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Potentiation by Febrifugine of Host Defense in Mice against *Plasmodium berghei* NK65

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ABSTRACT. The effect of febrifugine, the main alkaloidal constituent of an antimalarial crude drug, *Dichroa febrifuga* Lour., on protective immunity in mice infected with erythrocytic stage *Plasmodium berghei* NK65 was investigated. Febrifugine was administered orally, at a dose of 1 mg/kg/day, to mice before and/or after they were infected intraperitoneally with 2×10^6 parasitized red blood cells. Then, mortality and the levels of parasitemia and plasma NO_3^- [a degradation product of nitric oxide (NO)] were monitored. Febrifugine significantly reduced the mortality and the level of parasitemia. The plasma NO_3^- concentration began to rise within 2 days after treatment with febrifugine and declined to normal in 2 days when the mice were treated orally with febrifugine once a day for 3 consecutive days before parasite infection. This antimalarial activity of febrifugine was reduced by both N^G-monomethyl-L-arginine and aminoguanidine. These results indicate that the increased production of NO by febrifugine plays an important role in host defense against malaria infection in mice. BIOCHEM PHARMACOL **58**;10:1593–1601, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. *Plasmodium berghei* NK65; malaria; febrifugine; antimalarial activity; nitric oxide; host defense

NO§ is a short-lived messenger molecule produced by three different isoforms of NOS [1]. NO participates in intracellular signaling when produced in small amounts by constitutive NOS (NOS 1 and NOS 3) [1–3]. Immunological and inflammatory stimuli induce a transcriptionally inducible isoform of NOS (NOS 2) that can produce a large amount of NO over long periods [1]. In the latter case, NO can exert cytotoxic or cytostatic actions toward tumor cells, viruses, microbes, and bacterial protozoa [1]. Recently, it was shown that inflammatory cytokines such as INF-γ inhibit the infection of sporozoites into murine hepatocytes by enhancing NO production, and this effect is abolished by NOS inhibitors [4–8]. Moreover, the antimalarial effect of quinine is potentiated strongly by the simultaneous use of desferrioxamine B [9, 10], an iron chelator that triggers the generation of NO in macrophages [11, 12]. Therefore, the enhancement of NO production in activated macrophages substantially contributes to host defense against parasitic infection [13].

Although the quinazolone alkaloid febrifugine (Fig. 1) has been isolated from *Dichroa febrifuga* Lour. (Saxifragaceae) as a malariacidal constituent [14, 15], the mechanism of antimalarial activity of febrifugine *in vivo* has not been clarified. In a previous paper, we reported that orally administrated febrifugine potentiates the lipopolysaccharide-activated NO production in BCG-induced mouse peritoneal macrophages [16]. To elucidate the correlation between the antimalarial activity of febrifugine and the promoting activity of NO production by febrifugine, we examined the mortality and the levels of parasitemia and plasma NO_3^- (a degradation product of NO) in mice infected with the erythrocytic stage of *Plasmodium berghei* strain NK65 and treated orally with febrifugine. The effect of NOS inhibitors on the antimalarial activity of febrifugine also was examined.

MATERIALS AND METHODS

Reagents

Febrifugine was isolated from the roots of *D. febrifuga* Lour. (Saxifragaceae) [16], and its dihydrochloride (Fig. 1) was used throughout the experiment. Nitrate reductase was obtained from Boehringer Mannheim Japan. NADPH was purchased from the Oriental Yeast Co. Ltd. FAD, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and H_3PO_4 were from the Wako Pure Chemical Co. L-NMA hydrochloride and aminoguanidine hemisulfate were obtained from the Sigma Chemical Co.

Animals and Parasite Infection

Male ICR mice (6 weeks of age) were purchased from SLC-Japan. Mice were housed in groups of 15 animals in plastic cages with a light–dark rhythm of 12 hr–12 hr and

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§ *Abbreviations*: BCG, bacillus Calmette–Guérin; IFN, interferon; L-NMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; and PRBC, parasitized red blood cells.

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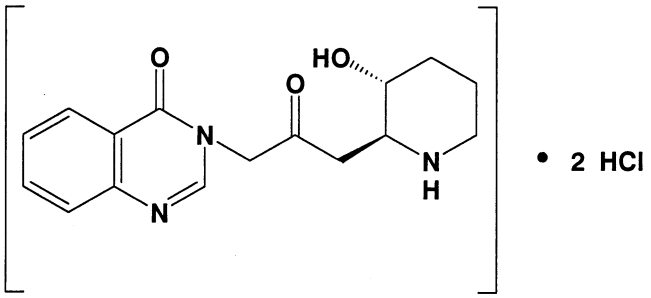

FIG. 1. Chemical structure of febrifugine 2HCl.

had free access to food and water *ad lib*. An adaptation period to these conditions of at least 1 week was used for the experiments. *P. berghei* strain NK65 was donated by Professor Dr. Y. Wataya, Faculty of Pharmaceutical Sciences, Okayama University, and was maintained as previously described [17, 18]. Mice were infected i.p. with 2×10^6 *P. berghei* NK65 PRBC, and mortality and parasitemia were monitored at the indicated times. The level of parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. The percentage of parasitemia was calculated as follows: parasitemia (%) = [(number of infected erythrocytes)/(total erythrocytes)] × 100.

Plasma Collection

Mice were anesthetized with ether, and heparinized blood samples were collected by intraventricular exsanguination. Blood plasma was separated by centrifugation and stored at −20° until used for determination of nitrate (NO_3^-) level.

Plasma NO_3^- Determination

Plasma NO_3^- concentrations were measured by the method of Giovannoni *et al.* [19] with modification. In brief, 200 μL of plasma sample was incubated at 37° for 90 min with 4 μL of reaction mixture (20 U/mL of enzyme nitrate reductase, 2.5 mM NADPH, and 250 mM FAD in phosphate-buffered saline at pH 7.4), and then plasma proteins were removed by filtration (centrifuged at 12,000 *g* for 90 min) using a molecular cut filter (MW: 10,000 kDa, Ultrafree-MC™, Millipore). The filtered samples (80 μL) were transferred into a 96-well flat-bottom plate (Becton Dickinson), and mixed with 80 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 3% H_3PO_4) [20]. After chromophore was formed at room temperature for 10 min, absorbance was determined at 510 nm using an Immunoreader (InterMed). NO_3^- concentrations in plasma were determined by comparison with serially diluted $NaNO_3$ solutions in plasma from non-infected mice.

Administration of Febrifugine and NOS Inhibitors

Febrifugine dihydrochloride was dissolved in physiological saline and injected orally (1 mg/kg/day, 0.1 mL/10 g) into mice once daily for 3 consecutive days before (day −2 to day 0) and/or after (day 0 to +2) parasite infection (on day 0). In other cases, mice were injected orally with 450 mg/kg/day of aminoguanidine hemisulfate salt (dissolved in saline, 0.1 mL/10 g) twice daily for 5 consecutive days after parasite infection [21]. L-NMA hydrochloride (60 mg/kg/day, 0.1 mL/10 g) dissolved in saline was injected orally once daily for 4 consecutive days after parasite infection [6]. Control mice were treated similarly with saline alone.

Statistical Analysis

Statistical significance of differences in mortality between groups of mice was determined using a nonparametric Kruskal-Wallis test. Differences in the level of either plasma NO_3^- or parasitemia between control and experimental groups were analyzed by Student's *t*-test, and differences were considered significant if $P < 0.05$. The level of plasma NO_3^- and the percentage of parasitemia data are given as means ± SEM.

RESULTS

Survival and Parasitemia Experiment

P. berghei strain NK65 is a lethal murine malaria [17], and all infected mice die within 2–3 weeks [18]. When ICR mice were infected with *P. berghei* of 2×10^6 PRBC, the body weights of the mice decreased, and a severe anemia appeared 6 days after parasite infection; all these mice died within 10 days. Oral injection of febrifugine to the infected mice at a dosage of 1 mg/kg/day significantly prolonged the survival times in all administration patterns of febrifugine, as illustrated in Fig. 2, and the effect was most potent when the mice were treated for 5 consecutive days (day −2 to +2). The parasitemia was also reduced by febrifugine, and the parasite clearance activity was more potent in the 5 consecutive day treatment (Fig. 3). Febrifugine showed no toxicity to uninfected normal mice at a dose of 1 mg/kg/day in these treatment regimens, evaluated by the fact that the body and liver weights did not change (data not shown), and the activity of a plasma hepatic marker enzyme was not enhanced (alanine aminotransferase level: 19.2 ± 2.8 U/mL, saline alone, once a day for 10 days, N = 3; 18.5 ± 3.5 U/mL, 1 mg/kg/day of febrifugine, once a day for 10 days, N = 4) [16].

Effect of Febrifugine on Plasma NO_3^- Concentration in Mice Infected with P. berghei

Since NO is known to be essential in the host defense against *Plasmodium* parasites [4, 5, 22–24], plasma NO_3^- (degradation product of NO) concentrations were measured at regular time intervals after parasite infection. The plasma NO_3^- level in uninfected normal mice was 49.7 ± 3.9 μM (N = 4). Plasma NO_3^- concentrations in ICR mice were not altered by infection with *P. berghei* NK65 (Fig. 4, A–C). In this case, there were not enough data because all

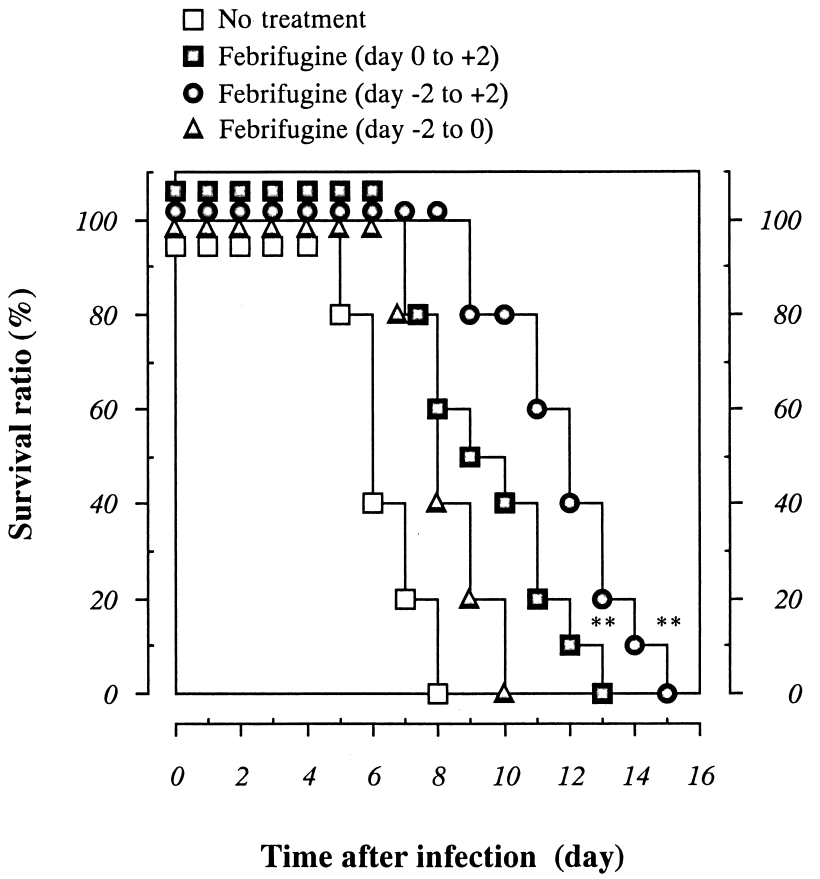

FIG. 2. Effect of febrifugine on acute mortality induced by infection with *P. berghei* NK65. All mice were infected i.p. with 2×10^6 PRBC on day 0, and were administered orally 0.2 mL saline (N = 10) or 1 mg/kg/day of febrifugine once daily for 3 consecutive days before (day −2 to 0, N = 10), after (day 0 to +2), or for 5 consecutive days (day −2 to +2, N = 10). Key: (**) $P < 0.01$ vs saline treatment.

the infected mice died by day 8 after infection. In contrast, febrifugine markedly increased the plasma NO_3^- concentrations in the infected mice. To examine the influence of administration timing on the enhancement of plasma NO_3^-, 1 mg/kg/day of febrifugine was administered orally with three different regimens: day −2 to day 0, day 0 to day +2, and day −2 to day +2. Febrifugine dramatically enhanced the NO_3^- level by 380% on day 1 in the day −2 to 0 treatment (Fig. 4A, N = 11, $P < 0.01$), 390% on day 3 in the day 0 to +2 treatment (Fig. 4B, N = 10, $P < 0.01$),

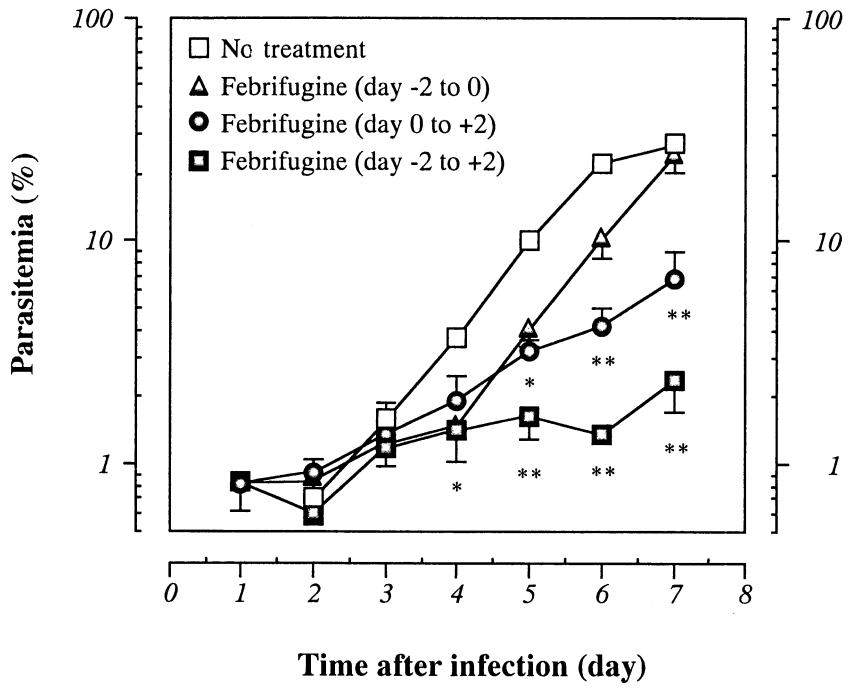

FIG. 3. Effect of febrifugine on parasitemia in mice infected with *P. berghei* NK65. Mice were infected i.p. with 2×10^6 PRBC on day 0, and then blood samples were collected at the indicated times. Parasitemia was assessed by microscopic examination of Giemsa-stained smears of tail blood. Groups of four infected mice were administered orally 0.2 mL saline alone (N = 10), or 1 mg/kg/day of febrifugine once daily for 3 consecutive days from day −2 to 0 (N = 10), or from day 0 to +2 (N = 10), or for 5 consecutive days from day −2 to +2. Data are expressed as means ± SEM. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs saline treatment.

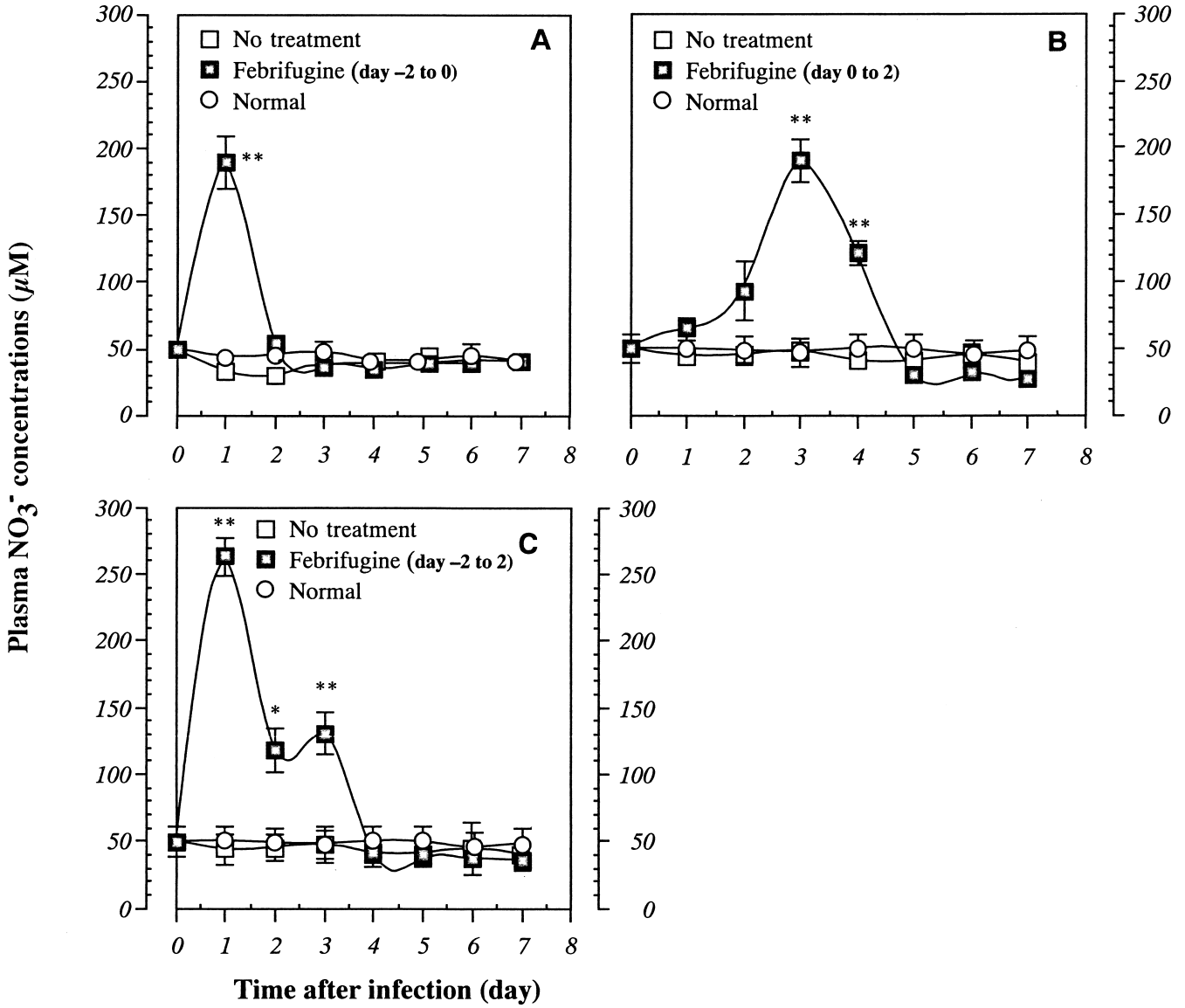

FIG. 4. Effect of febrifugine on plasma NO_3^- concentrations in mice infected with *P. berghei* NK65. Mice were infected i.p. with 2 × 10^6 PRBC on day 0, and then plasma samples were collected at the indicated times. NO_3^- concentrations in plasma were measured by using nitrate reductase (see Materials and Methods). Groups of four infected mice were administered orally 0.2 mL saline alone (no treatment, N = 16), or 1 mg/kg/day of febrifugine once daily for 3 consecutive days from day −2 to 0 (N = 11) (panel A), or from day 0 to +2 (N = 10) (panel B), or for 5 consecutive days from day −2 to +2 (N = 12) (panel C). Plasma NO_3^- concentrations in normal uninfected mice were monitored at the indicated times (normal, N = 5). Data are expressed as means ± SEM. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs saline treatment. Similar results were obtained in a duplicate experiment.

and 560% on day 1 in the day −2 to +2 treatment (Fig. 4C, N = 12, $P < 0.01$), respectively. In the last case, two peaks were observed on days 1 and 3, which might be the combined effect of the pre-treatment (day −2 to 0) and the post-treatment (day 0 to +2) (Fig. 4, A–C). The plasma NO_3^- concentration declined toward the normal level 4 days after parasite infection in this case (Fig. 4C). Plasma NO_3^- concentrations on day 2 were examined after various febrifugine doses in the day −2 to +2 treatment. Febrifugine enhanced these concentrations in a dose-dependent manner in the range of 0.3 to 1.0 mg/kg/day (Fig. 5). Febrifugine alone did not affect the level of plasma NO_3^- concentration in uninfected normal mice even at the higher doses and in other treatment regimens (data not shown).

The effects of long-term treatment of febrifugine (day −2 to +7) on survival time, parasitemia, and plasma NO_3^- concentration in mice infected with *P. berghei* were examined next. Febrifugine rescued all the infected mice by day 10 (Fig. 6A). However, mortality began to increase on day 11 (4 days after the last administration of febrifugine), and all the mice died by day 17. In this regimen, parasitemia was inhibited completely until day 10, but it increased dramatically on day 11 (Fig. 6C). Moreover, as shown in Fig. 6B, the pattern of enhancement of plasma NO_3^- concentration by febrifugine in this long-term treatment was similar to

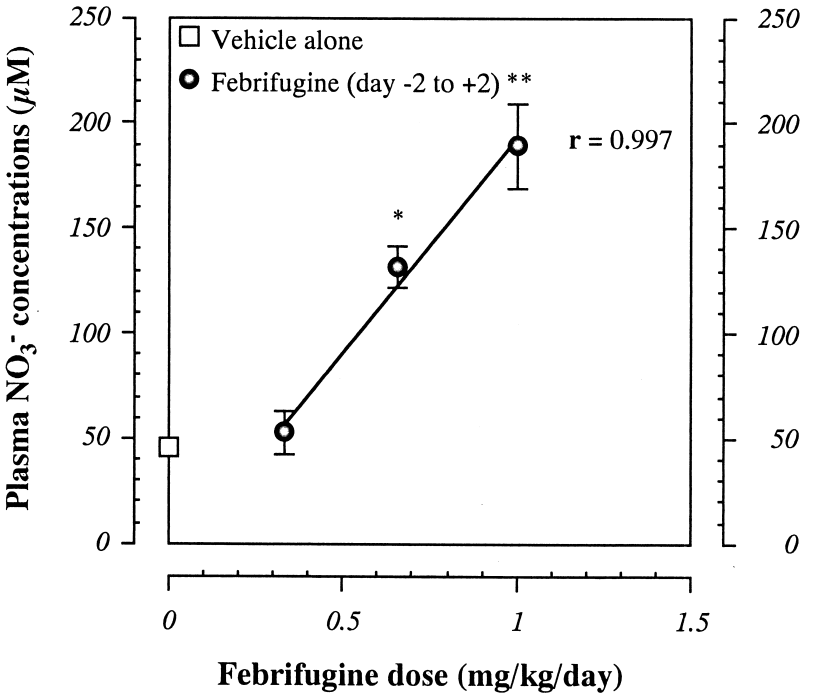

FIG. 5. Dose-dependent potentiation by febrifugine of plasma NO_3^- concentration in mice infected with *P. berghei* NK65. Mice were infected i.p. with 2×10^6 PRBC on day 0, and were administered orally 0.3 to 1 mg/kg/day of febrifugine once a day for 5 consecutive days (day −2 to +2, N = 14/group). Plasma samples were collected 2 days after infection, and the NO_3^- concentration in the plasma was measured. Data are expressed as means ± SEM. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs saline treatment.

that in the case of day −2 to +2 treatment (Fig. 4C). That is, the NO_3^- concentration initially peaked on day 1 after malaria infection and was elevated moderately on day 5.

Effects of NOS Inhibitors on the Antimalarial Activity of Febrifugine

To clarify the relationship between the enhancement of plasma NO_3^- concentration by febrifugine and the protective response against *P. berghei* NK65, the NOS inhibitors L-NMA and aminoguanidine were administered orally to the febrifugine-treated mice. L-NMA is a substrate inhibitor of NOS [4, 5], and aminoguanidine is a selective inhibitor of NOS 2 [25, 26]. Figure 7 shows plasma NO_3^- concentrations 2 days after parasite infection in seven groups of mice. Febrifugine enhanced the plasma NO_3^- concentration markedly, while co-injection of L-NMA (60 mg/kg/day for 4 consecutive days) or aminoguanidine (450 mg/kg/day for 8 consecutive days) with febrifugine markedly reduced it. At the same time, both NOS inhibitors shortened the survival time extended by febrifugine, and L-NMA was more effective than aminoguanidine (Fig. 8). L-NMA and aminoguanidine slightly reduced the survival time in the infected and febrifugine non-treated mice, respectively (Fig. 8). Furthermore, L-NMA and aminoguanidine aggravated the parasitemia improved by febrifugine (Fig. 9). L-NMA and aminoguanidine did not affect the parasitemia of the infected and febrifugine non-treated mice (Fig. 9).

DISCUSSION

There are some reports that malaria infection enhances plasma or serum NO_3^- concentration in experimental animals. Previous studies showed that serum concentration of NO_2^-/NO_3^- increased in NIH [27] and C57BL/6 [28] mice when they were infected with *P. chabaudi* AS. However, the plasma NO_3^- concentration in ICR mice infected with *P. berghei* NK65 has not been investigated. In the present study, the plasma NO_3^- concentration in ICR mice did not change after *P. berghei* NK65 infection. This observation partly supports the report by Asensio *et al.* [29]. That is, the level of NO_2^-/NO_3^- in urine of BALB/c mice infected with *P. berghei* did not increase at any monitoring time [29]. Although *P. chabaudi* AS and *P. berghei* NK65 are both murine malarias, the former is not lethal and the latter is lethal [18]. Therefore, the fact that the level of plasma NO_3^- did not increase in ICR mice infected with *P. berghei* NK65 might be ascribed to the difference of parasite and animal species.

The oral treatment of febrifugine at a dosage of 1 mg/kg/day for 5 consecutive days significantly reduced the parasitemia (Fig. 3) and prolonged the survival time of the infected mice (Fig. 2). Chien and Cheng [15] reported that subcutaneously injected febrifugine in the dose range of 1.25 to 10 mg/kg showed an antimalarial activity in mice lethally infected with *P. berghei*, but febrifugine also caused toxic death at a dose of 10 mg/kg. In our study, febrifugine administered orally at a dosage of 1 mg/kg/day did not show any significant toxicity to the mice, which was demonstrated by the fact that the body and liver weights did not change and the activity of a plasma hepatic marker enzyme was not enhanced; in addition, this dose was sufficient for the induction of antimalarial activity.

It is noteworthy that the NO_3^- concentration in the plasma of febrifugine-treated mice markedly increased during the infection. We previously reported that febrifugine

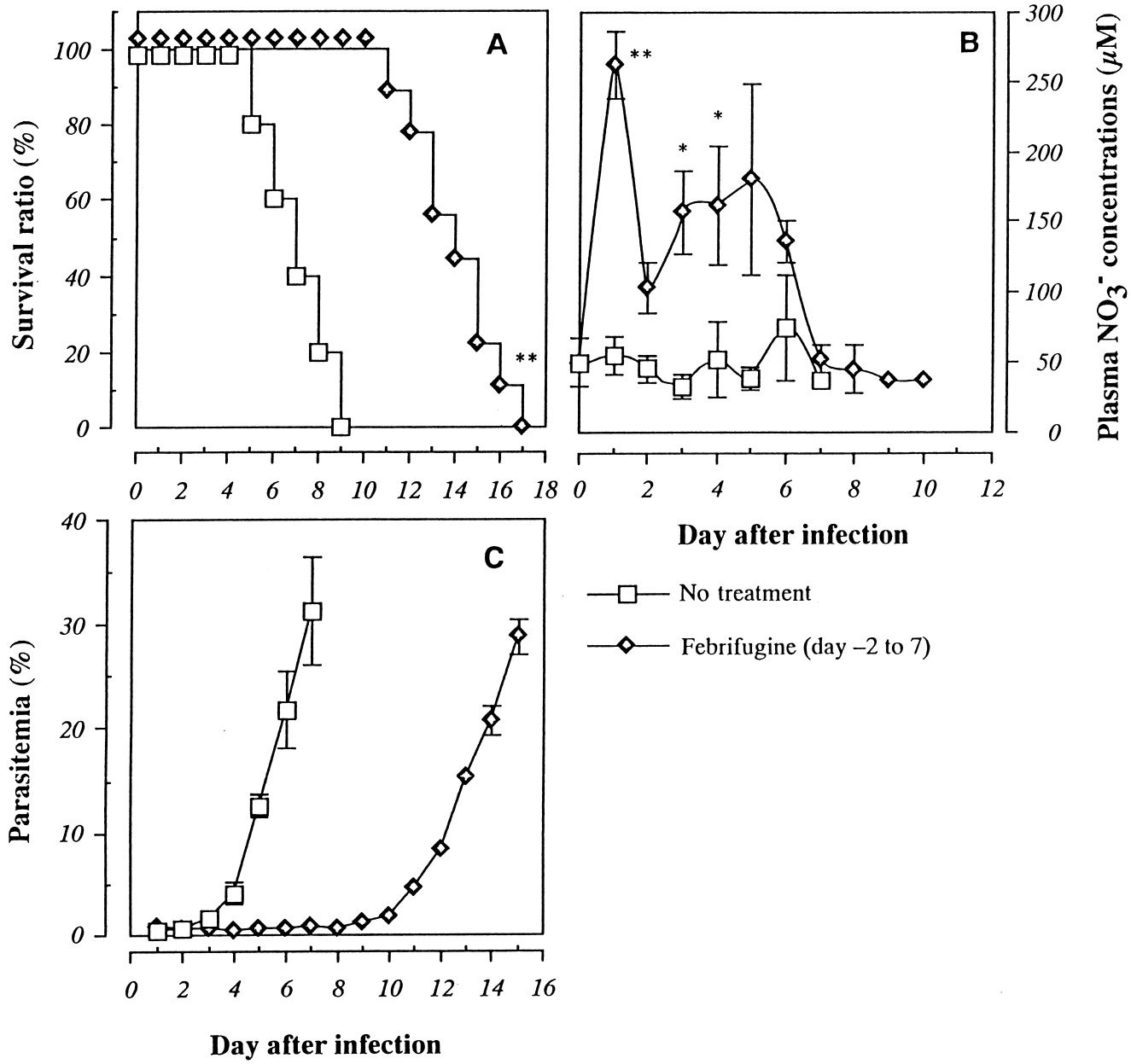

FIG. 6. Effects of long-term treatment of febrifugine on survival time, plasma NO_3^- concentration, and parasitemia in mice infected with *P. berghei* NK65. Mice were infected i.p. with 2×10^6 PRBC on day 0, and were administered orally 1 mg/kg/day of febrifugine once a day for 10 consecutive days (day −2 to +7, N = 10/group). Mortality (panel A) and parasitemia (panel C) were monitored at the indicated times. Plasma samples were collected at the indicated times, and the NO_3^- concentration (panel B) was measured by using nitrate reductase. Data are expressed as means ± SEM. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs saline treatment.

potentiates the lipopolysaccharide-activated stage of NO production in BCG-induced mouse peritoneal macrophages [16]. Thus, the increment of NO_3^- concentration in the plasma of febrifugine-treated mice is attributable to the potentiation of NO production in monocytes/macrophages. On the other hand, the NO production in the BCG-induced peritoneal macrophages was not enhanced when the macrophages were treated directly with febrifugine [16]. Moreover, febrifugine increased the plasma NO_3^- concentration in a characteristic manner with two peaks in the day −2 to +2 treatment (Fig. 4C). The first peak appeared on day 1, and the second peak appeared on day 3. Thus, the NO production in the mice began 3 days after the initial treatment with febrifugine (Fig. 4, A–C). Febrifugine did not alter the level of plasma NO_3^- in the uninfected normal mice (data not shown). These results indicate that febrifugine may act on T lymphocytes (i.e. Th1 cells) and potentiate the production of IFN-γ or interleukin-12, which activate monocytes *in vivo* and *in vitro* [10, 30].

To determine whether the potentiation of plasma NO_3^- concentration correlates with the antimalarial activity of febrifugine, mice were treated with L-NMA or aminoguanidine. Asensio *et al.* [29] have reported that intracranially injected L-NMA improves the mortality and cerebral ma-

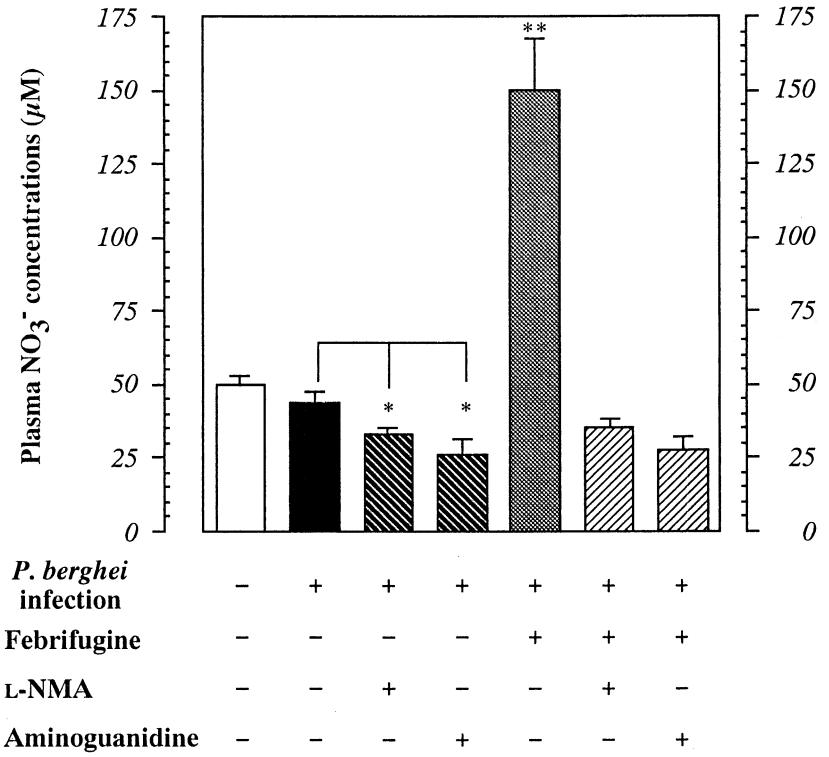

FIG. 7. Effects of NOS inhibitors on plasma NO_3^- concentration in mice infected with *P. berghei* NK65. Febrifugine-treated mice were administered orally 1 mg/kg/day of febrifugine (day −2 to +2) with L-NMA (60 mg/kg, day 0 to +2, N = 15) or 450 mg/kg/day of aminoguanidine twice daily for 3 consecutive days (day 0 to +2, N = 18). Plasma samples were collected 2 days after infection, and NO_3^- was measured. Data are expressed as means ± SEM. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs control mice.

laria in CBA/J mice infected with *P. berghei* ANKA. The protective role of NO against blood-stage *P. chabaudi* AS was also suggested by the finding that treatment of the infected mice with L-NMA or aminoguanidine results in aggravation of the parasitemia and mortality [21, 31]. In this experiment, L-NMA and aminoguanidine did not worsen the parasitemia of the infected mice (Fig. 9), but exacerbated the mortality of the mice (Fig. 8). Also, the improvements in survival time and parasitemia by febrifugine in the infected mice were reduced by these NOS inhibitors (Figs. 8 and 9). Moreover, both NOS inhibitors significantly reduced the plasma NO_3^- concentrations en-

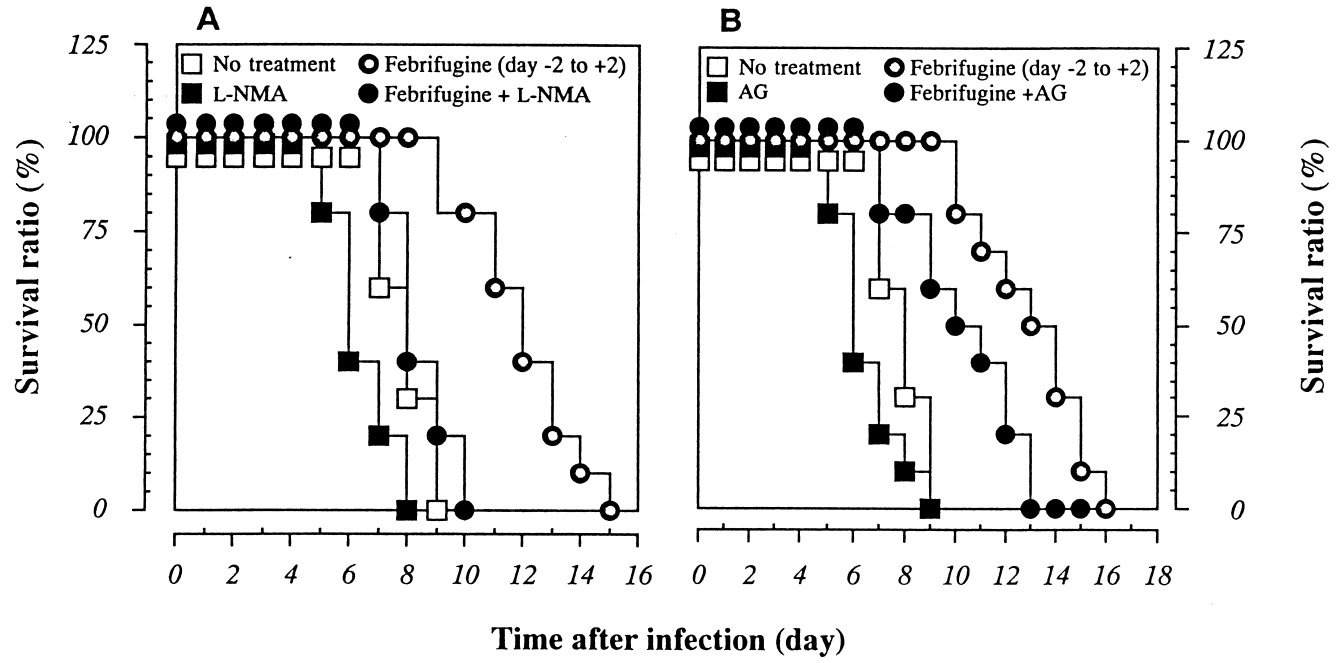

FIG. 8. Effects of NOS inhibitors on antimalarial activity of febrifugine. Groups of eight mice either were not treated or were treated orally with 1 mg/kg/day of febrifugine (day −2 to +2, N = 18) plus 60 mg/kg/day of L-NMA (panel A, day 0 to +3, N = 13) or 450 mg/kg/day of aminoguanidine (panel B, day 0 to +7, N = 15). All mice were infected i.p. with 2×10^6 PRBC on day 0, and the course of survival was measured at the indicated times. AG: aminoguanidine.

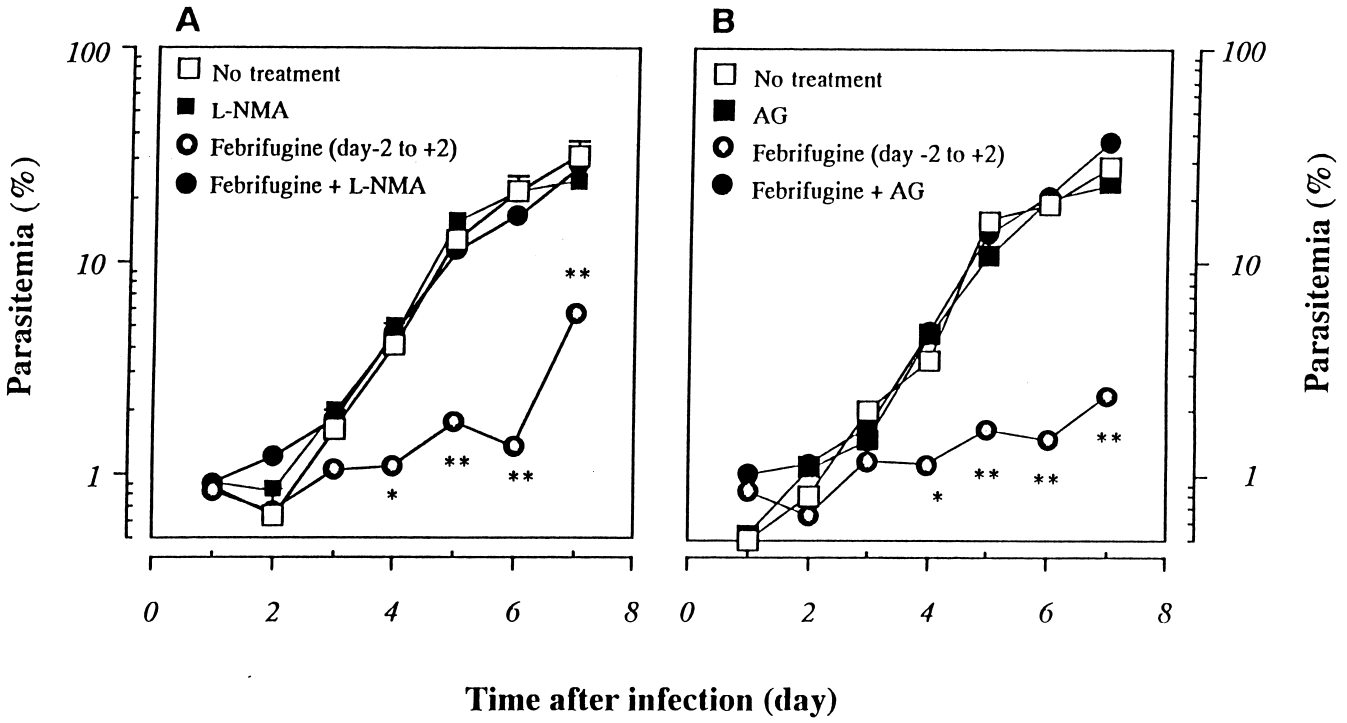

FIG. 9. Effects of NOS inhibitors on parasite clearance of febrifugine. Groups of eight mice were either not given or were given orally 1 mg/kg/day of febrifugine (day −2 to +2, N = 18) plus 60 mg/kg/day of L-NMA (panel A, day 0 to +3, N = 13) or 450 mg/kg/day of aminoguanidine (panel B, day 0 to +7, N = 15). All mice were infected i.p. with 2 × 10^6 PRBC on day 0, and the course of parasitemia was measured at the indicated times. Data are expressed as means ± SEM. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs control mice. AG: aminoguanidine.

hanced by febrifugine (Fig. 7). These results suggest that potentiation of NO production by febrifugine plays an important role in the host defense of the mice against infection with erythrocytic stage malaria of *P. berghei* NK 65.

The effects of long-term treatment of febrifugine on survival time, parasitemia, and plasma NO_3^- concentration in the infected mice were also examined. Febrifugine effectively rescued the infected mice from death (Fig. 6A), and the NO_3^- concentrations in the plasma were kept at a higher level during febrifugine treatment (Fig. 6B). But mortality began to increase 4 days after the last administration of febrifugine (Fig. 6A). These data indicated that febrifugine strengthens host defense against malaria infection in the mice and has a potential for malaria prophylaxis. *D. febrifuga* Lour. has been traditionally used in human malaria for a long time, and no resistant strains of malaria parasite have been reported [15]. In this study, we revealed febrifugine, a main antimalarial constituent of *D. febrifuga*, to potentiate NO production in acute immune responses, which might be a reason why no strains of malaria parasite resistant against *D. febrifuga* have been reported. Taken together, febrifugine is a promising source of a novel class of antimalarial drugs, especially in combination chemotherapy. Further investigation in line with this idea is underway.

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