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CLONING AND CHARACTERIZATION OF MEFLOQUINE-RESISTANT *PLASMODIUM FALCIPARUM* FROM THAILAND

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Abstract. Resistance to mefloquine in *Plasmodium falciparum* has begun to occur along the border of Thailand and Kampuchea. As a means of assessing the natural occurrence of mefloquine resistance, the admission and post-treatment parasite isolates from a mefloquine treatment failure were cloned and characterized. Clones from the admission isolate were susceptible to mefloquine in vitro (ID_{50} of 3.4 [2–5], G [95% CI] ng/ml) and showed a mixture of isozyme types for glucose phosphate isomerase (GPI types I and II). The post-treatment clones were resistant to mefloquine in vitro (ID_{50} of 17.3 [13–23] ng/ml) with only one isozyme (GPI type I) detected. These observations suggest that under mefloquine pressure a resistant parasite population was selected in the patient, indicating that the potential for mefloquine resistance already exists in the indigenous *P. falciparum* gene pool. In addition, the mefloquine-resistant clones showed decreased susceptibility in vitro to halofantrine suggesting possible cross-resistance to this new antimalarial drug currently under development.

Mefloquine hydrochloride, a 4-quinolinemethanol, is a relatively new single-dose blood schizonticide developed by the U.S. Army Antimalarial Drug Program.[1,2] It was first clinically evaluated in Thailand in 1975–1976.[3] At that time cure rates were 97%–100%. Subsequently mefloquine was used in limited field trials in Thailand with occasional RI type failures[4] observed (clearance of asexual parasitemia within 7 days of treatment, followed by recrudescence). Those treatment failures were attributed to reinfection or to pharmacokinetic variations in the host rather than true parasite resistance.[5] More recently, however, an RII type (marked reduction of asexual parasitemia, but no clearance) failure to mefloquine was reported[6] from a clinical drug trial involving Thai marines in eastern Thailand (E. F. Boudreau, personal communication). This same study has revealed 3 RI type failures with mefloquine given as a 1,000 mg single dose. Beginning in 1983 mefloquine in combination with sulfadoxine and pyrimethamine was introduced for large scale field trials in the treatment of falciparum malaria in Thailand. Mefloquine is the only clinically proven new antimalarial drug for treatment of multidrug-resistant falciparum malaria. Only 2 other schizonticidal drugs, halofantrine, a 9-phenanthrenemethanol, and enpiroline, a 4-pyridinemethanol, are under clinical development in the Walter Reed Army Institute of Research program. Both of these drugs are structurally related to mefloquine and quinine as aminoalcohols. The only structurally new antimalarial with prospects for clinical testing is quinghaosu (artemisinine) and its derivatives.[7] It is essential, therefore, that we study the natural emergence of parasite resistance to mefloquine and other structurally related and newly developed antimalarial drugs.

We have isolated and cloned the admission and recrudescent parasites from a Thai patient with a confirmed RI type failure to mefloquine. The *P. falciparum* parasite isolates and clones were evaluated for in vitro susceptibility to mefloquine, quinine, halofantrine and chloroquine. In addition, enzyme typing for glucose phosphate isomerase forms was done.

MATERIALS AND METHODS

Patient history

The patient was a 22-year-old Thai marine stationed at Pongnamron in eastern Thailand. He was admitted into a Royal Thai Navy–U.S.

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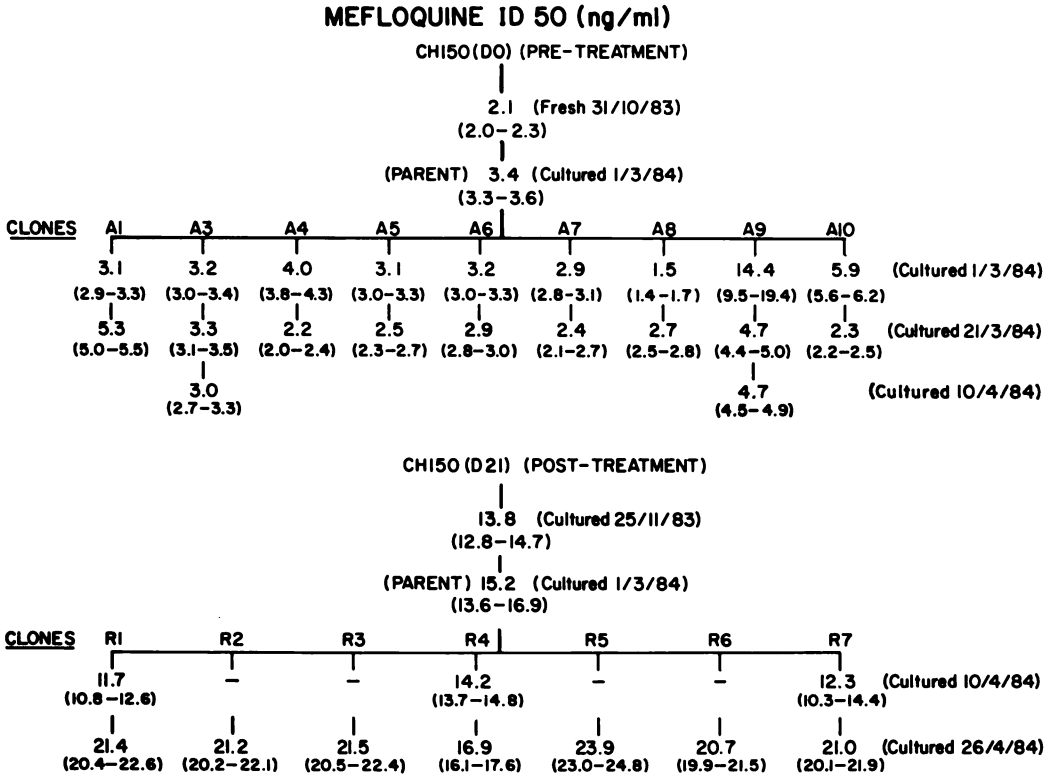

FIGURE 1. In vitro mefloquine susceptibility testing of original parasite isolates and clones. ID_{50} (95% CI).

EDITOR'S NOTE: After page proofs were set the senior author asked that changes be made in the above figure (he did not submit a revised figure) and in other parts of the article. He advised that the first two entries under CLONE A9 should be changed from 14.4 to 6.30 and from 9.5–19.4 to 5.0–6.8, respectively. These changes, Webster states, ". . . in no way alter(s) the conclusions of our paper and result from the discovery of an input error in the computer calculation. . . . The correct values suggest that A9 was not a mixture and corrections to page proof have been noted."

The requested deletions/changes have been made in the text and presumably they are correct. It should be noted, however, that referees have *not* seen the changes. The affair has been handled in this fashion because the index for Volume 34 was in press and withdrawal of the article for further review would have altered all subsequent pagination and significantly delayed the time of printing of the November issue. (Accommodation of these changes, under these specific circumstances, does not establish a precedent for those who would try to rewrite articles already in press. All letters of acceptance of MSs include the statement that *extensive revision of material in press will not be permitted.*)

Army clinical drug trial study at Ft. Taksin Hospital, Chantaburi, in October 1983. He had no previous malarial infection and no prior antimalarial drug treatment. His admission asexual parasite count was 5,760 parasites/mm^3 (*P. falciparum*). Mefloquine was administered as a 1,000 mg single dose. The maximum parasite count was 6,400/mm^3 with a fever clearance time of 36 hr and parasite clearance of 53 hr. On day 21 the infection recrudesced. Serum determinations showing adequate drug levels were achieved on day 3 (1,445 ng/ml) and day 7 (835 ng/ml) post-treatment. Curative treatment was achieved with a combination of quinine sulfate (650 mg q 8 hr × 7 days) and tetracycline HCL (500 mg q 8 hr × 7 days).

Parasites

Parasite isolates were obtained from the patient on day 0 prior to mefloquine treatment and when recrudescence occurred at day 21. Heparinized venous blood was obtained by venipuncture and aliquots taken for drug testing and

TABLE 1

Characteristics of P. falciparum *isolates and clones*

	Drug susceptibility (ID_{50} ng/ml)					Enzyme type		
	Mefloquine		Quinine	Halofantrine	Chloroquine	GPI I	GPI II	GPI I + II
Parasite source								
Uncloned admission isolate (CH150D0)	3.44		51.57	2.60	66.74			+ (I > II)
Clones								
DOA1	3.08	5.26*	57.13	1.15	122.16		+	
DOA3	3.21	3.28	25.03	0.96	66.74	+		
DOA4	4.04	2.20	47.51	0.62	89.19	+		
DOA5	3.15	2.49	70.27	0.90	90.14		+	
DOA6	3.17	2.92	64.13	0.99	93.25		+	
DOA7	2.91	2.40	37.34	0.46	76.20	+		
DOA8	1.55	2.66	43.81	0.85	57.16		+	
DOA9	6.30	4.71	62.87	1.13	99.27	+		
DOA10	5.90	2.34	59.40	0.86	87.84	+		
	3.44† (2.50–4.73)	3.00 (2.35–3.81)	49.76 (38.67–64.04)	0.85 (0.68–0.94)	85.01 (71.59–100.94)			
Uncloned recrudescence isolate (CH150D21)	15.24		77.87	4.47	68.65	+		
Clones								
D21R1	11.69		68.69	4.26	44.66	+		
D21R2	21.19		70.60	3.75	53.63	+		
D21R3	21.46		79.45	4.79	57.18	+		
D21R4	14.22		55.22	4.26	54.70	+		
D21R5	23.90		64.60	4.82	78.86	+		
D21R6	20.17		58.32	4.11	44.67	+		
D21R7	12.34		61.76	4.10	47.45	+		
	17.29 (13.13–22.76)		65.06 (58.05–72.92)	4.28 (3.94–4.65)	53.48 (44.53–64.23)			

* Second test of clones three weeks later.

† Geometric mean (95% CI).

cryopreservation. Day 0 parasites were tested for drug susceptibility using fresh blood and later following continuous culture. Day 21 parasites were tested for drug susceptibility using cultured fresh blood. Both isolates were cloned from continuous parasite cultures. The culture technique used was based on the petri dish method[8] and has been described.[9]

Preparation of parasite clones

Parasite isolates were maintained in continuous culture for 1 month and then cloned by a method of limiting dilution.[10,11] Basically the procedure was as follows: Diluted samples of cultured parasites, estimated to contain an average of 0.5 parasites per 100 μl of sample at a 2% hematocrit (HCT), were dispensed in microtiter plates (96-well, 8 × 10 matrix). Culture medium (100 μl) was changed every 2 days. Every 5 days 100 μl of fresh erythrocytes at a 1% HCT in complete medium was replaced instead of medium alone. Once parasites were detected on thick smear examination (≈3 weeks) the contents of positive wells were transferred to large microtiter plates (24-well, each well contained 1.5 ml fresh RBC at a 5% HCT). Once parasite growth was established the culture was continued in petri dishes. In using the limiting dilution technique it is possible to obtain a dilution culture that is in fact a "clonal mixture"[11] containing 2 or more parasites and thus not homogenous for the characters under study. The occurrence of a mixture would most likely result from an erythrocyte infected with 2 or more "ring" stages. An alternative approach to cloning involves microscopic selection from droplets containing single parasites, but this method, too, has certain disadvantages (e.g., not observing a "ring" in a crenated erythrocyte).[12]

In vitro drug susceptibility testing

The in vitro response of parasites to mefloquine and other antimalarial drugs was determined by a radioisotope microdilution technique[9,13] which measures the incorporation of [^{3}H]hypoxanthine into parasite nucleic acids in the presence of serial dilutions of the test drug. Concentration-response data were analyzed by a nonlinear regression function to determine the 50% inhibitory dose (ID_{50}). As an additional marker, the enzyme glucose phosphate isomerase (GPI) was studied by cellulose acetate electrophoresis of parasite lysates.[14] Two isozyme forms, GPI type I and GPI type II were identified for *P. falciparum*.[15]

RESULTS

Characterization of parasite isolates

In vitro susceptibility testing showed the parasite isolate obtained on admission prior to mefloquine treatment (CH150D0) to have a mefloquine ID_{50} of 2.13 ng/ml when cultured directly from the patient. Following 4 months continuous cultivation CH150D0 had an ID_{50} of 3.44 ng/ml (Fig. 1). The response to mefloquine appeared, therefore, to be consistently susceptible for this isolate. The ID_{50} of the admission isolate (CH150D0) was comparable to the value found in in vitro/in vivo correlated cases where treatment with mefloquine was successful (ID_{50} 3.57 [2.7–5.0], G [95% CI], n = 18). Enzyme typing of the primary isolate for GPI revealed a mixture of electrophoretic forms with GPI type I in greater proportion than GPI type II (Table 1). These results suggest that the admission isolate was a mixed population of parasites in which mefloquine-sensitive forms were the predominant drug phenotype.

The isolate collected at recrudescence on day 21 post-mefloquine treatment (CH150D21) had a mefloquine ID_{50} of 13.77 ng/ml when cultured directly from the patient (Fig. 1). CH150D21 was maintained in continuous culture for over 3 months and on retesting had a mefloquine ID_{50} of 15.24 ng/ml. We have observed that parasites with $ID_{50} > 10$ ng/ml are associated with mefloquine treatment failures.[9] Only 1 enzyme form, GPI type I, was observed for CH150D21 (Table 1). These observations on the CH150D21 isolate show a parasite population uniformly resistant to mefloquine and characterized by a single isozyme marker.

Characterization of clones

Clones (A1–A10) were prepared from the CH150D0 admission isolate. One of these, A2, was lost during culture. A consistently sensitive in vitro response to mefloquine was observed for 8 of the admission clones (Fig. 1). Despite the uniform in vitro response to mefloquine, enzyme typing revealed both GPI type I and type II or-

ganisms indicating that a mixture of clonal types were derived from the CH150D0 parasite isolate. The admission clones also were tested in vitro against quinine, halofantrine and chloroquine (Table 1). All the clones appeared resistant to chloroquine and quinine and susceptible to mefloquine and halofantrine.

There were 7 clones prepared from the mefloquine post-treatment parasite isolate. All of the clones had a high ID_{50} for mefloquine which remained high on subsequent testing (Fig. 1). An important distinction was the significantly ($P < 0.001$, Student's t-test) high halofantrine ID_{50} for the day 21 clones compared to those from day 0 (Table 1). Both halofantrine and mefloquine showed a 5-fold increase in ID_{50}. The geometric mean ID_{50} value for quinine was also significantly ($P < 0.03$) higher for the day 21 clones. Only GPI type I was detected in the day 21 clones. These observations suggested an absence of clonal diversity in the clones derived from the recrudescence parasite isolate.

DISCUSSION

The observations in this study suggest that the parasite isolate obtained prior to mefloquine treatment comprised a diverse parasite population in which the mefloquine-sensitive organisms were the predominant phenotype. Once mefloquine treatment was initiated it appeared that under selective drug pressure a mefloquine-resistant parasite population emerged. Consistent with these observations the post-mefloquine treatment isolate exhibited 1 isozyme type (GPI I) whereas the admission isolate showed both isozyme types (GPI I and GPI II).

When clones were prepared from the 2 original isolates the admission isolate showed variation among clones for isozyme type whereas there was no apparent diversity among clones from the recrudescence isolate for the characters examined. The CH150D21 clones also differed in their in vitro response to other antimalarial drugs compared to the CH150D0 clones. There was a significant increase in the mean ID_{50} of halofantrine suggesting possible cross-resistance for halofantrine and mefloquine. Similar observations have been made with other Thai *P. falciparum* isolates in which there was a positive correlation between high mefloquine and halofantrine ID_{50} values.[9] Both mefloquine and halofantrine have chemical structural similarities to quinine. This association with quinine provides a prospective basis for cross-resistance among these aminoalcohols in Thailand.

This study suggests that the potential for mefloquine resistance already exists in the indigenous *P. falciparum* gene pool. Once exposure to mefloquine is encountered these resistant forms grow with selective advantage and produce a stable drug phenotype. The implication for antimalarial chemotherapy from this study for mefloquine (and structurally related antimalarials) is ominous. There is an urgent need to further characterize and use the clones obtained in studies such as this one to investigate the biochemical and genetic basis of drug resistance in *P. falciparum*.

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