IN VIVO EVALUATION OF COMBINATION EFFECTS OF CHLOROQUINE WITH CEPHARANTHIN® OR MINOCYCLINE HYDROCHLORIDE AGAINST BLOOD-INDUCED CHLOROQUINE-RESISTANT *PLASMODIUM BERGHEI* NK 65 INFECTIONS

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Abstract: The combination effects of chloroquine with Cepharanthin® or minocycline hydrochloride were evaluated against a blood-induced infection with chloroquine-resistant *P. berghei* NK 65 in ICR mice. The infected mice in an untreated control group showed a progressively increasing parasitemia leading to mouse death. A two-day dosage of 20 mg base/kg of chloroquine alone produced little effect against *P. berghei* NK 65 infection, and all mice died from day 13 to 15 with an increasing parasitemia. A four-day dosage of 4 mg/kg of Cepharanthin® alone produced no antimalarial activity, and all mice died by day 10. A four-day dosage of 50 mg/kg of minocycline hydrochloride alone produced a slight effect, but all mice died by day 18. Furthermore, mice given chloroquine in combination with Cepharanthin® died from day 14 to 15. Mice given Cepharanthin® plus minocycline hydrochloride also died from day 15 to 17. On the other hand, infected mice treated with chloroquine plus minocycline hydrochloride survived during the experiment. All mice treated with chloroquine alone, minocycline hydrochloride alone, chloroquine plus Cepharanthin® or Cepharanthin® plus minocycline hydrochloride showed low parasitemia levels during drug administration and a few subsequent days, but then malaria parasites re-increased in the bloodstream of the treated mice until death. On the other hand, malaria parasites in the mice given chloroquine plus minocycline hydrochloride decreased on day 6 and then could not be detected by microscopic examination during the observation period. This finding strongly suggests that the combination effects of chloroquine and minocycline hydrochloride are worthy of evaluation in human malaria. The results also clearly demonstrate the necessity and importance of in vivo experiments in estimating the activities of drugs.

Key words: *Plasmodium berghei* NK 65, Cepharanthin®, minocycline, antimalarial activity, chloroquine-resistance

INTRODUCTION

Malaria is one of the most important tropical diseases in the world. Chloroquine resistance in the human malaria parasite *Plasmodium falciparum* arose first in South America and Southeast Asia (Harinasuta *et al.,* 1962; Young and Moore, 1961). It has now spread to all parts of the world where malaria is endemic and poses a major threat to the elimination of the disease. Although there are several strategies for controlling the disease, chemotherapy is the primary defense against malaria, and thus the worldwide emergence of chloroquine-resistant variants of parasite has stimulated the development of new treatments for malaria (Payne, 1987; Winstanley, 2000). The development of a new drug, however, is an extremely expensive and time-consuming process, and hence there have been efforts to re-evaluate the antimalarial activities of certain drugs already accepted for clinical use in patients with various non-malarial infectious diseases. White and Olliaro (1996) recently advocated the utility of combination chemotherapy as a rational approach to the containment of drug-resistant malaria. These trials focused on the detection of compounds that display killing activity against multiple drug-resistant malaria parasites in vitro (Haruki *et al.,* 2000; Lin *et al.,* 2001). However, the question remains as to which in vitro antimalarial effects can be induced in vivo. Since the discovery of murine malaria parasite *P. berghei* by Vincke and Lips (1948), the model of experimental murine malaria

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has proved to be very convenient both for detection of anti-malarial activity (Peters *et al.,* 1975) and investigations into the mode of action with *P. falciparum* in culture. Further-more, the mouse infected with *P. berghei* is generally con-sidered to be a valid model for the primary screening of drugs for eventual use against human malaria (WHO, 1973). In 1971, attempts to confirm the antimalarial activ-ity of *Artemisia annua* extracts in mice infected with *P. ber-ghei* led to the isolation of a plant constituent, artemisinin (Klayman, 1985). The use of drug-resistant strains of *P. berghei* can yield additional information concerning both the mode of action of a compound, and its potential value against drug-resistant strains of human malaria. Thus, we utilized the murine model to investigate the effects of chlo-roquine - in combination with drugs known to reverse chlo-roquine resistance in vitro (Haruki *et al.,* 2000; Lin *et al.,* 2001) - against blood-induced chloroquine-resistant *P. ber-ghei* NK 65 infections in ICR mice.

MATERIALS AND METHODS

Animals and parasites

All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine. Outbred male ICR mice, 7 weeks old, purchased from SLC Inc. (Hamamatsu, Japan), were used. Murine malaria parasites, chloroquine-resistant *Plasmodium berghei* (strain NK 65), were a gift from Pro-fessor Y. Wataya (Okayama University, Japan). The blood stage parasites were stored in a   80℃ deep freezer. For experiments, the parasites from frozen stock were injected into two mice. The mouse showing 10-15% of parasitemia was bled under ether anaesthesia to collect the parasitized blood. Experimental mice were given an intraperitoneal in-jection of the 10 parasitized blood. To determine the effect of the drug, treated mice were monitored for % parasitemia and days of survival relative to control mice up to day 30 post infection. Thin blood smears from the tail vein were prepared and Giemsa-stained throughout the observation period.

In vivo antimalarial activity of chloroquine against chloroquine-resistant P. berghei *NK 65 infections*

Twenty mice were infected intraperitoneally with 10 parasitized erythrocytes and divided into four per group, as well as an untreated control group, for activity assay of chloroquine diphosphate (Sigma Chemical Co., MO, USA). From day 4 after injection, the mice were given chloroquine orally at 20 mg base/kg body weight (kg) for 2 or 3 days, or 40 mg base/kg for 1 or 2 days, respectively, in the treated groups. An equivalent volume of distilled water was given orally to mice in the untreated, infected group for 3 days.

In vivo antimalarial activity of Cepharanthin[®] and minocy-cline hydrochloride against chloroquine-resistant P. berghei NK *65 infections*

Twenty-eight mice were infected intraperitoneally with 10 parasitized erythrocytes and divided into four per group, including an untreated control group, for activity assay of Cepharanthin[®] (an extract of *Stephania cepharantha*) and minocycline hydrochloride. Cepharanthin[®] and minocy-cline hydrochloride were purchased from Kaken-Shoyaku Co. (Tokyo, Japan) and Takeda Chemical Industries Ltd. (Osaka, Japan), respectively. From day 4 after injection, the mice were given Cepharanthin[®] at 0.2, 2 or 4 mg/kg in-traperitoneally once a day for 4 consecutive days. Mice in the other three groups were given minocycline hydrochlo-ride at 2.5, 25 or 50mg/kg intraperitoneally once a day for 4 consecutive days from day 4 after injection. An equivalent volume of sterile normal saline was given intraperitoneally to mice in the untreated control group.

Combination effects of the compounds against chloroquine-resistant P. berghei *NK 65 infections*

Twenty-eight mice were infected intraperitoneally with 10 parasitized erythrocytes and divided into four per group, including an untreated control group, for activity assay of the compounds in combination with or without chloroquine. From day 4 after injection, the mice in three groups were given chloroquine orally at 20 mg base/kg once a day for 2 days, and furthermore, to detect the activity of Cepharan-thin[®] and minocycline hydrochloride in combination with chloroquine, the mice in two out of three of the groups treated with chloroquine received Cepharanthin[®] at 4 mg/kg intraperitoneally once a day for 4 consecutive days and mi-nocycline hydrochloride at 50 mg/kg intraperitoneally once a day for 4 consecutive days. Furthermore, the mice in the other three groups were given Cepharanthin[®] at 4 mg/kg, minocycline hydrochloride at 50 mg/kg, or both drugs si-multaneously, intraperitoneally once a day for 4 consecutive days. An equivalent volume of water was given orally to mice in the untreated, infected group for 4 consecutive days.

RESULTS

In vivo antimalarial activity of chloroquine against chloroquine-resistant P. berghei *NK 65 infections*

As shown in Fig. 1A, mice in the untreated control group died from day 9 to day 11 post infection after a grad-ual body weight loss. Chloroquine treatment was somewhat effective against the parasites but it did not entirely eradi-cate them. All mice in the groups given the drug died after a gradual body weight loss as follows: mice given a two-day dosage of 20 mg base/kg died from day 12 to day 14, those given a three-day dosage of 20 mg base/kg died from
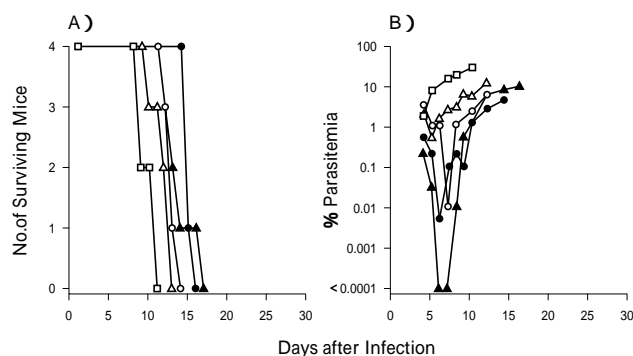
Fig. 1. Time-course changes of survival rate (A) and parasitemia in the bloodstream (B) of mice in response to treatment with chloroquine. From day 4 after injection of 10 *P. berghei* NK 65-parasitized erythrocytes, mice were given chloroquine orally at 20 mg base/kg b.wt. for 2 days ( ) or 3 days ( ), or 40 mg base/kg b.wt. for 1 day ( ) or 2 days ( ) in the treated group. An equivalent volume of distilled water was given orally in the untreated, infected mice ( ) for 3 days.
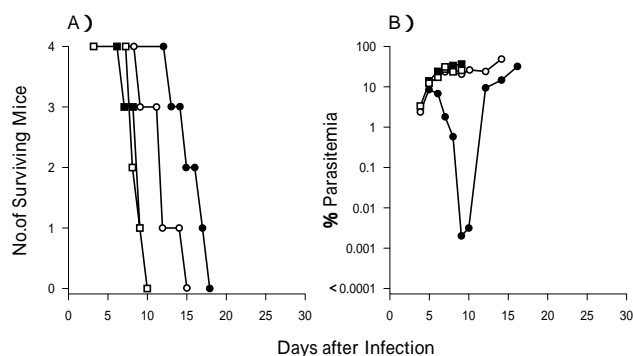

Fig. 2. Time-course changes of survival rate (A) and parasitemia in the bloodstream (B) of mice in response to treatment with minocycline hydrochloride. From day 4 after injection of10 *P. berghei* NK 65-parasitized erythrocytes, mice were given minocycline hydrochloride intraperitoneally at a dose of 2.5 ( ), 25 ( ) or 50 ( ) mg/kg b.wt. once a day for 4consecutive days. An equivalent volume of sterile normal saline was given intraperitoneally in the untreated, infected group ( ) for 4 consecutive days.

day 15 to day 16, those given a one-day dosage of 40 mg base/kg died from day 10 to day 13, and those given a two-day dosage of 40 mg base/kg died from day 12 to day 17. Malaria parasites appeared from day 4 post infection in the bloodstream of the control and treated groups (Fig. 1B). Parasitemia levels of mice in the control group gradually increased and all of the mice died by day 11. All of the mice treated with chloroquine showed low parasitemia levels during drug administration and the following few days. Especially, malaria parasites in the mice given a two-day dosage of 40 mg base/kg of chloroquine decreased on day 5 and then could not be detected by microscopic examination for two days. Malaria parasites, however, rebounded in the bloodstream of all treated mice and all eventually died.

In vivo antimalarial activity of Cepharanthin[®] and minocycline hydrochloride against chloroquine-resistant P. berghei *NK 65 infections*

Mice in the untreated control group died from day 8 to 10 post infection, showing a progressively increasing parasitemia (Figs. 2A and 2B). Four daily doses of 0.2, 2 or 4 mg/kg of Cepharanthin[®] from day 4 after parasite injection produced no antimalarial activity and all of the mice died by day 10, showing a pattern of parasitemia similar to that in the untreated control group (data not shown). A four-day dosage of 2.5 mg/kg of minocycline hydrochloride produced no effect, and all of the mice died by day 10. On the other hand, four daily doses of 25 or 50 mg/kg of minocycline hydrochloride produced a slight effect, but all of the mice died by day 17. Malaria parasites in the mice given a four-day dosage of 2.5 mg/kg of minocycline hydrochloride gradually increased until the mice died. A four-day dosage of 25 or 50 mg/kg of minocycline hydrochloride suppressed

the multiplication of parasites during drug administration and the following few days. Especially, malaria parasites in the mice given 50 mg/kg of minocycline hydrochloride decreased on day 6 but rebounded from day 10 in the bloodstream of all treated mice until death.

Combination effects of the compounds against chloroquine-resistant P. berghei *NK 65 infections*

Mice in the untreated control group died from day 9 to 11 post infection (Fig. 3A). A two-day dosage of 20 mg base/kg of chloroquine alone produced little effect against *P. berghei* NK 65 infection, and all of the mice died from day 13 to 15. Four daily doses of 4 mg/kg of Cepharanthin[®] alone produced no antimalarial activity, and all of the mice died by day 10. A four-day dosage of 50 mg/kg of minocycline hydrochloride alone produced a slight effect, but all of the mice died by day 18. Mice given Cepharanthin[®] plus minocycline hydrochloride died from day 14 to 17. Mice given chloroquine plus Cepharanthin[®] died from day 14 to 15. On the other hand, mice given chloroquine in combination with minocycline hydrochloride survived during the experiment. Malaria parasites appeared from day 4 in the bloodstream of the control and treated groups (Fig. 3B). Parasitemia levels of mice in the control and Cepharanthin[®]-treated groups gradually increased and all of the mice died. All mice treated with chloroquine alone, minocycline hydrochloride alone, Cepharanthin[®] plus minocycline hydrochloride and chloroquine plus Cepharanthin[®] showed low parasitemia levels during drug administration and the following few days. However, malaria parasites rebounded in the bloodstream of the treated mice until death. On the other hand, parasites in the mice treated with a combination
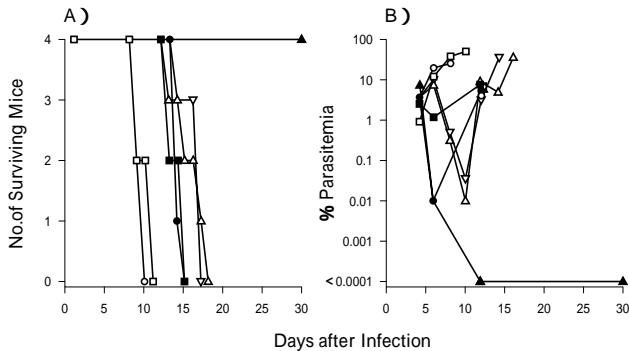
Fig. 3. Time-course changes of survival rate (A) and parasitemia in the bloodstream (B) of mice in response to the combined treatment. From day 4 after injection of 10 *P. berghei* NK 65-parasitized erythrocytes, mice in three groups were given chloroquine orally at a dose of 20 mg base/kg b.wt. once a day for 2 days, and then, except for mice in the chloroquine-medicated control group (   ), the remaining mice were further given the following drugs: Cepharanthin® at a dose of 4 mg/kg b.wt. intraperitoneally once a day for 4 consecutive days (   ) or minocycline hydrochloride at a dose of 50 mg/kg b.wt. intraperitoneally once a day for 4 consecutive days (   ). Additionally, Cepharanthin® alone (   ), minocycline hydrochloride alone (   ) or Cepharanthin® plus minocycline hydrochloride (   ) was given to the mice in the three other groups under the same treatment regimens. An equivalent volume of distilled water was given orally to the mice in the untreated, infected group (   ) for 4 consecutive days.

of minocycline hydrochloride and chloroquine decreased on day 6 and then could not be detected by microscopic examination during the observation period.

DISCUSSION

Recently, drugs enhancing the sensitivity of parasites to chloroquine were studied in resistant strains of rodent and human *Plasmodium* (Martin *et al.,* 1987; Bitonti and McCann, 1989; Peters *et al.,* 1990; Kyle *et al.,* 1993). In the present experiment, *P. berghei* NK 65 showed a chloroquine-resistant property, and hence this variant can be a useful model for research on drugs that enhance the sensitivity of parasites to chloroquine (Ishih *et al.,* 2003).

Lin *et al.* (2001) re-evaluated the antimalarial effects of three tetracyclines accepted for clinical use in patients with various non-malarial infectious diseases, and they reported that minocycline was effective against chloroquine-resistant *P. falciparum* in vitro. Their electron microscopic examination revealed a number of electron dense vesicles with a single membrane bound in the cytoplasm of minocycline-treated parasites (Lin *et al.,* 2001). The anti-parasite action of minocycline in vitro studies may be attributable to the high lipophilicity of this drug, but it is difficult

to explain whether the structural changes resulted from the specific action mechanism of tetracyclines. In our *P. berghei* NK 65 infected ICR model, all mice given minocycline hydrochloride alone showed low parasitemia levels during drug administration and the following few days, indicating the inhibitory effects of minocycline on parasite growth, but they died after re-increase of malaria parasites. In contrast, the combination of chloroquine with minocycline hydrochloride eventually produced a killing activity against *P. berghei* NK 65 parasites, and consequently, all mice survived during the experiment. Kim *et al.* (1998) evaluated a combination effect of 5-fluoroorotate and sulfamonomethoxine using this malaria isolate and reported a potent synergistic activity. By definition, chloroquine resistance reversal agents specifically target the chloroquine resistance mechanism and do not enhance the baseline activity of chloroquine against parasites (Martin *et al.,* 1987; Bitonti and McCann, 1989). In the present study, both chloroquine and minocycline hydrochloride showed moderate antimalarial activity against the present parasite isolate when given alone to the infected mice, and thus it remains unclear which drug enhanced the antimalarial activity of the other. This finding, however, strongly suggests that the combination of chloroquine and minocycline hydrochloride extends the clinical utility of chloroquine if the higher dose of minocycline hydrochloride is tolerated. Further studies are necessary to clarify the mechanisms of combination effects of these drugs.

Meanwhile, Haruki *et al.* (2000) reported that Cepharanthin®, widely used clinically in Japan, enhanced the activity of chloroquine against the resistant strain of *P. falciparum* in vitro. The mechanism by which Cepharanthin® increases the potency of chloroquine is unclear, but it may be related to enhanced accumulation of chloroquine. In our model, however, Cepharanthin® given approximately 20 times in clinical use (Harada *et al.,* 2001) produced neither combination effects with chloroquine nor its intrinsic antimalarial activity, suggesting the difference between in vitro and in vivo assay. The present results indicate that differences in assay systems, such as in vitro and in vivo methods, might lead to different and conflicting results for the antimalarial activities of drugs, and they indicate the necessity for and importance of in vivo experiments in the estimation of the activities of drugs.

REFERENCES

Bitonti, A.J. and McCann, P.P. (1989): Desipramine and cyproheptadine for reversal of chloroquine resistance in *Plasmodium falciparum*. Lancet, II, 1282-1283.

Harada K., Bando T., Yoshida H. and Sato M. (2001): Characteristics of antitumour activity of cepharantin against a human adenosquamous cell carcinoma cell line. Oral Oncol., 37, 643-651.

Harinasuta T., Migasen S. and Bunnag D. (1962): Chloroquine resistance in *Plasmodium falciparum* in Thailand. UNESCO First Regional Symposium on Scientific Knowledge of Tropical Parasites. Singapore, University of Singapore, 148-153.

Haruki, K., Bray, P.G., Ono, M. and Ward, S.A. (2000): Potent enhancement of the sensitivity of *Plasmodium falciparum* to chloroquine by the bisbenzylisoquinoline alkaloid Cepharanthin. Antimicrob. Agents Chemother., 44, 2706-2708.

Ishih, A., Suzuki, T., Watanabe, M., Miyase, T. and Terada, M. (2003): Combination effects of chloroquine with the febrifugine and isofebrifugine mixture against a blood-induced infection with chloroquine-resistant *Plasmodium berghei* NK 65in ICR mice. Phytother. Res., 17 (in press).

Kim, H.-S., Miyake, H., Arai, M. and Wataya, Y. (1998): A potent antimalarial activity of 5-fluoroorotate in combination with sulfamonomethoxine against *Plasmodium falciparum* in vitro and *Plasmodium berghei* in mice. Parasitol. Int., 47, 59-67.

Klayman, D.L. (1985) *Qinghaosu* (artemisinin): an antimalarial drug from China. Science, 228, 1049-1055.

Kyle, D.E., Milhous, W.K. and Rossan, R.N. (1993): Reversal of *Plasmodium falciparum* resistance to chloroquine in Panamanian *Aotus* monkeys. Am. J. Trop. Med. Hyg., 48, 126-133.

Lin, Q., Katakura, K., Oue, M., Kano, S. and Suzuki, M. (2001): Effects of minocycline against mefloquine-, chloroquine- and pyrimethamine-resistant *Plasmodium falciparum in vitro*. Jpn. J. Trop. Med. Hyg., 29, 343-348.

Martin, S.K., Oduola, A.M. and Milhous, W.K. (1987): Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science, 235, 899-901.

Payne, D. (1987): Spread of chloroquine resistance in *Plasmodium falciparum*. Parasitol. Today, 3, 241-246.

Peters, W., Portus, J.H. and Robinson, B.L. (1975): The chemotherapy of rodent malaria, XXII The value of drug-resistant strains of *P. berghei* in screening for blood schizontocidal activity. Ann. Trop. Med. Parasitol., 69, 155-171.

Peters, W., Ekong, R., Robinson, B.L., Warhurst, D.C. and Pan, X.-Q. (1990): The chemotherapy of rodent malaria. XLV. Reversal of chloroquine resistance in rodent and human *Plasmodium* by antihistaminic agents. Ann. Trop. Med. Parasitol., 84, 541-551.

Vincke, I.H. and Lips, M. (1948): Un nouveau plasmodium d'un rangeur du Congo (*Plasmodium berghei* n. sp.). Annals Societe Belgique Medicine Tropicale, 28, 97.

White, N.J. and Olliaro, P.L. (1996): Strategies for the prevention of antimalarial drug resistance: Rationale for combination chemotherapy for malaria. Parasitol. Today, 12, 399-401.

Winstanley, P.A. (2000): Chemotherapy for falciparum malaria: The armoury, the problems and the prospects. Parasitol. Today, 16, 146-15.

World Health Organization (1973): Chemotherapy of malaria and resistance to antimalarials. World Health Organization Technical Report Series, No.529.

Young, M.D. and Moore, D.V. (1961): Chloroquine resistance in *Plasmodium falciparum*. Am. J. Trop. Med. Hyg., 10, 317-320.