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Plasmodium falciparum: mefloquine resistance produced *in vitro**

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Camp and Smith strains of the human malaria parasite Plasmodium falciparum *became resistant to mefloquine after continuous cultivation in the presence of the drug. The 50% inhibitory dose (ID_{50}) values for mefloquine, as assessed by [^{3}H]hypoxanthine incorporation, were found to have increased 4-fold, from 3 μg/l to 12 μg/l. The ID_{50} values obtained by morphological examination of the cultures increased 10-fold. Resistance was stable in both strains either when grown in a drug-free medium or when kept frozen in liquid nitrogen. The mefloquine-resistant Camp strain remained sensitive to chloroquine and amodiaquine, and became slightly more resistant to quinine; there was increased sensitivity to pyrimethamine. The mefloquine-resistant Smith strain remained sensitive to amodiaquine and resistant to pyrimethamine; there was increased resistance to quinine, and an increase in sensitivity to chloroquine.*

Malaria continues to be one of the most widespread diseases in the world. Since 1974 there has been a resurgence of malaria in areas such as Central and South America, and south and south-east Asia where the disease had seemed to be declining (*1*). A major problem in malaria control is the development of drug-resistant strains of *Plasmodium falciparum*. Chloroquine resistance exists now in Latin America and Asia (*2–5*) and is developing in Africa (*6*, *7*). Resistance has also appeared against quinine (*8*) and the antifolates (*9*).

Mefloquine, DL-erythro-2,8-bis(trifluoromethyl)-α-(2-piperidyl)-4-quinolinemethanol hydrochloride (WR 142 490), is the only drug (at present available for experimental tests only) for the treatment and prophylaxis of multidrug-resistant falciparum malaria in man (*10*). Potential mefloquine resistance in falciparum malaria has to be considered since resistance can be induced in strains of the rodent malaria parasite, *P. berghei* (*11–12*). Emergence of human strains resistant to mefloquine would therefore lead to serious clinical and public health problems. Recently, a patient in Thailand with RII resistant falciparum malaria was identified (*13*).

We examined the induction of resistance to mefloquine *in vitro* in two strains of *P. falciparum* and determined their respective susceptibilities to other antimalarial drugs.

MATERIALS AND METHODS

Parasites

The multidrug-resistant Viet Nam Smith and the Malayan Camp strains of *P. falciparum* (*14*) were used as test organisms. Stock cultures were maintained as previously described (*14*). The culture medium consisted of RPMI 1640[a] supplemented with human A+ erythrocytes and 10% heat-inactivated human A+ plasma. Each culture flask contained a total volume of 5 ml and was flushed with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2. Stock cultures

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were always diluted to parasitaemias of either 0.1% (4-day growth) or 0.2% (for 3-day growth). Both strains attained final parasitaemias of 2.5–3.5%.

Drugs

Chloroquine diphosphate, mefloquine hydrochloride, amodiaquine hydrochloride, quinine sulfate, and pyrimethamine were obtained from the US Army Drug Development Program Inventory in powder form.

Induction of mefloquine resistance

Mefloquine was dissolved in 70% ethanol to a concentration of 1 g/l. Subsequent dilutions were then made with RPMI 1640 plus plasma (minus $NaHCO_3$) to achieve stock solutions of 200 to 500 times the required experimental concentrations. The solutions were divided among polypropylene tubes and frozen at −20 °C.

To produce resistance, both strains were continuously exposed to a starting mefloquine concentration of 5 μg/l. Culture medium containing the drug was changed daily and the parasites were subcultured with non-infected erythrocytes every 3–4 days. At the point where growth of the exposed lines attained the same level as those of the non-exposed parent lines, as determined by Giemsa-stained blood films, medium containing progressively higher concentrations (10, 15 and 20 μg/l) of mefloquine was added to the cultures. The mefloquine-resistant (MR) strains were unaffected by the concentration of mefloquine (13.5 μg/l) that is lethal to the parent strains.

Drug susceptibility tests

Two approaches were taken to determine drug sensitivities. One was the radioisotopic method of Desjardins et al. (*14*, *15*), using incorporation of [G-^{3}H]hypoxanthine[b] by the parasites as the index of parasite viability and antimalarial activity. Chloroquine, mefloquine, amodiaquine, and quinine were dissolved in 70% ethanol to a concentration of 1 g/l; pyrimethamine was dissolved in a 50/50 (vol/vol) mixture of dimethyl sulfoxide[c] and ethanol. Subsequent dilutions were made with complete culture medium. Each drug was serially diluted 2-fold in the microtitration plates for a total of seven concentrations over a 64-fold range. The final red blood cell suspension was 1% and the parasitaemia at the start of the experiment was 0.4–0.6%. In this test system the 50% inhibitory dose (ID_{50}) is defined as the drug concentration corresponding to 50% inhibition of the uptake of radiolabelled hypoxanthine by the parasites as analysed by nonlinear regression analysis (*15*).

Because our preliminary studies indicated a lack of correlation between hypoxanthine uptake and parasitaemia in the MR-strains versus mefloquine, a microscopic method to assess antimalarial activity was carried out. To the first two wells of a microtitration plate were added 200 μl of complete culture medium without drug; the remaining ten wells received 200 μl of culture medium containing a graded series of five drug concentrations (two wells for each drug concentration). Ten microlitres of a 50% suspension of parasitized erythrocytes were then added to all of the wells. Cultures that had been maintained in the presence of mefloquine were grown in drug-free medium for 48 h prior to introduction into the microtitration wells. The final erythrocyte suspension was 2.38% and the parasitaemia at the start of the experiment was 0.4–0.6%. After slight agitation to ensure uniform settling of the erythrocytes, the plates were placed in an airtight box, flushed with a gas mixture of 5% O_2, 5% CO_2, and 90% N_2, and placed in an incubator at 37 °C for a 48-h period with regeneration of the gas phase after 24 h. The ID_{50} values for the antimalarial drugs tested were determined by nonlinear regression analysis after counting the number of parasites per 10 000 erythrocytes at the end of the 48-h growth period. The ID_{50} in this test system is defined as the drug concentration producing 50% reduction of parasite growth as compared with the untreated controls.

RESULTS

The time course of development of resistance to different concentrations of mefloquine in the two strains is illustrated in Fig. 1. After one month of con-

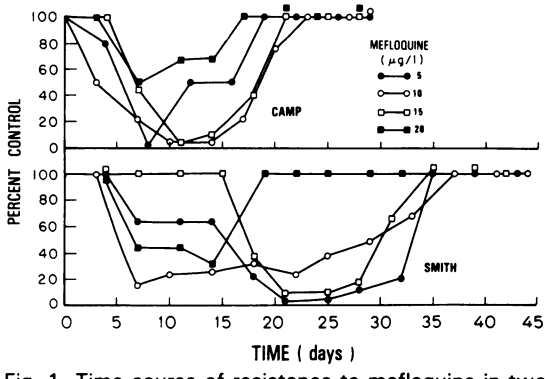

Fig. 1. Time course of resistance to mefloquine in two strains of *P. falciparum* in continuous culture. Parasitaemias of both parent lines ranged from 2.5% to 3.5%. Percent control represents the ratio of the growth of the drug-exposed line to the original culture line.

[b] New England Nuclear, Boston, MA, USA.

[c] Sigma Chemical Co., St. Louis, MO, USA.

tinuous culture in the presence of 5 μg/l of drug, comparable growth was achieved between the experimental lines and the strains from which they had been derived. Increasing the mefloquine concentrations to 10, 15, and then 20 μg/l produced a similar course of development of resistance at each successive concentration in both strains. Microscopic examination of the cultures revealed the presence of pigment.

The *in vitro* response of the Camp and MR-Camp strains to several antimalarial compounds, assessed by both radiolabelled hypoxanthine incorporation and microscopic examination, is presented in Table 1. The MR-Camp strain retained its susceptibility to chloroquine and amodiaquine, and became slightly more resistant to quinine. Most surprising was the increased sensitivity to pyrimethamine. Differences in

Table 1. Comparative susceptibilities of normal Camp strains and mefloquine-resistant Camp strains of *P. falciparum* against antimalarial drugs

Drug	ID_{50} (μg/l)[a]				Ratio of ID_{50} of MR-Camp to ID_{50} of parent Camp	
	Camp		MR-Camp[b]			
	^3H[c]	ME[c]	^3H	ME	^3H	ME
Chloroquine	8.5 (0.78)[d]	6.1 (0.32)	9.6 (0.88)	10.9 (1.2)	1.1	1.8
Mefloquine	4.9 (1.02)	3.4 (0.32)	12.0 (1.9)	34.0 (1.5)	2.4	10.0
Amodiaquine	8.1 (5.0)	3.7 (0.14)	7.4 (2.0)	5.2 (0.94)	0.91	1.4
Quinine	22.0 (3.2)	31.0 (3.6)	65.0 (4.1)	69.0 (5.4)	2.9	2.2
Pyrimethamine	505.0 (59.0)	325.0 (49.0)	28.0 (3.8)	13.0 (2.6)	0.05	0.04

[a] Mean of 4 determinations.
[b] Parasites used were from those grown in 20 μg/l mefloquine.
[c] ^3H, radiolabeled hypoxanthine; ME, microscopic examination.
[d] Numbers in parentheses are the standard error of the nonlinear regression analysis.

Table 2. Comparative susceptibilities of normal Smith strains and mefloquine-resistant Smith strains of *P. falciparum* against antimalarial drugs

Drug	ID_{50} (μg/l)[a]				Ratio of ID_{50} of MR-Smith to ID_{50} of parent Smith	
	Smith		MR-Smith[b]			
	^3H[c]	ME[c]	^3H	ME	^3H	ME
Chloroquine	68.0 (0.42)[d]	58.0 (2.4)	24.0 (1.7)	23.0 (2.5)	0.35	0.39
Mefloquine	3.5 (0.16)	3.9 (0.41)	12.0 (1.2)	36.0 (1.6)	3.4	9.3
Amodiaquine	9.2 (4.8)	5.7 (2.9)	6.2 (2.0)	10.2 (0.44)	0.67	1.8
Quinine	55.0 (5.7)	48.0 (3.2)	193.0 (15.0)	234.0 (12.0)	3.5	4.8
Pyrimethamine	>1355 (−)	>1000 (−)	>1355 (−)	>1000 (−)		

[a] Mean of 4 determinations.
[b] Parasites used were from those grown in 20 μg/l mefloquine.
[c] ^3H, radiolabeled hypoxanthine; ME, microscopic examination.
[d] Numbers in parentheses are the standard error of the nonlinear regression analysis; minus sign indicates that the value is out of the test range.

ID_{50} values for mefloquine versus MR-Camp obtained by the two methods were noted (Table 1): microscopic determinations gave higher ID_{50} values (34 μg/l) than did the radiolabelled hypoxanthine procedure (12 μg/l).

Table 2 presents the response of the Smith and MR-Smith strains to antimalarial compounds. The MR-Smith strain retained susceptibility to amodiaquine and resistance to pyrimethamine; resistance to quinine was increased while sensitivity to chloroquine was increased. Differences were noted in the ID_{50} values obtained by the two methods for mefloquine versus MR-Smith (Table 2): ID_{50} values obtained by morphological examination (ID_{50} = 36 μg/l) were greater than those obtained with hypoxanthine (ID_{50} = 12 μg/l).

When both strains were removed from drug pressure (mefloquine concentration = 20 μg/l) and grown in drug-free medium, the resistance to mefloquine was stable for up to six months and their respective sensitivities to pyrimethamine and chloroquine were retained. Resistance was also stable in both strains after storage in liquid nitrogen for six months (data not shown).

DISCUSSION

Our approach of continuous exposure of parasites to mefloquine to produce resistant lines *in vitro* is similar to that used by Nguyen-Dinh & Trager (*16*) for inducing resistance to chloroquine but differs from that of Brockelman et al. (*17*), who used discontinuous exposure to the drug. This study indicates that *P. falciparum* has the genetic capability of developing resistance to mefloquine. Furthermore, because of the changes in drug response demonstrated by our resistant strains (increased susceptibility to either chloroquine or pyrimethamine), it is possible that compounds whose efficacies have decreased over the years owing to development of drug resistance may again be of value.

Peters et al. (*11*) have shown that *P. berghei* can easily develop resistance to mefloquine, especially if the parasites were already resistant to chloroquine. In our studies with *P. falciparum*, mefloquine resistance came about more quickly in the chloroquine-sensitive strain than in the chloroquine-resistant one (Fig. 1).

Drug susceptibility studies by Kazim et al. (*12*) and Merkli et al. (*18*) with mefloquine-resistant *P. berghei* (derived from a chloroquine-sensitive parent line) showed a continued sensitivity to chloroquine, amodiaquine, and pyrimethamine but an increased resistance to quinine. On the other hand, Ager (personal communication) has observed that chloroquine-resistant *P. berghei* was cross-resistant to mefloquine and mefloquine-resistant parasites exhibited cross-resistance to chloroquine. In none of the *P. berghei* studies was there evidence for collateral susceptibility, i.e., increased susceptibility to one drug concomitant with development of resistance to another. Some evidence for this was shown by our MR-strains. MR-Camp became more sensitive to pyrimethamine than was the parent strain, and the MR-Smith strain became more sensitive to chloroquine; both showed increased resistance to quinine in varying degrees. Collateral susceptibility has been observed in trypanosomatids (*19*) where isometamidium and oxophenarsine had greater activity against clones of a *Leptomonas* resistant to acriflavinium, quinapyramine, diminazene, stilbamidine, and pentamidine than the parent strain.

The different results between the two methods for the determinations of ID_{50} for mefloquine versus MR-Camp and MR-Smith strains suggest that the change that occurred in these strains may be of a biochemical nature that limits the use of radiolabelled hypoxanthine as an index of parasite viability. On the other hand, the erythrocyte suspension used in the morphological assay was 2.38 times that of the radioisotopic assay which may indicate that much more mefloquine might be needed to exert parasite inhibition though the ID_{50} values of the other antimalarials tested were generally in agreement between the two methods.

In summary, we have demonstrated that strains of *P. falciparum* developed resistance to mefloquine *in vitro* and that the resistance appears to be stable in the absence of drug pressure. It is now possible to perform chemotherapeutic analysis and to investigate the mechanisms of drug resistance in malaria by comparisons of resistant strains and the sensitive strains from which they were derived.

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RÉSUMÉ

PLASMODIUM FALCIPARUM: RÉSISTANCE INDUITE *IN VITRO* À LA MÉFLOQUINE

Le paludisme reste une des maladies les plus répandues dans le monde. Un des problèmes cruciaux de la lutte antipaludique est l'apparition de souches de *Plasmodium falciparum* résistantes aux médicaments.

La méfloquine (WR 142 490) est le seul médicament récent dont on dispose actuelle pour le traitement et la prophylaxie du paludisme humain à falciparum polypharmacorésistant. Une résistance potentielle de ce parasite à la méfloquine doit être envisagée vu qu'une résistance induite est possible chez des souches de *P. berghei*, agent du paludisme des rongeurs. L'induction d'une méfloquino-résistance a été étudiée *in vitro* chez deux souches de plasmodies et leur sensibilité respective à d'autres antipaludéens a été mesurée.

On a employé comme organismes d'épreuve des souches multirésistantes, la souche Camp de Malaisie et la souche Smith du Viet Nam. Le milieu de culture était du RPMI 1640 supplémenté d'érythrocytes humains A+ et de 10% de plasma humain A+ inactivé par la chaleur. Chaque flacon de culture contenait au total 5 ml et l'on y a fait circuler un courant gazeux constitué de 5% de O_2, 5% de CO_2 et 90% de N_2.

Pour l'étude de la résistance, la méfloquine a d'abord été mise en solution dans l'éthanol à 70% puis diluée avec le milieu de culture (sans $NaHCO_3$) de façon à obtenir des solutions-mères 200 à 500 fois plus concentrées que la solution nécessaire aux expériences. La solution finale a été répartie en tubes de polypropylène et congelée à -20 °C. Pour induire la résistance chez les deux souches exposées en permanence à une concentration de méfloquine, on les a d'abord fixées à 5 µg/l. Quand les lignées exposées atteignaient un niveau de développement semblable à celui de la lignée parentale non exposée, les concentrations de médicament dans le milieu étaient augmentées progressivement (10, 15 et 20 µg/l).

Deux méthodes ont été employées pour déterminer la pharmacosensibilité. La première, la méthode radioisotopique, utilise comme indicateur de l'activité antipaludique l'incorporation d'hypoxanthine tritiée [G-^{3}H] par le parasite. La seconde, la méthode microscopique, consiste à compter le nombre de parasites pour 10 000 érythrocytes après 48 heures de culture. Les médicaments soumis à l'épreuve sont la chloroquine (diphosphate), la méfloquine (chlorhydrate), l'amodiaquine (chlorhydrate), la quinine et la pyriméthamine (sulfate). Dans les deux méthodes, la dose inhibitrice médiane (DI_{50}) de chaque médicament a été calculée par régression non linéaire.

Après un mois de culture ininterrompue en présence de 5 µg/l de méfloquine, on a obtenu un développement comparable des lignées expérimentales et des souches dont elles étaient issues. L'augmentation de la concentration à 10, 15 puis 20 µg/l de méfloquine a provoqué l'accroissement de la résistance chez les deux souches, dans des temps comparables.

On a constaté pour les deux souches que la DI_{50} mesurée au moyen de l'hypoxanthine marquée avait été multipliée par 4, alors qu'elle avait décuplé d'après l'examen morphologique. La résistance s'est montrée stable chez les deux souches, qu'elles soient cultivées pendant six mois sur milieu exempt de médicament ou conservées le même temps dans l'azote liquide.

La souche Camp résistante à la méfloquine est restée sensible à la chloroquine et à l'amodiaquine et est devenue légèrement plus résistante à la quinine; on a noté une sensibilité accrue à la pyriméthamine. La souche Smith, également résistante à la méfloquine, est restée sensible à l'amodiaquine et résistante à la pyriméthamine tandis qu'on observait une augmentation de la résistance à la quinine et de la sensibilité à la chloroquine.

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