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Decrease in susceptibility of *Plasmodium falciparum* to mefloquine in continuous culture*

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Chloroquine-resistant P. falciparum, *line FCK from Thailand, was tested for susceptibility to mefloquine in continuous culture. The drug resulted in 50% schizont inhibition at the level of 8.0 nmol/ml of culture when tested for the first time. After three months of discontinuous exposure to mefloquine at increasing levels, the sensitivity of the parasite decreased. A concentration as high as 128 nmol/ml eventually was required for 50% inhibition of growth.*

The occurrence of chloroquine resistance in *Plasmodium falciparum* in Thailand has increased rapidly since it was first reported by Harinasuta in 1961 (*1*). More recent data from this country has shown that about 99% of isolates are resistant to chloroquine (*2*). Chemoprophylaxis and treatment of chloroquine-resistant *P. falciparum* has been possible with a combination of pyrimethamine and sulfadoxine (*3*). However, it has been reported recently that higher doses of this drug are required for treatment, and the cure rates are lower than was previously the case.[a] This indicates that *P. falciparum* in Thailand is also developing resistance to this drug combination.

Synthesis and screening of 60 000 compounds by the US Army has given rise to one promising drug, mefloquine. It is an analogue of quinine and of the experimental drug WR 30090, which was used successfully to treat chloroquine-resistant falciparum malaria in Viet Nam (*4*). Single-dose treatment with mefloquine gave satisfactory results and it was also found that this drug actively suppresses both falciparum and vivax malaria. It is well tolerated and produces no side effects at a dose as high as 500 mg/kg of body weight (*5*). *In vitro* studies of responsiveness of chloroquine-resistant *P. falciparum* to mefloquine showed that concentrations of 1.0 nmol per ml of blood could readily inhibit development of schizonts in all of the cultures tested in Thailand (*6*).

Using the Petri dish continuous culture system developed by Jensen & Trager (*7*), we have observed that a concentration as low as 0.05 nmol/ml of medium inhibited the growth of about half the population of *P. falciparum*. Healthy ring forms would develop into schizonts if the mefloquine was removed and fresh erythrocytes were added to the culture. These surviving parasites appeared to tolerate mefloquine when treatment was repeated. It was therefore of interest to investigate whether or not selection for resistance to mefloquine was occurring.

MATERIALS AND METHODS

P. falciparum line FCK was isolated from a patient in Kanchanaburi, west Thailand, in January 1979. Tests for response to chloroquine (*8*) revealed that the isolate was resistant to chloroquine at the level of 1 nmol/ml of blood. The parasites were maintained in continuous culture by the Petri dish cultivation method described by Jensen & Trager (*7*). They were subcultured regularly every 4 days in erythrocytes group O, the percentage parasitaemia on day 0 always being 0.2%. The culture medium consisted of RPMI 1640, with the addition of 50 g of $NaHCO_3$ and 100 ml of heat-inactivated (56 °C, 30 min) human serum, group O, per litre. This medium is referred to below as control medium.

Mefloquine hydrochloride was obtained as a 1 mmol/litre sterile solution from the World Health Organization, Geneva, Switzerland. The stock solution was diluted with sterile, deionized water to 100 times the required concentration. A working solution was made by further dilution with RPMI + $NaHCO_3$, but without serum. The final required concentrations were obtained by adding different volumes of the working solution to the control medium. Precaution was taken that not more than 0.1 ml of the working solution was used per 10 ml of the control medium in order to maintain the human serum : RPMI + $NaHCO_3$ ratio within a narrow range.

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The parasite responses to mefloquine were tested in replicated glass-covered vials (2 cm x 3 cm), each containing 0.1 ml of erythrocytes, with parasitaemia of 0.3%, suspended in 0.9 ml of control medium. We replaced the control medium with the test medium (control medium + mefloquine) on the second day of subculture. Generally, the cultures were exposed to the drug for 48 hours. Giemsa-stained thin blood films were made before drug treatment and 48 hours afterwards. Four replicates were used for each drug level and for the controls.

To produce drug tolerance, the cultures were exposed to mefloquine at a concentration of 0.04 nmol/ml for 48 hours. Cultures that showed healthy ring forms were subcultured with non-infected, fresh erythrocytes and control medium and maintained without drug for several cycles. Cultures were treated again with mefloquine at a 2–4 times higher concentration.

After three months the mefloquine-treated line (designated FCL-R) was unaffected by this antimalarial at a concentration of 32 nmol/mol, and its sensitivity to mefloquine was then compared with that of the original line (FCK-S). Mefloquine was added to cultures of both lines at concentrations of 8, 16, 32, 64 and 128 nmol/ml.

Evaluation of mefloquine inhibition was done by direct counting of parasites in thin blood films made before drug treatment (day 2) and 48 hours after treatment (day 4). The multiplication factor (MF) of the parasites is N (day 4)/N (day 2) where N is the number of parasites/10 000 red blood cells on the day indicated. Because the multiplication is exponential, a logarithmic transformation is used:

$$\log \mathrm{MF} = \log N\,(\text{day } 4) - \log N\,(\text{day } 2)$$

The 11 groups of rates (log MF), excluding the group with all zeros, were found to have homogenous variances (Bartlett's test: $\chi^2 = 39.09$, $0.5 > P > 0.1$) and so two-way ANOVA was carried out to test the effects of drug concentration and culture line. The highest concentration was omitted from the analysis because of the constraint in group variance.

RESULTS

The *in vitro* responses to mefloquine of the FCK line, before (FCK-S) and after production of drug tolerance (FCK-R) are summarized in Fig. 1. The sensitive line, which has never been exposed to mefloquine, showed a steady drop in the rate of population increase with increase in the concentration of the drug. Complete inhibition occurred at a concentration of 128 nmol/ml.

The FCK-R line was not inhibited by any of the lower concentrations (8–64 nmol/ml). Only at 128 nmol/ml was inhibition first evident. The mean multiplication factor with two days of treatment at that concentration was 0.089 which was higher than that of the sensitive line at 128 nmol/ml. Table 1 shows a significant effect difference between parasite lines ($F_{1,30} = 21.64$, $P < 0.001$), indicating that line FCK-R had reduced susceptibility to mefloquine. The change in the sensitivity of the FCK-R line is indicated by the significant interaction mean square. Only at the highest concentration was there a marked depression in multiplication.

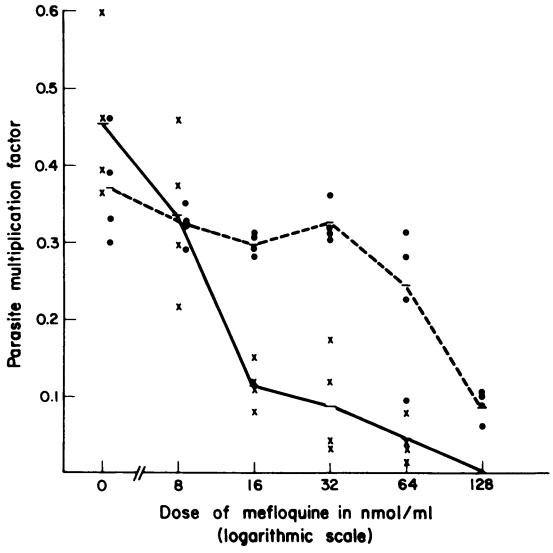

Fig. 1. Response of the two lines of *P. falciparum* to mefloquine in continuous cultures. Parasite population increases during 48 h of drug treatment are presented as the common logarithm of the multiplication factor (MF). Closed circles represent log MF of FCK-R, the line that was repeatedly exposed to mefloquine; crosses represent the FCK-S line that was not exposed to mefloquine. The lines connect the means at each dose level.

Table 1. Analysis of variance of population growth rates (log multiplication factor) of *P. falciparum* lines FCK-R and FCK-S when treated with mefloquine at various levels

Source of variation	df	Mean square	*F*	*P*
Line FCK-R *versus* FCK-S	1	104 448	21.64	0.001
Mefloquine concentration	4	101 44	21.02	0.001
Interaction	4	140 673	29.14	0.001
Error	30	4 827		
Total	39			

DISCUSSION

Short-term *in-vitro* tests of the sensitivity of chloroquine-resistant *P. falciparum* to mefloquine have shown, in most cases, that mefloquine is fully effective in inhibiting the parasite's growth. Rieckmann (*6*) reported that at 1.0 nmol/ml of blood no schizonts were observed in any of his 15 test cultures. This was confirmed by López Antuñano & Wernsdorfer (*9*), who obtained 99.9% schizont inhibition by mefloquine at a level as low as 2 pmol/5 μl of blood (0.4 nmol/ml).

Our results obtained with continuous cultures also showed that the chloroquine-resistant *P. falciparum* line FCK was very susceptible to mefloquine when it was exposed to this drug for the first time. However, we found that the surviving ring stages could be revived and would develop into schizonts. This phenomenon is comparable to the *in vivo* situation where drug treatment is terminated and medical follow-up ceases. In order to imitate this drug abuse situation, which may lead to tolerance or resistance to antimalarials, we did not expose *P. falciparum* cultures to the drug continuously as specified in the experimental design of Nguyen-Dinh & Trager (*10*). Recrudescence occurred, with the chance that the surviving parasites had become resistant to the drug.

The misuse of chloroquine and the pyrimethamine–sulfadoxine combination by local people has given rise to resistance to both drugs. In consequence, we are concerned that when mefloquine is commercially available, it too might soon become ineffective. Our results showed that the "misuse" of mefloquine in culture, i.e., at a concentration lower than prescribed and with early termination of treatment, caused a decrease in sensitivity to mefloquine within three months.

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RÉSUMÉ

DIMINUTION DE LA SENSIBILITÉ À LA MÉFLOQUINE CHEZ *PLASMODIUM FALCIPARUM* EN CULTURE CONTINUE

Les difficultés qui sont rencontrées dans le traitement du paludisme à *P. falciparum* en Thaïlande sont attribuées à une pharmacorésistance des parasites. Des données récentes obtenues par application de la méthode de Rieckmann ont révélé que 99% des isolements étaient résistants à la chloroquine. En outre, on a signalé que la chimioprophylaxie et le traitement au moyen de la pyriméthamine et de la sulfadoxine deviennent moins satisfaisants.

L'introduction d'un médicament nouvellement synthétisé, le WR 30090 (méfloquine), qui est un analogue de la quinine, a permis un traitement efficace par une dose unique. Cependant nous avons observé que lorsqu'on éprouvait de la méfloquine contre *P. falciparum* établi en culture continue, il y avait un petit nombre de jeunes parasites qui semblaient tolérer ce médicament. Il importait donc de rechercher si une sélection de parasites résistants à la méfloquine se produisait.

P. falciparum utilisé dans la présente étude a été isolé d'un malade dans la province de Kanchanaburi, à l'ouest de la Thaïlande, en janvier 1979. Les épreuves destinées à déterminer les réponses de cette souche à la chloroquine ont montré qu'elle était résistante à la concentration de 1 nmol/ml de sang. Le parasite a été maintenu en culture continue dans du milieu RPMI 1640 avec tampon HEPES, additionné de 10% de sérum humain. Le chlorhydrate de méfloquine obtenu auprès de l'OMS a été ajouté aux cultures à la concentration initiale de 40 pmol/ml de milieu. Les cultures ont été soumises à la pression du médicament pendant 48 heures puis transférées et remises en culture avec des érythrocytes non infectés en suspension dans du milieu exempt de médicament. La culture a de nouveau été traitée par la méfloquine à une concentration augmentant chaque semaine. Après trois mois, la lignée traitée par la méfloquine (FCK-R) n'était plus affectée par cet antipaludique à une concentration de 32 nmol. Sa sensibilité à la méfloquine a alors été comparée à celle de la lignée originelle (FCK-S) aux concentrations de 8, 16, 32, 64 et 128 nmol.

L'évaluation a été faite par l'analyse de la variance du facteur de multiplication (FM) du parasite [log FM = log N (jour 4)–log N (jour 2)]. La souche sensible qui n'avait jamais été exposée à la méfloquine a présenté un abaissement régulier du taux d'accroissement de la population, correspondant à la concentration du médicament.

La lignée FCK-R n'était inhibée par aucune des concentrations les plus faibles (8–64 nmol). Ce n'est qu'à 128 nmol que le médicament manifestait un effet inhibiteur. Il y avait une différence significative de l'effet selon la lignée de parasites ($F_{1,30}$ = 21,64, P<0,001), indiquant qu'une

lignée FCK-R avait une sensibilité réduite à la méfloquine. C'est seulement à la plus haute concentration qu'on observait un fléchissement marqué de la multiplication.

Les résultats de nos expériences ont clairement montré que la «mauvaise utilisation» de la méfloquine dans la culture, c'est-à-dire à une concentration plus basse que celle qui est prescrite, avec un arrêt précoce du traitement, entraîne facilement une diminution de la sensibilité à la méfloquine dans les trois mois.

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