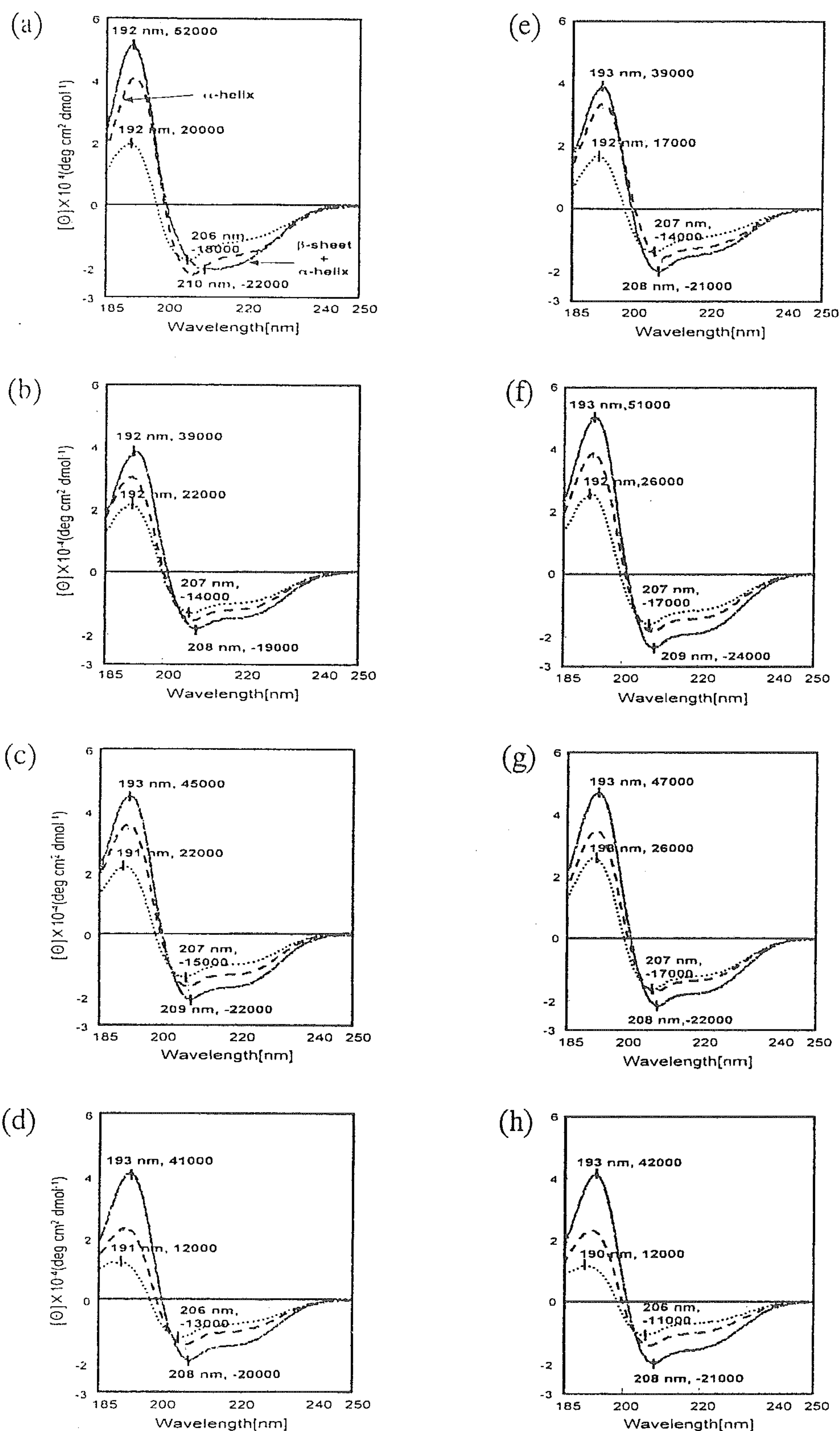

*Fig. 2. CD Spectral changes of in the absence of chloroquine and in the absence ((a-d) for **1-4**, respectively) and presence of chloroquine (1 eq. mol) ((e-h) for **1-4**, respectively) . [peptide] = 1 mg/1 mL, at 20 °C. ——: in n-butanol-HFIP = 5:5 (= v/v). – – –: in n-butanol-HFIP = 2:8 (= v/v). - - - -: in HFIP.*

The SVMNT peptide, **2**, exhibits the intensifying of CD bands by the addition of n-butanol (Fig. 2b). This indicates the stabilization of the α-helical conformation with decreasing the dielectric constant of the solution. The band intensity is further increased in the presence of chloroquine (Fig. 2f). In this case, chloroquine stabilizes the α-helical conformation by electrostatic and hydrogen bond interactions.

For the SVMET peptide, **3**, the three bands are gradually increasing in intensity with increasing n-butanol concentration (Fig. 2c). Very similar spectral change was observed even in the presence of chloroquine. Therefore in this sequence, CD spectroscopy is not probably suitable to study the interaction between the peptide, **3**, and chloroquine (Fig. 2g).

The CD spectra of a CQS sequence, **4**, were intensified by the addition of n-butanol (Fig. 2d). The spectral change was not affected by the addition of chloroquine (Fig. 2h). This suggests that **4** does not interact with chloroquine, although further spectral studies, such as ^{1}H NMR, are required for confirmation.

By the comparison of three CQR and one CQS sequences, we have realized structural effects of the Lys76Thr mutation. The spectral change indicates that the Thr residue probably gives an interaction site with chloroquine molecules when transporting the anti-malarial drug from the digestive vacuole.

Acknowledgements

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The Japanese Peptide Society (2003)

Solution-Phase Synthesis and Structural Analysis of Multiple Antigenic Peptides Having Partial Sequences of *Plasmodium Falciparum* Enolase

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We have compared the structures of Lys- and Glu-branched multiple antigenic peptides (MAPs). A short epitopic sequence from Plasmodium falciparum enolase was anchored to the branched core. Circular dichroism spectral study indicated that the Lys-branched MAP significantly induces α-helical conformation in the epitope parts. While the Glu-branched MAP shows only unordered feature even in a trifluoroethanol solution.

Introduction

Malaria is caused by protozoal infection, and is the most serious tropical disease. Especially, one of the causative parasites, *Plasmodium falciparum* (*P. f.*) has been paid attention due to its virulency [1].

Multiple antigenic peptides (MAPs) are widely used to generate site-specific antibodies [2,3], and are expected to be good candidates for efficient synthetic vaccine toward malaria. However, the structural information of the branching core in MAPs is quite few [4]. In the present study, we have clarified how a branching core can stabilize or destabilize the epitopic sequences on a MAP.

Materials and Methods

The peptides were synthesized by solution-phase method using fragment condensation and stepwise coupling procedures. All the peptide compounds used in this study are summarized in Figure 1. In the syntheses of MAPs, we have employed a convergent procedure where four or two protected NL7 sequences are ligated to the Lys- and Glu-branched core with strong but clean coupling reagents, HATU/HOAt [5]. The deprotection of protecting groups on MAPs (3-5) was carried out with trifluoromethanesulfonic acid-thioanisole-trifluoroacetic acid mixture [6]. As an example, the synthetic procedure of **4** is shown in Figure 2. All the peptides were purified by reprecipitation and chromatography. The purity and structure for prepared compounds were confirmed by melting point, specific rotation, ^{1}H-NMR

and mass spectroscopy. **2**: ESI-MS (m/z) calced for $C_{38}H_{64}N_{10}O_{15}$: 900.5 (monoisotopic mass). Found, 901.5 ($[M+H]^{+}$). **3**: ESI-MS (m/z) calced for $C_{85}H_{143}N_{23}O_{31}$: 1982.03 (monoisotopic mass). Found, 992.3 ($[M+2H]^{2+}$). **4**: MALDI-TOF-MS (m/z) calced for $C_{85}H_{143}N_{23}O_{31}$: 3937.5 (monoisotopic mass). Found, 3936.8 ($[M]^{+}$).

Circular dichroism (CD) spectra were taken on a Jasco J-700 spectropolarimeter in a trifluoroethanol (TFE) solution. Obtained spectra were expressed as mean residue molar ellipticity ($[\theta]_R$, deg.cm^2.dmol^{-1}).

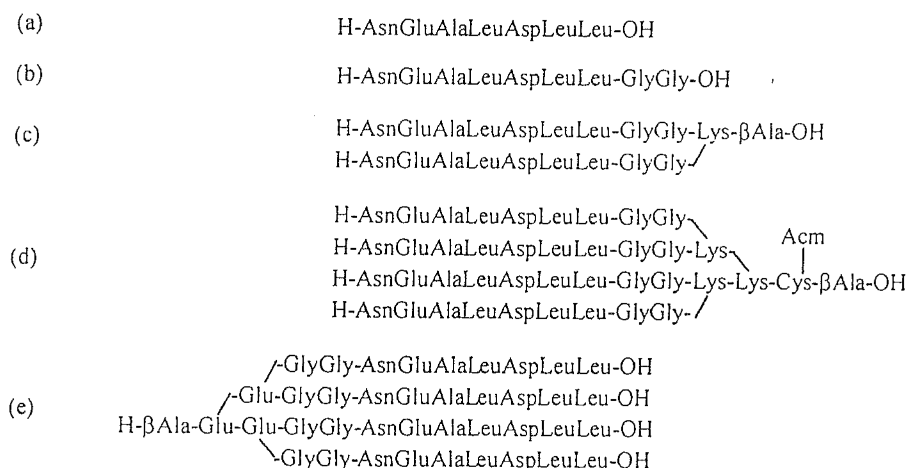

*Figure 1. Schematic structure of the peptides used in this study. (a) H-NL7-OH (**1**), an epitope peptide from the sequence of Plasmodium falciparum enolase, (b) H-NL7-GG-OH (**2**), the epitopic sequence having a -Gly-Gly- linker, (c) di-epitope and (d) tetra-epitope MAPs with Lys branching (**3** and **4**, respectively), (e) tetra-epitope MAP with Glu branching (**5**).*

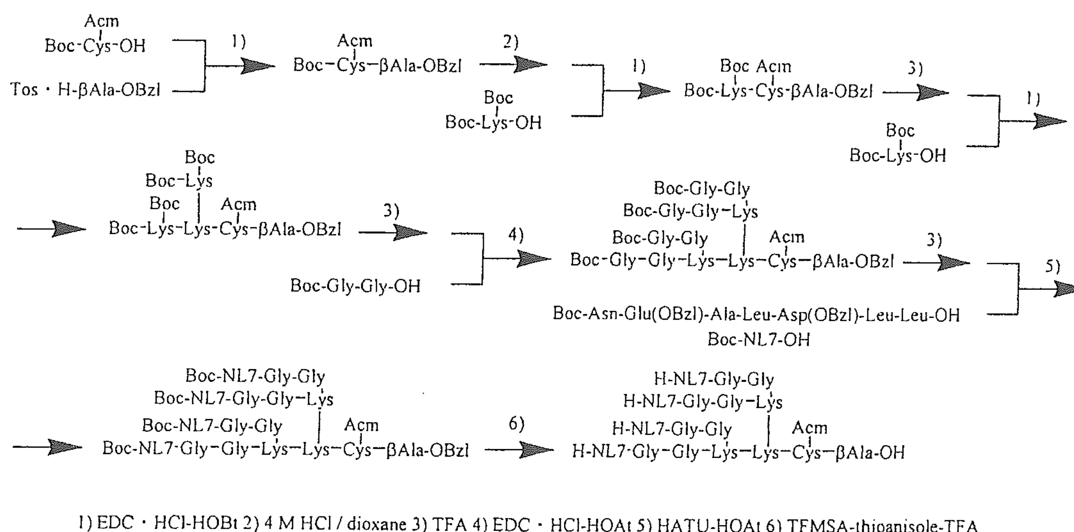

*Figure 2. Synthetic strategy of tetra-epitope MAP with Lys-branching core (**4**).*

Results and Discussion

We first compared the branching cores for Lys- and Glu-type MAPs (**4** and **5**) by using CD spectroscopy (Figure 3a). The CD spectrum of **4** indicated that the Lys-core induces α-helix in a short epitopic sequence, NL7. While in the case of **5**,

the Glu-core does not show any stabilization of the MAP structure.

Figure 3b shows CD spectra of the tetra-, di-, and mono-epitope compounds (**1-4**). Interestingly by the addition of a -Gly-Gly- sequence to **1**, clear bands appeared at around 190, 205, and 220 nm as observed in the spectrum of **2**. This indicates weak but significant α-helix conformation in **2** with the extension of -Gly-Gly- linker. Initially, this linker was expected to be a loose connector between the epitopic sequence and the branching core in MAP compounds, **3-5**.

Figure 3b also indicates that four-branched MAP (**4**) has the most stable helical structure among the compounds, **1-4**. Therefore, the number of epitopic sequences is indeed an important factor in the stabilization of the whole MAP structure.

For Lys-type MAP compounds, **3-4**, we have studied CD spectra at various temperatures and concentrations. Figure 4 shows the temperature dependence of the molar ellipticity at 222 nm ($[\theta]_{222}$) for the peptides. With increasing temperature from -10 to +50 °C, band intensities was decreased by 32, 39, and 42% for **2-4**, respectively.

Figure 5 shows the CD spectral change of **2-4** with diluting concentration of the peptide. Interestingly, the mean residue ellipticity was increased by 54, 67, and 23% for **2-4**, respectively. Thus we have found that higher concentration of MAP solution destabilize α-helical structure in these peptides, especially in the di-epitope compounds, **3**.

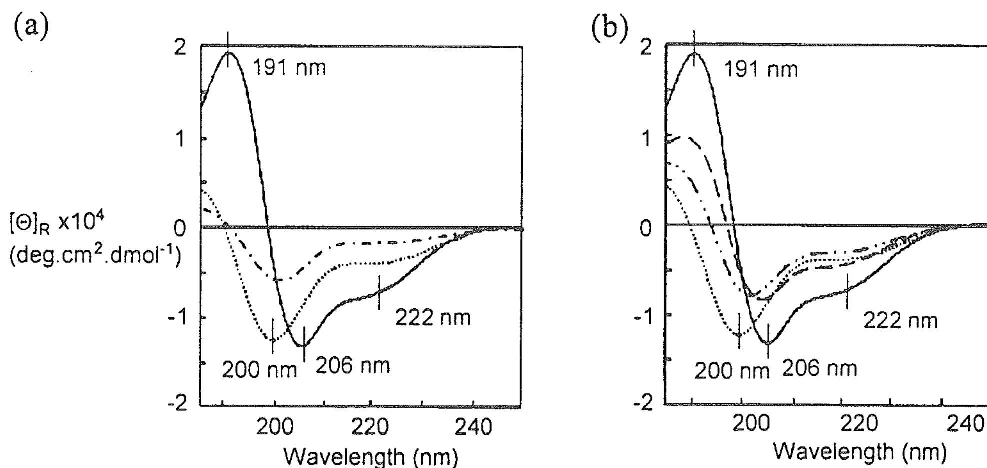

Figure 3. Comparison of CD spectra in TFE (cell length 0.1 mm, 1.0 mg/mL at 20 ℃). The NL7 epitopic sequence with (2, ------) and without (1, –··–··–) -Gly-Gly- linker. Di-epitope (3, — — — —) and tetra-epitope (4, ———) MAPs with Lys branching. Glu-branched (5, –·–·–) MAP.

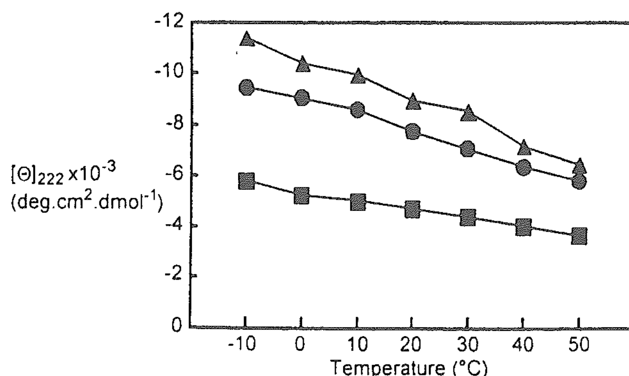

Figure4. Temperature dependence of CD band intensity at 222 nm for 3 (●), 4 (▲), and 2 (■). (TFE solution, cell length 0.1 mm, 1.0 g/mL).

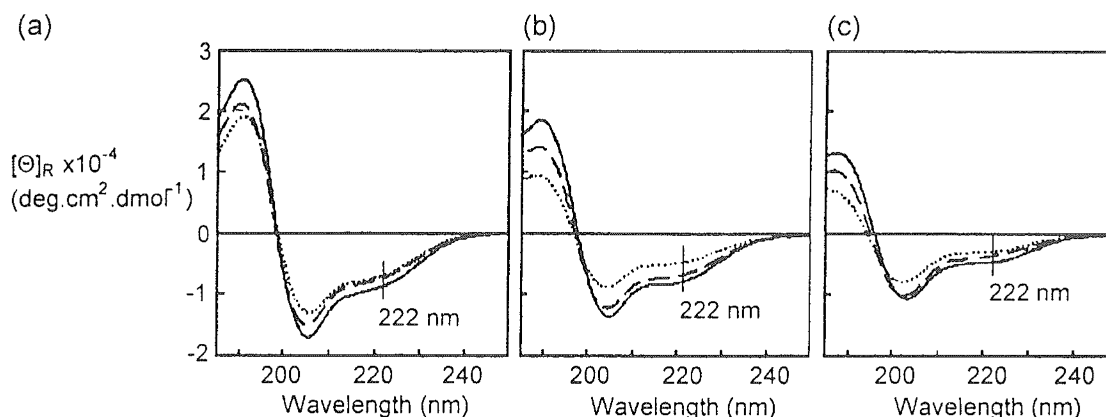

*Figure 5. Concentration dependent CD spectra for (a) **4**, (b) **3**, and (c) **2** in TFE. Measurement conditions: cell length 0.1 mm, 1.0 mg/mL (•••••); cell length 0.5 mm, 0.5 mg/mL (— — — —); cell length 1.0 mm, 0.1 mg/mL (———).*

Summary

We have successfully prepared the Lys- and Glu-branched MAPs by solution peptide synthesis. By comparison of CD spectra, we have found that the conformation of the epitope sequence is significantly stabilized/destabilized by (a) Lys- or Glu-branching core, (b) temperature, (c) concentration. Especially the di-epitope compound **3**, was influenced. Therefore we should consider very carefully the design for synthetic peptide antigens.

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In-vitro uptake of vitamin A by *Plasmodium falciparum*

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The vitamin-A uptake of *Plasmodium falciparum* was investigated by culturing a standard isolate of the parasite (FCR-3) with ^{3}H-labelled vitamin A, at concentrations of the vitamin corresponding to those normally present in human serum. The ^{3}H-labelled vitamin A accumulated in the parasites from each culture in a parasitaemia-dependent manner. The radioactivity detected in the parasites increased with parasite maturation from the ring to the late-trophozoite stage. In addition, most of the radioactivity incorporated into the parasite cells was in the cytoplasm. The accumulation of vitamin A in the cytoplasm of late trophozoites indicates that *P. falciparum* may use vitamin A, from its human host, as an antioxidant, to protect itself from oxidative stress while intra-erythrocytic. The amount of the vitamin taken up by the parasite *in vitro* is small compared with the deficit that sometimes causes severe hypovitaminosis A in malaria cases. Consumption of vitamin A by the parasites together with the systemic decreases in non-enzymatic antioxidants that are seen in malaria may together cause this characteristic hypovitaminosis.

In humans, vitamin-A deficiency may exacerbate the morbidity and mortality associated with several infectious diseases (Scrimshaw *et al.*, 1968; Sommer *et al.*, 1983). In particular, hypovitaminosis A has been reported as a factor involved in the aggravation of malaria (Rosales *et al.*, 2000) and significant decreases in the serum concentration of vitamin A are often observed in severe malaria (Thurnham and Singkamani, 1991; Stürchler *et al.*, 1987). In rodent models at least, serum concentrations of vitamin A appear to be inversely correlated with levels of parasitaemia (Krishnan *et al.*, 1976). Malaria-associated hypovitaminosis A has been attributed to the hepatic dysfunction that is widely observed among patients with advanced malarial infection (Davis *et al.*, 1998). Such dysfunction can lead to the inhibition of retinol (vitamin A) release from retinol-binding protein (RBP) in the liver, regardless of the pre-infection retinol status of the patient. However, since RBP is found not only in the liver but also in several other organs and tissues and particularly in serum, the impaired release of retinol from RBP in the liver is unlikely to be the sole cause of the severe hypovitaminosis A often observed in malaria cases (Rosales *et al.*, 1996). The aim of the present study was to determine whether uptake of vitamin A by *Plasmodium falciparum* could be contributing to the vitamin-A deficiency.

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MATERIALS AND METHODS

Plasmodium falciparum Culture

The FCR-3 strain of *P. falciparum* was cultured (Trager and Jensen, 1976) at 37°C in a computerized, mixed-gas incubator (Astec, Fukuoka, Japan) in an atmosphere of 90% N_2, 5% O_2 and 5% CO_2. The culture medium — RPMI 1640 adjusted to pH 7.4 (Gibco BRL, Grand Island, NY) and supplemented with 10% (v/v) normal human serum and 10 μg gentamicin/ml — was changed daily, and fresh human erythrocytes (of blood type O) were added when required. The intra-erythrocytic parasite stages were synchronized with 5% D-sorbitol (Lambros and Vanderberg, 1979) before each experiment. The parasite-infected erythrocytes were incubated in 6-cm-diameter culture dishes at 5% haematocrit until the parasitaemia reached approximately 15%.

Preparation of ^{3}H-labelled Vitamin A

The ^{3}H-labelled vitamin A [with a specific activity of 72.878 disintegrations/min (dpm)/pmol] was bought, from NEN Life Science Products (Boston, MA), as [11,12-^{3}H(*N*)]-all-*trans* retinol. This ^{3}H-retinol and 'cold' (i.e. non-radiolabelled) retinol were mixed, and the ^{3}H-retinol then separated from ^{3}H-retinal and ^{3}H-retinyl esters, by chromatography on open columns of de-activated aluminium oxide and elution, with diethyl ether/hexane (50:50 by volume), into glass tubes. The solvent was evaporated. The residue was firstly mixed with 100 μl ethanol and a small amount of fatty-acid-free bovine serum albumin (BSA) and then dissolved in 5 ml phosphate-buffered saline (PBS). This stock solution was stored in the dark at −80°C until used.

Growth-inhibition Assays

To see if the addition of vitamin A inhibited the in-vitro multiplication of the parasites, cultures containing various concentrations (0–64 μM) of 'cold' retinol were incubated in duplicate for 4 days. Levels of parasitaemia were estimated, every 24 h (as percentages of erythrocytes infected), by the preparation and microscopical examination of Giemsa-stained smears. The counts (of infected cells/10,000 erythrocytes) were repeated at least three times for each culture.

Assays of Vitamin-A Uptake

In the initial investigations of vitamin-A uptake, the cultures were either left undiluted (with a 'high' parasitaemia of 15%) or diluted with RPMI medium and normal erythrocytes to give intermediate (7.5%) or low (3.75%) levels of parasitaemia. Normal (uninfected) erythrocytes in RPMI medium were used as the negative control. The parasite and control cultures were supplemented with 1 μM ^{3}H-vitamin A and then incubated for 24 h at 37°C, in a conventional incubator containing an AnaeroPack® gas-regulation pouch (Mitsubishi Gas Chemical Company, Tokyo; Mizuno *et al.*, 2000). For the subsequent time-course experiments, cultures with intermediate (7.5%) parasitaemias were similarly incubated for 4, 8, 12 or 24 h.

After incubation, the erythrocytes in each culture were pelleted and lysed with PBS containing 0.05% saponin. The parasite cells in the lysate, having been freed from the erythrocytes, were precipitated by centrifugation (4000 × *g* for 10 min at 4°C) and then the cell pellets were washed twice in 100 volumes of PBS. The final parasite pellet was mixed with a scintillation cocktail in a glass vial, and the radioactivity measured by liquid-scintillation counting in a Tri-Carb 2700TR unit (Packard Instruments, Downers Grove, IL).

To rule out non-specific binding of the retinol outside the parasite cell membrane, the parasite cells that had been cultured with ^{3}H-retinol were separated into cell-membrane and cytoplasmic fractions. For this, the parasite cells were disrupted using several cycles of freezing (in a mix of dry ice and ethanol) and thawing (in a water-bath at 37°C) and then a brief sonication (using a microtip and several 5- to 10-s bursts). The lysate

produced was centrifuged (15,000 × *g* for 30 min at 4°C) to produce a supernatant solution (the cytoplasmic fraction) and a pellet (the cell-membrane fraction). A sample of each fraction was smeared, stained with Giemsa and microscopically examined. The radioactivity in each fraction was measured as described above.

All the experiments involving the radioisotope were done in the area designated for such investigations, at the Research Institute of the International Medical Centre of Japan, in Tokyo.

RESULTS

Although incubation for 4 days with higher concentrations clearly inhibited the multiplication of *P. falciparum* FCR-3, it appeared that retinol at the concentrations typically observed in human serum (1–3 μM) had no significant effect on the parasite *in vitro* (Fig. 1). Retinol at 1 μM was therefore used in all of the subsequent experiments.

The radioactivity levels detected in the parasite cells from the 24-h cultures increased with (initial) level of parasitaemia (Fig. 2).

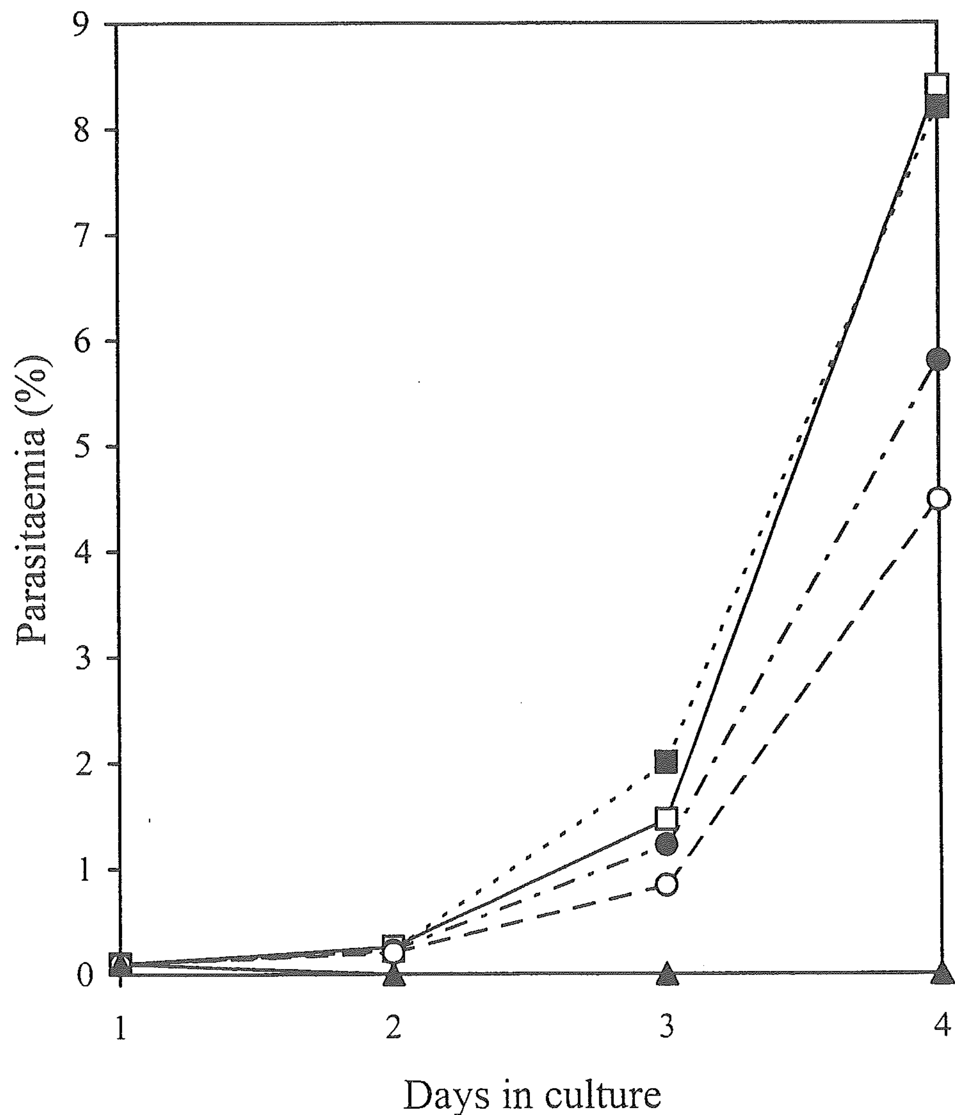

FIG. 1. The multiplication *in vitro* of *Plasmodium falciparum* FCR-3 in medium containing 0 (■), 2 (□), 8 (●), 32 (○) or 64 (▲) μM retinol. The results shown are the mean parasitaemias seen in triplicate cultures.

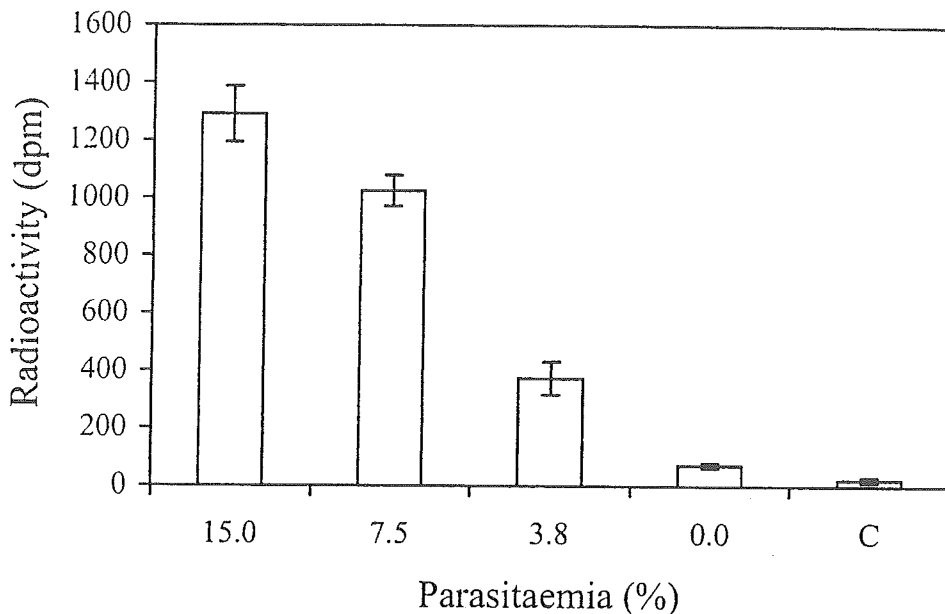

FIG. 2. Retinol uptake over 24 h by *Plasmodium falciparum* FCR-3 *in vitro*. Cultures with high (15%), intermediate (7.5%) or low (3.75%) parasitaemias, or control cultures with uninfected erythrocytes (i.e. a 0% parasitaemia) were cultured with 1 μM ^{3}H-labelled retinol. As a further control (C), infected erythrocytes were cultured without ^{3}H-labelled retinol. Results show the mean levels of activity, in disintegrations/min (dpm), found in the parasites isolated from triplicate cultures. The vertical lines indicate S.D.

Only a negligible amount of radioactivity was detected in the corresponding sample from the parasite-free, negative-control culture (Fig. 2). The level of radioactivity recorded in the culture that initially had a 15% parasitaemia, which contained 1.88×10^8 parasite-infected erythrocytes, was 1300 dpm. Since the specific activity of the labelled retinol was 72.878 dpm/pmol, approximately 1 pmol of ^{3}H-retinol had been incorporated for every 10^7 parasite-infected erythrocytes during the 24-h incubation.

Radioactivity increased with incubation time up to 24 h (Fig. 3), even though, in the synchronized cultures used, there was no multiplication of parasites within 24 h, only maturation of the parasites from ring stages to late trophozoites. After incubation for 24 h, almost all of the parasites were at the late-trophozoite stage.

When the parasites were divided into membrane and cytoplasmic fractions, only 30% of the radioactivity was detected in the membrane fraction (Fig. 4). (Transfer of radioactivity from the membrane fraction to the cytoplasmic during the brief sonication is an unlikely possibility.)

DISCUSSION

Although retinol at concentrations equivalent to those normally found in human serum inhibits the in-vitro development of several culture-adapted strains of *P. falciparum* (Davis *et al.*, 1998), it had no apparent effect on FCR-3 (present study) or the chloroquine-resistant strain investigated by Samba *et al.* (1992). The susceptibility of *P. falciparum* to retinol therefore appears to vary with the strain of the parasite involved, or perhaps with the length of the period since the strain was isolated. As the use of adjunctive retinol therapy in the clinical management of malaria is being considered (Davis *et al.*, 1998), it would be useful to know more about the in-vivo effects on the parasite of retinol in the serum.

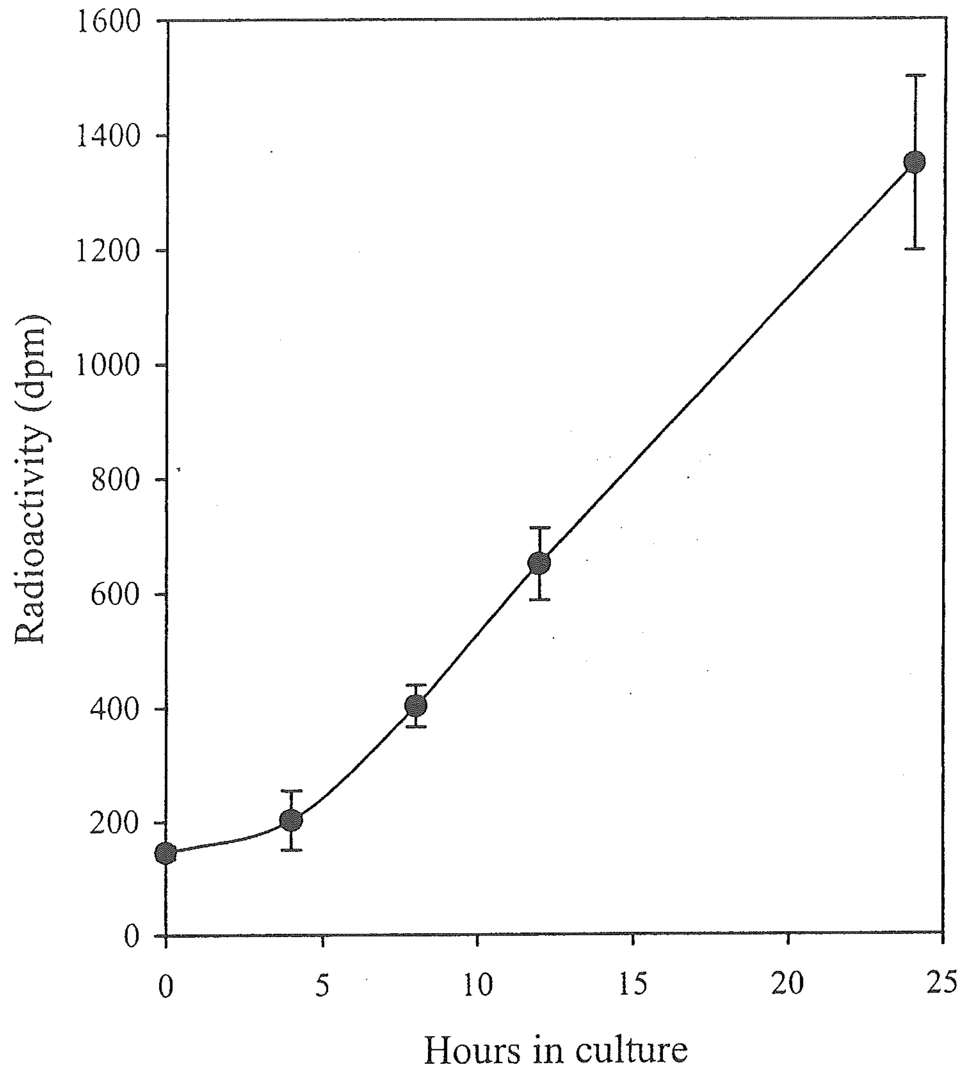

FIG. 3. Time course of retinol uptake by *Plasmodium falciparum* FCR-3 *in vitro*. The cultures were incubated with 1 μM ^{3}H-labelled retinol for 4, 8, 12 or 24 h, before the total radioactivity in the parasite cells from each culture was measured. Results show the mean levels of activity, in disintegrations/min (dpm), found in the parasites isolated from triplicate cultures. The vertical lines indicate S.D.

The present results indicate that malarial parasites can incorporate retinol during their development, at least *in vitro*. If the level of incorporation observed (i.e. 1 pmol taken up by 10^7 parasite-infected erythrocytes every 24 h) occurred in an adult case of severe malaria with 15% parasitaemia and 30% haematocrit, the parasites would daily remove about 0.1 μmol retinol from their host's serum. This theoretical loss of retinol from the serum is small compared with the deficits causing severe hypovitaminosis A in some malaria patients, although parasite uptake may contribute to such deficits. The mechanism that lies behind most of the fall in serum concentrations of retinol seen in malaria-associated hypovitaminosis A remains unknown. The hypovitaminosis may largely be attributable to changes in the host's metabolism, presumably related to changes in the cytokine profile in the presence of *P. falciparum* infection (Frankenburg *et al.*, 1998).

Plasma concentrations of several non-enzymatic antioxidants (vitamin A, carotenoids and vitamin E) are depressed in children who present with acute, uncomplicated, *P. falciparum* malaria (Metzger *et al.*, 2001).

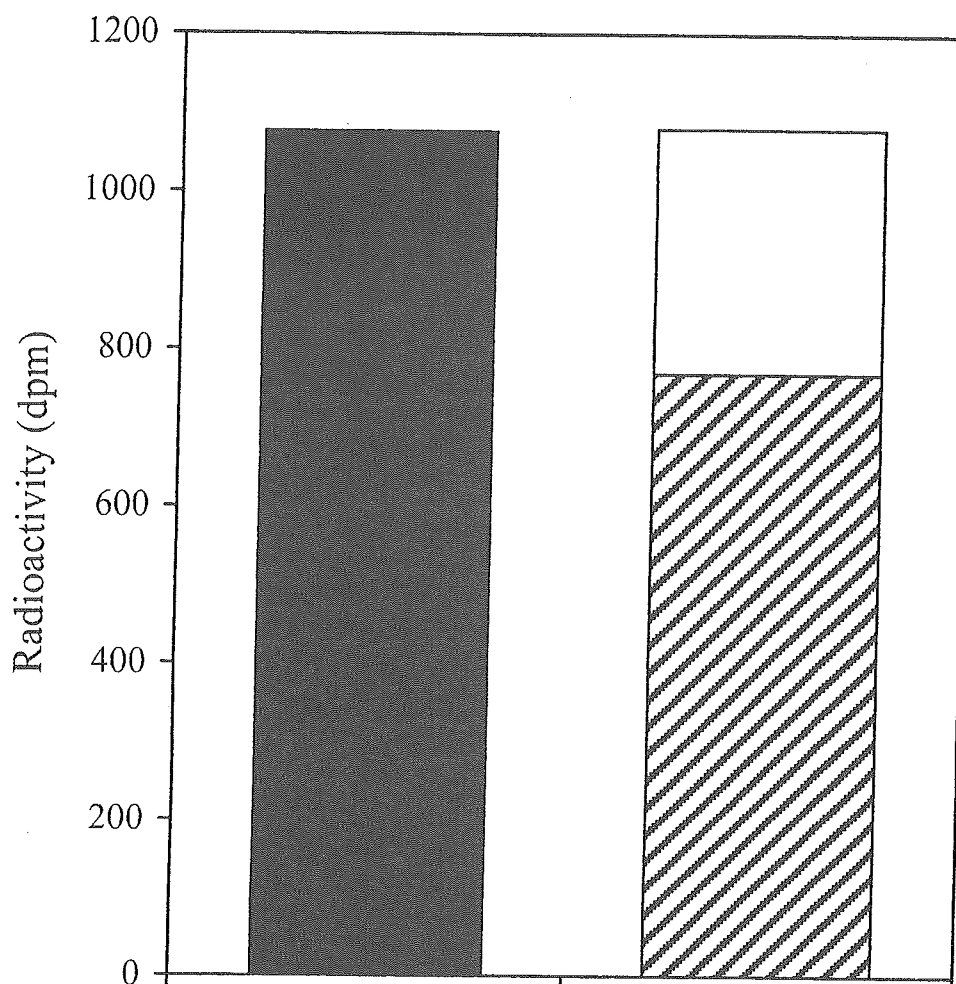

FIG. 4. Localization of radioactivity in cells of *Plasmodium falciparum* FCR-3 that had been cultured with 1 μM ^{3}H-labelled retinol for 24 h. The bars indicate the mean levels of radioactivity, in disintegrations/min (dpm), in whole cells (■) and the cytoplasmic (▨) and membrane fractions (□). The mean values and (S.D.), from triplicate cultures, were 1076.39 (163.78), 768.11 (127.53) and 311.71 (93.54) dpm/culture, for the whole cells, cytoplasmic fractions and membrane fractions, respectively.

A reduced reserve of vitamin E (α-tocopherol) in the erythrocyte membrane may contribute to erythrocyte loss in severe malaria (Griffiths *et al.*, 2001) and tumor necrosis factor-α, a major cytokine involved in the pathogenesis of malaria (Clark *et al.*, 1987; Kern *et al.*, 1989), decreases the cellular concentrations of another antioxidant, glutathione, in various tissues and cells (Ishii *et al.*, 1992), including erythrocytes (Mohan *et al.*, 1992). Depressed concentrations of these non-enzymatic anti-oxidants in infected erythrocytes may lead to the intra-erythrocytic parasites being subject to additional oxidative stress. These parasites are vulnerable to such stress, especially as they generate superoxide (Clark *et al.*, 1989; Becker *et al.*, 1994). It seems possible that the parasites take up vitamin A from human serum to compensate for the increased oxidative stress. This hypothesis is supported by the observation, in the present study, that uptake of vitamin A by the late-trophozoite stages is greater than that of the ring stages (Fig. 3). The late trophozoites metabolise host haemoglobin and produce high levels of reactive oxygen species (Griffiths *et al.*, 2001).

Although the influence of pre-existing hypovitaminosis cannot be ruled out, the systemic decrease in non-enzymatic anti-oxidants together with the consumption of vitamin A by the parasites themselves may

cause the hypovitaminosis A seen in malaria patients. Further studies will be conducted to clarify whether vitamin A is consumed only as an antioxidant or whether it is metabolised into other materials in the parasite cell.

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COMPARATIVE CLINICAL TRIAL OF TWO-FIXED COMBINATIONS DIHYDROARTEMISININ-NAPTHOQUINE-TRIMETHOPRIM (DNP®) AND ARTEMETHER-LUMEFANTRINE (COARTEM®/ RIAMET®) IN THE TREATMENT OF ACUTE UNCOMPLICATED FALCIPARUM MALARIA IN THAILAND

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Abstract. An open randomized comparison of two-fixed dose artemisinin derivative-containing combination regimens was conducted in adults with acute uncomplicated multidrug resistant falciparum malaria in Thailand. DNP®, a combination of dihydroartemisinin with napthoquine and trimethoprim developed recently in China, has been evaluated in China, Vietnam, Cambodia and Thailand. This study was performed to compare the safety, tolerability and efficacy of DNP® and artemether-lumefantrine/ Coartem®. One hundred and thirty eligible uncomplicated falciparum malaria patients were enrolled into the study. Patients were randomly assigned in a 2:1 ratio into group A, which received DNP® one tablet twice a day for one day; and group B, which received Coartem®/ Riamet® four tablets twice a day for 3 days. The cure rates at 28-day were 99% and 97% in group A and group B, respectively. No serious adverse events occurred. We concluded that both DNP® and Coartem®/ Riamet® were safe, well tolerated and highly efficacious in the treatment of acute uncomplicated falciparum malaria in Thailand.

INTRODUCTION

Malaria has always been a major killer in the tropics. It remains one of the largest global health care problems of the 21st century. Multidrug resistance and compliance with treatment are still major obstacles to be confronted. Efficacious and easily administered drugs are urgently needed. With problems of multidrug resistant falciparum malaria in Thailand (Bunnag and Harinasuta, 1987; Looareesuwan *et al*, 1992a; Nosten *et al*, 1996), the rationale of combining drugs with independent modes of action and different resistance mechanisms is to improve therapeutic efficacy and to prevent or delay the emergence or development of resistance (Peters, 1990; White, 1998). Recent guidelines in treating malaria strongly recommend the use of combinations of artemisinin derivatives with other antimalarial agents (WHO, 2001).

Recently, clinical trials of a combination of artesunate with mefloquine has proved this to be a very effective and well-tolerated regimen (Looareesuwan *et al*, 1992b; 1994; 1996a; Nosten *et al*, 1994; Price *et al*, 1995; 1997), therefore this regimen has been chosen for the treatment of multidrug resistant falciparum malaria in Thailand. However, some patients cannot tolerate the adverse effects of mefloquine.

Coartem®/ Riamet®, an oral fixed-dose combination tablet, consisting of 20 mg of artemether (half-life circa 1 hour) and 120 mg of lumefantrine (half-life 3 days), was developed in China and has been registered for use in many countries (von Seidlein, *et al*, 1997; Hatz *et al*, 1998; van Vugt *et al*, 1998; 1999; Looareesuwan *et al*, 1999; van Agtmael *et al*, 1999; Bakshi *et al*, 2000; Lefevre *et al*, 2001). The currently recommended treat-

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ment is a six-dose regimen given over three days (Looareesuwan *et al*, 1999; van Agtmael *et al*, 1999; Lefevre *et al*, 2001).

Dihydroartemisinin, an active metabolite of all artemisinin derivatives, has also been evaluated in Thailand for the treatment of falciparum malaria since 1994 (Klayman, 1985; Looareesuwan *et al*, 1996b; Wilairatana *et al*, 1998). *In vitro*, it is 2-3 times more active than the derivatives and can reduce parasitemia by 90% within 24 hours after administration. Clinical trials in Thailand revealed 80-90% cure rates depending on the dosage and duration of treatment (Looareesuwan *et al*, 1996b; Wilairatana *et al*, 1998).

DNP® (compound napthoquine), which consists of 160 mg of dihydroartemisinin, 400 mg of naphthoquine and 200 mg of trimethoprim, was also developed in China, and has been used in clinical trials in China, Vietnam, Cambodia and Thailand in over 1,000 patients (Wilairatana *et al*, 2002). The results of these studies have shown good efficacy (Professor GQ Li, personal communication). This drug was donated free of charge by Professor GQ Li, Republic of China. The advantages of this fixed combination drug are the short duration of treatment and ease of administration (one tablet is given 12 hours apart for 1 day). The aim of this study was to determine the safety, tolerability and efficacy of DNP® and Coartem®/ Riamet®.

MATERIALS AND METHODS

All patients who fulfilled the inclusion criteria [acute uncomplicated falciparum malaria, either sex (if female, a pregnancy test had to be negative before enrollment into the study), positive asexual forms of *P. falciparum* in blood smear, weight more than 40 kg and age more than 14 years, able to take oral medication, agree to stay in the hospital for at least 28 days, informed consent provided by patients or guardians must be obtained] were included in the trial. They were admitted to the Bangkok Hospital for Tropical Diseases and remained there for 28 days to exclude reinfection and to assess the safety, tolerability and efficacy of DNP® and Coartem®/ Riamet®. We excluded severe malaria, according to the WHO criteria (2000), severe vomiting not allowing oral medication, pregnancy or lactation, significant concomitant systemic diseases or disease requiring therapy, and ingestion of other antimalarials in the past 14 days, or presence of urine sulphonamides or 4-aminoquinolones upon admission. This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Clinical evaluation, including vital signs and full physical examination including neurological examination, were evaluated every day for the first 7 days, and weekly thereafter. Body temperature, pulse and respiratory rates were recorded every 4 hours and blood pressure was measured every 12 hours throughout the study. Laboratory measurements, including hematological and biochemistry examination, were performed at baseline, and on days 7, 14, 21 and 28. Parasitological examinations, consisting of thick and thin blood films, were also evaluated every 12 hours until the film was negative, then daily for 28 days. Blood films were considered negative if no parasites were seen in 200 oil-immersion microscopic fields. The malaria parasite count per µl was obtained by calculation against 1,000 red blood cells or 200 white blood cells in thin and thick blood film, respectively.

Each patient was randomly assigned in a ratio 2:1 into groups A:B as follows: group A received DNP® (compound naphthoquine) one tablet orally twice a day for one day (total dose = 2 tablets); group B received Coartem®/ Riamet® 4 tablets orally twice a day for 3 days (total dose = 24 tablets).

The parameters of the response to treatment were the 28-day cure rate, parasite clearance time and fever clearance time. The cure rate at day 28 (cured patients/ evaluable patients x 100%) was defined as the absence of parasite recrudescence during 28 days of in-patient follow-up. Parasite clearance time (PCT) was defined as the time from the start of treatment until the first negative blood film and blood films remaining negative for the next 24 hours. Fever clearance time (FCT) was taken as the period from the start of treatment until the oral temperature decreased to 37.5°C and remained below this temperature for the next 48 hours. Side-effects were defined as signs and symptoms that occurred or became more severe after drug administration. If there were RI, RII or RIII failure (World Health Organization, 1973), standard antimalarial regimens of the hospital would be given. All patients were to be treated symptomatically, as indicated according to the standard practice of the hospital.

We performed statistical analysis using the

Epi Info Version 6.04 (USD, Inc, Stone Mountain, GA, USA) software package. The means of various parameters for the 2 groups were compared by Student t-test, while using the chi-square test or Fisher's exact test where appropriate for comparing categorical variables. Two-tailed tests were used throughout and a p-value < 0.05 was considered statistically significant.

RESULTS

A total of 130 eligible patients (89 in group A and 41 in group B) admitted to the Bang Hospital for Tropical Diseases were enrolled i the study. These two groups were compara before treatment in all demographic, clinical a laboratory characteristics except for the high i cidence of liver enlargement in group A, whi did not interfere with the objective of the stuc (Table 1). The baseline clinical signs and symp toms of both groups were also similar and re flected the underlying malaria infection.

After treatment, 16 patients (12%) withdrew from the study [9 (10%) and 7 (17%) in group A

Table 1
Clinical and laboratory characteristics of study groups before treatment.

	Group A[a] (n = 89)	Group B[b] (n = 41)	p-value
Male: Female	57 : 32	30 : 11	0.31
Age			
Mean (SD)	25.4 (12.1)	28.3 (13.5)	0.21
Min-Max	14 - 62	14 - 73	
Height			
Mean (SD)	155.5 (12.7)	159.6 (7.5)	0.10
Min-Max	130 - 185	147 - 172	
Weight			
Mean (SD)	50.5 (9.6)	50.5 (8.0)	0.98
Min-Max	40.0 - 87.4	40.0 - 75.5	
Fever			
Duration (days)	5.6 (5.1)	3.9 (4.7)	0.07
Highest temperature	37.9 (0.9)	37.7 (0.9)	0.19
No. of patients with			
Hepatomegaly (%)	16 (18)	16 (39)	< 0.01
Splenomegaly (%)	13 (15)	4 (10)	0.45
First malaria attack	45 (51)	15 (37)	0.17
Initial parasite count			
Geometric mean			0.38
Min-max	14 - 203,720	80 - 172,380	
Laboratory data [Mean (SD)]			
Packed cell volume (%)	34.1 (7.1)	36.4 (6.8)	0.09
WBC count (per µl)	5.6 (4.9)	5.7 (1.8)	0.83
BUN (mg/dl)	14.2 (5.9)	13.5 (6.8)	0.59
Cr (mg/dl)	0.88 (0.18)	0.91 (0.20)	0.53
TB (mg/dl)	1.3 (1.2)	1.7 (1.4)	0.12
DB (mg/dl)	0.36 (0.58)	0.57 (0.53)	0.06
SGOT (U/l)	41.2 (52.6)	44.6 (40.4)	0.72
SGPT (U/l)	31.0 (25.9)	41.0 (39.8)	0.39
Albumin (g/dl)	3.9 (0.4)	3.7 (0.4)	0.11
AP (U/l)	107.5 (58.8)	120.1 (48.3)	0.25

WBC = white blood cell.
[a]DNP® (compound naphthoquine) one tablet orally twice a day for one day (total dose = 2 tablets).
[b]Coartem®/ Riamet® 4 tablets orally twice a day for 3 days (total dose = 24 tablets).

Table 2
Therapeutic responses.

	Group A[a] (n = 89)	Group B[b] (n = 41)	p-value
No. of patients with 28-days' follow-up	80	34	0.26
No. (%) cured at 28 days	79	33	0.53
Recrudescence on day	28	21	
Fever clearance time			
Mean (SD)	32.8 (27.7)	41.2 (37.3)	0.35
Min-Max	4 - 156	4 - 144	
Parasite clearance time			
Mean (SD)	43.9 (17.4)	48.1 (15.1)	0.18
Min-Max	9 - 124	22 - 104	
No. of patients with *P. vivax*	1	0	

[1]DNP® (compound naphthoquine) one tablet orally twice a day for one day (total dose = 2 tablets).
[2]Coartem®/ Riamet® 4 tablets orally twice a day for 3 days (total dose = 24 tablets).

and group B, respectively] for reasons unrelated to their treatments. All had negative blood films and were well upon discharge from hospital. Thus, 114 out of 130 patients (88%) remained in the hospital for a full 28 days' follow-up. Only patients who were followed for 28 days were included in calculations of drug efficacy.

Table 2 shows the parasitological and clinical responses to the treatments. The cure rate at 28-day revealed high efficacy in both groups (99% and 97% in groups A and B, respectively). Two cases (one in each group) had RI responses. These two cases and another patient treated with DNP® who had positive asexual forms of *Plasmodium vivax* in blood smears were successfully treated with the standard antimalarial regimens of the hospital. Similar to the cure rate, both fever and parasite clearance times showed no significant differences. Parasitemia in most of the patients was not detected in their blood smears within 72 hours. Fig 1 shows the percent reduction in parasitemia after treatment.

No deterioration in clinical or biochemical responses, or deaths, occurred after treatment in either group. In addition, there were no serious adverse events and neurological or neuropsychiatric manifestations during treatment and during the 28-day period. Some minor symptoms, such as nausea, headache, dizziness occurred in 4, 5 and 7 cases in group A and in 2, 2 and 4 cases in group B, respectively. However, these signs and symptoms could not be differentiated from malaria signs and symptoms since they disappeared simultaneously with fever within 1-4 days after treatment.

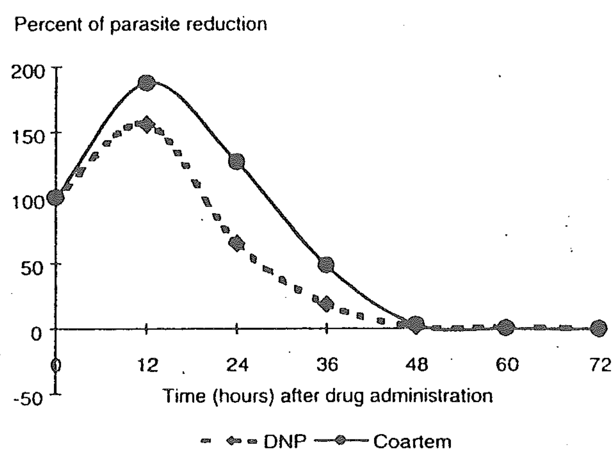

Fig 1–Mean parasitemia as compared to parasitemia at the onset of treatment.

DISCUSSION

Artemisinin derivatives are potent, rapidly-acting antimalarials that can reduced parasitemia by more than 90% within 24 hours in uncomplicated malaria cases. However, the rate of recrudescence within 28 days when used alone can be as high as 10-100%, depending upon dosage, duration of treatment and severity of disease (Arnold *et al*, 1990; Bunnag *et al*, 1991; Hien *et al*, 1991; Li *et al*, 1994). Artemisinin derivatives are often

combined with other long-acting antimalarials, (in group A combined with naphthoquine, dihydroartemisinin and trimethoprim and in group B combined with lumefantrine), to improve efficacy and patient compliance. The rationale underlying the use of combinations is the same as that underlying the standard multidrug treatments for tuberculosis, patients with HIV and most cases of cancer. The advantages of adding artemisinin derivatives to the combination are as follows: the rapid killing of parasites by the artemisinin derivatives accelerates the therapeutic response, prevents dangerous early treatment failure in cases of high-grade resistance, reduces the parasite biomass and provides gametocytocidal activity (Looareesuwan *et al*, 1999). The benefits of adding an appropriate and suitable long-acting drug is to prevent recrudescence by killing residual parasites, to reduce the chance of a resistant mutant surviving, and in addition the long-acting antimalarial might protect the artemisinin derivatives from the emergence of resistance in low-transmission areas. Combined administration of artemisinin derivatives and longer-acting antimalarials, such as pyronaridine and mefloquine, have been being evaluated in uncomplicated malaria in many countries. At present, Roll Back Malaria, under the World Health Organization, recommends the use of artemisinin-based combinations. In Thailand, the combinations (*eg* quinine-tetracycline, artesunate-mefloquine and artemether-lumefantrine) have been registered for use and recommended for the treatment of multidrug resistant falciparum malaria. However, minor adverse effects are relatively common with quinine-tetracycline and artesunate-mefloquine. Currently, there is no fixed combination of artesunate and mefloquine available in the market. DNP® and Coartem®/ Riamet® have the advantage of being fixed combinations. Moreover, DNP® has a short duration of treatment and possibly lower cost than the other drugs.

In this study, all patients responded satisfactorily to the two treatment regimens with similarly high levels of efficaciousness (99% and 97% cure rates at 28 days, respectively). The combinations of the individual drugs and the role of synergy between naphthoquine, dihydroartemisinin and trimethoprim remain to be determined. Interestingly, with the shorter duration (1-day treatment with 2 doses) of DNP®, when compare with the 3-day treatment with 6 doses of Coartem®/ Riamet®, might give better patient compliance to complete the treatment course.

In conclusion, this study indicated that DNP® is as effective and well-tolerated as Coartem®/ Riamet®, and may be an alternative treatment to the standard combination treatment of uncomplicated multidrug resistant falciparum malaria, such as in Thailand. However, additional studies in special groups (in children, pregnant women and field trials) are needed in order to get more information about DNP® in general practice.

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IN VITRO SUSCEPTIBILITY OF *PLASMODIUM FALCIPARUM* ISOLATES TO CHLOROQUINE AND MEFLOQUINE IN SOUTHEASTERN MINDANAO ISLAND, THE PHILIPPINES

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Abstract. Although the presence of multi-drug-resistant falciparum malaria has been reported in the Philippines, the distribution of drug-resistant malaria parasites has not yet been determined in Mindanao Island. *In vitro* susceptibility of *P. falciparum* to both chloroquine and mefloquine was assessed to forecast the spread of drug-resistant parasites in various foci in southeastern Mindanao Island. Of the 33 isolates of *P. falciparum* successfully tested, 10 (30%) were susceptible, 12 (36%) showed decreased susceptibility (80 nM $\leq IC_{50} <$ 114 nM), and 11 (33%) were resistant ($IC_{50} \geq$ 114 nM) to chloroquine. Ten (91%) of the resistant isolates and 9 (75%) of those with decreased susceptibility were from northern and northwestern Davao del Norte Province. Chloroquine-susceptible isolates were found among patients in the eastern parts of Davao del Norte and Davao Oriental provinces. Seven isolates from several foci in the study area were all mefloquine- susceptible ($IC_{50} <$ 10 nM). This is the first report indicating the potential emergence of chloroquine-resistant *P. falciparum* on Mindanao Island, which is presently regarded as a drug-susceptible area.

INTRODUCTION

Malaria is one of the major fatal parasitic diseases endemic to many tropical and subtropical regions of the world. Global distribution of drug-resistant *Plasmodium falciparum* is making malaria treatment increasingly difficult (White, 1996; Alrajhi *et al,* 1999). Chloroquine had been the most reliable antimalarial drug for more than three decades until the emergence and spread of drug-resistant *P. falciparum* rendered its application ineffective in much of the world (White, 1996; Alrajhi *et al,* 1999). However, chloroquine remains the drug of choice in a number of malaria-endemic foci for the treatment of uncomplicated malaria (Okonkwo *et al,* 2001; Thanh *et al,* 2001).

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In the Philippines, malaria is a major public health problem; about 11 million people live in endemic areas. Approximately 31% of the population lives in high malaria transmission areas (Pilarita *et al,* 1999). Shute *et al* (1972) first reported *in vitro* amodiaquine resistance of *P. falciparum* isolates in the Philippines. As of 1996, between 23 to 39% of all cases of falciparum malaria in Palawan Island were reported to be resistant to chloroquine (Baird *et al,* 1996). Although the existence of chloroquine-resistant parasites in the northeastern Philippines has been reported, local distribution of drug-resistant isolates on Mindanao Island has not yet been evaluated. Because the island is close to Indonesia, where drug-resistant malaria parasites are very prevalent (Verdrager *et al,* 1976; Pribadi *et al,* 1992), it is important to survey the drug susceptibility of isolates in the Mindanao region. In this study, the *in vitro* susceptibility of *P. falciparum* isolates to chloroquine and mefloquine was assessed to clarify the current distribution of drug-resistant falciparum malaria on Mindanao Island.