

SHORT REPORT: FAILURE TO SELECT FOR CHLOROQUINE- OR MEFLOQUINE-RESISTANT *PLASMODIUM BERGHEI* THROUGH DRUG PRESSURE IN *ANOPHELES STEPHENSI* MOSQUITOES

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Abstract. We investigated whether chloroquine- or mefloquine-resistant *Plasmodium berghei* could be selected through drug pressure applied during continuous cyclical transmission in *Anopheles stephensi* mosquitoes. Mosquitoes were infected by feeding them on mice previously inoculated with a drug-sensitive clone of *P. berghei* ANKA. Mosquitoes ingested mefloquine or chloroquine with the infectious blood-meal, or by feeding on a drug-treated (uninfected) mouse 4 or 10 days after the infectious blood-meal. Twenty-two days after being infected, mosquitoes transmitted sporozoites to uninfected mice. Blood from these animals was used to infect naive mice that were then used to reinitiate the mouse/mosquito/mouse cycle. A total of 20 passages through mosquitoes were completed while under drug pressure. Drug-resistance levels were assessed in the initial clone and after 20 passages through mosquitoes. None of 18 “sub-clones” of parasites showed significant increases in chloroquine or mefloquine resistance, suggesting that exposure of sporogonic stage *Plasmodium* to chloroquine or mefloquine will not result in the development of drug resistance.

The use of antimalarial drugs is a fundamental component of malaria control strategies; however, the presence of drug-resistant *Plasmodium falciparum* hampers malaria control in many areas.^{1,2} Mechanisms leading to the development of resistance to antimalarial agents have been extensively reviewed.² The potential role of mosquitoes in the development of drug resistance has not been fully evaluated. Results from several studies suggest that exposure of sporogonic stage parasites to antimalarial drugs could contribute to the development of resistance.^{3–5} Recently, Fonseca and others⁶ found that mefloquine-resistance could be selected through drug pressure applied during continuous cyclical transmission. In this study, we determined if exposure of sporogonic-stage *Plasmodium berghei* ANKA to chloroquine or mefloquine affected the development of resistance to these compounds.

Procedures used for the maintenance of the *P. berghei* ANKA clone in mice, for passage through mosquitoes, and for the preparation and administration of chloroquine (CHL) and mefloquine (MEF) have been described.^{5,7} The maintenance and care of experimental animals used in this study complies with the National Institutes of Health guidelines for the humane use of laboratory animals. We maintained a total of 18 distinct parasite “sub-clones” for 20 passages through mosquitoes. These parasite sub-clones originated from the *P. berghei* ANKA clone. During each passage, a given parasite sub-clone was exposed to CHL or MEF (10 or 40 mg drug/kg mouse body weight, with controls receiving diluent only) either with the infectious blood meal (Day 0), or 4 (Day 4), or 10 (Day 10) days later. In brief, 18 groups of 5 mice each were infected with 10⁶ *P. berghei*-infected erythrocytes. Four days later, mice in the Day 0-treated groups were treated with MEF or CHL. Mice in the Day 4/Day 10 groups were not treated at this time. Ninety min later, all mice were anesthetized and the 5 mice in each group were placed for 30 min on a screened cage containing approximately 200 mosquitoes.⁷ Mosquitoes in the Day 4 and Day 10 treated groups were exposed to drugs by re-feeding them on drug-treated (uninfected) mice 4 or 10 days

after the infectious blood-meal. After feeding was completed, unengorged mosquitoes were removed from the cages. Blood samples were obtained from the mice in order to confirm that MEF or CHL was present.⁵ Mosquitoes were allowed to transmit *P. berghei* to mice 22 days after being infected. Mosquitoes infected with a given sub-clone were fed on 3 naive mice for 30 min. Fourteen days after being infected, blood samples were collected from the mice, pooled, and examined for *P. berghei*.^{5,7} Blood samples (0.5 mL) were prepared in glycerin and stored for subsequent analysis of drug-resistance levels. The remaining blood was diluted with PBS and 10⁶ infected erythrocytes injected into a naive mouse in order to synchronize gametogenesis. Blood from these mice was then used to re-initiate the mouse/mosquito/mouse cycle.

Resistance levels for each parasite sub-clone were assessed using Peters’ 4-day technique.⁸ Blood from a given sub-clone was injected into 3 naive mice (0.2 mL each). Blood from these mice was collected 7 days later, pooled, and diluted in PBS so that 10⁶ infected erythrocytes were inoculated intraperitoneally (i.p.) into each of 25 recipient mice. These mice were divided into 5 groups of 5 mice each. The mice in each group received an i.p. injection of CHL or MEF once a day for 4 days. Mice treated with CHL received doses of 0, 1.68, 2.40, 3.43, or 4.90 mg base drug/kg body weight (5 mice per treatment), while mice treated with MEF received doses of 0, 2.81, 3.75, 5.00, or 6.67 mg/kg. Blood smears were made 24 hr after the last treatment and the number of infected erythrocytes per 200 cells determined. Drug-resistance levels were determined by calculating the median dose required to eliminate 50% of the parasites. An index of resistance was obtained by dividing the ED₅₀ value of the treated group by the ED₅₀ value of the control group.

An *in vitro* microassay confirmed the presence of drug (CHL or MEF) in mice that were used to expose mosquitoes to the drugs.⁵ Using a 24-hr microassay, we observed inhibition of growth and/or killing of *P. falciparum* parasites in

TABLE 1

Drug resistance levels in eighteen "sub-clones" of the *Plasmodium berghei* ANKA clone after 20 passages through *Anopheles stephensi* mosquitoes under drug pressure*

Drug	Date of resistance exposure	Dose (mg/kg)	ED ₅₀		Formula	Index	
			(mg/kg)	r Value			
CHL	Day 0	0	2.24	0.97	$Y = -6.76X + 7.37$		
		10	2.04	0.95	$Y = -6.14X + 6.90$	0.91	
		40	1.63	0.98	$Y = -4.68X + 6.00$	0.73	
	Day 4	0	2.18	0.97	$Y = -7.10X + 7.10$		
		10	2.08	0.98	$Y = -6.30X + 7.00$	0.95	
		40	2.02	0.95	$Y = -3.35X + 6.02$	0.93	
	Day 10	0	2.08	0.99	$Y = -6.14X + 6.95$		
		10	2.13	0.96	$Y = -7.04X + 7.31$	1.02	
		40	3.53	0.89	$Y = -7.55X + 9.13$	1.70	
	MEF	Day 0	0	2.99	0.97	$Y = -6.23X + 7.97$	
			10	3.60	0.89	$Y = -7.76X + 9.32$	1.20
			40	4.12	0.93	$Y = -4.08X + 7.51$	1.38
Day 4		0	5.74	0.80	$Y = -4.28X + 8.24$		
		10	8.75	0.61	$Y = -7.07X + 11.66$	1.52	
		40	3.54	0.80	$Y = -4.20X + 7.30$	0.62	
Day 10		0	5.13	0.81	$Y = -4.46X + 8.17$		
		10	5.74	0.62	$Y = -7.89X + 10.99$	1.12	
		40	3.53	0.89	$Y = -7.55X + 9.13$	0.69	

* Parasites in each experimental group were exposed to chloroquine (CHL) or mefloquine (MEF) (10 or 40 mg/kg) with the infectious blood meal (Day 0), or 4 (Day 4) or 10 (Day 10) days later. Twenty passages through *An. stephensi* mosquitoes were completed. Drug-resistance levels of twentieth-passage parasites were assessed in infected chloroquine resistant (ICR) mice using a modification of Peters' 4-day technique.⁸

all samples obtained from drug-treated mice, while control sera had no effect on parasite growth.

No significant changes in *P. berghei* resistance levels to CHL or MEF were noted after 20 passages through mosquitoes (Table 1). These results clearly demonstrate that exposure of sporogonic stage *P. berghei* ANKA to CHL or MEF in this model system does not lead to the development of resistance in subsequent blood-stage parasites.

These results are in contrast to those obtained by Fonseca and others,⁶ who were able to select for resistance by exposing sporogonic stage *P. berghei* to mefloquine during continuous cyclical transmission. Why results from these studies differ is not clear; however, Fonseca and others used lower drug doses (5 and 25 mg/kg) than we used (10 and 40 mg/kg) and conducted their studies using *Anopheles gambiae* mosquitoes. Fonseca and others⁶ determined that mefloquine-resistance had developed after a single drug dose failed to prevent the appearance of erythrocytic stage parasites; however, they did not quantify the degree of resistance that emerged. In this study, we precisely quantified resistance levels in all 18 *P. berghei* ANKA sub-clones that we developed. Although the resistance index in the different sub-clones was never identical (Table 1), no consistent patterns were evident that suggest drug resistance had emerged.

These results suggest that the emergence of resistance to chloroquine and/or mefloquine is due to selection of resistant asexual parasites in the vertebrate host, with subsequent transmission of the resistant parasites by mosquitoes, and that the selection of resistance does not occur within the anopheline vector.

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