^{51}-release assay. The data represent the mean % cytotoxicity from three mice ± SEM. Asterisk (*) indicates anti-NK-1.1-treatment significantly reduced ($P < 0.001$) splenic NK activity compared with the appropriate control.

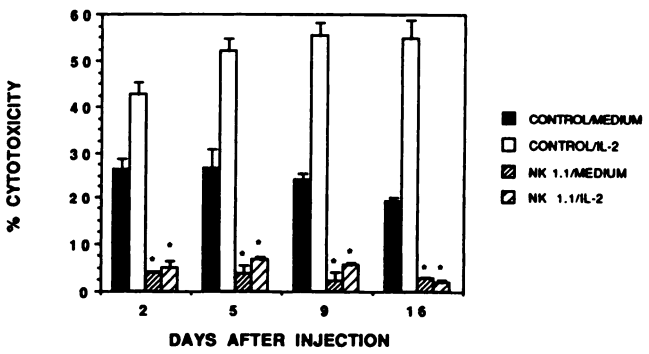

Fig. 3. Sustained in vivo depletion of splenic NK activity in mice treated with mAb to NK-1.1. Bg/+ mice were injected (i.v.) with 200 μg of control ascites (■,□) or anti-NK-1.1 mAb (▨,▨) and splenic NK activity was monitored 2, 5, 9, and 16 days after treatment. In NK assays, splenic effectors were incubated in vitro with medium (■,▨) or 50 U IL-2/well (□,▨) for 6 h. Data are expressed as the mean % cytotoxicity ± SEM from three mice. Asterisk (*) denotes NK-1.1-treatment significantly reduced ($P < 0.001$) splenic NK activity compared with appropriate ascites-treated control.

Splenic NK Activity in NK-Depleted *C. neoformans*-Infected Mice

Splenic NK activity was monitored during the course of *C. neoformans* infection in anti-NK-1.1-treated and ascites control-treated mice (Fig. 4). Both bg/bg and bg/+ mice treated with anti-NK-1.1 had significantly lower ($P < 0.001$) splenic NK activity (range in % cytotoxicity was 0.1–3.2%) than their respective controls throughout the 14 day study. In *C. neoformans*-infected bg/+ control mice, NK activity was significantly higher ($P \leq 0.02$) on day 14 than on days 1, 3, and 7 (Fig. 4).

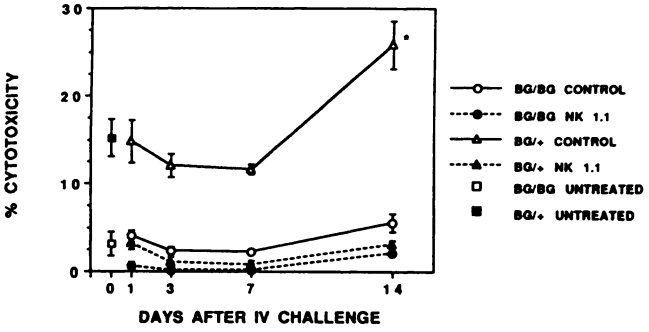

Fig. 4. Effect of anti-NK-1.1-treatment on augmentation of splenic NK activity during *C. neoformans* infection in bg/bg (○,●) and bg/+ (△,▲) mice. Mice were treated (i.v.) with 200 μg of anti-NK-1.1 (●,▲) or 200 μg of control ascites (○, △) 2 days prior to (day-2) i.v. challenge with 8×10^3 *C. neoformans* (day 0). Untreated bg/bg (□) and bg/+ (■) mice were also used as controls. Each data point represents the mean % cytotoxicity at an E:T of 100:1 ± SEM as assessed in a 6-h Cr^{51}-release assay. Six mice were used per group for each timepoint.

Effect of NK-1.1 mAb Treatment on the Susceptibility of Bg/+ and Bg/bg Mice to *C. neoformans*

The effect of NK cell depletion on the susceptibility to cryptococcosis was assessed. Both bg/bg and bg/+ mice were treated with either anti-NK-1.1 mAb or control ascites (day -2), i.v. challenged with 8×10^3 *C. neoformans* (day 0), and their internal organs were cultured on days 1, 3, 7, and 14 (Fig. 5). Anti-NK-1.1-treated bg/+ mice had significantly higher ($P < 0.05$) *C. neoformans* CFU in their lungs on days 3 and 7 after cryptococcal challenge than control bg/+ mice (1.5 and 1.4 logs greater, respectively). By day 14, similar numbers of *C. neoformans* were cultured from the lungs of anti-NK-1.1-treated and control bg/+ mice. Conversely, no differences in *C. neoformans* CFU in the lungs of anti-NK-1.1-treated and control bg/bg mice were observed at any culture timepoint. Anti-NK-1.1-treatment did not alter the growth of *C. neoformans* in the spleens of either bg/bg or bg/+ mice at any culture timepoint (Fig. 5). Additionally, no differences between anti-NK-1.1-treated and control mice (bg/bg or bg/+) were observed in the liver, kidneys, and brain at any culture interval.

DISCUSSION

Results from these studies show that: 1) splenic NK activity is augmented in bg/+, but not in bg/bg mice during cryptococcal infection; 2) the kinetics of NK cell augmentation was dependent on the dose of *C. neoformans* injected; 3) in vivo administration of mAb NK-1.1

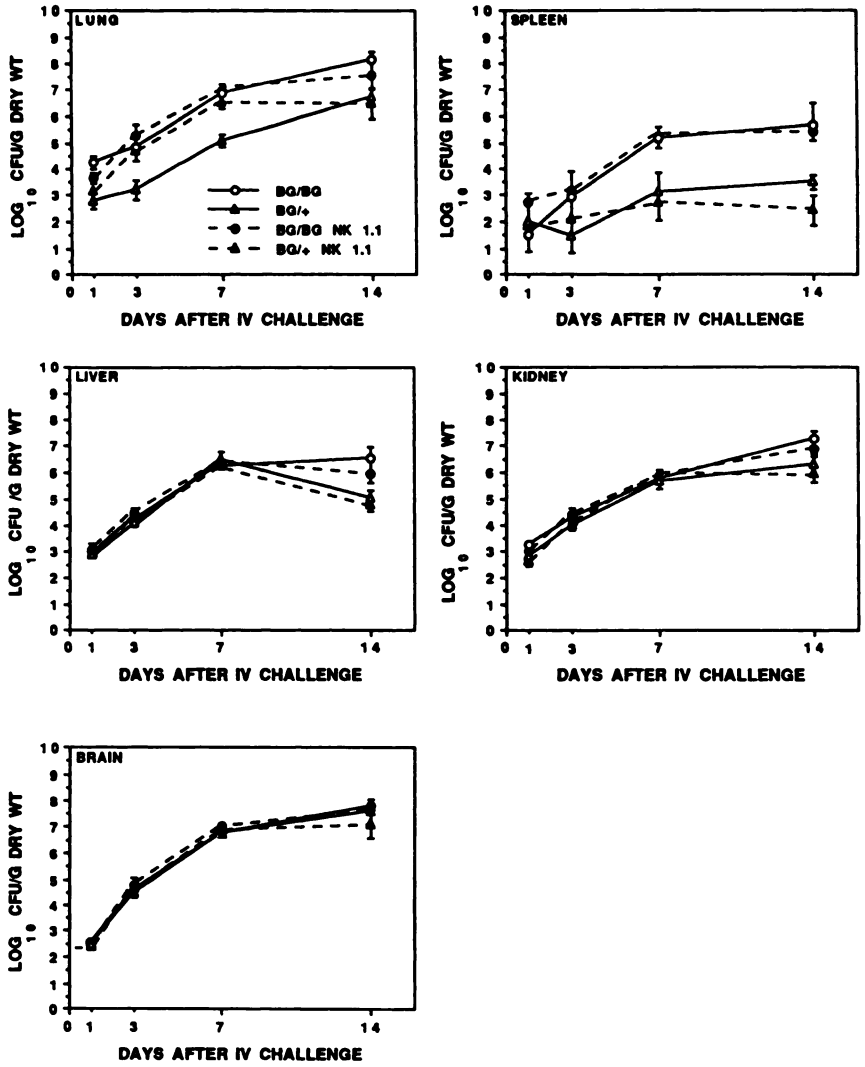

Fig. 5. Effect of anti-NK-1.1-treatment on recovery of *C. neoformans* from internal organs of infected bg/bg (○,●) and bg/+ (△,▲) mice. Mice were treated (i.v.) with 200 μg of anti-NK-1.1 (●,▲) or 200 μg of control ascites (○,△) 2 days prior to (day-2) i.v. challenge with 8 × 10^3 *C. neoformans* (day 0). Each data point represents the mean Log_{10} CFU/g dry wt ± SEM from six mice per group for each timepoint.

to bg/+ mice resulted in depletion of NK cells and abrogation of the augmented NK cell activity observed during *C. neoformans* infection; 4) an early but transient enhancement of the growth of *C. neoformans* was observed in the lungs of NK-1.1-treated bg/+ mice; 5) in vivo depletion of NK cells did not appear to alter the number of *C. neoformans* in the spleen or other extrapulmonary organs of bg/+ mice; and 6) in vivo depletion of NK cells from bg/bg mice did not enhance their susceptibility to *C. neoformans*.

These studies clearly demonstrate the splenic NK activity is augmented following infection of bg/+ mice with *C. neoformans*. The kinetics of splenic NK cell modulation in bg/+ mice was dependent on the dose of *C. neoformans* administered. Splenic NK activity was augmented earlier (day 7) in bg/+ mice challenged with a higher inoculum (10^5), than with a lower challenge inoculum (10^4). Despite apparent differences in the kinetics of NK modulation, the increase in % cytotoxicity (200% and 172% increase), was similar after both challenge inocula (10^5, 10^4, respectively). These data suggest that alterations in the regulation of NK cells occurs during *C. neoformans* infection and that these alterations may vary depending on the severity of the infection. Augmented NK cell activity following *C. neoformans* infection, which was dose dependent, has also been observed in nu/nu and nu/+ BALB/c mice [33]. Although other studies have indicated that NK cells from

bg/bg mice can be augmented in vivo under some experimental conditions [4,16,23], cryptococcal infection did not augment the splenic NK cell activity of bg/bg mice.

The mechanism by which *C. neoformans* augmented NK cell activity was not pursued in these experiments. The functional activity of resting NK cells, however, is readily enhanced by cytokines like IL-2 and IFN [1,11,36]. In a previous study we reported that treatment of nu/nu and nu/+ BALB/c mice with mAb to IFN-γ abrogated the augmentation of NK cell activity induced by *C. neoformans* infection. In addition to augmenting splenic NK activity, *C. neoformans* infection was also immunosuppressive. Suppressed NK activity was associated with progressive disease and was only observed late in the course of infection (day 21). Other investigators have demonstrated that NK cell activity can be suppressed by both T cells and Mϕs [5,38]. While it is not known if either of these cell populations mediated the suppression of NK cell activity observed in these studies, induction of T_S cell networks by *C. neoformans* is well described [25].

In these studies, the NK competence of bg/+ mice appeared to correlate with reduced *C. neoformans* CFU in the lungs and spleen of infected bg/+ mice at most culture timepoints compared with infected NK-deficient bg/bg mice (Fig. 1, Table 1). Our culture data from *C. neoformans*-challenged bg/bg and bg/+ mice corroborated the work of Hidore and Murphy [13] which showed that bg/bg mice had an early enhanced susceptibility to systemic cryptococcosis as indicated by higher *C. neoformans* CFU in their lungs and spleen 3 days after i.v. challenge than bg/+ mice. Due to the multiple defects associated with the beige gene, however, it was unclear which cellular defect (NK cell, PMN, and/or Mϕ) was responsible for the enhanced susceptibility of bg/bg mice to *C. neoformans* infection. Hidore and Murphy reported that in vivo peritoneal exudate responses, in vitro phagocytosis, and in vitro growth inhibition of *C. neoformans* by PMNs and Mϕs were similar between bg/bg and bg/+ mice suggesting that the multiple phagocytic cell defects in bg/bg mice were not responsible for altered in vivo clearance of *C. neoformans*. Conversely, we reported in a previous study that in situ inflammatory responses to *C. neoformans* were delayed in bg/bg mice 14 days after i.v. challenge indicating that the defects in PMNs and Mϕs associated with the beige gene has a visible impact (in vivo) on the susceptibility of beige mice to systemic cryptococcosis [34].

The reason for the apparent differences in phagocytic cell competence between these studies is not known. During the course of cryptococcal infection a complex series of events including chemotaxis to the site of infection, phagocytosis, killing, and the release and response to a variety of biological response modifiers is involved in in vivo clearance of this pathogen. Delays in any one of a combination of these events in beige mice could result in reduced in vivo clearance of *C. neoformans* from infected organs. To date, it is not known if phagocytic cells from bg/bg and bg/+ mice differ in their capacity to generate and respond to cytokines involved in inducing and maintaining a host immune response in vivo.

To directly assess the role of NK cells in in vivo resistance to *C. neoformans* in this study, we treated bg/bg and bg/+ mice with mAb to NK-1.1 to specifically deplete NK cells in vivo. In vivo treatment with mAb NK-1.1 resulted in a sustained depletion of NK cell activity. These data confirm the work of other investigators which also demonstrated sustained depletion of NK cells in the spleen and lungs following mAb NK-1.1-treatment [18,20]. In the latter studies and ours, NK cell activity in NK depleted mice could not be enhanced by IL-2, IFN, or IFN-inducers [18–20]. Moreover, the depletion is specific for NK cells and does not alter the in vivo distribution of T-cell subsets in the spleen nor cellular and humoral immune responses [35].

In these studies, anti-NK-1.1-treatment of bg/+ mice resulted in increased burden of *C. neoformans* in the lungs. Enhanced pulmonary susceptibility of NK-depleted bg/+ mice was early (days 3 and 7) and transient. This suggests that NK cells can play an early role in host defense against *C. neoformans* in the lungs. Moreover, *C. neoformans* CFU in the lungs of NK-depleted bg/+ mice were similar to those in infected bg/bg mice indicating that the increased CFU in the lungs of bg/bg mice was very likely due to defects in NK cells and not to defects in Mϕ and PMN function. By day 14, however, any protective effect related to early NK cell function was negated since no differences in lung CFU were observed in anti-NK-1.1-treated and ascites control bg/+ mice. Thus, increased CFU in the lungs of bg/bg mice observed late in the infection (day 14) are probably due to defects in PMN and Mϕ function. This was corroborated by histopathological examination of *C. neoformans*-infected tissues which revealed reduced and delayed phagocytic cell function in the lungs of bg/bg mice 14 days after i.v. challenge when compared with bg/+ mice [34].

The administration of mAb NK-1.1 did not appear to affect the growth of *C. neoformans* in the spleen or other internal organs of bg/+ mice. Previous work in bg/bg and bg/+ mice associated the presence of functional NK cells in bg/+ mice with enhanced clearance of *C. neoformans* from both the lungs and spleen of bg/+ compared with bg/bg mice [13]. Results from this study indicate that in the spleen, enhanced clearance observed in bg/+ mice is apparently not due to the presence of functional NK cells and that increased CFU in the spleens of bg/bg mice is probably related to defects in PMNs and Mϕs. In vivo

depletion of NK cells also did not alter growth of *C. neoformans* in the liver, kidneys, and brain of bg/+ mice. These data extend and confirm the observations of Lipscomb et al. [20] who reported that anti-NK-1.1 mAb-treated wild-type C57BL/6 mice had higher *C. neoformans* CFU in the lungs, but not extrapulmonary organs, 24 h after i.v. challenge than saline-treated controls. In the latter study, however, organ CFU were not monitored after 24 h [20]. Depletion of NK cells from C57BL/6 mice in the latter study also did not result in altered mortality rates following i.v. challenge with *C. neoformans* compared with infected controls nor altered clearance of *C. neoformans* from the lungs, spleen, or brain on days 7 and 28 after intratracheal challenge [20].

NK cells from bg/bg mice have reduced lytic capacity, however, the defect is not complete and bg/bg NK cells can be activated by IFN, IFN inducers, injection of tumors, and infection with virus or parasites [4,14, 16,23]. Additionally, in vitro levels of splenic NK activity from uninfected bg/bg mice against YAC-1 targets increase as the assay incubation period increases (unpublished observations) indicating that the lytic capability of bg/bg NK cells may be partially related to delays in the kinetics of target cell lysis. Based on the latter observations, we delineated whether the NK cells in bg/bg mice had any impact on clearance of *C. neoformans*. In this study, the growth of *C. neoformans* in all the internal organs in anti-NK-1.1-treated and control bg/bg mice was similar indicating that bg/bg NK cells did not affect the in vivo growth of *C. neoformans*.

In summary, we have shown that NK cell activity is modulated during cryptococcal infection in bg/+ mice. Also, NK cells very likely play an early role in host defense against *C. neoformans* which appears to be limited to the lung. As cryptococcal infection progresses, however, other effector cells in the immune system appear to play a more predominant role in controlling *C. neoformans*.

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