CHLOROQUINE EFFICACY IN *PLASMODIUM BERGHEI* NK65-INFECTED ICR MICE, WITH REFERENCE TO THE INFLUENCE OF INITIAL PARASITE LOAD AND STARTING DAY OF DRUG ADMINISTRATION ON THE OUTCOME OF TREATMENT

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Abstract. We examined whether the initial number of parasites inoculated and the starting day of medication post-infection influenced the antimalarial efficacy of chloroquine (CQ) against *Plasmodium berghei* NK65 infection in ICR mice. Male ICR mice were inoculated intraperitoneally with 1×10^5, 1×10^6, 1×10^7, 1×10^8 *P. berghei* NK65-parasitized erythrocytes (pRBC). In the treated group, all mice received an oral dose of 20 mg/kg of CQ base for 4 days starting on day 0 after infection. From day 3, Giemsa-stained thin blood smears from tail vein blood were used to assess parasitemia. Mice in the untreated control in each group showed a progressive increase in parasitemia leading to death. Treatment of mice, inoculated with 1×10^5, 1×10^6 and 1×10^7 pRBC, with CQ showed a marked effect. All the mice survived during the experiment. During the observation period, malaria parasites could not be detected on microscopic examination. Conversely, mice inoculated with 1×10^8 pRBC showed little response to CQ treatment, and all mice showed a progressive increase in parasitemia and ultimately died. In another experiment, mice infected with 1×10^3 and 1×10^5 pRBC were treated with an oral four-day dosage of 20 mg/kg of CQ base from days 2, 3 or 4 post-infection. Treatment of mice, inoculated with 1×10^3 pRBC, with CQ from days 2 and 3 showed a marked effect. All mice survived during the experiment. However, treatment from day 4 showed a limited derease in parasitemia and all the mice ultimately died. On the other hand, treatment from day 2 showed a marked effect against 1×10^5 *P. berghei* NK65-infected mice, but treatment from days 3 or 4 was only slightly effective and all the mice died with an increasing parasitemia. The present results indicate that in *in vivo* antimalarial drug-assay systems, several factors, sush as initial parasite load and starting time of treatment may influence the drug response in the host.

INTRODUCTION

Malaria is one of the major health problems of the world, and chemotherapy is a widely used strategy in its control. Although it is true that the pharmacokinetics of drugs differ between humans and mice, it is also true that all mammalian *Plasmodium* species have comparable life cycles and are sensitive to the same drugs (Landau and Gautret, 1998). Four rodent Plasmodia species (*P. berghei, P. yoelii, P. chabaudi* and *P. vinckei)* infect inbred mouse strains with varying degrees of morbidity and mortality (Stevenson, 1989), and the lethal infections are better suited for investigating possible new chemotherapeutic interventions. The rodent malaria parasite *Plasmodium berghei* causes lethal infections in mice. The outbred albino mouse inoculated with *P. berghei* is generally considered to be a valid model for the primary and large-scale screening of drugs for eventual use against human malaria. *In vivo* antimalarial activity is commonly determined by the 4-day test against *P. berghei* strain (Peters *et al*, 1975).

Antimalarial drug resistance is on the increase. Currently combination chemotherapy is advocated as a rational approach to the containment of drug-resistant malaria (White and Olliaro, 1996). Despite the spread of chloroquine-resistant strains to many regions, chloroquine is

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still considered to play a valuable role in the treatment of acute malaria infections. New antimalarial drug-combinations are rapidly being developed and the search for drugs that can either potentiate or reverse chloroquine-resistance is also going on. The use of drug-resistant strains of rodent malaria parasites can yield additional information concerning both the mode of action of a compound, and its potential value against drug-resistant strains of human malaria. Therefore, a credible *in vivo* screening system needs to be established for testing the efficacy of these drugs against drug-resistant malaria. In this study, we investigated whether the initial number of parasites inoculated and/or the starting day of medication after inoculation influence the antimalarial efficacy of chloroquine against *P. berghei* NK65 infection in ICR mice.

MATERIALS AND METHODS

Animals and parasites

Outbred male ICR mice, 7 weeks old, purchased from SLC Inc (Hamamatsu, Japan), were used. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine. The rodent malaria parasite, *Plasmodium berghei* (strain NK65), was kindly supplied by Professor S Waki (Gunma Prefectural College of Health Sciences, Japan), and was maintained by serial blood passage in mice and blood stage parasites were stored at -80ºC. Initially, two mice were inoculated with the parasites from frozen stock, and the mouse showing 10-15% parasitemia was bled under ether anesthesia to collect the parasitized blood. Experimental mice were given an intraperitoneal injection of the parasitized blood. All mice were fed ad libitum on a commercial diet (LabDiet; PMI Nutrition International, Mo, USA) and water.

Compounds

Chloroquine diphosphate (CQ) was purchased from Sigma Chemical (MQ, USA), and dissolved in distilled water for administration to mice in a volume of 75 µl/10 g body weight (b.wt.).

Experimental design

Effects of the initial number of parasites inoculated. Mice were inoculated intraperitoneally with

$1x10^5$, $1x10^6$, $1x10^7$ or $1x10^8$ *P. berghei* NK65-parasitized erythrocytes (pRBC), and randomized into four mice per group for both treated and untreated groups. All mice in the treated groups were given a four-day oral dose of 20 mg/kg of CQ base from day 0 after infection, except for untreated controls which received a corresponding volume of distilled water. An equivalent volume of distilled water was given in the untreated, inoculated mice from day 0. From day 3 post-infection, Giemsa-stained thin blood smears made by bleeding via the tail vein were used to determine the course of infection throughout the observation period (expressed as % parasitemia). Mice were monitored for % parasitemia and days of survival relative to control mice up to 42 days post-infection (dpi).

Effects of the starting day of medication after parasite inoculation. In the subsequent experiment, mice were inoculated intraperitoneally with $1x10^3$ or $1x10^5$ pRBC, and randomized into four mice per group for both treated and untreated groups. The treated groups received a four-day oral dosage of 20 mg/kg of CQ base from days 2, 3 or 4 post-infection. A corresponding volume of distilled water was given in the untreated but incoculated mice from day 2. From the starting day of CQ administration, thin blood smears from the tail vein blood were used to monitor the course of infection throughout the observation period. Mice were monitored for % parasitemia and days of survival relative to the control mice up to 42 dpi.

RESULTS

Effects of the initial number of parasites inoculated

After inoculated intraperitoneally with $1x10^5$, $1x10^6$, $1x10^7$ or $1x10^8$ pRBC, mice in the untreated control showed a progressively increasing parasitemia, and all the mice died by day 11 (Fig 1). Treatment with a four-day oral dosage of 20 mg/kg of CQ base from day 0 after infection against mice inoculated with $1x10^5$, $1x10^6$ or $1x10^7$ pRBC showed a marked effect on the reduction of parasitemia, and all the mice survived during the experiment. Malaria parasites in the bloodstream of the mice could not be detected on microscopic examination during the obser-
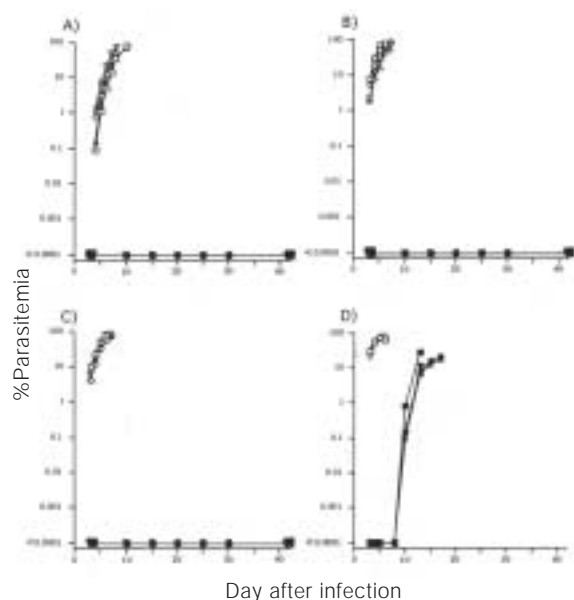
Fig 1–Time-course changes of parasitemia in the bloodstream of *Plasmodium berghei* NK65-infected ICR mice in response to treatment with chloroquine. Mice were intraperitoneally given 10^5 (A), 10^6 (B), 10^7 (C), 10^8 (D) *P. berghei* NK65-parasitized erythrocytes and were randomized into four per group. Infected mice were orally given chloroquine at a dose of 20 mg base/kg b.wt. once a day for 4 days from day 0 of infection (closed symbols). Control group of mice received an equivalent volume of distilled water (open symbols). Each symbol represents an individual mouse.
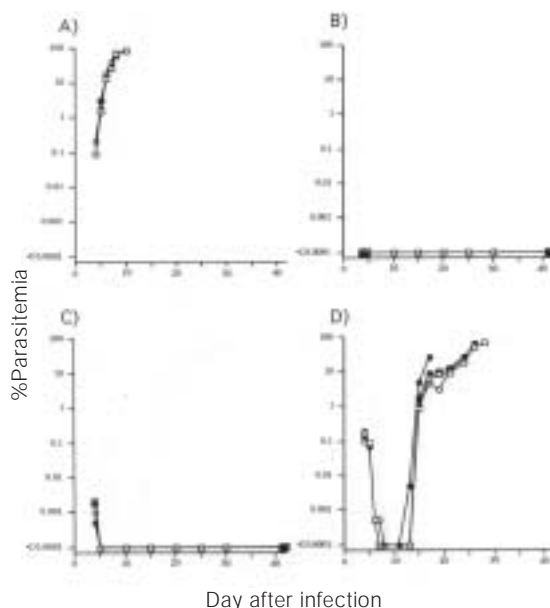
Fig 2–Parasitemia profile in the bloodstream of ICR mice intraperitoneally given 10^3 *Plasmodium berghei* NK65-parasitized erythrocyes. Infected mice were treated orally with a four-day dosage of 20 mg/kg b.wt. of chloroquine base in the treated group from days 2 (B), 3 (C) or 4 (D) after inoculation with parasitized erythrocytes. An equivalent volume of distilled water was given in the untreated, inoculated mice from day 2 (A). Each symbol represents an individual mouse.

vation period. However, for mice inoculated with 1×10^8 pRBC, the CQ treatment lowered the parasitemia to undetectable levels up to day 8 of infection but did not entirely eradicate it. From day 10 post-infection, parasitemia levels gradually increased and all the mice died by day 22 of infection.

Effects of the starting day of medication after parasite inoculation

Mice inoculated intraperitoneally with 1×10^3 or 1×10^5 pRBC were treated orally with a four-day dosage of 20 mg/kg of CQ base from days 2, 3 or 4 after infection. Untreated control mice inoculated with 1×10^3 pRBC showed a progressive increase in parasitemia, and all the mice died by day 12 (Fig 2). Treatment with CQ from day 2 post-infection showed a marked effect on mice inoculated with 1×10^3 pRBC and all the mice

survived the experiment without an increase in parasitemia. Malaria parasites in the bloodstream of the mice were not detectable by a microscopic examination during the observation period. Treatment with CQ from day 3 also showed a remarkable effect and all the mice survived the experiment with no increase in parasitemia. On day 4 of infection, less than 0.01% parasite was observed, but after medication it could not be detected by microscopic examination. Treatment from day 4 in mice with more than 0.1% parasitemia gave a decline in parasite load but not total parasite clearance. After medication, the malaria parasites decreased and could not be detected microscopically from days 7 to 11. On days 13 to 15, parasites reappeared and all the mice died by day 28 with increasing parasitemia.

All the mice infected with 1×10^5 pRBC died by day 11, showing a progressive increase in parasitemia (Fig 3). Treatment with CQ from day 2 showed a marked effect against 1×10^5 *P.*
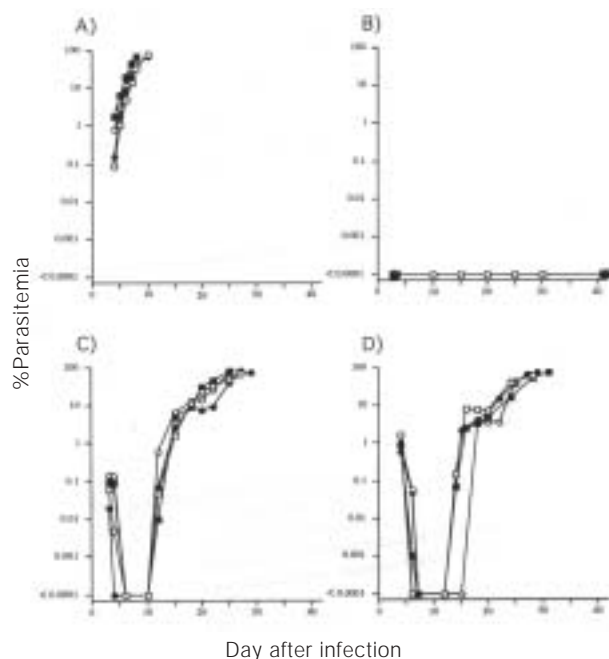
Fig 3–Parasitemia profile in the bloodstream of ICR mice intraperitoneally given 10^5 Plasmodium berghei NK65-parasitized erythrocytes. Infected mice were treated orally with a four-days 2 (B), 3 (C) or 4 (D) after inoculation with parasitized erythrocytes. An equivalent volume of distilled water was given in the untreated, inoculated mice from day 2 (A). Each symbol represents an individual mouse.

berghei NK65 infection, and all the mice survived during the experiment without an increase in parasitemia. However, treatment from day 3 or 4 initially achieved a decrease in parasitemia but all the mice died by day 32. Mice in both groups had more than 0.01% parasitemia on the starting day of CQ administration. After medication, malaria parasites decreased to undetectable levels for several days, but reappeared in the bloodstream and all the mice died with an increasing parasitemia.

DISCUSSION

In the classical 4-day test, mice were inoculated with approximately 10^7 parasitized erthrocytes intraperitoneally in the original experiments, but later intravenous infection via a tail vein was adapted (Peter et al, 1975). The infected mice were then treated once daily for 4 successive days from the same day of parasite inoculation. In the present experiments, we first examined whether or not the initial number of inoculated parasites influenced the antimalarial efficacy of CQ against Plasmodium berghei NK65 infection in ICR mice. The infected mice were treated orally with a four-day dosage of 20 mg/kg of CQ base from day 0 after infection of pRBC. Mice inoculated with less than 1×10^7 pRBC showed a marked response to treatment. All the mice survived; there was total parasite clearance from the bloodstream during the observation period. However, for mice inoculated with 1×10^8 pRBC, CQ treatment resulted in a transient parasite clearance, but all the mice died with a progressively increasing parasitemia. The sensitivity of P. berghei NK65 to CQ was determined from the duration of the prepatency of the infected mice which were treated with a single dose of CQ of 20, 10, 5 or 2.5 mg/kg, and the minimum effective dose of CQ was shown to be 10 mg/kg (Beaute-Lafitte et al, 1994). In principle, it seems that increasing drug concentrations eventually lead to better therapeutic resolution. The present levels of CQ administered to mice seem to be sufficient to clear parasites. However, the result of CQ treatment for mice inoculated with 1×10^8 pRBC suggests that a complex relationship exists between the parasite load and the efficacy of the drug. Landau et al (1990) recently proposed the presence of latent merozoites being able to penetrate into the erythrocytes at other times than schizogony. Free merozoites in the circulation are believed not to be vulnerable to the action of antimalarial drugs (Peters, 1998). Merozoites have been shown to be resistant to CQ (Cambie et al, 1991), and their latent forms may therefore penetrate into erythrocytes. Further studies are necessary to clarify the relationship between the parasite load in the host, CQ bioavailability and/or its effectiveness, and the host responses, including immune mechanisms in the course of medication.

In this study, the parasitemia levels on the day of starting the medication influenced the outcome of CQ treatment. After CQ treatment, all mice harboring less than 0.01% parasitemia achieved total parasite clearance and all of them survived. On the other hand, CQ treatment for mice with more than 0.01% parasitemia reduced parastemia to some extent but did not achieve

total parasite clearance. These results suggest that there is a threshold level of parasitemia in the bloodstream of mice, affecting the outcome of CQ treatment in *P. berghei* NK65 infection. This level should be determined in order to establish the relationship between the parasite load and drug response. Recently, drugs enhancing the sensitivity of parasite to CQ were researched in resistant strains of rodent and human *Plasmodium* (Martin *et al*, 1987; Bitonti and McCann, 1989; Peters *et al*, 1990; Kyle *et al*, 1993). The present results suggest that in antimalarial drug studies, either alone or in combination, it is more useful to evaluate the ideal time to initiate treatment in mice. In our *P. berghei* NK65 infected ICR model, the febrifugine and isofebrifugine mixture isolated from *H. macrophylla* var. *Otaksa* leaves enhanced the activity of CQ against *P. berghei* NK65 *in vivo* (Ishih *et al*, 2003). Whereas mice harboring more than 1% parasitemia could not be treated by CQ alone, combination of CQ with febrifugine and isofebrifugine mixture, decreased parasitemia to undetectable levels on microscopic examination during the observation period. On re-evaluating the antimalarial effects of tetracyclines, Lin *et al* (2001) reported the effectiveness of minocycline against CQ-resistant *P. falciparum in vitro.* In a previous study, treatment of mice harboring more than 1% parasitemia with a combination of CQ and minocycline hydrochloride also decreased the malaria parasites (Ishih *et al,* 2004). These experiments demonstrate that the above-mentioned *in vivo* method might be useful in detecting the combination effects of other drugs with CQ.

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