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Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine

(drug resistance/malaria/P-glycoprotein/chloroquine)

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ABSTRACT **Two chloroquine-resistant cloned isolates of *Plasmodium falciparum* were subjected to mefloquine selection to test if this resulted in alterations in chloroquine sensitivity and amplification of the *pfmdr1* gene. The mefloquine-resistant lines derived by this selection were shown to have amplified and overexpressed the *pfmdr1* gene and its protein product (Pgh1). Macrorestriction maps of chromosome 5, where *pfmdr1* is encoded, showed that this chromosome has increased in size in response to mefloquine selection, indicating the presence of a gene(s) in this area of the genome that confers a selective advantage in the presence of mefloquine. Concomitant with the increase in mefloquine resistance was a corresponding increase in the level of resistance to halofantrine and quinine, suggesting a true multidrug-resistance phenotype. The mefloquine-selected parasite lines also showed an inverse relationship between the level of chloroquine resistance and increased *pfmdr1* gene copy number. These results have important implications for the derivation of amplified copies of the *pfmdr1* gene in field isolates, as they suggest that quinine pressure may be involved.**

Plasmodium falciparum is the causative agent of the most severe form of human malaria, and the ability of this parasite to develop resistance to antimalarial agents, such as chloroquine, makes it difficult to select appropriate drugs for both prophylaxis and treatment. Chloroquine resistance in *P. falciparum* involves a decrease in chloroquine concentration in the parasite, and the rate of chloroquine efflux has been shown to be 40- to 50-fold more than in sensitive isolates (1). Other studies have suggested that decreased influx of chloroquine in resistant parasites is responsible for the phenotype (2).

A P-glycoprotein homologue 1 (Pgh1) encoded by the *pfmdr1* gene (3, 4) has been suggested to be involved in the chloroquine-resistance phenotype (5). The Pgh1 protein is localized on the membrane of the digestive vacuole of *P. falciparum* (6), and heterologous expression of this protein in Chinese hamster ovary (CHO) cells confers a chloroquine-sensitive phenotype involving increased accumulation of the drug in the lysosomes (H. Van Es, S. Karcz, F. Chu, A.F.C., P. Gros, and E. Schürr, unpublished work). These results have suggested that Pgh1 is involved either directly or indirectly with the concentration of chloroquine in the *P. falciparum* food vacuole.

The possible role of Pgh1 in concentrating chloroquine in the digestive vacuole is consistent with results of the selection for increased levels of chloroquine resistance obtained with three cloned lines of *P. falciparum* (7). The chloroquine pressure caused deamplification of the *pfmdr1* gene and decreased expression of the protein. It was also found that concomitant with the increase in chloroquine resistance was a decrease in the level of mefloquine resistance. In another study, selection for mefloquine resistance in the W2 isolate of *P. falciparum* showed a decrease in chloroquine resistance (8) and amplification of the *pfmdr1* gene (4). All these results suggested an inverse relationship between chloroquine and mefloquine resistance and a direct link with the level of Pgh1 expression. In this study we have selected two cloned lines of *P. falciparum* for increased mefloquine resistance and show that this has resulted in increased expression of the *pfmdr1* gene, a decrease in chloroquine resistance, and cross-resistance to halofantrine and quinine.

MATERIALS AND METHODS

Parasites. The *P. falciparum* isolate K1 (Thailand) was obtained from G. Knowles (Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea), and clone K1A2 from G. Brown (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) was used in all experiments described. Clone W2mef was obtained from D. Kyle (Walter Reed Army Institute of Medical Research, Washington) and clone 3D7 was from David Walliker (Department of Genetics, University of Edinburgh).

Drug Assays. Parasites at an initial parasitemia of 1% were grown at 2% hematocrit in erythrocytes for 72 hr with dilutions of the appropriate drug. All assays were done in duplicate, and fresh drug was added with each daily medium change. The final parasitemia was assayed on the FACScan as described (9). The concentration of the drug that inhibits growth to 50% (IC_{50}) was determined graphically. Parasites were grown in RPMI 1640/Hepes/5.8% $NaHCO_3$/10% human serum (10).

Pulsed-Field Gel Electrophoresis (PFGE) and Chromosome Mapping. All PFGE experiments were done in a contoured clamp homogeneous electric field apparatus (11). Chromosome mapping was as described (12), and sizes were determined by comparison with chromosomes from *Saccharomyces cerevisiae* and bacteriophage λ concatamers (Promega). DNA probes were labeled with [α-^{32}P]dATP and hybridized as described (7).

Quantitation of Pgh1 with Immunoblotting. Parasites were synchronized with 5% sorbitol and allowed to develop to trophozoite stage. We had reported (6) that between 2.5×10^5 and 5×10^6 trophozoites give a linear signal in immunoblotting experiments with affinity-purified anti-Pgh1 antibodies. Therefore, 5×10^5 trophozoites, purified free of uninfected erythrocytes by Percoll gradients, were separated by SDS/PAGE, and immunoblots were probed with affinity-purified anti-Pgh1 and anti-Pfhsp70 antibodies followed by ^{125}I-labeled protein A. The intensity of the signal was determined

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Abbreviation: PFGE, pulsed-field gel electrophoresis.

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by using a PhosphorImager (model 400; Molecular Dynamics) and Image Quant software.

RESULTS

Selection for Mefloquine-Resistant *P. falciparum*. We have shown that selection for increased chloroquine resistance was linked to deamplification and decreased expression of the *pfmdr1* gene, as well as a decrease in the level of mefloquine resistance (7). This result suggested that the expression level of the *pfmdr1* gene may influence the sensitivity of *P. falciparum* to mefloquine. To test this hypothesis, two cloned isolates of *P. falciparum*, K1 and W2mef, were subjected to increased mefloquine pressure, resulting in cell lines designated K1mef and W2mef^2 that could grow in medium containing mefloquine at 25 ng/ml and 53 ng/ml, respectively. Finally, these cell lines were grown in medium containing mefloquine at 90 ng/ml to obtain W2mef^3 and medium containing mefloquine at 75 ng/ml to derive K1mef^2. It was shown that the cell lines were derived from the original parental lines by hybridization of *Acc* I-cut genomic DNA to the repetitive probe 7H8/6 (data not shown) (13).

Chromosome 5 Increases in Size During Mefloquine Selection. Intact chromosomes of the parental lines K1 and W2mef and the mefloquine-selected progeny, K1mef, K1mef^2, W2mef^2, and W2mef^3, were separated by PFGE and blotted to a nylon filter for hybridization with radioactively labeled *pfmdr1* (Fig. 1). Chromosome 5, which contains the *pfmdr1* gene (3), has increased in size in both K1mef and K1mef^2 by ≈60 kb and 100 kb, respectively, as the mefloquine concentration was increased. The same chromosome in W2mef^2 and W2mef^3 has also increased in size ≈100 and 200 kb, respectively. W2mef^2 clearly has a mixture of two populations of parasites containing a different-sized chromosome 5; the minor form is the same size as that in W2mef^3. However, under increased selection with mefloquine all of the parasites contain the larger form of the chromosome.

To further analyze the alterations in size of chromosome 5 *Sma* I digests of chromosomes from K1, K1mef, K1mef^2, W2mef, W2mef^2, and W2mef^3, as well as *Bgl* I digests of the K1 series, were separated on PFGE gels and probed with the *pfmdr1* gene (Fig. 2). The *pfmdr1* gene hybridizes to an ≈900-kb *Sma* I fragment in K1; however, in K1mef and K1mef^2 the *Sma* I fragment has increased in size to ≈940 and 970 kb, respectively. A *Bgl* I digest of the same chromosomes probed with the *pfmdr1* gene reveals in K1 a 100-kb *Bgl* I fragment, whereas in K1mef it has increased in size to 140 kb. K1mef has two *Bgl* I fragments that encompass the *pfmdr1* gene, of 140 and 180 kb, showing that it is a mixture of two subpopulations, each of which contains a slightly different-sized chromosome 5 that is not detectable in the *Sma* I digests or PFGE of whole chromosomes. A *Sma* I digest of chromosomes from W2mef, W2mef^2, and W2mef^3 revealed very similar results to those seen in the K1-selected cell lines (Fig. 1). In W2mef a *Sma* I fragment of 1 mb is detected by the *pfmdr1* gene, and this increases in size to 1.1 mb and 1.2 mb in W2mef^2 and 1.2 mb in W2mef^3.

To determine the nature of the size increase of chromosome 5 that was selected by the mefloquine pressure we generated a physical map of the chromosomes from both the parental and drug-selected cell lines (Figs. 3 and 4). This technique revealed that chromosomes 5 in K1, K1mef, and K1mef^2 were identical along the length of the chromosome, except for an increase in size of the *Bgl* I fragment in K1mef and K1mef^2 that is defined by the *pfmdr1* gene (Fig. 3). The restriction map of chromosome 5 from W2mef, W2mef^2, and W2mef^3 (Fig. 4) is very different to that in K1 and its derived cell lines. W2mef has two 100-kb amplicons defined by the *Bgl* I sites, and these contain the *pfmdr1* gene. Chromosomes 5 from W2mef^2 and W2mef^3 are identical in their restriction map compared with W2mef, except for the number of amplicons encompassed by the *Bgl* I sites. W2mef^2 has a mixture of two different sized chromosomes 5, each with two and three *pfmdr1*-containing amplicons, whereas the chro-

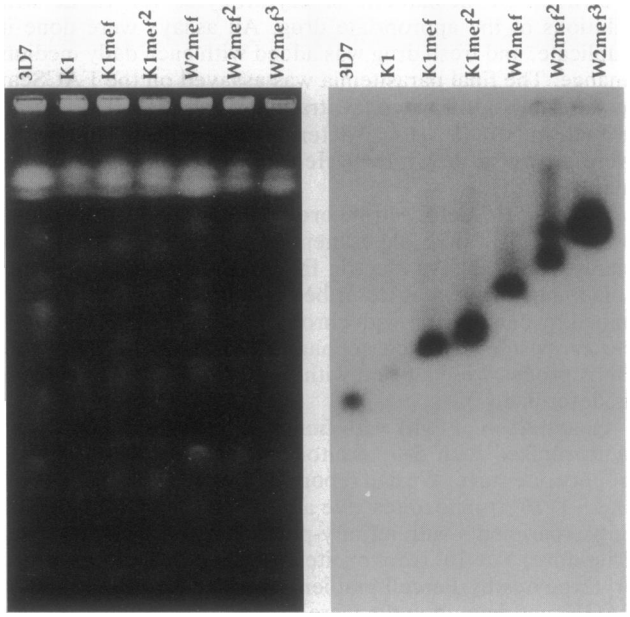

FIG. 1. Mefloquine pressure on cloned isolates K1 and W2mef selects for a larger chromosome 5. Intact chromosomes were separated by PFGE for 72 hr at 140 V with a pulse time of 225 sec. Chromosomal DNA in the gel was blotted to a nylon membrane and hybridized to the *pfmdr1* gene.

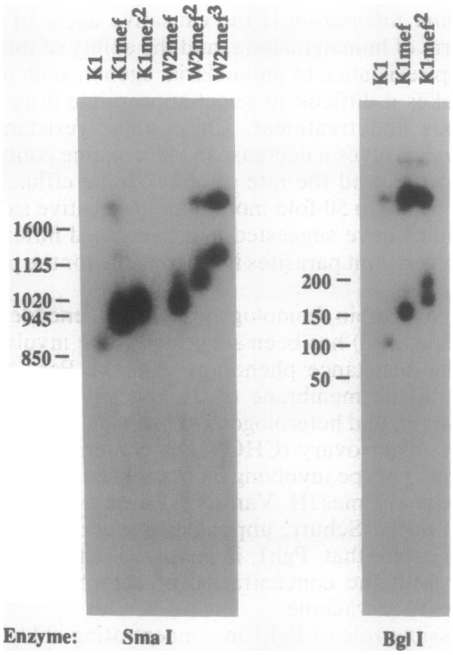

FIG. 2. DNA surrounding the *pfmdr1* gene is amplified during mefloquine selection. Chromosomes from each isolate were cut with *Sma* I or *Bgl* I (as specified on the gel) and separated by PFGE on a contoured clamp homogeneous electric field apparatus for, in the case of the *Sma* I digest, 15 hr with a pulse time of 60 sec followed by 9 hr with a pulse time of 90 sec at 200 V for both pulse settings; for *Bgl* I digests, the protocol was 16 hr with a pulse time of 20 sec at 160 V. The digests were blotted to nylon membrane and probed with the *pfmdr1* gene.

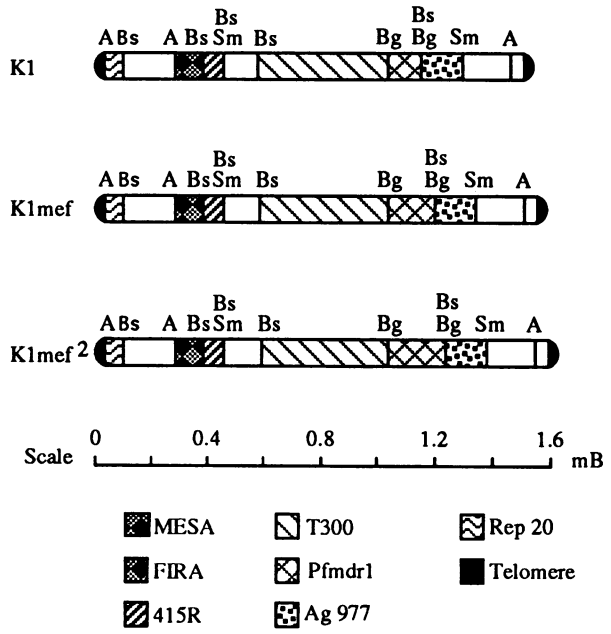

FIG. 3. Physical map of chromosome 5 from K1, K1mef, and K1mef^2. Restriction digests of whole chromosomes fractionated by PFGE were blotted and probed with various cloned fragments as shown in the code at the bottom: MESA, mature erythrocyte surface antigen; FIRA, falciparum interspersed repeat antigen; Ag, 977, antigen 977; Rep 20, repeat 20. Sizes of fragments were calculated by comparison with *S. cerevisiae* chromosomes and bacteriophage λ DNA concatamers. A, *Apa* I; Bg, *Bgl* I; Bs, *Bss*HII; Sm, *Sma* I. Shaded regions indicate those restriction fragments that hybridize to the probes in the key.

mosome 5 of W2mef^3 has four amplicons. These results strongly suggest that the mefloquine selection is inducing an increase in the size of chromosome 5 around the *pfmdr1* gene and that the parasites containing the larger chromosome 5 have a selective advantage under mefloquine pressure.

The *pfmdr1* Gene Is Amplified and Overexpressed in the Mefloquine-Selected Cell Lines. The macrorestriction maps of the mefloquine-selected lines of *P. falciparum* suggest regions, which include the *pfmdr1* gene, of the chromosome

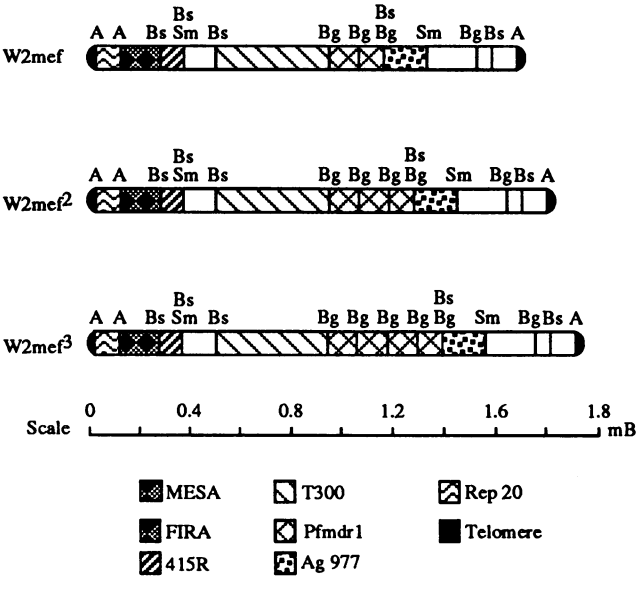

FIG. 4. Physical map of chromosome 5 from W2mef, W2mef^2, and W2mef^3. The method and key to the probes used to generate this macrorestriction map are as described in Fig. 3.

have been amplified. To confirm this hypothesis and determine the extent of amplification we digested genomic DNA from the different parasite lines with *Bam*HI and *Eco*RI and separated the fragments by agarose gel electrophoresis, transferred them to a nylon membrane, and hybridized them with the *Bam*HI and *Eco*RI 1.6-kb cloned *pfmdr1* gene fragment (3). The K1-cloned isolate has been shown to have a single copy of the *pfmdr1* gene (3), and the extent of hybridization of the *pfmdr1* gene to DNA from these isolates was used to determine the *pfmdr1* gene copy number in the other parasite lines. The 1.6-kb *Bam*HI–*Eco*RI fragment of the *pfmdr1* gene hybridized to a fragment of the same size in genomic DNA in all of the isolates; however, the mefloquine-selected lines K1mef, K1mef^2, W2mef^2, and W2mef^3 all appear to have increased copies of the *pfmdr1* gene compared with their parental cell lines. This same filter was reprobed with the calmodulin gene (14) as a control for loading and to act as a reference to determine the *pfmdr1* gene copy number by quantitation using a PhosphorImager. The results suggest that K1 has a single copy of the gene, and K1mef and K1mef^2 have 1.9 and 2.4 copies of the gene, respectively. W2mef, W2mef^2, and W2mef^3 have 2, 2.6, and 3.4 copies of the *pfmdr1* gene.

The protein product of the *pfmdr1* gene has been termed Pgh1, and it has been shown to be overexpressed in direct correlation with the gene copy number in a number of different isolates (6, 7). To show that the amplification of the *pfmdr1* gene correlated with increased expression of the Pgh1 protein product we used parental lines, and the mefloquine-selected parasite lines to detect the 160-kDa Pgh1 protein. The levels of Pgh1 appear to increase in the cell lines subjected to mefloquine pressure (Table 1).

K1 has a single *pfmdr1* gene and, as expected, it expresses the same level of Pgh1 as does other isolates such as 3D7 that have a single copy of the *pfmdr1* gene (data not shown). However, K1mef and K1mef^2 express 2- and 2.6-fold increased levels of the Pgh1 protein (Fig. 5). Similarly, W2mef expresses ≈2-fold increased levels of Pgh1, whereas the mefloquine-selected cell lines W2mef^2 and W2mef^3 express 2.6- and 4.2-fold increased levels of the protein. Therefore, the amplification of the *pfmdr1* gene selected by mefloquine pressure has resulted in increased expression of the protein product of the gene.

Amino acid differences in the *pfmdr1* gene have been linked to chloroquine resistance (5), and both K1 and W2mef have been shown (5) to have Tyr-86 rather than Asp-86. To determine whether any alterations had occurred in this region of the *pfmdr1* gene during the mefloquine selection we isolated a 400-bp region by PCR (15) and sequenced across the region encoding amino acid 86. K1 and its mefloquine-selected daughter lines all encoded Tyr-86, whereas W2mef, W2mef^2, and W2mef^3 all encoded Phe-86 in the *pfmdr1* gene. The *pfmdr1* gene of W2mef has previously been shown to encode Tyr-86, suggesting that a subpopulation may have grown out of the original W2mef that differed in sequence at this position. Nevertheless, it is clear from the PCR sequencing results that no alteration in this amino acid is involved in the increased mefloquine resistance that has been selected.

Mefloquine Selection Results in Halofantrine and Quinine Cross-resistance and a Decrease in Chloroquine Resistance. We had reported (7) that selection for increased chloroquine resistance resulted in decreased mefloquine resistance and deamplification of the *pfmdr1* gene. This result suggested that selection for increased mefloquine resistance resulted in decreased chloroquine resistance and amplification of the *pfmdr1* gene. To test this hypothesis we determined the level of resistance of the different mefloquine-selected cell lines and the parental isolates to both chloroquine and mefloquine, as well as the other quinoline-containing drugs–halofantrine, amodiaquine, and quinine. Cells were grown in various

Table 1. Summary of drug sensitivities of isolates

Strain	IC_{50}, ng/ml					Pgh1 expression*
	Mefloquine	Halofantrine	Chloroquine	Quinine	Amodiaquine	
K1	22.4	7.8	446.9	127.1	19.9	1 ± 0.1
K1mef	50.1	12	246.1	295.3	16.2	2 ± 0.3
K1mef^2	91.2	13.2	316.3	288.4	19.9	2.6 ± 0.4
W2mef	58.88	12.9	194.98	239.9	12.9	1.9 ± 0.4
W2mef^2	51.29	12.9	125.89	190.6	12.9	2.6 ± 0.5
W2mef^3	83.18	26.8	98.8	309	15.1	4.2 ± 0.6

*These figures are from five independent experiments and are normalized to Pgh1 expression in strain 3D7.

concentrations of each drug, and the IC_{50} of each was calculated (Table 1).

The K1 cloned line had an IC_{50} value for mefloquine of 22.4 ng/ml, whereas K1mef and K1mef^2 had increased resistance to mefloquine with IC_{50} values of 50.1 ng/ml and 91.2 ng/ml, respectively. However, the level of chloroquine resistance of the two mefloquine-selected cell lines decreased compared with the K1 parent with IC_{50} values for chloroquine of 446.9, 246.1, and 309 ng/ml for K1, K1mef, and K1mef^2, respectively. The IC_{50} values for mefloquine of W2mef (58.88 ng/ml) and W2mef^2 (51.29 ng/ml) were similar; however, W2mef^3 (83.18 ng/ml) increased, whereas the IC_{50} values for chloroquine showed an inverse pattern. This result together with previous results from chloroquine-selected mutants (7) strongly suggests an inverse correlation with the expression level of Pgh1 in chloroquine resistance and a positive correlation with mefloquine resistance.

Some studies in malaria endemic areas have suggested that resistance to mefloquine, halofantrine, and quinine is linked (16–18). We analyzed the relationship of the increased mefloquine resistance in the *in vitro* selected cell lines to their sensitivity to halofantrine, quinine, and amodiaquine by determining the IC_{50} values of each drug for each of the parasite lines (Table 1). The IC_{50} value of K1 and the mefloquine-selected cell lines K1mef and K1mef^2 for halofantrine increased as the parasite lines became more resistant to mefloquine. Similarly, quinine resistance of the K1 series of cell lines also increased parallel to the increase in mefloquine resistance. In contrast, the IC_{50} values for amodiaquine in all three of these cell lines were virtually identical. The results obtained with W2mef, W2mef^2, and W2mef^3 were very similar to that seen with K1 and its mefloquine-selected cell lines. Halofantrine resistance paralleled the increase in resistance to mefloquine, as did quinine resistance in going from W2mef to the more mefloquine-resistant cell line W2mef^3. Amodiaquine was again almost identical for all three of the W2mef parasite lines. These results suggest that either *pfmdr1* or a closely linked gene plays a role in resistance to mefloquine, halofantrine, and quinine in these cell lines.

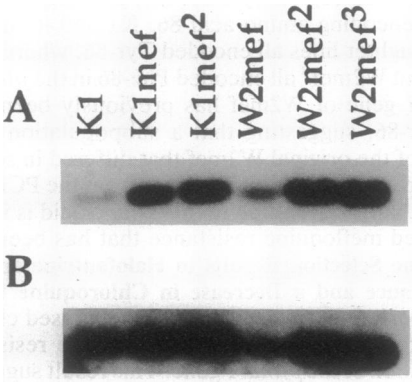

FIG. 5. Amplification of the *pfmdr1* gene during mefloquine selection. Genomic DNA from K1, K1mef, K1mef^2, W2mef, W2mef^2, and W2mef^3 was digested with *Eco*RI and *Bam*HI, the fragments were separated by agarose gel electrophoresis, and the gel was blotted to a nylon membrane. The filter was probed with a 1.6-kb *pfmdr1* gene probe (*A*) followed by a 1.3-kb calmodulin probe (*B*) to quantitate the gene copy number by PhosphorImage analysis.

DISCUSSION

Previously we have shown that selection for increased chloroquine resistance results in not only deamplification and decreased expression of the *pfmdr1* gene but also a decrease in resistance to mefloquine (7). These results suggested an inverse relationship between chloroquine and mefloquine resistance and a possible role for the protein product of the *pfmdr1* gene. To test this we subjected two cloned isolates of *P. falciparum* to mefloquine drug pressure and have shown that the *pfmdr1* gene is amplified, resulting in increased expression of the protein product (Pgh1) and decreased resistance to chloroquine, as well as increased resistance to quinine and halofantrine. These results suggest that overexpression of Pgh1 may play a role in the mechanism of resistance to mefloquine, halofantrine, and quinine.

The gradual increase in amplification of the area of the genome surrounding the *pfmdr1* gene in response to mefloquine pressure strongly suggests that a gene is present within this genomic region that confers a selective advantage in the presence of mefloquine. The sizes of the K1 and W2mef amplicons containing *pfmdr1* are ≈50 kb and 100 kb, respectively, and consequently a gene(s) other than *pfmdr1* could be involved in the mefloquine-resistance phenotype. However, it has been shown (3) that the amplicon in the IndoChina 1 *P. falciparum* isolate was <20 kb; thus, we believe that the selective pressure is probably being directed on *pfmdr1*, rather than a closely linked gene.

Amplification of the *pfmdr1* gene in field isolates has been shown to arise in multiple independent events, suggesting that this area was under strong selective pressure (19). It is unlikely that mefloquine use had selected the amplification events seen in these field isolates, as they were obtained before mefloquine was in use. The observation that the K1mef and W2mef lines are cross-resistant to quinine suggests that amplification and overexpression of the *pfmdr1* gene that has been observed in field isolates may result from quinine drug pressure. A study in the Cameroon showing a linkage of mefloquine and quinine resistance before mefloquine was used in this country supports this possibility (17, 18).

Transfection of the *pfmdr1* gene into Chinese hamster ovary (CHO) cells has suggested that Pgh1 is involved in concentration of chloroquine into the food vacuole of *P. falciparum*, either acting as a direct chloroquine transporter or indirectly in regulation of the pH of the acidic vacuole (H. Van Es, S. Karcz, F. Chu, A.F.C., P. Gros, and E. Schürr, unpublished work). This function is consistent with the inverse relationship between amplification of the *pfmdr1*

gene and decreased chloroquine resistance, as increased expression of the Pgh1 protein would increase accumulation of chloroquine and consequently decrease levels of chloroquine resistance. The inverse relationship between chloroquine and mefloquine resistance is more difficult to reconcile mechanistically. It is possible, that Pgh1 is a transporter of mefloquine. The selection of increased expression of the protein appears inconsistent with this possibility, unless it results in the expression of higher protein levels on the plasma membrane of the parasite rather than the food vacuole membrane (6, 7, 21); this would result in increased transport of mefloquine out of the cell and, consequently, increased resistance. It is also possible that Pgh1 is a target of action of mefloquine or in an indirect way affects the accumulation of mefloquine into the food vacuole.

The observation that selection for mefloquine resistance also results in cross-resistance to halofantrine agrees with results obtained with field isolates from Thailand (22); this has shown linkage of amplification of the *pfmdr1* gene, mefloquine, and halofantrine resistance. The documentation of quinine and mefloquine cross-resistance in malaria endemic areas (16) is consistent with the acquisition of cross-resistance to quinine by the K1mef and W2mef lines we have selected. This result also demonstrates a true multidrug resistant phenotype between mefloquine, halofantrine, and quinine in *P. falciparum*.

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