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POSSIBLE INVOLVEMENT OF IFN-γ IN EARLY MORTALITY OF *PLASMODIUM BERGHEI* NK65-INFECTED BALB/c MICE AFTER FEBRIFUGINE TREATMENT

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Abstract. Parasitemia patterns, survival and cytokine levels of *Plasmodium berghei* NK65-infected BALB/c mice, treated orally with the alkaloidal mixture of febrifugine and isofebrifugine at a dose of 1 mg/kg twice a day for 4 consecutive days were monitored. Whereas the untreated mice showed a progressive increase in parasitemia and ultimate death, the alkaloid mixture-treated group showed a transient suppression of parasitemia during the course of treatment. However, the parasitemia increased on discontinuation of treatment, leading to earlier death of mice in the treated group than in the infected but untreated controls. Mice in the infected but untreated group displayed a significant elevation in serum IFN-γ levels during the first week post-infection (pI) and from Day 14 pI, relative to the levels in the uninfected controls. In contrast, although mice in the alkaloid mixture-treated group displayed no significant elevation in serum IFN-γ levels during the first week pI, they showed considerable levels on Day 14 pI. There were no significant differences in serum IL-4 levels among the groups. The titers of the parasite-specific IgG1, IgG2a, IgG2b and IgG3 were significantly elevated from Day 11 pI in both the treated and untreated groups. There was a significant difference in survival duration between the IFN-$\gamma^{-/-}$ mutant and BALB/c mice. IFN-$\gamma^{-/-}$ mutant mice showed a decrease in parasitemia levels while receiving medication, which was significantly lower than those of the treated BALB/c mice. The results of the present study suggest that although IFN-γ is significant for protective immunity in mice with malaria infection, it may play an adverse role post-medication, causing earlier mortality of treated BALB/c mice.

INTRODUCTION

Malaria is one of the most important tropical diseases in the world. The current malaria control strategy in most countries relies on disease management using chemotherapy, but its impact is seriously hampered by the spread of antimalarial drug resistance (Teklehaimanot and Bosman, 1999). The widespread emergence of chloroquine-resistant variants of *Plasmodium falciparum* has stimulated research for new and more effective treatments against malaria (Payne, 1987; Winstanley, 2000). Plants have always been considered a rich source of new drugs. Since approximately 80% of the world's population depends on botanical medicines for their primary health care needs (Phillipson and Wright, 1991), it is of great interest to investigate the

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folk medicine exhibiting potent antimalarial activity. Febrifugine is an active antimalarial compound isolated 50 years ago from the Chinese herb *changshan* (*Dichroa febrifuga* Lour), which has been used as an antimalarial in Chinese traditional medicine for more than 2,000 years (Koepfli *et al*, 1947). Properties of febrifugine and its derivatives have been intensively studied especially for antimalarial activity in murine models (Chien and Cheng, 1970; Jiang *et al*, 2005). Recrudescence of the parasites after treatment with febrifugine or its analogs seems to be a key factor causing mouse mortality. Immunological and biological mechanisms leading to the host's death, however, have not yet been elucidated.

An understanding of host-parasite interactions in malaria is an important prerequisite for improving chemotherapy and also for vaccine design. In order to gain a better insight into *in vivo* interactions, various murine model systems have been developed. Four rodent Plasmodia species (*Plasmodium berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei*) infect inbred mouse strains with varying degrees of morbidity and mortality (Stevenson, 1989). Although none of these models exactly reflects the infection in humans, they provide valuable insight into the mechanisms of immunity and immunopathogenesis in human malaria. Recently, we demonstrated that ICR or BALB/c mice infected with *P. yoelii* 17XL can survive after treatment with febrifugine and isofebrifugine isolated from the leaves of *Hydrangea macrophylla* var. *Otaksa* (Ishih *et al*, 2003, 2004). IFN-γ and parasite-specific antibodies seemed to play a critical role in the control of this lethal malaria infection in mice after medication (Ishih *et al*, 2004). However, when we used a different rodent malaria strain, *P. berghei* NK65, the medication with the mixture did not rescue the mice (Ishih *et al*, 2003). The infected ICR mice showed low parasitemia levels during the drug treatment period, but all mice died following recrudescence of the malaria parasites. Murata *et al* (1999) reported that oral treatment with febrifugine significantly reduces the level of parasitemia and prolongs the survival of male ICR mice infected with *P. berghei* NK65.

Malaria is similar to many other infections in that the immune effector mechanisms that are required to eliminate it are also inherently damaging to host tissues. Relatively little is known about immune responses associated with infection after medication, and thus it is of interest to further extend studies regarding the role of cytokines in lethal malaria infection after medication. *Plasmodium berghei* NK65 and *P. yoelii* 17XL are known to cause fatal malaria infections in BALB/c mice. In the present study, we investigated the production of cytokines, IFN-γ and IL-4, and of malaria-specific antibodies in *P. berghei* NK65-infected BALB/c mice treated with the alkaloidal mixture of febrifugine and isofebrifugine. The outcomes of infection after drug treatment were examined in genetically engineered IFN-γ deficient mice on a BALB/c mouse background.

MATERIALS AND METHODS

Animals and parasites

All animal experiments were performed according to *The Rules for Animal Experimentation and the Guidelines for the Care and Use of Laboratory Animals*, Hamamatsu University School of Medicine. Inbred male BALB/c mice, 8 weeks old, supplied by CLEA Japan (Hamamatsu, Japan), were used as hosts. Inbred IFN-γ deficient (IFN-$\gamma^{-/-}$) mice were supplied from Jackson Laboratories (ME, USA). The rodent malaria parasite, *Plasmodium berghei* NK65, a previous gift from Professor Y Wataya (Okayama University, Japan), and blood stage parasites were stored at -80ºC.

Alkaloidal mixture of febrifugine and isofebrifugine

The febrifugine and isofebrifugine mixture was prepared from the dried leaves of *H. macrophylla* var. *Otaksa* using a porous poly-

mer gel (Mitsubishi Diaion HP-20; Mitsubishi Chemical, Tokyo, Japan) column and a cation exchanger (Amberlyst 15; Organo, Tokyo, Japan) column (Ishih *et al*, 2001). For oral administration to mice, the alkaloid fraction was dissolved in 0.4% Cremophor EL (Sigma, St Louis, MO) solution at a volume of 75 μl/10 g body weight (b.wt.).

Treatment of infected BALB/c mice with the alkaloidal mixture

For the experiments, blood stage *P. bergehi* NK65 parasites from frozen stock were inoculated intraperitoneally (IP) into two BALB/c mice which served as donor mice. The mouse showing 10-15% parasitemia was bled under ether anesthesia to collect the parasitized blood. Using the parasitemia and erythrocyte density of the donor mouse, the parasitemia was adjusted downwards using physiological saline. The experimental mice were infected IP with 10^5 parasitized erythrocytes, and were randomized into treated and untreated groups of 6 mice each (Experiment 1). From Day 4 post-infection, the treated group was given orally the alkaloidal mixture using a metal catheter at 1 mg/kg b.wt., twice a day for 4 consecutive days. Similarly, a corresponding volume of 0.4% Cremophor EL solution was given orally to mice in the untreated but infected group. All mice were fed on a commercial diet (LabDiet, PMI Nutrition International, MO) and given water *ad libitum*. Following infection, parasitemia levels were monitored through Giemsa-stained thin blood smears made by bleeding via the tail vein throughout the observation period.

Cytokine assays

Four uninfected BALB/c mice were sacrificed at the time of infection. Four infected mice in the untreated group were bled under ether anesthesia on Days 4, 6, 8, 11, 14 and 20 post-infection (pI) and the mice in the treated group were sacrificed on Days 6, 8, 11 and 14 pI (Experiment 2). Each blood sample was collected in a tube and centrifuged. The resultant serum was stored at -20ºC until assay. The levels of IFN-γ were determined by sandwich ELISA (Yoshida *et al*, 1995). For the assay, the following combinations of capture and biotinylated mAbs were used as recommended by the manufacturer (PharMingen, San Diego, CA): R4-6A2 and XMG1.2 for IFN-γ, and 11B11 and BVD6-24G2 for IL-4. The amount of cytokines was calculated using standard murine recombinant cytokine curves run on the same immunoplate.

Preparation of malarial antigens

The malarial antigens were prepared from the erythrocytic stages of *P. berghei* NK65. Mice with high parasitemia were bled by cardiac puncture. Heparinized red blood cells (RBCs) were washed twice in phosphate buffered saline, pH 7.2 (PBS). The RBCs were then passed through a two-layered column of glass beads (150-210 μm; Polysciences, Warrington, PA) and CF 11 cellulose (Whatmann International, Maidstone, UK) to remove the white blood cells from the red blood cells (RBCs) by the method of Goldman *et al* (1992). After washing twice in PBS, the packed RBCs were diluted three-fold and lysed with an equal volume of 0.1% saponin in PBS. Twenty minutes later, the lysed RBCs were washed in PBS at least four times. The pellet was diluted two-fold with PBS and the freeze/thaw procedure was repeated six times. The preparation was sonicated with UR-200P (Tomy Seiko, Tokyo, Japan) until the membranes were disrupted. After centrifugation, the supernatant was collected and passed through a 0.45 μm filter unit (Millipore, Bedford, MA). The resulting preparations were aliquoted and stored at -80ºC until use. The protein concentration was determined with Bio-Rad reagents (Bio-Rad Laboratories, Herculus, CA) using bovine serum albumin as a standard. The RBCs collected from normal, uninfected animals were prepared by the same procedure and used as a control.

Malaria-specific antibody assays

Malaria-specific antibodies (Abs) were measured in the sera of infected mice (Experiment 2). ELISA plates (Sumitomo Bakelite, Tokyo, Japan) were coated with soluble malarial antigens in 0.1 M carbonate-bicarbonate buffer, pH 9.6. For titration of anti-malarial Ig isotypes, serial dilutions of serum in PBS containing 0.05% Tween 20 and 3% BSA were applied to the antigen-coated plates. Biotinylated anti-mouse IgG1 (Zymed, San Francisco, CA), IgG2a, IgG2b (Immunotech, FL) and IgG3 (Cybus Biotech, Chandlers Ford, UK) were used to detect the respective isotypes of mouse Ig. The reactions were visualized by peroxidase conjugated streptavidin (Zymed) and the substrate, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma, St Louis, MO). The absorbance of individual wells was determined using a Microplate Reader MTP-120 (Corona, Ibaraki, Japan) at a wavelength of 415 nm (cutoff value; 0.21 at 415 nm).

Infection of IFN-$\gamma^{-/-}$ mice with blood-stage *P. berghei* NK65

Inbred male BALB/c and IFN-$\gamma^{-/-}$ mice, consisting of 4 mice of each strain, were used in this experiment (Experiment 3). Experimental mice were infected at the age of 10 weeks, by IP inoculation of 10^5 parasitized erythrocytes. From Day 4 post-inoculation, the mice were given febrifugine and isofebrifugine orally using a metal catheter at a dose of 1 mg/kg b.wt. twice a day for 4 consecutive days. Similarly, a corresponding volume of 0.4% Cremophor EL solution was given orally to mice in the untreated but infected group. All mice were fed on a commercial diet (LabDiet, PMI Nutrition International, MO) and water *ad libitum*.

Statistical analysis

Differences in mice survival were analyzed using the log-rank test. The Mann-Whitney *U* test was performed to assess differences in cytokine and antibody production and parasitemia. P-values less than 0.05 were considered significant.

RESULTS

Outcomes of *P. berghei* NK65 infected BALB/c mice receiving placebo or the alkaloidal mixture (Experiment 1)

Parasitemia of the group treated with the alkaloid mixture was significantly lower on Days 6 and 9 pI, compared with untreated controls (Mann-Whitney *U* test, $p<0.05$) (Fig 1A). Although the treated mice had a decrease in parasitemia while on the medication and for a few days post-treatment, the parasites rebounded, leading to mouse death. A longer survival duration was observed in the untreated group than in the treated group (log-rank test, $p< 0.01$) (Fig 1B). Six mice in the untreated control died during Days 19 to 25 pI with gradual weight loss and progressive parasitemia. However, the six mice given the alkaloid mixture died during Days 14 to 16 pI and had marked weight loss between Days 11 and 14 pI. A significant difference (Mann-Whitney *U* test, $p<0.05$) was observed in body weight between the treated and untreated groups on Days 9 and 11 pI (Fig 1C).

Analysis of cytokines during infection (Experiment 2)

The levels of cytokines in the sera were compared between the infected controls and the alkaloid-treated mice after infection. The mean value for serum IFN-γ in the normal mice was 85.8 ± 28.5 pg/ml (mean ± SD, n=4). The mice in the untreated group displayed significantly higher serum IFN-γ levels on Days 4, 6 and 8 pI than the uninfected animals (Fig 2). On Day 11 pI, the IFN-γ levels in the untreated mice decreased, but on Days 14 and 20 pI the levels increased significantly. The mice in the alkaloid-treated group displayed significantly higher serum IFN-γ levels on Day 14 pI than the uninfected animals. There were no

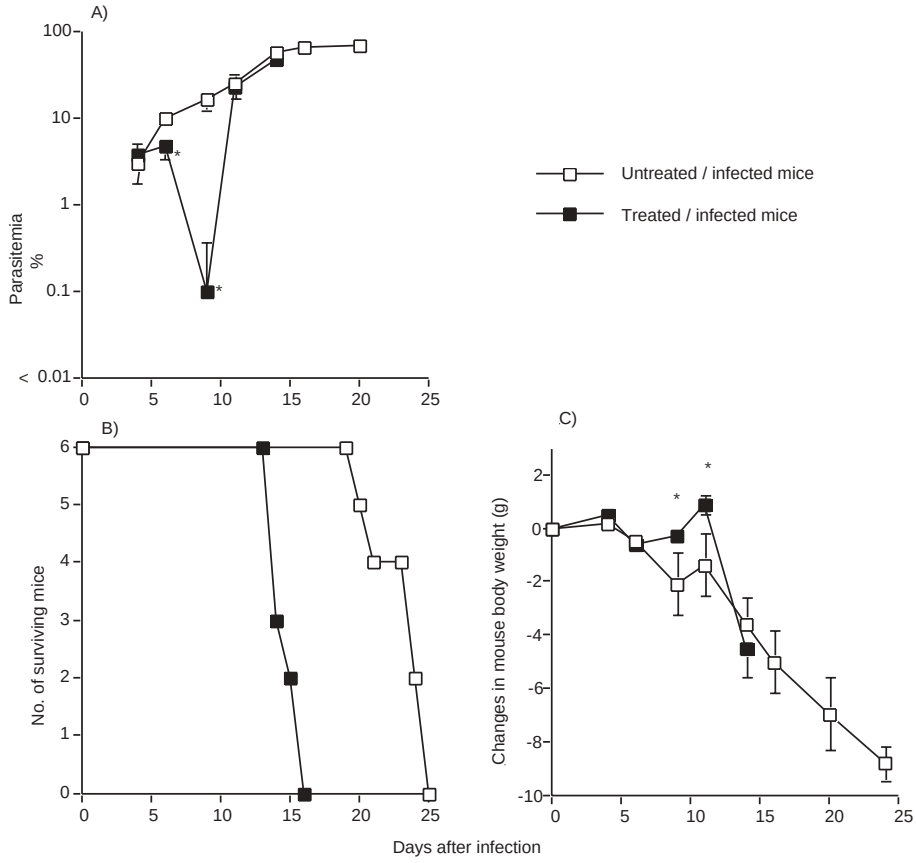

Fig 1–Time-course changes of parasitemia (A), survival rate (B), and body weight (C) of male BALB/c mice inoculated with 10^5 *P. berghei* NK65-parasitized erythrocytes after oral treatment with the febrifugine and isofebrifugine mixture at a dose of 1 mg/kg b.wt., twice a day for 4 consecutive days from Days 4 to 7 after infection (Experiment 1). Mice in the infected but untreated control group received a corresponding volume of 0.4% Cremophor EL solution. Results of body weight and parasitemia are expressed as mean values with standard deviation. Statistically significant differences between the treated mice and the infected but untreated controls (Mann-Whitney *U* test, $p < 0.05$) indicated by asterisk.

significant differences in serum IL-4 levels among the three groups (data not shown).

Analysis of antibody levels (Experiment2)

The production of the subclasses (IgG1, IgG2a, IgG2b and IgG3) of parasite-specific antibodies (Abs) during *P. berghei* NK65 infection was evaluated in both the alkaloid-treated mice and the infected but untreated animals (Fig 3). On Day 8 pI, the levels of malaria-specific IgG1 in the infected but untreated mice and those of parasite-specific IgG1, IgG2a and IgG3 in the alkaloid-treated mice tended to elevate, but the difference was not significant. On Day 11 pI and thereafter, the titers of the four subclasses of Abs in the infected but untreated mice and the levels of IgG2a, IgG2b and IgG3 in the alkaloid-treated mice were significantly elevated. The titers of IgG1 in the alkaloid-treated mice elevated significantly on Day 14 pI.

Infection of IFN-$\gamma^{-/-}$ mice with blood-stage *P. berghei* NK65 (Experiment 3)

Longer survival duration was observed in the infected and treated IFN-$\gamma^{-/-}$ mice than in

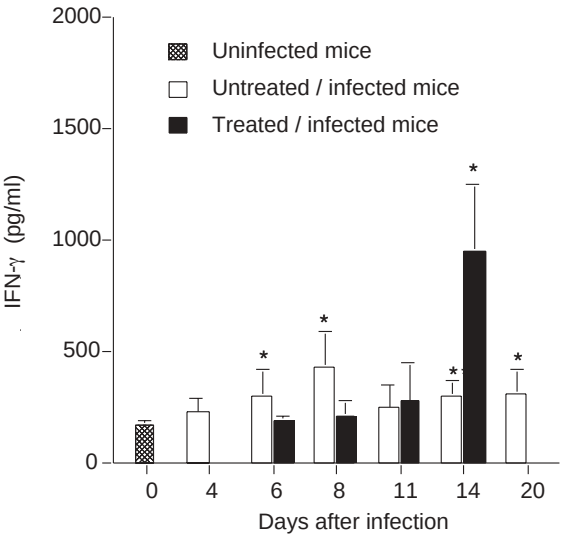

Fig 2–Serum IFN-γ levels determined by ELISA. Male BALB/c mice inoculated with 10^5 *P. berghei* NK65-parasitized erythrocytes were treated orally with the febrifugine and isofebrifugine mixture at a dose of 1 mg/kg b.wt., twice a day for 4 consecutive days from Days 4 to 7 after infection (Experiment 2). Mice in the infected but untreated control group received a corresponding volume of 0.4% Cremophor EL solution. Four normal mice were used as uninfected controls. Following infection of the parasites, 4 mice in each group were sacrificed under ether anesthesia at various time points post-infection. Data represent mean values with standard deviation for 4 mice per time point. Significantly different from the value of the uninfected control mice (Mann-Whitney *U* test, $p < 0.05$) indicated by asterisk.

the infected and treated BALB/c mice (log-rank test, $p< 0.01$) (Fig 4A). The infected and treated BALB/c mice died during Days 13 to 15 pI. In contrast, the infected and treated IFN-$\gamma^{-/-}$ mice died during Days 16 to 24 pI. All the infected and treated BALB/c mice had a marked suppression of parasitemia during medication with the alkaloid-mixture (Fig 4B). The alkaloid treatment also suppressed parasitemia in the IFN-$\gamma^{-/-}$ mice, but this level of suppression was significantly less than that of the BALB/c mice (Mann-Whitney *U* test, $p<0.05$).

DISCUSSION

Febrifugine, identified as a natural anti-malarial compound, failed to stop recrudescence of malaria parasites in infected mice, leading to subsequent host mortality, the underlying mechanisms of which have not yet been clarified. *Plasmodium berghei* NK65 is a lethal rodent malarial strain. The progression of this infection varies considerably depending on the mouse strain (Eling *et al*, 1997). IFN-γ is considered to be potentially involved in the pathogenesis of primary *P. berghei* NK65 infection since anti-IFN-γ treatment prolongs survival of infected CBA mice (Waki *et al*, 1992). The enhancement of nitric oxide (NO) production in activated macrophages contributes substantially to host defense against parasitic infection (Liew, 1993). Febrifugine has been reported to alter NO production in activated macrophages and is suggested to play an important role in host defense of ICR mice against infection with erythrocytic stage parasites of *P. berghei* NK65 (Murata *et al*, 1999). In the present study, using a different mouse strain, BALB/c, a marked suppression of parasitemia was observed while giving alkaloid medication, but no mouse recovered from *P. berghei* NK65 infection. The alkaloid-treatment shortened the period of survival of *P. berghei* NK65-infected BALB/c mice compared to that of the infected but untreated animals. This observation is inconsistent with a study by Murata *et al* (1999) in which febrifugine treatment prolonged survival of infected ICR mice. The hepatotoxicity with higher concentrations of the alkaloids is known (Ishih *et al*, 2002), but the present results show no acute hepatotoxicity in the dead mice (data not shown). The discrepancy in length of survival could be attributed to a difference in immunological re-

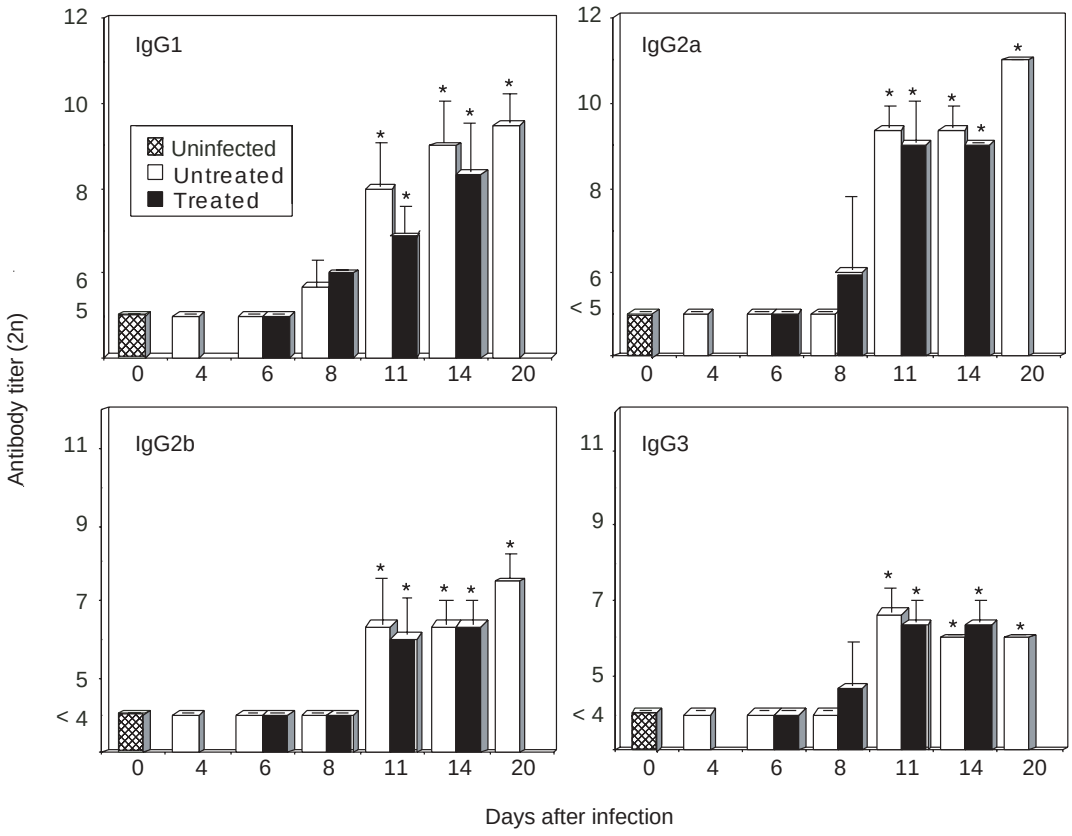

Fig 3–Titers of the malaria-specific IgG1, IgG2a, IgG2b and IgG3 subclasses determined by ELISA in serum samples of male BALB/c mice inoculated with 10^5 *P. berghei* NK65-parasitized erythrocytes after oral treatment with the febrifugine and isofebrifugine mixture at a dose of 1 mg/kg b.wt., twice a day for 4 consecutive days from Days 4 to 7 after infection (Experiment 2). Mice in the infected but untreated control group received a corresponding volume of 0.4% Cremophor EL solution. Four normal mice were used as uninfected controls. Following infection of the parasites, 4 mice in each group were sacrificed under ether anesthesia at various time points post-infection. Data represent mean values with standard deviation for 4 mice per time point. Significantly different from the value of the uninfected control mice (Mann-Whitney *U* test, $p < 0.05$) indicated by asterisk.

sponses in the mouse strains used. NO has been suggested to have both a protective and pathogenic role in malaria. In appropriate amounts at appropriate times during the inflammatory response NO can be protective, whereas a sustained NO response that fails to eliminate the pathogen can result in host tissue damage (Riley *et al*, 2006). Further studies on NO production are required in *P. berghei* NK65-infected BALB/c mice with or without drug treatment.

BALB/c mice treated with the alkaloid mixture showed a rebound in parasitemia after transient suppression of parasite growth during medication use. It is noteworthy that the parasitemia levels just before death in the treated group were lower than those of the untreated controls. Waki *et al* (1992) reported that *P. berghei* NK65-infected CBA mice died with greater parasitemia and more severe anemia after treatment with anti-IFN-γ. In the present experiments, higher levels of IFN-γ

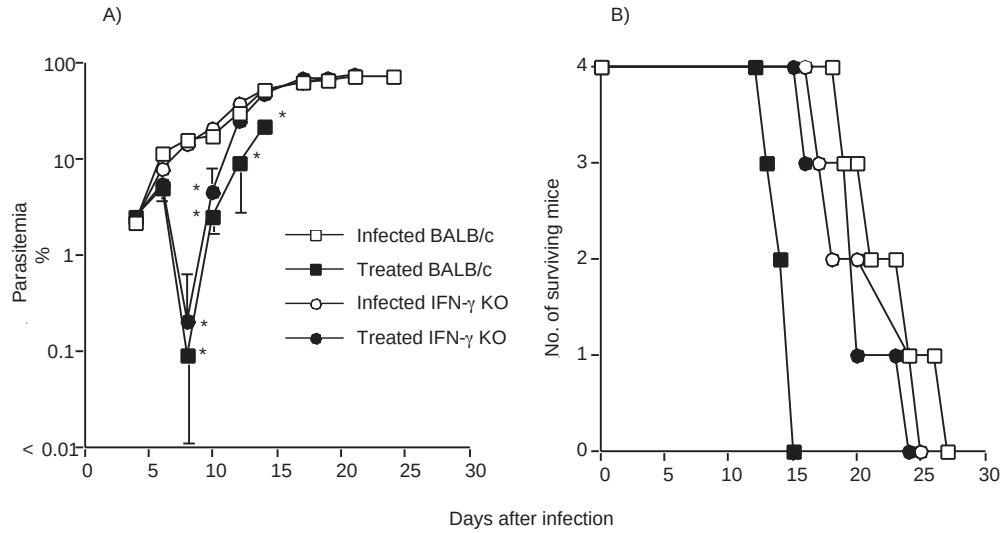

Fig 4–Time-course changes of survival rate (A) and parasitemia (B) of mice after oral treatment with the febrifugine and isofebrifugine mixture (Experiment 3). Male BALB/c and IFN-γ deficient (IFN-γ KO) mice on the BALB/c background were inoculated with 10^5 *P. berghei* NK65-parasitized erythrocytes. Infected mice were given the mixture at a dose of 1 mg/kg b.wt., twice a day for 4 consecutive days from Days 4 to 7 after infection. Results of parasitemia are expressed as mean values with standard deviation. Significantly different from the value of the infected and treated IFN-γ KO mice (Mann-Whitney *U* test, $p < 0.05$) indicated by asterisk.

were seen in the infected and alkaloid-treated group than in the infected but untreated group on Day 14 pI. Enhanced IFN-γ production may have resulted in acceleration of mouse mortality. Rebound parasite multiplication may serve as a secondary boosting effect on immune mechanisms. IFN-$\gamma^{-/-}$ mice showed no significant differences in survival duration or parasitemia patterns between the infected but untreated controls and the infected and treated mice, hence the potential involvement of IFN-γ in mouse death after medication use is implied. Yoshimoto *et al* (1998) observed that IL-12 p40 was produced in the culture supernatant of spleen cells and in the sera of C57BL/6 mice infected with *P. berghei* NK65. This suggests that IL-12 is involved in the pathogenesis of liver injury via IFN-γ production rather than protection in infected C57BL/6 mice. In the present study, IL-12 p70 production in the sera was enhanced after parasite inoculation but no significant difference in production was seen at different times between the infected but untreated and the infected and treated groups (data not shown). Thus, further studies are required to elucidate the precise role of IFN-γ in *P. berghei* NK65-infected BALB/c mice after drug treatment.

In CBA mice immunized by repeated inoculation of *P. berghei* NK65 followed with chemotherapy, significantly high anti-plasmodial IgG1 and IgG2a titers were observed (Akanmori *et al*, 1994). All immunized mice survived a challenge inoculation of parasite, showing a marked rise in IgG2a titer alone, but there were no elevations of Ig isotypes with primary *P. berghei* NK65 infection in CBA mice which died by the 10th day pI. In the present study, *P. berghei* NK65-infected BALB/c mice in the untreated control and in the alkaloid treated group died between Days 19 and 25

pI, and between Days 14 and 16 pI, respectively, with gradual weight loss and progressively increasing parasitemia. In both groups, the titers of parasite-specific IgG1, IgG2a, IgG2b and IgG3 were elevated significantly from Day 11, but the Ig isotypes did not control the infection. Long *et al* (2002) reported that *P. berghei* NK65-infected mice which received subcurative treatments with sulfadiazine became immunized, which induced a considerable level of protective immunity. The resolution of a primary *P. berghei* NK65 infection in BALB/c mice might be carried out by continuous chemotherapy with febrifugine of rebounded parasites, which should result in further elevation of specific Ig isotypes involved in the control of parasites.

In the present study, accelerated mortality of *P. berghei* NK65- infected BALB/c mice after treatment with the febrifugine and isofebrifugine mixture partly seems to be due to the IFN-γ-related mechanisms but not due to drug side effects, because the IFN-$\gamma^{-/-}$ mutant mice showed significantly longer survival duration than BALB/c mice did and autopsy showed no suspected toxicity. It is possible that this accelerated mortality of *P. berghei* NK65- infected BALB/c mice may be caused by other known antimalarial drugs such as chloroquine and pyrimethamine through IFN-γ-related mechanisms. It is therefore necessary to clarify the effects of antimalarial drugs on the etiology of lethal *P. berghei* NK65 infection which may contribute valuable information regarding the development of chemotherapy and vaccine design in human malaria.

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