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Gamma Interferon Production Is Critical for Protective Immunity to Infection with Blood-Stage *Plasmodium berghei* XAT but Neither NO Production nor NK Cell Activation Is Critical

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We have examined the roles of gamma interferon (IFN-γ), nitric oxide (NO), and natural killer (NK) cells in the host resistance to infection with the blood-stage malarial parasite *Plasmodium berghei* XAT, an irradiation-induced attenuated variant of the lethal strain *P. berghei* NK65. Although the infection with *P. berghei* XAT enhanced NK cell lytic activity of splenocytes, depletion of NK1.1$^+$ cells caused by the treatment of mice with anti-NK1.1 antibody affected neither parasitemia nor IFN-γ production by their splenocytes. The *P. berghei* XAT infection induced a large amount of NO production by splenocytes during the first peak of parasitemia, while *P. berghei* NK65 infection induced a small amount. Unexpectedly, however, mice deficient in inducible nitric oxide synthase (iNOS$^{-/-}$) cleared *P. berghei* XAT after two peaks of parasitemia were observed, as occurred for wild-type control mice. Although the infected iNOS$^{-/-}$ mouse splenocytes did not produce a detectable level of NO, they produced an amount of IFN-γ comparable to that produced by wild-type control mouse splenocytes, and treatment of these mice with neutralizing anti-IFN-γ antibody led to the progression of parasitemia and fatal outcome. CD4$^{-/-}$ mice infected with *P. berghei* XAT could not clear the parasite, and all these mice died with apparently reduced IFN-γ production. Furthermore, treatment with carrageenan increased the susceptibility of mice to *P. berghei* XAT infection. These results suggest that neither NO production nor NK cell activation is critical for the resistance to *P. berghei* XAT infection and that IFN-γ plays an important role in the elimination of malarial parasites, possibly by the enhancement of phagocytic activity of macrophages.

Both cell-mediated immunity and humoral immunity play important roles in the mechanisms of defense against intracellular pathogens. Although these defense mechanisms largely depend on antigen-specific T helper (Th) cell activation, early innate mechanisms associated with natural killer (NK) cells, cytokines, and nitric oxide (NO) produced by phagocytic cells are also important.

Cytolytic activity and gamma interferon (IFN-γ) production of NK1.1$^+$ cells were shown to be important for innate resistance to a variety of pathogens (1). Strains of mice resistant to *Plasmodium chabaudi* infection were reported to exhibit high NK cell activity (1), and NK cells were suggested to play a role in protection from malarial parasites (14, 23, 34, 39). However, resistance to *P. chabaudi* and *P. vinckei petteri* infection was shown not to be impaired in beige mutant C57BL/6 mice with reduced NK cell activity (19, 33, 45). Thus, the role of NK cells in the protection against malarial infection has not been elucidated.

NO produced by the activation of inducible nitric oxide synthase (iNOS) has been indicated to be an important effector molecule to kill a variety of pathogens, since iNOS antagonists inhibited macrophage killing of pathogens in vitro and in vivo (6, 16, 20). NO production pathways have been shown to be activated by IFN-γ or lipopolysaccharide in various types of cells, including macrophages (37), endothelial cells (24), and hepatocytes (21). Resistance to blood-stage *P. chabaudi* AS infection was reported to correlate with the amount of NO produced by splenocytes at an early stage of the infection (12). Moreover, adoptive transfer of a Th1 clone was shown to protect the host from *P. chabaudi* AS infection, and an NO-dependent mechanism was asserted to play a critical role in the protection, because treatment with an iNOS inhibitor, aminoguanidine, made mice susceptible to the infection and the increase in susceptibility was correlated with reduction in serum NO_2^- level (12). Furthermore, administration of recombinant interleukin-12 (IL-12) was reported to promote resistance to *P. chabaudi* AS infection via an NO-dependent mechanism (36). We have recently shown that IL-12 produced by splenocytes plays an important role in protection against infection with *P. berghei* XAT, an irradiation-induced attenuated variant of the lethal strain *P. berghei* NK65, through stimulation of IFN-γ production (46). In liver-stage infection, IFN-γ produced by CD8$^+$ T cells was shown to stimulate NO production of liver cells (31). The authors of that study proposed that the NO production is critical for the destruction of infected hepatocytes. The administration of recombinant IL-12 was indicated to cure *P. yoelii* sporozoite infection of mice by stimulating the production of IFN-γ and NO (30).

In the present study, we have examined the roles of IFN-γ, NO, and NK1.1$^+$ cells in the host defense against *P. berghei* XAT infection by using iNOS-deficient (iNOS$^{-/-}$) mice and mice depleted of NK1.1$^+$ cells by treatment with anti-NK1.1. Our results indicate that neither NO nor NK1.1$^+$ cells play a crucial role in the resistance against *P. berghei* XAT infection,

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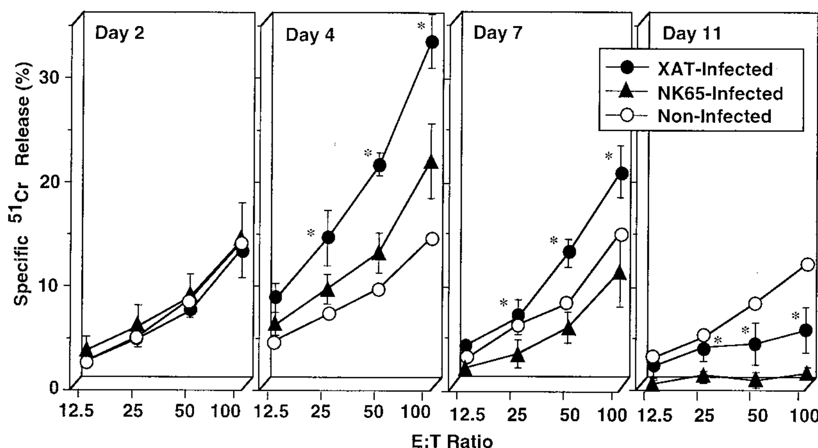


FIG. 1. Increased NK cell lytic activity in splenocytes caused by the infection with blood-stage *P. berghei* XAT but not with *P. berghei* NK65. After i.v. inoculation of normal mice with 10^4 PRBC, splenocytes were obtained at various time intervals and assayed for NK cell lytic activity by using YAC-1 cells as a target. The percentages of spontaneous ^{51}Cr release in assays of splenocytes obtained on days 2, 4, 7, and 11 were 5.7, 6.1, 10.7, and 5.7% of the maximum release, respectively. Data are means ± SD for three mice. *, $P < 0.05$, compared with data for NK65-infected mice. These results were confirmed to be reproducible by performing a repeat experiment. E:T, effector-to-target-cell.

although the *P. berghei* XAT infection induced NO production and NK cell activation more efficiently than the infection with *P. berghei* NK65. IFN-γ was indicated to play a critical role in the resistance against *P. berghei* XAT infection. Although the cells that produced IFN-γ in mice with *P. berghei* XAT infection were not formally identified, $CD4^+$ cells were suggested to play a role.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). $iNOS^{-/-}$ mice backcrossed onto C57BL/6 mice were kindly provided by J. D. MacMicking and C. Nathan (Cornell University Medical College, New York, N.Y.) and J. S. Mudgett (Merck Research Laboratories, Rahway, N.J.) (17); in some experiments, we also used $iNOS^{-/-}$ mice purchased from Jackson Laboratory, Bar Harbor, Maine (15). $CD4^{-/-}$ mice on C57BL/6 background were a generous gift from T. W. Mak (26) (University of Toronto, Toronto, Ontario, Canada). C57BL/6 mice were used as controls in all experiments. Mice were used for experiments at 6 to 10 weeks of age.

Culture media. RPMI 1640 (JRH Biosciences, Lenexa, Kans.) supplemented with 10% fetal calf serum (Summit Biotechnology, Fort Collins, Colo.), 5×10^{-5} M 2-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan), and kanamycin (100 μg/ml) (Meiji Seika, Tokyo, Japan) was used. Eagle's minimal essential medium (JRH Biosciences) was used for cell washing.

Parasite infection. For malarial infection, mice were injected intravenously (i.v.) with erythrocyte (RBC) suspension containing 10^4 RBC parasitized (PRBC) with a lethal strain, *P. berghei* NK65, or its irradiation-induced attenuated variant *P. berghei* XAT (41). Parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. The percent parasitemia was calculated as follows: parasitemia (%) = [(number of infected RBC)/(total number of RBC counted)] × 100.

NK cell activity. YAC-1 lymphoma cells were labeled with ^{51}Cr by incubation at 37°C (5% CO_2) for 1 h with $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, Ill.) and washed extensively. The ^{51}Cr-labeled YAC-1 cells (10^4 cells) were incubated at 37°C (5% CO_2) for 4 h with effector splenocytes in 0.2 ml of RPMI 1640 medium in a round-bottom 96-well plate (Corning, New York, N.Y.). ^{51}Cr released into the supernatant was estimated in a gamma counter (1470 WIZARD; Wallac, Turku, Finland). The activity was assayed at effector-to-target-cell ratios of 100:1, 50:1, 25:1, and 12.5:1. The percent specific ^{51}Cr release was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. The maximum release was obtained by target cell lysis with 1% Triton X-100 (Wako). In all experiments, spontaneous release did not exceed 11% of the maximum release.

Depletion of NK cells in vivo with monoclonal antibody (MAb). To deplete NK cells in vivo, each mouse was injected intraperitoneally (i.p.) with rat anti-mouse NK1.1 (PK136, rat immunoglobulin G1 [IgG1]) at 0.3 mg/injection once daily for 3 consecutive days before the day of the parasite inoculation and then once daily every other day for 20 days. Anti-NK1.1 was purified from ascites on a protein G column (Pharmacia, Uppsala, Sweden). Normal rat IgG (Sigma, St. Louis, Mo.) was used as a control.

FACScan analysis. One million splenocytes were stained with phycoerythrin (PE)-labeled anti-NK1.1 (PK136, rat IgG1; PharMingen, San Diego, Calif.) or PE-labeled anti-IL-2Rβ (TM-β1, rat IgG2b; PharMingen) and analyzed for NK ($NK1.1^+$ or $IL\text{-}2R\beta^+$) cells on a FACScan by using Lysis II software (Becton Dickinson, Mountain View, Calif.) for data analysis.

Detection of NO production. Splenocytes were incubated for 72 h at 6×10^6 cells/ml without addition of parasite antigen in RPMI 1640 medium. Culture supernatants were assayed for NO_2^- by the Griess reaction (12). Briefly, 100 μl of the supernatant was incubated with 100 μl of Griess reagent for 5 min at room temperature, and NO_2^- concentration was determined by measuring the optical density at 550 nm (OD_{550}) in reference to the OD_{550} of standard $NaNO_2$ solution.

Assay for IFN-γ production by splenocytes. Splenocytes were incubated for 48 h at 6×10^6 cells/ml without addition of parasite antigen in RPMI 1640 medium, and culture supernatants were assayed for IFN-γ in a sandwich enzyme-linked immunosorbent assay (ELISA) by using two different clones of rat MAbs against mouse IFN-γ (R4-6A2, rat IgG1, and XMG1.2, rat IgG1; PharMingen) according to the manufacturer's instructions.

Neutralization of IFN-γ in vivo with MAb. To neutralize IFN-γ in vivo, each mouse was injected i.p. with rat anti-mouse IFN-γ (XMG1.2, rat IgG1) (4) at 0.2 mg/injection once daily for 4 consecutive days starting on the day of the parasite inoculation and then twice a week for 3 weeks. Anti-IFN-γ was purified from ascites on a protein G column. Normal rat IgG was used as a control.

Treatment of mice with CGN. Carrageenan (CGN) type II (Sigma) in sterile phosphate-buffered saline (PBS) was injected i.p. into each mouse at 1 mg/injection on days −9, −7, −5, −3, −1, +1, +3, +5, +7, and +9 in relation to the day of PRBC inoculation.

Assay for phagocytosis. Ten million splenocytes obtained from *P. berghei* XAT-infected mice and uninfected mice treated with CGN or PBS were incubated at 37°C (10% CO_2) for 2 h on a culture dish, and then nonadherent cells were removed by washing three times with warmed Eagle's minimal essential medium. Fluorescein isothiocyanate (FITC)-conjugated beads (Fluoresbrite Plain YG 0.75-μm Microspheres; Polysciences, Inc., Warrington, Pa.) were added to the adherent cells and incubated at 37°C (5% CO_2) for 2 h. These adherent cells were then collected with cold 5 mM EDTA–PBS and stained with PE-labeled anti-Mac-1 (M1/70, rat IgG2b; Serotec, Oxford, United Kingdom), followed by analysis for the cells containing FITC-conjugated beads.

Statistical analysis. Statistical analysis was performed by using Student's *t* test.

RESULTS

No effect of NK cell lytic activity on the resistance to *P. berghei* XAT infection. We first compared splenocytes from C57BL/6 mice infected with *P. berghei* XAT to those from *P. berghei* NK65-infected mice for NK cell lytic activity using YAC-1 cells as a target. The NK cell lytic activity was significantly increased by *P. berghei* XAT infection; the peak activity was observed on day 4 after the inoculation of the parasites, and the activity gradually decreased thereafter (Fig. 1). In

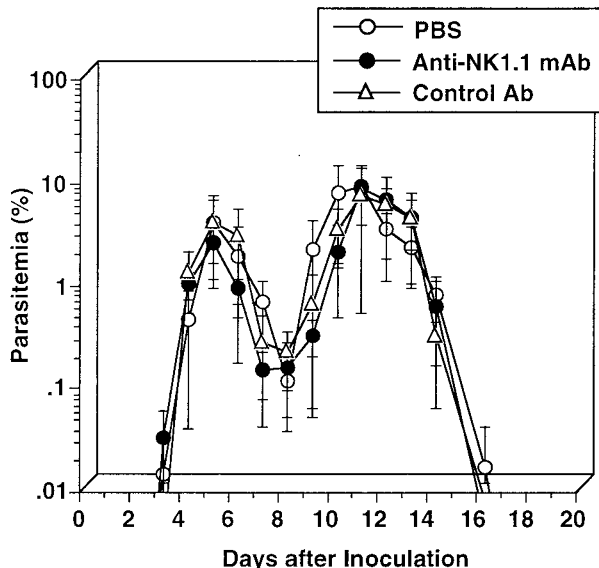
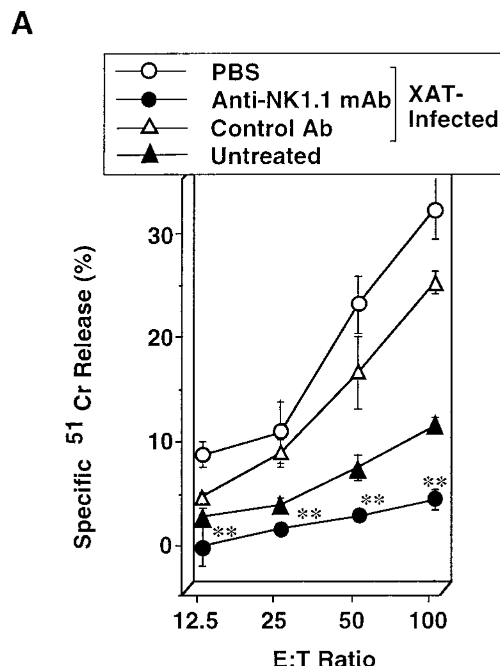


FIG. 2. No effect of NK cell depletion on the resistance to infection with blood-stage *P. berghei* XAT. NK1.1$^+$ cells were depleted by treatment with anti-NK1.1 MAb at 0.3 mg/injection/mouse once daily for 3 consecutive days before the day of inoculation i.v. of 10^4 PRBC and then every other day for 20 days. Normal rat IgG was used as a control antibody. Parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. Data are means ± SD for five mice. These results were confirmed to be reproducible by performing a repeat experiment.

contrast, *P. berghei* NK65 infection barely increased the activity on day 4, and the activity was rapidly decreased thereafter (Fig. 1). We repeated the experiments and obtained essentially the same results. These results suggest the correlation of NK cell lytic activities with the host resistance against *P. berghei* XAT and *P. berghei* NK65 infections. To examine further the role of NK1.1$^+$ cells, mice were treated with anti-NK1.1 antibody to deplete NK1.1$^+$ cells, infected with *P. berghei* XAT, and assayed for parasitemia. Unexpectedly, the parasites were cleared in mice treated with anti-NK1.1 antibody after these mice showed temporal kinetics of parasitemia similar to that for control mice (Fig. 2). To confirm the depletion of NK1.1$^+$ cells in these mice, NK cell lytic activities for splenocytes from these mice were assayed 4 days after the infection. The activity was confirmed to be abrogated by the anti-NK1.1 treatment (Fig. 3A). Neither NK1.1$^+$ cells nor IL-2Rβ$^+$ cells were detected in these splenocytes (data not shown). NK$^+$ cells are known to produce IFN-γ. Therefore, we also assayed IFN-γ production by splenocytes from anti-NK1.1-treated mice 4 days after the *P. berghei* XAT infection. In our preliminary experiments, IFN-γ production of splenocytes reached the maximum 4 days after the infection and decreased rapidly thereafter. We observed no significant difference in IFN-γ production between anti-NK1.1-treated mice and mice treated with control antibody or PBS (Fig. 3B). These results were all confirmed to be reproducible in experiments performed twice. These results suggest that NK cell activity is not critical for the host resistance against infection with *P. berghei* XAT.

Splenocytes from mice infected with *P. berghei* XAT produced more NO than those from *P. berghei* NK65-infected mice. We next analyzed in vitro NO production of splenocytes from mice infected with *P. berghei* XAT or *P. berghei* NK65. For splenocytes obtained 4 days after *P. berghei* XAT infection, significant NO production was observed, and the peak production was observed for splenocytes obtained 6 days after the



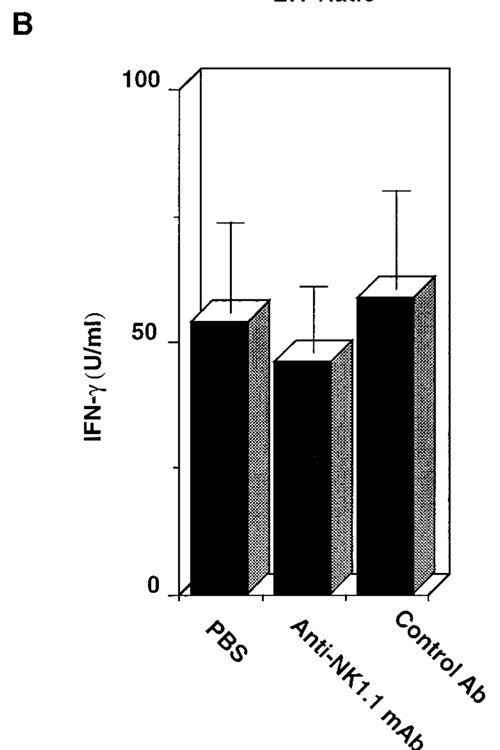


FIG. 3. NK cell lytic activity was reduced in mice treated with anti-NK1.1 without impairment of IFN-γ production. Mice were injected i.p. at 0.3 mg/injection/mouse with anti-NK1.1 once daily for 3 consecutive days before the day of the 1×10^4 PRBC i.v. inoculation and then every other day for 20 days. (A) Spleens were obtained 4 days after the inoculation of parasites and assayed for NK cell lytic activity by using YAC-1 cells as a target. E:T, effector-to-target-cell. The percent spontaneous ^{51}Cr release in the assay was 8.7% of the maximum release. Data are means ± SD for three mice. (B) Splenocytes obtained 4 days after the parasite inoculation were cultured without addition of parasite antigen for 48 h, and the culture supernatants were assayed for IFN-γ by using an ELISA. Data are means ± SD for three mice. **, $P < 0.01$, compared with the data in PBS- or control antibody-treated mice. These results were confirmed to be reproducible by performing a repeat experiment.

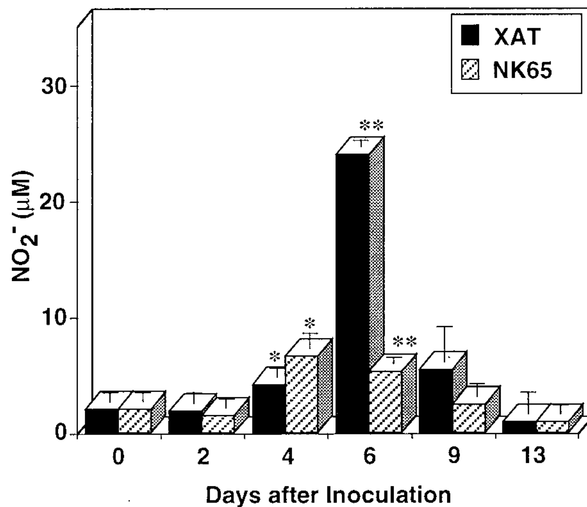


FIG. 4. Splenocytes obtained from mice infected with blood-stage *P. berghei* XAT produced more NO than those obtained from mice infected with *P. berghei* NK65. After i.v. inoculation of 10^4 PRBC, splenocytes were obtained at various intervals and cultured in vitro without addition of parasite antigen for 72 h. The culture supernatants were assayed for NO_2^-. Data are means ± SD for three mice. *, $P < 0.05$, and **, $P < 0.01$, compared with the data for splenocytes obtained on day 0. Similar experiments were performed three times, and essentially the same results were obtained.

parasite inoculation (Fig. 4), coincident with the first peak of parasitemia. In contrast, only a weak, though significant, enhancement of NO production was observed for splenocytes obtained from mice 4 and 6 days after the inoculation with *P. berghei* NK65 (Fig. 4). The experiment was performed twice, and essentially the same results were obtained both times. The difference in NO production on days 4 and 6 postinfection was further confirmed in additional experiments performed four times. These results indicate that *P. berghei* XAT infection induced NO production in an early phase of infection more efficiently than *P. berghei* NK65 infection. Thus, NO production by splenocytes seems to correlate with the different degrees of resistance to *P. berghei* XAT and *P. berghei* NK65 infection.

No difference in parasitemia between iNOS$^{-/-}$ mice and wild-type control mice inoculated with *P. berghei* XAT. To examine the role of NO in the host defense against *P. berghei* XAT infection, we inoculated *P. berghei* XAT into iNOS$^{-/-}$ mice and wild-type control mice and assayed for parasitemia. Surprisingly, iNOS$^{-/-}$ mice showed temporal kinetics of parasitemia similar to that in wild-type control mice and cleared parasites (Fig. 5A). The results were confirmed in experiments carried out twice by using the same protocol. We also obtained similar results in experiments in which 10^5 PRBC were inoculated (data not shown). The results were essentially the same for two different strains of iNOS$^{-/-}$ mice obtained from different sources (data not shown). We inoculated PRBC with *P. berghei* XAT into more than 30 iNOS$^{-/-}$ mice in total; however, all these mice cleared the parasites. NO production by splenocytes from iNOS$^{-/-}$ mice was confirmed not to be induced by the *P. berghei* XAT infection, although infected wild-type control mice produced a large amount of NO (Fig. 5B). The results were confirmed in three repeated experiments. Inability of iNOS$^{-/-}$ mice infected with *P. berghei* XAT to produce NO was also confirmed by stimulation of their splenocytes with lipopolysaccharide (data not shown). These results

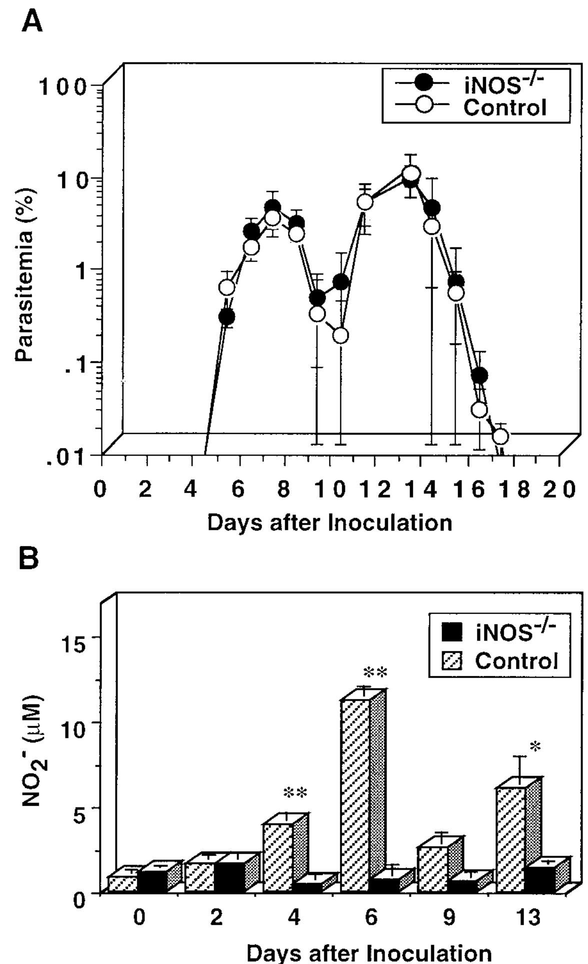


FIG. 5. Comparable susceptibilities of iNOS$^{-/-}$ mice and wild-type control mice to the infection with blood-stage *P. berghei* XAT. (A) After iNOS$^{-/-}$ mice or wild-type control mice were inoculated i.v. with 10^4 PRBC, parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. Data are means ± SD for five mice. (B) After iNOS$^{-/-}$ mice or wild-type control mice were inoculated i.v. with 10^4 PRBC, splenocytes were obtained at various intervals and cultured in vitro without addition of parasite antigen for 72 h. The culture supernatants were assayed for NO_2^-. Data are means ± SD for three mice. *, $P < 0.05$, and **, $P < 0.01$, compared with the data for splenocytes obtained on day 0. These experiments were performed three times, and similar results were obtained.

suggest that NO production is not critical for the defense against *P. berghei* XAT infection.

Important role of IFN-γ in the resistance of iNOS$^{-/-}$ mice to infection with blood-stage *P. berghei* XAT. To examine the mechanism of clearing of the parasite in iNOS$^{-/-}$ mice, we analyzed IFN-γ production by their splenocytes. IFN-γ production by wild-type control mouse splenocytes was increased after the inoculation of *P. berghei* XAT and peaked on day 4 (Fig. 6). iNOS$^{-/-}$ mouse splenocytes also produced a level of IFN-γ comparable to that produced by wild-type control mouse splenocytes, although the peak response was delayed by 2 days (Fig. 6). Since IFN-γ was demonstrated to play a critical role in protective immunity against *P. berghei* infection in wild-type control mice (42, 43), we next examined whether neutralization of IFN-γ by anti-IFN-γ antibody made iNOS$^{-/-}$ mice susceptible to *P. berghei* XAT infection. Parasitemia in iNOS$^{-/-}$ mice treated with anti-IFN-γ antibody progressively

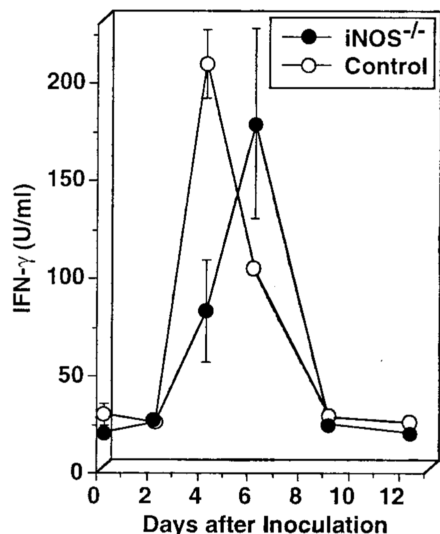


FIG. 6. Comparable production of IFN-γ in splenocytes from iNOS$^{-/-}$ mice and in those from wild-type control mice caused by the infection with blood-stage *P. berghei* XAT. After iNOS$^{-/-}$ mice or wild-type control mice were inoculated i.v. with 10^4 PRBC, splenocytes were obtained at various intervals and cultured in vitro without addition of parasite antigen for 48 h. The culture supernatants were assayed for IFN-γ by using an ELISA. Data are means ± SD for three mice. Similar results were obtained in two successive experiments.

increased, and all mice eventually died (Fig. 7). On the other hand, the treatment with control antibody did not affect the parasitemia. We obtained essentially the same results in three repeated experiments. These results indicate that IFN-γ plays a critical role in the host defense against *P. berghei* XAT infection in iNOS$^{-/-}$ mice.

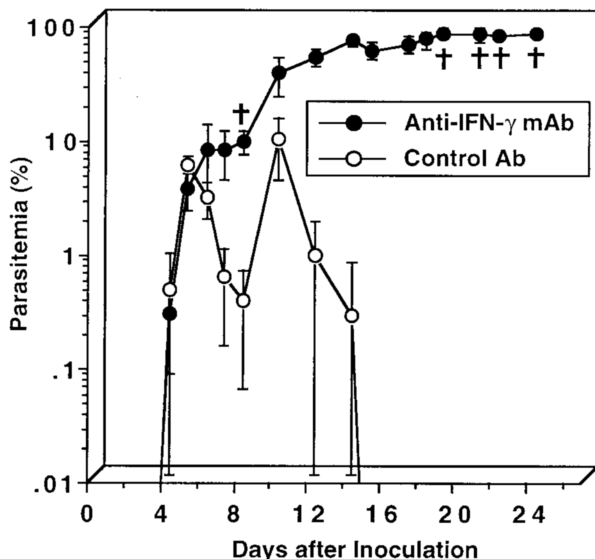
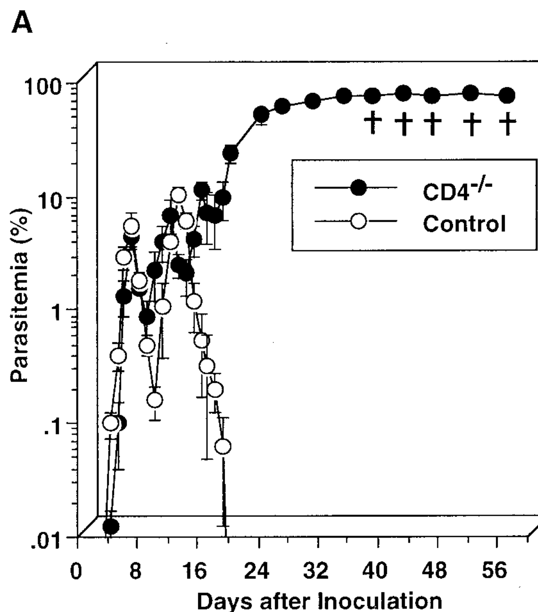


FIG. 7. Important role for IFN-γ in the host resistance of iNOS$^{-/-}$ mice against the infection with blood-stage *P. berghei* XAT. After iNOS$^{-/-}$ mice were inoculated i.v. with 10^4 PRBC, endogenously produced IFN-γ was neutralized by treatment at 0.2 mg/mouse with anti-IFN-γ once daily for 4 consecutive days starting from the day of the inoculation and then twice a week for 3 weeks. Parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. Normal rat IgG was used as a control antibody. Data are means ± SD for five mice. †, days on which individual mice died. We obtained essentially the same results in three successive experiments.



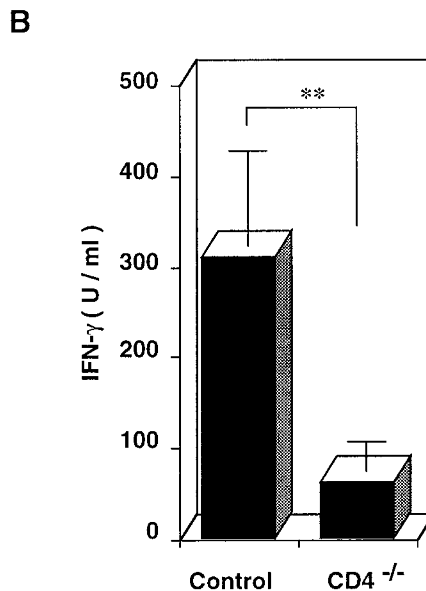


FIG. 8. Increased susceptibility to blood-stage *P. berghei* XAT infection of CD4$^{-/-}$ mice with reduced IFN-γ production. (A) After CD4$^{-/-}$ mice or wild-type control mice were inoculated i.v. with 10^4 PRBC, parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. Data are means ± SD for five mice. †, days on which individual mice died. (B) Splenocytes were obtained on day 4 after the parasite inoculation and cultured in vitro without addition of parasite antigen for 48 h. The culture supernatants were assayed for IFN-γ by using an ELISA. Data are means ± SD for three mice. **, $P < 0.01$. The results presented in both panels A and B were confirmed to be reproducible in two successive experiments.

Role of CD4$^+$ T cells in IFN-γ production in *P. berghei* XAT infection. We examined the role of CD4$^+$ T cells in IFN-γ production in *P. berghei* XAT infection using CD4$^{-/-}$ mice. When CD4$^{-/-}$ mice were injected with 10^4 PRBC, the PRBC progressively increased in number after small peaks in parasitemia were observed; all these mice eventually died (Fig. 8A). When splenocytes from CD4$^{-/-}$ mice inoculated with PRBC

were assayed for IFN-γ production in vitro, spleen cells obtained from both $CD4^{-/-}$ mice and wild-type mice on day 4 after the parasite inoculation were found to show peak responses in IFN-γ production. The results for their peak responses are shown in Fig. 8B. These results indicate that $CD4^+$ T cells play a critical role in the production of IFN-γ, which plays an important role in the clearance of *P. berghei* XAT.

Impairment of *P. berghei* XAT clearance by treatment of mice with CGN. Macrophage function has been suggested to play an important role in the defense mechanisms against malarial infection (2, 32, 35). To investigate the macrophage involvement in the mechanism of defense against *P. berghei* XAT infection, we examined the effect of CGN treatment on parasitemia of mice infected with *P. berghei* XAT, since CGN was reported to block macrophage function (11, 27). Parasitemia was progressively increased in four of six mice treated with CGN (Fig. 9A). We obtained similar results in the repeat experiments performed according to the same protocol. Splenocytes obtained from *P. berghei* XAT-infected mice treated with CGN produced amounts of IFN-γ and NO comparable to those produced by splenocytes from infected mice which had not received the CGN injection. Splenocytes from infected mice treated and not treated with CGN produced 206.3 ± 17.7 and 202.2 ± 67.0 U of IFN-γ/ml and 12.1 ± 4.3 and 13.9 ± 2.2 μM NO_2^-, respectively, 6 days after the parasite inoculation (values are means ± standard deviations [SD] for three mice). To examine the effect of the CGN treatment on macrophage phagocytic function, adherent spleen cells from infected and uninfected mice treated with CGN were obtained 14 days after the parasite inoculation, incubated with FITC-conjugated beads, and analyzed for their phagocytosis on a FACScan. The percents Mac-1^{high} macrophages containing FITC-conjugated beads for both infected and uninfected mice treated with CGN were significantly reduced compared with those for control mice (Fig. 9B). These results suggest that phagocytic function of macrophages is important for the host defense against the *P. berghei* XAT infection.

DISCUSSION

NK cells play an important role in the innate resistance to a variety of pathogens through their target cell lysis and IFN-γ production (1) and were suggested to be involved in the host resistance to *P. falciparum* in humans (23, 39), to *P. berghei* in rats (34), and to *P. chabaudi* in mice (9). However, beige mutant mice with reduced NK activity were shown to resolve *P. chabaudi* and *P. vinckei petteri* infection like the control mice did (19, 33, 45), suggesting that NK cell activity is not crucial for the resistance. The treatment with anti-NK1.1 has been shown to increase the mortality of mice infected with blood-stage *P. chabaudi* (14). Treatment with anti-asialo GM1 was also reported to increase the parasitemia of mice infected with blood-stage *P. chabaudi* AS (19) and *P. yoelii* coincident with impairment in IFN-γ production, especially in the early phase of infection (8). Our present results suggest that $NK1.1^+$ cells do not play a critical role in the resistance to *P. berghei* XAT infection. In addition, in our present experiments, $CD4^{-/-}$ mice showed increased susceptibility to the infection and their IFN-γ production was shown to be impaired during *P. berghei* XAT infection. The parasitemia was increased after initial small fluctuations in $CD4^{-/-}$ mice, although in wild-type control mice treated with anti-IFN-γ it increased consistently without regression. The findings may indicate that IFN-γ produced by cells other than $CD4^+$ cells, such as NK cells, plays a role in the defense mechanisms during an early phase of infection with *P. berghei* XAT. The above results also indicate that

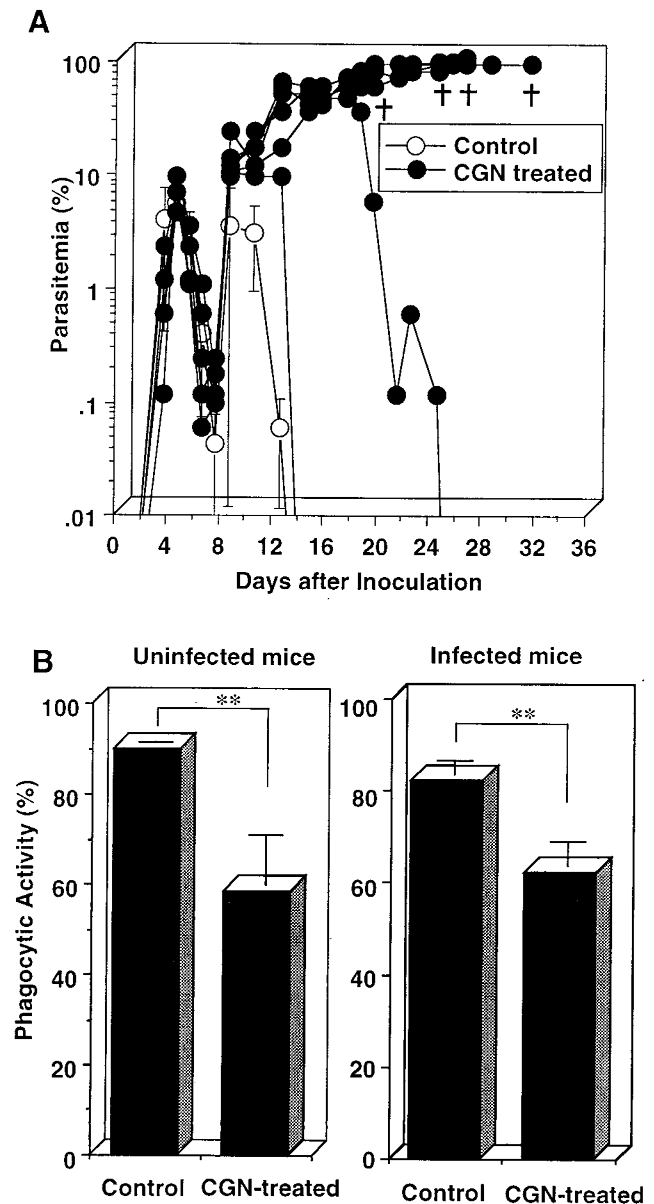


FIG. 9. Increased susceptibility of mice treated with CGN to blood-stage *P. berghei* XAT infection with reduced phagocytic activity of spleen macrophages. (A) Mice treated with CGN as described in Materials and Methods were inoculated i.v. with 10^4 PRBC, and parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. †, days on which individual mice died. (B) Adherent spleen cells obtained from CGN-treated uninfected mice and also from mice infected for 14 days were incubated with FITC-conjugated beads for 2 h. Phagocytic activity was assayed by FACScan analysis with gating on a Mac-1^{high} population. Data are means ± SD for eight mice. **, $P < 0.01$. These results were confirmed to be reproducible in two successive experiments.

$CD4^+$ cells play a more important role in the resistance against *P. berghei* XAT infection than $NK1.1^+$ cells, although NK cells may play a role in an early phase of the infection and the involvement of NK cells in the defense mechanism could vary depending on the kind of malarial parasite.

The present results show that *P. berghei* XAT infection induced a larger amount of NO production by splenocytes as compared with *P. berghei* NK65 infection. NO production by splenocytes was apparently increased in mice infected with *P.*

berghei XAT, and peaked 6 days after the inoculation, coincident with the first peak of parasitemia. In contrast, NO production of splenocytes was only weakly increased by the infection with *P. berghei* NK65. The mechanism of the difference in NO production between splenocytes infected with lethal and nonlethal strains remains unknown. However, it is possible that the activation of macrophages downstream to IFN-γ stimulation may be impaired in the *P. berghei* NK65 infection. The correlation of the NO production with host resistance observed in the present experiments seems to suggest an important role of NO in mechanisms of defense against *P. berghei* XAT infection, as reported for *P. chabaudi* AS infection (12). Surprisingly, however, iNOS$^{-/-}$ mice infected with *P. berghei* XAT showed a profile of parasitemia, in terms of both temporal kinetics and percent PRBC, similar to that of wild-type control mice, and all the mice used in our experiments recovered from infection. In the present experiments, we used iNOS$^{-/-}$ mice obtained from two different sources to confirm the results, and essentially the same results were obtained. Splenocytes from iNOS$^{-/-}$ mice were confirmed not to produce NO as a result of the infection with *P. berghei* XAT (Fig. 2). These results indicate that NO is not critical for the protective immunity to *P. berghei* XAT infection. iNOS$^{-/-}$ mice were shown to display increased susceptibility to infection with *Listeria monocytogenes* (17), *Leishmania major* (44), or *Mycobacterium tuberculosis* (18). These results indicate that NO plays at least some role in defense mechanisms against these pathogens. Although macrophages from these mice were defective in killing of *Toxoplasma gondii* in vitro, iNOS$^{-/-}$ mice were shown to survive the acute infection and the protective role of NO in the late stage of the infection was indicated to be tissue specific (29). Taken together, these results suggest a possibility that iNOS$^{-/-}$ mice develop an alternative pathway(s) of pathogen clearance. In our previous experiments, IL-12 was shown to play an important role in the clearance of *P. berghei* XAT (46). It is possible that IL-12-dependent mechanisms play a compensatory role in the clearance of *P. berghei* XAT in iNOS$^{-/-}$ mice. In *T. gondii* infection, IL-12 was shown to be able to enhance protection in the absence of an NO pathway by engaging both IFN-γ-dependent and -independent pathways (13). In our present study, IFN-γ was shown to play a critical role in the parasite clearance in iNOS$^{-/-}$ mice. These results indicate that there is some important mechanism(s) downstream to IFN-γ other than the activation of iNOS for the clearance of *P. berghei* XAT. Induction of respiratory burst and upregulation of natural resistance-associated macrophage protein 1 (NRAMP1) could be possible mechanisms. NRAMP1$^{-/-}$ mice showed impaired resistance to infection with intracellular parasites (40).

The in vivo role of NO in host resistance to blood-stage *P. chabaudi* AS was previously investigated by treating resistant C57BL/6 mice with iNOS inhibitors (12, 38). The treatment with aminoguanidine was shown to reduce serum NO_3^- levels in *P. chabaudi* AS-infected mice to a level similar to that observed in uninfected control mice, and mortality was increased to 80% without affecting parasitemia (12). Parasitemia with *P. chabaudi* AS in mice depleted of CD4$^+$ T cells that received transferred Th1 cells was also demonstrated to be increased by treatment with the iNOS inhibitor L-N$^\gamma$ monomethyl arginine (L-NMMA), although these mice cleared the parasites without the L-NMMA treatment (38). These results might be caused by adverse side effects of these iNOS inhibitors. Aminoguanidine was reported to bind to reactive aldehydes formed by oxidative stress during malarial infection (3, 5, 10, 25).

NO has also been suggested to play a crucial role in protection from blood-stage malarial parasites in humans. Plasma NO levels were reported to increase in patients infected with *P. falciparum* and *P. vivax* (7, 22), and the duration of coma due to cerebral malaria was reported to be short in children with high plasma NO levels (7). Moreover, NO-generating compounds were shown to be able to kill blood-stage *P. falciparum* in vitro (28). It is possible that involvement of iNOS in the clearance of malarial parasites varies depending upon the kind of parasite.

To investigate the involvement of macrophages in the host resistance to *P. berghei* XAT infection, we examined the effect of the macrophage-toxic substance CGN (11, 27) on parasitemia. Splenocytes obtained from *P. berghei* XAT-infected mice treated with CGN produced amounts of IFN-γ and NO comparable to those produced by splenocytes from mice which had not been treated with CGN. However, treatment with CGN was shown to increase the parasitemia, resulting in high mortality of mice infected with *P. berghei* XAT, as previously reported to occur in infection with other parasites (27). The CGN treatment did not affect the first peak of parasitemia (Fig. 9A). The finding suggests that the mechanism(s) of resistance to *P. berghei* XAT during the early phase of the infection is different from that during the second peak of parasitemia. The CGN treatment may affect only the mechanism involved in the late phase. Thus, the function, possibly phagocytic activity, of macrophages activated by IFN-γ may play an important role in the resistance to *P. berghei* XAT infection.

Taken together, the present results suggest that neither NO production nor NK cell activation plays a critical role in the resistance to blood-stage *P. berghei* XAT infection, although both NO production and NK cell activity correlate with the resistance of mice to infections with *P. berghei* XAT and *P. berghei* NK65. IFN-γ was indicated to play an important role in the protective immunity through macrophage activation. Although the cells that produce IFN-γ in *P. berghei* XAT infection were not formally identified, CD4$^+$ cells were indicated to play an important role in the IFN-γ production.

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