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A Monoclonal Antibody to Gamma Interferon Blocks Augmentation of Natural Killer Cell Activity Induced during Systemic Cryptococcosis

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These studies demonstrate that the cytotoxic activity of splenic natural killer (NK) cells is augmented in both *nu/nu* and *nu/+* mice during systemic cryptococcosis. Both the kinetics and the regulation of NK cell activity differed in *Cryptococcus neoformans*-infected *nu/nu* and *nu/+* mice. Greater augmentation was observed following challenge with 10^5 cells than with smaller inocula, and augmented NK cell activity was not always associated with enhanced control of systemic cryptococcosis. Infection with a nonencapsulated strain of *C. neoformans* induced an early but transient increase in splenic NK cell activity in *nu/nu* and *nu/+* mice. Injection of capsular polysaccharide induced a transient augmentation of splenic NK cell activity in *nu/+* mice but caused a persistent increase in splenic NK cell activity in *nu/nu* mice. In vivo treatment with monoclonal antibody to gamma interferon abrogated the augmentation of splenic NK cell activity induced during cryptococcal infections in both *nu/nu* and *nu/+* mice and enhanced the susceptibility of *nu/+* mice to *C. neoformans* to a greater extent than it did that of *nu/nu* mice. These results suggest that gamma interferon is an important mediator of resistance to *C. neoformans*.

Cryptococcosis is a fungal disease of increasing clinical importance. Because *Cryptococcus neoformans* is prominent in the environment, exposure to airborne organisms is probably common. Cryptococcal infections in the general population are thought to be primarily subclinical; however, in immunocompromised individuals, *C. neoformans* is a major cause of disseminated fungal disease (19, 29). The apparent high rate of exposure in conjunction with the low incidence of clinical disease in the general population suggests that innate immune responses play an important role in host defense against cryptococcal infections.

Numerous in vitro studies have demonstrated that polymorphonuclear neutrophils and macrophages (Mϕ) play an important role in innate immunity to *C. neoformans* (8, 11, 13, 21). Recent studies suggest that natural killer (NK) cells may also be involved in innate host defenses against cryptococcal infections. Murine NK cells bind to and inhibit the growth of *C. neoformans* in vitro (26, 27). Additionally, several in vivo studies have suggested that NK cells may be involved in the clearance of *C. neoformans* from infected tissues. Beige (*bg/bg*) mice, which have defects in NK cells, polymorphonuclear neutrophils, and Mϕ (4, 32), had more *C. neoformans* in their lungs and spleen 3 days after infection than did immunocompetent *bg/+* mice (15, 32). Reduced numbers of *C. neoformans* in the lungs and spleen were also observed following adoptive transfer of NK cell-enriched spleen cells to cyclophosphamide-treated mice as compared with mice receiving NK cell-depleted spleen cells (14). Cauley and Murphy (6) also reported that *nu/nu* mice, which have higher NK cell activity than do *nu/+* mice, had an early enhanced clearance of cryptococci from infected tissues. In all of these in vivo studies, enhanced clearance of *C. neoformans* from infected tissues was observed in the mouse model that was either NK cell competent or had greater NK cell activity. These results suggest that NK cells may be important effector cells in early host resistance to *C. neoformans*.

Previous in vivo studies assessed NK cell activity prior to cryptococcal challenge and not during the course of *C. neoformans* infection. The immunomodulatory effects of cryptococcal infection are well documented (reviewed in reference 24) and influence innate, humoral, and cell-mediated immune responses. Given the diversified range of effects that *C. neoformans* and cryptococcal antigens have on the immune system, we felt that it was important to monitor NK cell activity during the course of a cryptococcal infection. In addition, we assessed whether the effects of a *C. neoformans* infection on NK cell activity differed between T-cell-deficient (*nu/nu*) and immunocompetent (*nu/+*) hosts. In this study, we show that (i) splenic NK cell activity was augmented during the course of a cryptococcal infection in both *nu/nu* and *nu/+* mice, (ii) augmentation of splenic NK cell activity was dependent on the dose of encapsulated *C. neoformans* injected, (iii) the kinetics of splenic NK cell augmentation during *C. neoformans* infection differed in *nu/nu* and *nu/+* mice, (iv) augmented splenic NK cell activity was also induced by a nonencapsulated strain of *C. neoformans* and by capsular polysaccharide, (v) in vivo augmentation of splenic NK cell activity in both *nu/nu* and *nu/+* mice could be abrogated by treatment with monoclonal antibody (MAb) to gamma interferon (IFN-γ), (vi) in vivo treatment with MAb to IFN-γ enhanced the susceptibility of *nu/+* mice to *C. neoformans* to a greater extent than it did that of *nu/nu* mice, and (vii) increased NK cell activity was not always associated with enhanced control of *C. neoformans* growth in vivo.

MATERIALS AND METHODS

Mice. Inbred germ-free *nu/nu* and *nu/+* BALB/c mice between 8 and 12 weeks of age were used in this study.

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Animals were obtained from the University of Wisconsin Gnotobiotic Laboratory (Madison) and were maintained in accordance with National Institutes of Health guidelines. On the day each experiment was started, mice were removed from the germ-free isolator, fed sterilized food and water, and maintained in sterile cages with filter bonnets in a laminar flow hood.

Target cells and medium. The tissue culture medium used in all experiments 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.

Yeast cultures and cryptococcal antigens. An encapsulated strain of *C. neoformans* (SLHA; serotype A) and a nonencapsulated strain (M7) were used in this study (5). The encapsulated strain was a human clinical isolate obtained from the State Laboratory of Hygiene at the University of Wisconsin. The nonencapsulated strain was provided by G. Bulmer, University of Oklahoma, Oklahoma City. Yeast cells were maintained on Sabouraud's dextrose agar and passaged weekly. For inoculation, yeast cells 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 nonpyrogenic saline, counted on a hemacytometer, and adjusted to the appropriate inoculum. For verification of the number of viable cells, the inoculum was serially diluted in phosphate-buffered saline, plated on Sabouraud's dextrose agar, and incubated at 37°C for 48 h, and CFU were determined. Capsular polysaccharide was prepared from *C. neoformans* SLHA by an ethanol precipitation procedure (3). The concentration of polysaccharide was determined by the anthrone reaction (18), while protein was assessed by the Lowry procedure (23). Capsular preparations contained 1.7 mg of polysaccharide per ml and 250 μg of protein per ml. Mice were given either viable *C. neoformans* (strain SLHA or M7, 0.1 ml) or capsular polysaccharide (0.2 ml) by the intravenous (i.v.) route.

Antibody depletion. The rat immunoglobulin G1 MAb to IFN-γ was produced as ascites in pristane-treated *nu/nu* BALB/c mice injected with hybridoma R4-6A2 (American Type Culture Collection, Rockville, Md.). MAb R4-6A2 was purified by high-pressure liquid chromatography and was a gift of C. Czuprynski, University of Wisconsin, Madison. The concentration of rat immunoglobulin G1 was quantified by an enzyme-linked immunosorbent assay. Mice were given 200 μg of MAb R4-6A2 in 0.15 ml of nonpyrogenic saline or saline without the antibody by the intraperitoneal (i.p.) route 1 day prior to (day −1) i.v. challenge with 10^4 *C. neoformans* (day 0) and 100 μg (0.1 ml) 10 days after cryptococcal challenge.

NK cell assay. Splenic NK cell activity was assessed in a standard 4-h Cr^{51} release assay as previously described (2). In brief, spleens were aseptically removed and placed in 10 ml of RPMI 1640, and single-cell suspensions were prepared. Spleen cell preparations from infected mice were divided into two portions. One portion (2.5 ml) was used to assess *C. neoformans* CFU as described below; splenic effector cells for NK cell assays were prepared from the second portion (7.5 ml). In NK cell assays, spleen cells from saline-treated controls were run concomitantly with spleen cells from *C. neoformans*-challenged or polysaccharide-injected mice. Spleen cell preparations for NK cell 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-to-target (E:T) ratios 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 for 4 h at 37°C in a humidified incubator (5% CO_2). Supernatants were collected by using a 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 12% maximum release. Under the clean microisolator conditions used to house *nu/nu* and *nu/+* mice, the splenic NK cell activity of saline-treated controls remained stable over the 2-week experiment. For assessment of the number of viable *C. neoformans* cells in splenic effector cell preparations from infected mice, a portion of the effector cell preparation was serially diluted and plated on Sabouraud's dextrose agar, and CFU were determined.

Microbial enumeration. The kidneys, liver, lungs, and brain were removed and homogenized in 5 ml of phosphate-buffered saline. Homogenates were serially diluted in phosphate-buffered saline and plated in duplicate on Sabouraud's dextrose agar, and colonies were counted after incubation for 48 h at 37°C. Data are expressed as the mean $\log_{10}$ number of *C. neoformans* cells per gram (dry weight) of each tissue homogenate from three to six mice per group for each culture interval. Dry weight was obtained by placing 1 ml of tissue homogenate in an aluminum pan and drying it at 60°C for 24 h. Statistical differences in *C. neoformans* CFU between *nu/nu* mice and their *nu/+* littermates were determined with Student's *t* test and analysis of variance.

Competitive inhibition. A standard 4-h Cr^{51} release assay was set up as described above at an E:T ratio of 100:1, with the following modifications. Splenic effector cells were prepared from *nu/nu* mice given saline (0.1 ml; i.p.) or poly(I · C) (100 μg; 0.1 ml; i.p.) 24 h prior to the assay. The splenic effector cell concentration was 200,000 cells in 0.1 ml. Unlabeled ("cold") *C. neoformans* or YAC-1 tumor cells were added to wells (0.05 ml) so that the ratio of labeled ("hot") tumor targets to cold targets (hot-to-cold [H:C] ratio) was 1:1, 1:10, or 1:100 (2,000, 20,000, or 200,000 competitors, respectively; 0.05 ml). Cytotoxicity was assessed as described above. Control wells (H:C ratio, 1:0) contained effector cells, chromated YAC-1 tumor targets, and no competitor. The addition of an unlabeled competitor did not alter spontaneous or maximum release from chromated YAC-1 targets.

RESULTS

Augmentation of splenic NK cell activity. Athymic (*nu/nu*) mice challenged i.v. with 10^5 encapsulated yeast cells had augmented splenic NK cell activity as early as day 3, and further increases were evident on days 7 and 10 (Fig. 1A). By day 14, all remaining *nu/nu* mice had died. Following i.v. challenge with 10^5 yeast cells, *nu/+* mice also had augmented splenic NK cell activity as early as day 3; however, the activity appeared to peak earlier in *nu/+* mice (day 7) than in *nu/nu* mice and then declined on days 10 and 14 (Fig. 1B). While augmented NK cell activity was greater in *nu/nu* mice than in *nu/+* mice (10^5), the increase in percent cytotoxicity over that in the appropriate saline-treated control was similar (286 and 270%, respectively; Fig. 1A).

When the challenge inoculum was reduced 10-fold to 10^4,

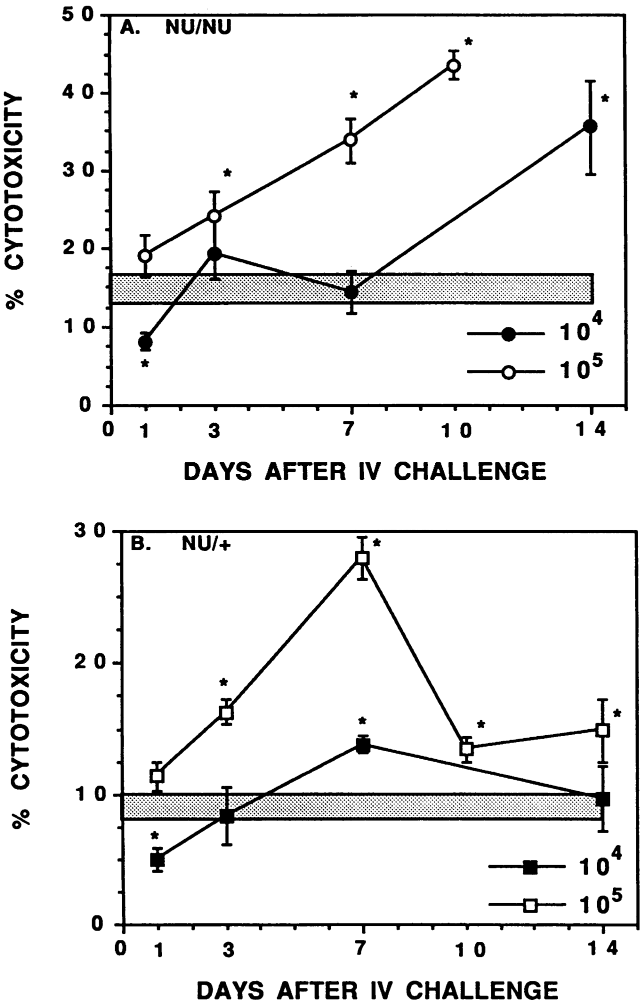

FIG. 1. Augmentation of splenic NK cell activity during *C. neoformans* infection. Data are expressed as the mean percent cytotoxicity ± standard error of the mean for three or six mice (following i.v. challenge with 10^4 or 10^5 *C. neoformans* SLHA cells, respectively). The E:T ratio was 100:1. The range of splenic NK cell activity in saline-treated controls, which were assayed on the same day as were *C. neoformans*-challenged mice, is shown by the stippled box. Asterisks indicate that splenic NK cell activity in *C. neoformans*-infected mice was significantly different ($P < 0.05$) from that in saline-treated mice assayed on the same day. Results are representative of two experiments at each challenge inoculum. (A) Splenic NK cell activity in *nu/nu* mice. (B) Splenic NK cell activity in *nu/+* mice.

the kinetics of splenic NK cell modulation were altered in both *nu/nu* and *nu/+* mice. Splenic NK cell activity was suppressed on day 1 but returned to control levels on day 3 in both *nu/nu* and *nu/+* mice (Fig. 1B). Augmented splenic NK cell activity was observed on days 7 and 14 for *nu/+* and *nu/nu* mice, respectively. At this lower challenge inoculum, the overall magnitude of the increase in percent cytotoxicity was reduced (230 and 150% increases for *nu/nu* and *nu/+* mice, respectively; Fig. 1).

Pathogenesis of *C. neoformans* SLHA in *nu/nu* and *nu/+* mice. The spleen was cultured to determine whether modulated NK cell 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. By day 3 after challenge with 10^5 yeast cells, higher *C. neoformans* CFU were observed in the spleens of *nu/nu* mice than in those of *nu/+* mice; however, the increased CFU in *nu/nu* mice were not statistically significant from the CFU in *nu/+* mice until day 10 (Table 1). The course of systemic cryptococcosis in the lungs and brains of *nu/nu* and *nu/+* mice challenged with 10^5 yeast cells was progressive, and no differences in the numbers of *C. neoformans* CFU in the lungs and brains of *nu/nu* and *nu/+* mice were observed at any culture time point (Table 1).

When the inoculum was reduced 10-fold to 10^4, differences in *C. neoformans* CFU between *nu/nu* and *nu/+* mice became evident at early times after challenge. Significantly higher numbers of *C. neoformans* CFU were observed in the spleens of *nu/nu* mice than in those of *nu/+* mice by day 3, and the increased CFU in *nu/nu* mice were evident throughout the 14-day study (Table 1). Additionally, at the lower challenge inoculum (10^4), *nu/+* mice had a greater capacity to control the growth of *C. neoformans* in their lungs and brains than did *nu/nu* mice. Athymic (*nu/nu*) mice had significantly higher ($P < 0.05$) *C. neoformans* CFU than did *nu/+* mice on days 7 and 14 in the brain and on day 14 in the lungs (Table 1).

Competitive inhibition. Cold-target competitive inhibition experiments were carried out to assess whether the presence of *C. neoformans* in splenic effector cell preparations from infected mice modulated NK cell cytolysis of YAC-1 cells. Splenic NK effector cells were obtained from *nu/nu* BALB/c mice injected with saline or poly(I · C) to enhance NK cell activity in vivo (36). Quantitation of *C. neoformans* CFU in splenic effector cell preparations from *nu/+* mice challenged i.v. with 10^5 yeast cells demonstrated that few viable cryptococci (≤2 cells per well) were present in the cytotoxicity assay on days 1, 3, 7, and 10 and that there were ≈500 cells per well on day 14. On days 1 and 3 after i.v. challenge with 10^5 yeast cells, *C. neoformans* CFU in *nu/nu* splenic effector preparations were ≤2 cells per well, and on days 7 and 10, the CFU did not exceed 1,000 cells per well. No *C. neoformans* CFU were detected in splenic effector cell preparations from *nu/+* mice infected with 10^4 yeast cells at any culture time point. Cryptococci were not detected in splenic effector cell preparations from *nu/nu* mice challenged with 10^4 yeast cells until day 14, when there were ≤350 *C. neoformans* cells per well. On the basis of the latter data, H:C ratios of 1:1, 1:10, and 1:100 were chosen to assess the effect of *C. neoformans* on NK cell cytotoxicity. A 1:1 H:C target ratio would represent maximal competition by *C. neoformans* in cytotoxicity assays with spleen cells from infected mice, while 1:10 and 1:100 H:C target ratios would represent a 10- to 100-fold excess of *C. neoformans*. When unlabeled (cold) YAC-1 targets were incorporated into the cytotoxicity assay, lysis of chromated YAC-1 targets was dramatically inhibited at H:C target ratios of 1:1 (30 to 50% inhibition), 1:10 (60 to 70% inhibition), and 1:100 (85 to 93% inhibition) (Fig. 2A and B). When *C. neoformans* (SLHA) was used as the cold competitor, no inhibition of the cytotoxicity of splenic effector cells from saline-treated mice was observed at an H:C target ratio of 1:1; however, 20% inhibition and 81% inhibition were observed at H:C target ratios of 1:10 and 1:100, respectively (Fig. 2A). Although *C. neoformans* reduced the cytotoxicity of spleen cells from poly(I · C)-treated mice at a 1:1 H:C ratio, significantly reduced lysis ($P < 0.05$) was observed only at H:C ratios of 1:10 (23% reduction) and 1:100 (22% reduction) (Fig. 2B).

In vivo induction of splenic NK cell activity by infection with

TABLE 1. Pathogenesis of an encapsulated strain of *C. neoformans* for *nu/nu* and *nu/+* BALB/c mice

Inoculum[a]	Day(s) after i.v. challenge	No. of *C. neoformans* CFU in[b]: Spleen		Lungs		Brain	
		nu/nu	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*
10^5	1	4.1 ± 0.3	4.3 ± 0.1	4.6 ± 0.5	4.5 ± 0.3	3.3 ± 0.1	3.5 ± 0.1
	3	5.3 ± 0.2	4.5 ± 0.5	5.3 ± 0.3	5.2 ± 0.4	5.4 ± 0.1	5.2 ± 0.1
	7	5.9 ± 0.6	5.1 ± 0.2	6.9 ± 0.6	7.1 ± 0.1	8.2 ± 0.2	8.0 ± 0.1
	10	6.4 ± 0.4[c]	5.0 ± 0.1	7.8 ± 0.5	7.8 ± 0.3	8.8 ± 0.2	8.5 ± 0.1
	14	—[d]	6.2 ± 0.6	—	8.5 ± 0.3	—	8.9 ± 0.2
10^4	1	1.9 ± 0.6	1.4 ± 0.6	3.1 ± 0.2	3.1 ± 0.2	1.1 ± 0.5	1.6 ± 0.3
	3	1.5 ± 0.7[c]	0	4.7 ± 0.2	4.8 ± 0.2	3.9 ± 0.2	4.2 ± 0.2
	7	2.7 ± 0.8[c]	0.6 ± 0.6	6.6 ± 0.4	6.4 ± 0.4	6.7 ± 0.1[c]	5.2 ± 0.8
	14	5.4 ± 0.3[c]	0.8 ± 0.5	7.7 ± 0.2[c]	5.9 ± 0.4	8.1 ± 0.1[c]	7.6 ± 0.1

[a] Mice were challenged i.v. with either 10^4 or 10^5 *C. neoformans* SLHA cells.
[b] Data are expressed as the mean $\log_{10}$ *C. neoformans* CFU per gram (dry weight) ± standard error of the mean for three or six mice challenged with 10^4 or 10^5 cells, respectively. Results are representative of two experiments at each challenge dose.
[c] Nude mice had significantly higher CFU ($P < 0.05$) than did similarly treated *nu/+* mice at the indicated times.
[d] —, Mortality. Six nude mice died 9 to 12 days after i.v. challenge.

a nonencapsulated strain. We assessed whether infection with a nonencapsulated strain (M7) of *C. neoformans* would also result in augmented splenic NK cell activity. Splenic NK cell activity was enhanced in both *nu/nu* and *nu/+* mice as early as 1 day after i.v. challenge with 10^4 *C. neoformans* M7 cells (Fig. 3). By day 3 in *nu/nu* mice and day 7 in *nu/+* mice, splenic NK cell activities had returned to the levels observed with spleen cells from saline-injected controls.

Clearance of nonencapsulated *C. neoformans* M7 from infected *nu/nu* and *nu/+* mice. In addition to assessing splenic NK cell activity, we also cultured the spleens, lungs, and brains of M7-infected mice (Table 2). Similar declining numbers of *C. neoformans* M7 cells were cultured from the spleens of both *nu/nu* and *nu/+* mice. Clearance of the nonencapsulated strain from the lungs and brains of both *nu/nu* and *nu/+* mice was also evident (Table 2).

Augmentation of splenic NK cell activity by capsular polysaccharide. From the previous experiments, it was unclear whether the inability of *nu/nu* and *nu/+* mice to maintain augmented levels of splenic NK cell activity following i.v. challenge with strain M7 was related to the lack of a capsule or the inability of the nonencapsulated strain to produce a progressive infection. To assess whether the capsule was capable of augmenting splenic NK cell activity, we injected *nu/nu* and *nu/+* mice i.v. with 250 μg of capsular polysaccharide obtained from encapsulated strain SLHA.

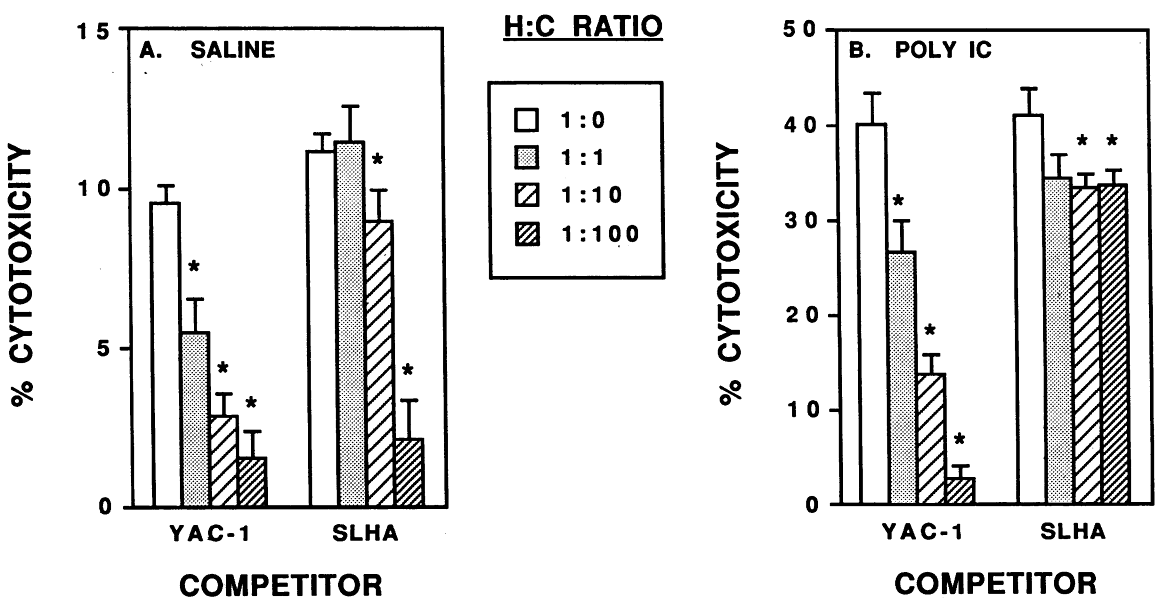

FIG. 2. Cold-target competitive inhibition assay. Splenic effector cells from *nu/nu* mice treated in vivo with either saline (0.1 ml; i.p.) or poly(I · C) (100 μg; 0.1 ml; i.p.) were incubated with chromated (hot) YAC-1 tumor targets. Unlabeled (cold) YAC-1 or *C. neoformans* (strain SLHA) cells were added at H:C ratios of 1:0 (no cold targets), 1:1, 1:10, and 1:100. Cytotoxicity was assessed at E:T ratios (hot) of 100:1. Data represent the mean percent cytotoxicity ± standard error of the mean for six mice. Asterisks denote a significant decrease ($P < 0.05$) in the percent cytotoxicity as compared with that in controls with no cold competitor (H:C ratio, 1:0). Results are representative of three experiments.

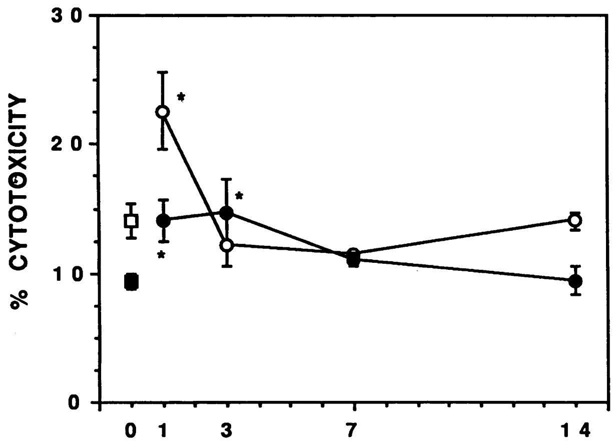

FIG. 3. In vivo induction of splenic NK cell activity following infection with a nonencapsulated strain of *C. neoformans*. *nu/nu* (○) and *nu/+* (●) BALB/C mice were challenged i.v. with 10^4 *C. neoformans* M7 cells, while *nu/nu* (□) and *nu/+* (■) controls were given saline. Saline-treated controls were assayed on the same day as were *C. neoformans*-challenged mice. Since splenic NK cell activity from saline-treated controls was stable over the 2-week study, splenic NK cell activity in control mice is shown on day 0. Each datum point represents the mean percent cytotoxicity ± standard error of the mean for an experiment in which three mice were used for each time point. Asterisks denote results significantly greater ($P < 0.05$) than those for the respective saline-treated controls assayed on the same day.

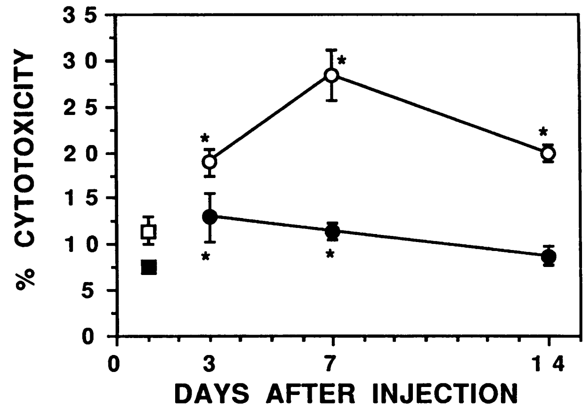

FIG. 4. Augmentation of splenic NK cell activity by cryptococcal capsular polysaccharide. *nu/nu* (○) and *nu/+* (●) mice were injected i.v. with 250 μg of polysaccharide, while *nu/nu* (□) and *nu/+* (■) controls received saline. Saline-treated controls were assayed on the same day as were *C. neoformans*-challenged mice. Since splenic NK cell activity from saline-treated controls was stable over the 2-week study, splenic NK cell activity in control mice is shown on day 0. Data are expressed as the mean percent cytotoxicity ± standard error of the mean for an experiment in which three mice were used for each time point. Asterisks denote percent cytotoxicity significantly greater ($P < 0.05$) than that in the respective saline-treated controls assayed on the same day.

Enhanced splenic NK cell activity ($P < 0.05$) was observed 3 days after the injection of capsular polysaccharide, and this augmentation persisted on days 7 and 14 ($P < 0.05$) (Fig. 4). In *nu/+* mice, an early enhancement of splenic NK cell activity occurred on days 3 and 7 ($P < 0.05$), and a return to the level of splenic NK cell activity observed in saline-treated *nu/+* control mice occurred on day 14.

In vivo treatment with MAb to IFN-γ abrogates augmented splenic NK cell activity. Since IFN-γ is one of the major regulatory factors of NK cell activity both in vivo and in vitro (20, 36), we considered the possibility that the augmented splenic NK cell activity of *C. neoformans*-infected mice may be due to the ability of a *C. neoformans* infection to induce IFN-γ production. To test this hypothesis, we gave *nu/nu* and *nu/+* mice 200 μg of an anti-IFN-γ MAb 1 day prior to (day −1) and 100 μg 10 days after (day 10) i.v. challenge with 6×10^3 *C. neoformans* SLHA cells (day 0). Control mice were given saline prior to cryptococcal challenge. At the lower *C. neoformans* challenge inoculum of 6×10^3 cells, peak splenic NK cell activity in saline-treated control *nu/nu* and *nu/+* mice occurred on day 14 after i.v. challenge. The administration of the anti-IFN-γ MAb completely abrogated the augmentation of splenic NK cell activity observed in both *nu/nu* and *nu/+* mice 14 days after i.v. challenge with *C. neoformans* (Table 3). We had also observed splenomegaly in both *nu/nu* and *nu/+* mice 14 days after *C. neoformans* challenge (Table 3). Treatment with the anti-IFN-γ MAb reduced splenomegaly in *nu/nu* but not *nu/+* mice during the course of systemic cryptococcosis (Table 3).

Pathogenesis of *C. neoformans* in IFN-γ-depleted mice. We also assessed whether the injection of an MAb to IFN-γ would alter the susceptibility of *nu/nu* and *nu/+* mice to systemic cryptococcosis. Significantly higher ($P < 0.05$) numbers of *C. neoformans* CFU were observed only in the livers of anti-IFN-γ MAb-treated *nu/nu* mice on days 3, 7, and 14 after cryptococcal challenge, as compared with saline-treated *nu/nu* mice (0.4, 0.7, and 1.3 log units higher, respectively) (Fig. 5). Conversely, in vivo treatment with the MAb to IFN-γ did not alter the growth of *C. neoformans* in the spleens and lungs (Fig. 5) or the kidneys and brains (data

TABLE 2. Pathogenesis of a nonencapsulated strain of *C. neoformans* for *nu/nu* and *nu/+* mice

Day(s) after i.v. challenge[a]	No. of *C. neoformans* CFU in[b]:					
	Spleen		Lungs		Brain	
	nu/nu	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*
1	3.5 ± 0.3	3.6 ± 0.1	3.2 ± 0.3	3.1 ± 0.1	1.2 ± 0.6	0.7 ± 0.7
3	3.3 ± 0.3	3.8 ± 0.1	0.8 ± 0.8	0.8 ± 0.8	0.6 ± 0.6	0.7 ± 0.7
7	2.2 ± 1.1	3.1 ± 0.2	0	0	0	0.6 ± 0.6
14	2.3 ± 1.2	2.0 ± 1.0	0	0	0	0

[a] Mice were challenged i.v. with 10^4 *C. neoformans* M7 cells.
[b] Data are expressed as the mean $\log_{10}$ *C. neoformans* CFU per gram (dry weight) ± standard error of the mean for three mice. Results are representative of two experiments.

TABLE 3. Abrogation of splenic NK cell augmentation and splenomegaly during a *C. neoformans* infection by in vivo administration of an anti-IFN-γ MAb

Genotype	Treatment[a]	*C. neoformans* infection[b]	% Cytotoxicity[c]	Spleen % body weight[d]
nu/nu	None	−	15.6 ± 1.7	0.48 ± 0.03
	Saline	+	29.3 ± 3.3[e]	0.96 ± 0.16[e]
	Anti-IFN-γ MAb	+	9.7 ± 2.3[f]	0.64 ± 0.01[e,f]
nu/+	None	−	8.5 ± 0.4	0.46 ± 0.02
	Saline	+	13.2 ± 0.6[e]	0.64 ± 0.01[e]
	Anti-IFN-γ MAb	+	8.0 ± 0.1[f]	0.65 ± 0.03[e]

[a] Mice were given either nonpyrogenic saline or the anti-IFN-γ MAb as described in Materials and Methods and challenged i.v. with 6 × 10^3 *C. neoformans* SLHA cells.
[b] Mice were assayed 14 days after i.v. challenge with *C. neoformans* SLHA.
[c] Data are expressed as the mean percent cytotoxicity ± standard error of the mean for an experiment in which three mice were used per group. The E:T ratio was 100:1.
[d] The spleen as a percentage of body weight was calculated as follows: spleen wet weight (g)/body weight (g) × 100.
[e] Significantly higher ($P < 0.05$) than the respective untreated control.
[f] Significantly lower ($P < 0.05$) than the respective saline-treated control.

not shown) of *C. neoformans*-infected *nu/nu* mice (as compared with saline-treated controls) at any culture time point. By day 14 after *C. neoformans* infection, *nu/+* mice treated with the anti-IFN-γ MAb had significantly higher ($P < 0.05$) *C. neoformans* CFU in the spleens, lungs, and livers than did saline-treated *nu/+* mice (1.9, 1.4, and 2.4 log units higher, respectively). No differences in the numbers of *C. neoformans* cells were observed in the brains and kidneys of *nu/+* mice treated with either the MAb to IFN-γ or saline at all culture time points (data not shown).

DISCUSSION

The results of this study demonstrate that NK cell activity is augmented in both *nu/nu* and *nu/+* mice during infection with encapsulated *C. neoformans*. The kinetics and duration of splenic NK cell augmentation were dose dependent. Maximal and prolonged enhancement of NK cell activity was observed after *nu/nu* mice were challenged i.v. with 10^5 *C. neoformans* cells. Additionally, the immunomodulatory effects of cryptococcal infection on NK cell activity differed between *nu/nu* and *nu/+* mice, since the apparent peak in NK cell activity was observed earlier (day 7) in *nu/+* mice than in *nu/nu* mice (day 14). Also, *nu/+* mice, but not *nu/nu* mice, had the capacity to down regulate NK cell activity late (day 14) in the course of systemic cryptococcosis. Moreover, the increase in percent cytotoxicity over that in saline controls was greater in *nu/nu* mice than in *nu/+* mice following i.v. challenge with 10^4 *C. neoformans* cells. Collectively, these data suggest that the modulation and regulation of NK cell activity that occur during a *C. neoformans* infection are dependent upon the immune status of the host and the severity of the infection.

Murphy and co-workers (16, 26, 27) have shown that murine NK cells can bind to and inhibit the growth of *C. neoformans* in vitro. Competitive inhibition assays were performed to assess whether *C. neoformans* in spleen effector cell preparations from infected mice would inhibit NK cell cytolysis of YAC-1 tumor targets by competing for binding sites on NK cells. Overall, our findings suggest that

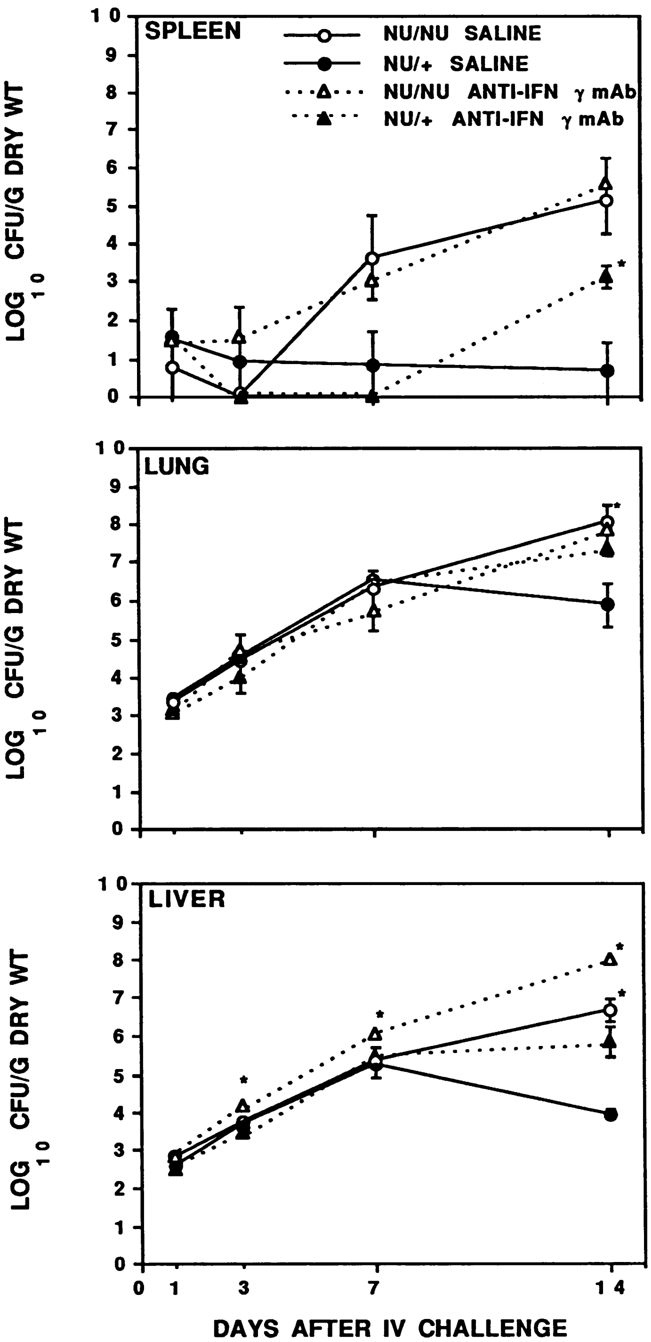

FIG. 5. Pathogenesis of *C. neoformans* in *nu/nu* and *nu/+* mice treated in vivo with an anti-IFN-γ MAb. Mice were treated with saline or the anti-IFN-γ MAb as described in Materials and Methods and challenged i.v. with 6 × 10^3 *C. neoformans* SLHA cells. Each datum point represents the mean $\log_{10}$ *C. neoformans* CFU per gram (dry weight) ± standard error of the mean for an experiment with three mice at each time point. When not visible, error bars were smaller than the symbol. Asterisks indicate that anti-IFN-γ MAb-treated mice had significantly higher ($P < 0.05$) *C. neoformans* CFU than did respective saline-treated controls.

NK cell activity is unaffected by the small numbers of viable *C. neoformans* cells present in cytotoxicity assays with spleen cells from infected mice.

Cryptococci exist in nature in an nonencapsulated to poorly encapsulated state (9, 30). It is this poorly encapsulated state which, upon inhalation into the lungs and production of a capsule (9, 12), is thought to initiate a cryptococcal infection. Thus, we assessed whether a nonencapsulated strain of *C. neoformans* could also modulate NK cell activity. Infection with a nonencapsulated strain of *C. neoformans* caused an early but transient increase in NK cell activity in both *nu/nu* and *nu/+* mice, suggesting that cell wall components can modulate the activity of NK cells. From this study, it was unclear whether the inability of *nu/nu* and *nu/+* mice to maintain augmented splenic NK cell activity was related to the lack of capsule production or the inability of the nonencapsulated strain to produce a progressive infection.

The ability of capsular polysaccharide to suppress the immune response is well established. Investigators have shown that capsular polysaccharide can impair leukocyte migration (22), inhibit phagocytosis (35), and reduce humoral responses (20, 25). Data from our study clearly demonstrate that capsular polysaccharide can also be a potent augmentor of NK cell activity, particularly in *nu/nu* mice. Several important differences in the responses of *nu/nu* and *nu/+* mice to capsular polysaccharide were observed. First, the kinetics of NK cell augmentation varied; the splenic NK cell activity in *nu/+* mice peaked earlier (day 3) than did that in *nu/nu* mice (day 7). Second, the magnitude of the increase in NK cell activity after polysaccharide challenge was greater in *nu/nu* mice than in *nu/+* mice, as compared with saline controls; the maximal percent increase in NK cell activity observed for *nu/nu* mice on day 7 was 250%, while only a 160% increase in NK cell activity was observed for *nu/+* mice on day 3. Finally, augmented NK cell activity persisted in *nu/nu* mice but not in *nu/+* mice. The reason why augmented NK cell activity did not persist in *nu/+* mice is unclear but may be related to the altered clearance of capsular polysaccharide and/or the ability of T cells to control NK cell responses in *nu/+* mice.

From our data, it is also apparent that endogenous IFN-γ plays a role in augmenting NK cell activity in both *nu/nu* and *nu/+* mice during a cryptococcal infection. This conclusion was supported by the lower splenic NK cell activity 14 days after *C. neoformans* challenge in mice treated with an MAb to IFN-γ than in mice injected with saline. There is no evidence as yet to indicate which lymphoid cell population is responsible for endogenous IFN-γ production. $CD4^+$ lymphocytes are typically thought to be the primary producers of IFN-γ and may have been partially responsible for IFN-γ production in T-cell-competent *nu/+* mice. The production of IFN-γ in *C. neoformans*-infected *nu/nu* mice suggests that cells other than $CD4^+$ lymphocytes secreted IFN-γ. Two potential candidates are NK cells and $\gamma\delta$-T cells, since both are capable of IFN-γ secretion and are present in *nu/nu* mice (7, 17, 34). Early in vivo production of IFN-γ by a T-cell-independent mechanism (1) has also been observed following infection with *Listeria monocytogenes* (1, 28).

Our study also demonstrates that IFN-γ plays an important role in resistance to *C. neoformans* in a T-cell-competent host (*nu/+* mice). The enhanced susceptibility of anti-IFN-γ MAb-treated mice was not observed until late in the infection and was observed only in organs rich in lymphoid cells and/or tissue Mϕ such as the spleen, lungs, and liver. The type of cells responsible for the enhanced clearance of *C. neoformans* from *nu/+* mice is not known, but several lines of evidence suggest the involvement of Mϕ. In a previous study, we reported that large numbers of Mϕ were evident in inflammatory foci in the lungs and livers of *C. neoformans*-infected *nu/+* mice 14 days after i.v. challenge (33). In vitro studies have demonstrated that Mϕ activated by IFN-γ have enhanced cidal activity for *C. neoformans* (11, 21). In contrast to *nu/+* mice, *nu/nu* mice treated with an MAb to IFN-γ had increased burdens of *C. neoformans* in the liver but showed no alteration of the growth of *C. neoformans* in the other internal organs. Enhanced growth of *C. neoformans* in the livers of *nu/nu* mice was evident as early as day 3 and persisted on days 7 and 14. In *nu/nu* mice, IFN-γ was apparently produced in sufficient quantities to augment NK cell activity in vivo. The type of effector cell responsible for the enhanced clearance of *C. neoformans* in the livers of *nu/nu* mice is not known but is likely to be Mϕ, since NK cell activity is generally very low in the liver (14). In a previous study, we reported that *C. neoformans*-infected *nu/nu* mice could develop low-level chronic inflammation consisting of Mϕ in their livers (33). Induction of this chronic inflammatory response, which could be induced by the production of IFN-γ, may explain the lower numbers of *C. neoformans* CFU in the livers of saline-treated *nu/nu* mice than in those of anti-IFN-γ MAb-treated *nu/nu* mice.

Previous studies by other investigators have shown an association between NK cell activity and in vivo clearance of *C. neoformans* from the spleens and lungs of infected mice (14, 15). Despite dramatically enhanced NK cell activity during the course of a *C. neoformans* infection, in this study we observed no strong association between enhanced in vivo clearance of *C. neoformans* and enhanced NK cell activity. In fact, at times, enhanced growth of *C. neoformans* corresponded to enhanced NK cell activity while enhanced clearance of *C. neoformans* coincided with reduced or declining NK cell activity. One possible explanation for the lack of a correlation between enhanced NK cell activity and enhanced control of *C. neoformans* growth is that as NK cells become activated they may become less effective in controlling *C. neoformans* growth. Data from our competitive inhibition assays suggest that *C. neoformans* is a more effective competitor when effector cell preparations contain resting or nonactivated (Fig. 2A) rather than activated (Fig. 2B) NK cells. Thus, as NK cells become activated during the course of a cryptococcal infection, their affinity for *C. neoformans* in vivo may be reduced. From this study, it is clear that more work is needed to define the in vivo role of NK cells in immunity to *C. neoformans*.

Immunocompetent and congenitally immunodeficient gnotobiotic mice are excellent animal models with which to study immunity to *C. neoformans*. It is well established that infection with a variety of microorganisms can modulate NK cell activity, as well as other components of the immune system (10, 31). Conventionally reared athymic mice have an enhanced susceptibility to infection with viruses and *Pneumocystis carinii* (31, 37) that could result in the modulation of their NK cell activity. We were interested in the capacity of *C. neoformans* to modulate NK cell activity in the absence of these complicating factors. To eliminate these variables, we used germ-free mice as a "microbially controlled" model system. Microbially controlled conditions make studies of murine responses to systemic cryptococcosis particularly relevant, since the mice manifest host responses that are not altered by opportunistic infections.

In summary, these data demonstrate that *C. neoformans* infection augments NK cell activity and that the augmentation appears to be associated with endogenous IFN-γ production in both *nu/nu* and *nu/+* mice. Moreover, IFN-γ production in a T-cell-competent host is an important mediator of resistance to *C. neoformans*. Further studies are needed to define the role of NK cells in host defense, immunoregulation, and immunopathology during *C. neoformans* infections.

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REFERENCES

1. **Bancroft, G. J., R. D. Schreiber, G. C. Bosma, M. J. Bosma, and E. R. Unanue.** 1987. A T cell-independent mechanism of macrophage activation by interferon-γ. J. Immunol. **139:**1104–1107.
2. **Bartizal, K. F., C. Salkowski, and E. Balish.** 1983. The influence of a gastrointestinal microflora on natural killer cell activity. J. Reticuloendothel. Soc. **33:**381–390.
3. **Bhattacharjee, A. K., K. J. Kwon-Chung, and C. P. J. Glaudemans.** 1978. On the structure of the capsular polysaccharide from *Cryptococcus neoformans* serotype C. Immunochemistry **15:**673–679.
4. **Brandt, E. J., R. T. Swank, and E. K. Novak.** 1981. The murine Chédiak-Higashi mutation and other murine pigmentation mutations, p. 99–117. *In* M. E. Gershwin and B. Merchant (ed.), Immunologic defects in laboratory animals, vol. 1. Plenum Press, New York.
5. **Bulmer, G. S., M. D. Sans, and C. M. Gunn.** 1967. *Cryptococcus neoformans*. I. Nonencapsulated mutants. J. Bacteriol. **94:**1475–1479.
6. **Cauley, L. K., and J. W. Murphy.** 1979. Response of congenitally athymic (nude) and phenotypically normal mice to *Cryptococcus neoformans* infection. Infect. Immun. **23:**644–651.
7. **De Geus, B., M. Van den Enden, C. Coolen, L. Nagelkerken, P. Van der Heijden, and J. Rozing.** 1990. Phenotype of intraepithelial lymphocytes in euthymic and athymic mice: implications for differentiation of cells bearing a CD3-associated γδ T cell receptor. Eur. J. Immunol. **20:**291–298.
8. **Diamond, R. O., R. K. Root, and J. E. Bennett.** 1972. Factors influencing killing of *Cryptococcus neoformans* by human leukocytes *in vitro*. J. Infect. Dis. **125:**367–376.
9. **Farhi, F., G. S. Bulmer, and J. R. Tacker.** 1970. *Cryptococcus neoformans*. IV. The not-so-encapsulated yeast. Infect. Immun. **1:**526–531.
10. **Fitzgerald, P. A., and C. Lopez.** 1986. Natural killer cells active against viral, bacterial, protozoan and fungal infections, p. 107–131. *In* E. Lotzova and R. B. Herberman (ed.), Immunobiology of natural killer cells, vol. II. CRC Press, Inc., Boca Raton, Fla.
11. **Flesch, I. E., G. Schwamberger, and S. H. E. Kaufmann.** 1989. Fungicidal activity of IFN-γ-activated macrophages. Extracellular killing of *Cryptococcus neoformans*. J. Immunol. **142:**3219–3224.
12. **Granger, D. L., J. R. Perfect, and D. T. Durack.** 1985. Virulence of *Cryptococcus neoformans*. Regulation of capsule synthesis by carbon dioxide. J. Clin. Invest. **76:**508–516.
13. **Granger, D. L., J. R. Perfect, and D. T. Durack.** 1986. Macrophage-mediated fungistasis *in vitro*: requirements for intracellular and extracellular cytotoxicity. J. Immunol. **136:**672–680.
14. **Hidore, M. R., and J. W. Murphy.** 1986. Correlation of natural killer cell activity and clearance of *Cryptococcus neoformans* from mice after adoptive transfer of splenic nylon wool-nonadherent cells. Infect. Immun. **51:**547–555.
15. **Hidore, M. R., and J. W. Murphy.** 1986. Natural cellular resistance of beige mice against *Cryptococcus neoformans*. J. Immunol. **137:**3624–3631.
16. **Hidore, M. R., and J. W. Murphy.** 1989. Murine natural killer cell interactions with a fungal target, *Cryptococcus neoformans*. Infect. Immun. **57:**1990–1997.
17. **Janis, E. M., S. H. E. Kaufmann, R. H. Schwartz, and D. M. Pardoll.** 1989. Activation of T cells in the primary immune response to *Mycobacterium tuberculosis*. Science **244:**713–716.
18. **Kabat, E. A., and M. M. Mayer (ed.).** 1961. Experimental immunochemistry, p. 526–537. Charles C Thomas, Publisher, Springfield, Ill.
19. **Kofler, H., and P. Fritsch.** 1989. Cryptococcosis in the immunocompromised host. Curr. Probl. Dermatol. **18:**193–202.
20. **Kozel, T. R., W. F. Gulley, and J. Cazin, Jr.** 1977. Immune response to *Cryptococcus neoformans* soluble polysaccharide: immunological unresponsiveness. Infect. Immun. **18:**701–707.
21. **Levitz, S. M., and D. J. DiBenedetto.** 1988. Differential stimulation of murine resident peritoneal cells by selectively opsonized encapsulated and acapsular *Cryptococcus neoformans*. Infect. Immun. **56:**2544–2551.
22. **Louria, D. B., T. Kaminski, and G. Finkel.** 1963. Further studies on immunity in experimental cryptococcosis. J. Exp. Med. **117:**509–520.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
24. **Murphy, J. W.** 1989. Immunoregulation in cryptococcosis, p. 319–399. *In* E. Kurstak (ed.), Immunology of fungal disease. Marcel Dekker, Inc., New York.
25. **Murphy, J. W., and G. C. Cozad.** 1972. Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque technique. Infect. Immun. **5:**896–901.
26. **Murphy, J. W., and D. O. McDaniel.** 1982. *In vitro* reactivity of natural killer (NK) cells against *Cryptococcus neoformans*. J. Immunol. **28:**1577–1583.
27. **Nabavi, N., and J. W. Murphy.** 1985. In vitro binding of natural killer cells to *Cryptococcus neoformans* targets. Infect. Immun. **50:**50–57.
28. **Nakane, A., A. Numata, M. Asano, M. Kohanawa, Y. Chen, and T. Minagawa.** 1990. Evidence that endogenous gamma interferon is produced early in *Listeria monocytogenes* infection. Infect. Immun. **58:**2386–2388.
29. **Perfect, J. R.** 1989. Cryptococcosis. Infect. Dis. Clin. North Am. **3:**77–102.
30. **Powell, K. E., B. A. Dahl, R. J. Weeks, and F. E. Tosh.** 1972. Airborne *Cryptococcus neoformans*: particles from pigeon excreta compatible with alveolar deposition. J. Infect. Dis. **125:**412–415.
31. **Quimby, F. W.** 1989. Immunodeficient rodents. A guide to their immunobiology, husbandry and use. National Academy Press, Washington, D.C.
32. **Salkowski, C. A., and E. Balish.** Can. J. Microbiol., in press.
33. **Salkowski, C. A., and E. Balish.** J. Leukocyte Biol., in press.
34. **Scala, G., J. Y. Djeu, P. Allavera, T. Kasahara, J. R. Ortaldo, R. B. Herberman, and J. J. Openheim.** 1986. Cytokine secretion and noncytotoxic functions of human large granular lymphocytes, p. 134–144. *In* E. Lotzova and R. B. Herberman (ed.), Immunobiology of natural killer cells, vol. II. CRC Press, Inc., Boca Raton, Fla.
35. **Small, J. M., and T. G. Mitchell.** 1989. Strain variation in antiphagocytic activity of capsular polysaccharides from *Cryptococcus neoformans* serotype A. Infect. Immun. **57:**3751–3756.
36. **Talmadge, J. E., R. B. Herberman, M. A. Chirigos, A. E. Maluish, M. A. Schneider, J. S. Adams, H. Philips, G. B. Thurman, L. Varesio, C. Long, R. K. Oldham, and R. H. Wiltrout.** 1985. Hyporesponsiveness to augmentation of murine natural killer cell activity in different anatomical compartments by multiple injections of various immunomodulators including recombinant interferons and interleukin 2. J. Immunol. **135:**2483–2491.
37. **Walzer, P. D., C. K. Kim, M. J. Linke, C. L. Pogue, M. J. Huerkamp, C. E. Chrisp, A. V. Lerro, S. K. Wixson, E. Hall, and L. D. Shultz.** 1989. Outbreaks of *Pneumocystis carinii* pneumonia in colonies of immunodeficient mice. Infect. Immun. **57:**62–70.