Amplification of a Gene Related to Mammalian mdr Genes in Drug-Resistant *Plasmodium falciparum*

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The malaria parasite *Plasmodium falciparum* contains at least two genes related to the mammalian multiple drug resistance genes, and at least one of the *P. falciparum* genes is expressed at a higher level and is present in higher copy number in a strain that is resistant to multiple drugs than in a strain that is sensitive to the drugs.

HUMAN MALARIA IS WIDESPREAD throughout the tropical world and is a major human health problem in both morbidity and mortality. Infection with drug-resistant strains of *Plasmodium falciparum* is an evolving public health problem in nearly all endemic areas (*1*). Parasites resistant to high levels of chloroquine have necessitated the use of less effective and more expensive antimalarial drugs, but the parasites are becoming resistant to these other antimalarial drugs also. Neither the mechanism of action of the aminoquinolines nor the mechanism of resistance to these compounds is completely understood (*2*). Resistance of *P. falciparum* to chloroquine is associated with increased drug efflux (*3*), and this efflux can be reversed by verapamil and other compounds (*4*). Resistance to mefloquine can be reversed by penfluridol (*5*).

The finding that drug efflux and drug resistance can be reversed by verapamil is similar to findings in studies of drug resistance in mammalian tumor cells (*6*). The mechanism of resistance in tumor cells has been defined as increased drug efflux that is mediated by increased expression of the P-glycoprotein (*7*). This drug efflux can be inhibited by simultaneous administration of verapamil and related compounds (*8*). The sequence similarity of P-glycoprotein to bacterial transport proteins has led to the model that the P-glycoprotein is an adenosine triphosphate (ATP)–dependent efflux pump responsible for decreasing drug accumulation in resistant cells (*8*). The P-glycoprotein is encoded by the multiple drug resistance gene (mdr gene), which is amplified either at the DNA level or RNA expression level in tumor lines resistant to multiple drugs.

Thus one mechanism of drug resistance in *P. falciparum* may be similar to multidrug resistance in mammalian cells, namely the mediation of drug efflux by an ATP-dependent efflux pump (*3, 4*). We tested this model by using sequences that are conserved in the mammalian P-glycoproteins and several bacterial transport proteins (*9, 10*) to identify putative mdr-like genes in *P. falciparum*. Two primers based on conserved protein sequences shared in the mouse mdr, human mdr, and the bacterial hemolysin B(HlyB) proteins (*9, 10*) were synthesized. The sense primer was based on a nine–amino acid homology found in position 1066–1075 of the murine mdr gene, and the antisense primer was based on a seven–amino acid homology found in position 1198–1204 (*10*). The codon usage was based on the preferred codon usage for *P. falciparum* (*11*).

These primers were incubated with DNA extracted from the W2 strain of *P. falciparum* under conditions of the polymerase chain reaction (*12*). Only those reactions that contained primers, enzyme, and *P. falciparum* template DNA showed the presence of an amplified sequence. The mdr gene primers did not amplify a sequence in human DNA. This is the predicted result, because human codon usage is different from that of the parasite, and the sequences chosen as primers would not be able to prime the human sequence. In using the mdr primers, there was an additional amplified band approximately 600 bp long and some other minor bands ranging in size from 300 to 1000 bp. The two major bands, approximately 450 and 600 bp in length, were used for further analysis. The identity of the other bands remains to be determined.

The sequence of the 600-bp (Pfmdr1) and 450-bp (Pfmdr2) fragments and the predicted amino acid sequences were compared with the sequence of murine mdr gene

Fig. 1. Identification of mdr-like genes in *Plasmodium falciparum*. Comparison of the deduced amino acid sequence of Pfmdr1 and Pfmdr2 to the deduced amino acid sequence of murine mdr gene (*9*); mdr1a represents residues amino acids 425 to 562 and mdr1b represents residues 1068 to 1207 (*9*). The boxed (Pfmdr1) and shaded (Pfmdr2) areas indicate sequence identity between the *P. falciparum* genes and at least one of the two halves of the murine mdr gene. The selection of primers is discussed in the text. The two oligonucleotides synthesized (Biosearch 8750) with degeneracies in parentheses are (5′ to 3′) for sense strands, GT(A,T)-GG(A,T)-(C,A)G(T,A)-TC(T,A)-GG(T,A)-GG(T,A)-AAA-TC(T,A)-AC (degree of degeneracy, 256), and for the antisense strand, ATC-TAA-(T,A)GC-(T,A)GA-(T,A)GT-(T,A)GC-TTC-AT (degree of degeneracy, 16). Two additional primers containing synthetic Eco RI (sense) and Hind III (antisense) restriction sites were used in the cloning of the Pfmdr1 gene. The total polymerase chain reaction was extracted with phenol-chloroform and either cloned directly (Pfmdr2) or after restriction digestion (Pfmdr1) into the pBluescript vector at appropriate restriction sites (Eco RV for Pfmdr2 and Eco RI–Hind III for Pfmdr1). Transformants containing the putative mdr genes were selected by hybridization with radiolabeled polymerase chain reaction products, which were extracted from low melt agarose gels. Plasmids containing putative mdr genes were sequenced by using a modification of the dideoxynucleotide chain termination method with Sequenase enzyme (*16*). The data presented represents sequences of three independent clones for each gene. GenBank accession number for the nucleotide sequences of Pfmdr1 (600 bp) and Pfmdr2 (450 bp) are M24850 and M24851, respectively.

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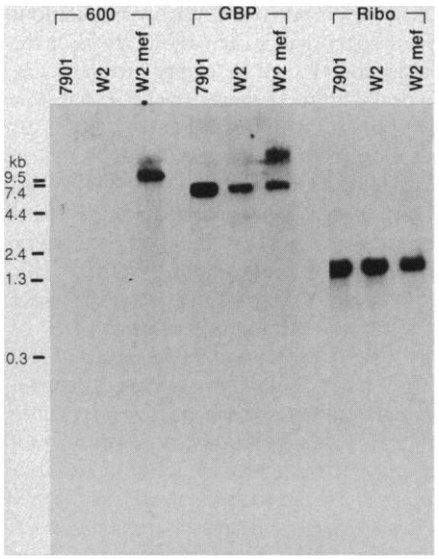

Fig. 2. RNA hybridization analysis with Pfmdr1. RNA was extracted from W2, W2 mef, and 7901. The median inhibitory concentrations to chloroquine are 40 to 60 ng/ml for W2, 20 to 30 ng/ml for W2 mef, and 3 to 5 ng/ml for 7901; and to mefloquine are 2 to 3 ng/ml for W2, 8 to 9 ng/ml for W2 mef, and 1 to 3 ng/ml for 7901. Cultivated parasites were washed and resuspended in RPMI supplemented with 30% human plasma. This suspension was layered onto a 75% to 76% Percoll gradient and centrifuged at 1200*g* for 30 min. The middle layers of concentrated parasitized red blood cells were washed twice in RPMI and suspended in a lysis buffer (50 m*M* Hepes, 2 m*M* EDTA, and 100 m*M* NaCl). Sarcosyl (10%) was added to a final concentration of 1% followed by vigorous mixing. The aqueous phase was repeatedly extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) until there was no interface material. The nucleic acid was precipitated with ethanol after the addition of sodium acetate (*p*H 5.2) to 0.3*M*. Equal amounts of total nucleic acid (10 μg) were resolved in triplicate on a 1.3% agarose gel in 0.66*M* formaldehyde. The RNA was transferred to nitrocellulose membranes, and hybridization was performed with three different probe molecules, Pfmdr1, the glycophorin binding protein (GBP) cDNA (*17*), and an rDNA probe. Autoradiograms of the RNA blots are shown after hybridization.

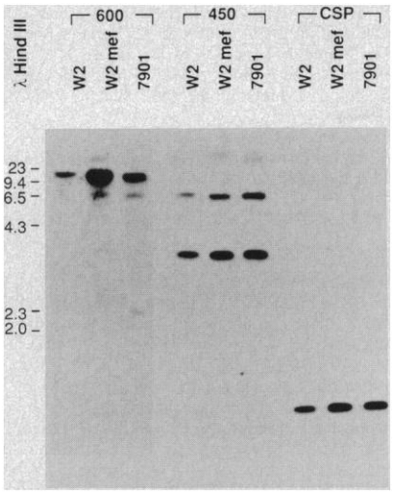

Fig. 3. DNA hybridization analysis of Pfmdr1 and Pfmdr2. DNA was extracted from strains W2, W2 mef, and 7901. Eco RI–digested DNA (1 μg) from each strain was resolved in triplicate on a 1% agarose gel and transferred to nitrocellulose membranes. Hybridization was performed as in Fig. 4. Three probes were used: Pfmdr1 (600 fragment), Pfmdr2 (450 fragment), and the CSP gene (*15*). Autoradiograms of the DNA blots after hybridization are shown. Quantitative densitometry was performed with a densitometer and the data are expressed as a ratio of the 450 or 600 hybridizing bands to the band hybridizing to the CSP probe. For the 450 fragment these values are 1.31 for W2, 1.47 for W2 mef, and 1.37 for 7901; for the 600 fragment the values are 0.62 for W2, 2.28 for W2 mef, and 0.92 for 7901.

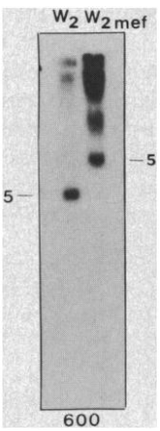

Fig. 4. Chromosome analysis of Pfmdr1. Agarose blocks containing *P. falciparum*–infected erythrocytes were prepared by standard techniques (*18, 19*). Separation of chromosomes was done at 150 mA (constant current), for 48 hours with a 2-min pulse interval at 16°C in a Beckman Geneline system. DNA was transferred to nitrocellulose membranes, and hybridization was performed according to the manufacturer's instructions. Chromosome 5 was confirmed by hybridization of parallel lanes with a known chromosome marker, MESA antigen (*18*).

(Fig. 1). The highlighted (boxed or shaded) regions indicate identical amino acids between the mouse mdr1 protein and Pfmdr1 (59% identity) or Pfmdr2 (58% identity). The Pfmdr1 fragment contains an additional 45 amino acids not in the mouse mdr1 gene or Pfmdr2. Pfmdr1 and Pfmdr2 are similar to each other (59% identity) at the amino acid level, but have little similarity at the nucleotide level. In the mammalian systems, there are also multiple mdr genes (three in rodents and two in humans) that are members of a closely related family (*13*), whereas in the *P. falciparum* system at least two different genes code for similar proteins.

If the mechanism of these putative mdr genes in *P. falciparum* is similar to that of mdr genes in human multidrug-resistant tumor cells, the model predicts that the gene would be expressed at a higher level in drug-resistant cells than in drug-sensitive cells. For this analysis, we used the cloned W2 and W2 mef lines of *P. falciparum*. W2 mef was derived from W2 by stepwise selection in increasing concentrations of mefloquine (*14*). RNA analysis reveals that in the W2 mef clone there is a significantly increased expression of an RNA molecule which hybridizes to Pfmdr1 (600 fragment) (Fig. 2). Equivalent amounts of RNA were analyzed from the W2, W2 mef, and 7901 strains, as demonstrated by the hybridization with probes specific for ribosomal RNA and the cloned glycophorin-binding protein cDNA. The Pfmdr1 transcript is longer than the glycophorin-binding protein transcript, which encodes a protein of 130 kD. This result is consistent with the Pfmdr1 gene encoding a protein of 150 to 170 kD. The use of RNA markers indicates that the size of the transcript is between 7 and 8 kb, which is larger than the 5-kb transcript of most mammalian mdr genes. The larger size of the transcript may be due to larger 5′ and 3′ untranslated regions in the *P. falciparum* mRNA. Longer exposure showed that the Pfmdr1 mRNA is also present in W2 and 7901 but at significantly reduced levels. This result indicates that the Pfmdr1 gene is expressed in both drug-sensitive and drug-resistant parasites.

In many of the mammalian tumor lines expressing high levels of mdr mRNA, there is also an amplification of the corresponding mdr gene. In DNA analysis with the same strains as used above, the W2 mef clone showed a two- to fourfold increase in the copy number of the Pfmdr1 gene when compared to its parent clone W2 and the 7901 strain (Fig. 3). There was no difference in the DNA copy number of Pfmdr2 (450 fragment) among the strains tested. Two other sensitive strains, 3D7 and Honduras, have a single copy of Pfmdr1. Quantitative densitometry analysis was performed with the single-copy circumsporozoite gene (CSP) as an internal standard (*15*).

The 600-bp Pfmdr1 sequence was also mapped by the pulsed-field gel technique (Fig. 4). In this case, two cloned parasites were compared, W2 and W2 mef. In W2, the 600-bp fragment maps to chromosome 5, whereas in the W2 mef strain it maps to a region containing several of the larger chromosomes of *P. falciparum*. Hybridization with a probe specific for another chromosome 5 marker (MESA antigen) indicated that chromosome 5 has increased in size, as expected with an internal amplification of the Pfmdr1 gene. In addition, as with the Southern blot, there is increased hybridization intensity in the W2 mef strain, again in agreement with this sequence being amplified.

The conclusion from these experiments is that *P. falciparum* contains at least two genes that are similar in sequence to the mammalian mdr genes and that at least one of these genes is expressed at a higher level and is present in higher copy number in one *P. falciparum* strain that is resistant to multiple drugs. These data are consistent with the

possibility that one mechanism of drug resistance in *P. falciparum* is similar to that of multidrug resistance in mammalian cells. Other mechanisms of drug resistance may also exist (*2*) and analyses of field isolates will be necessary to determine the importance of these observations to natural drug resistance in *P. falciparum*.

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Monitoring the AIDS Epidemic in the United States: A Network Approach

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Respondents in the 1988 General Social Survey (GSS) were asked to scan their acquaintance networks to identify all those who had been a victim of a homicide or had acquired immunodeficiency syndrome (AIDS). Estimates of the sex, race, age, and regional breakdowns for homicides in the last year and for people with AIDS were compared with official statistics. The GSS estimates for the distribution of homicide victims replicate the official statistics quite well. The GSS estimates for AIDS cases suggest that the data provided to the Centers for Disease Control may underestimate by a substantial margin the prevalence of AIDS in the white population of higher socioeconomic status, overstate the relative prevalence of the disease in the minority populations, underestimate the prevalence of the disease in the Midwest, and overstate it for the East.

MONITORING THE SPREAD OF AIDS throughout the population of the United States has posed a special challenge to public health officials interested in bringing AIDS under control. Almost from the onset of the epidemic in the early 1980s, it has been recognized that the incidence of AIDS was highly selective in its geographic and social distribution, both in this country and abroad. The central mechanisms that transmit the disease—certain sexual practices, sharing of needles among drug users, and contaminated blood products—have focused attention on social processes such as mate selection and social intimacy in which these mechanisms are embedded.

In the United States, a key method for monitoring the spread of the disease has been the collation of reports of AIDS cases from local and state departments of public health by the Centers for Disease Control (CDC) in Atlanta (*1*). This data-gathering method has itself been subject to socially based distortions arising out of the controversial nature of the disease with respect both to its biological nature and to its socially stigmatizing implications (*2*). The highly decentralized nature of the CDC reporting system makes it vulnerable to systematic distortions and overt manipulations by interested parties at various levels of the loosely constructed reporting hierarchy (*3*). To deal with these issues, the CDC has now developed a "family of surveys" in an attempt to monitor the levels and trends of human immunodeficiency virus (HIV) infection (*4*). Such surveillance of HIV infection will not replace the need to devise independent methods for monitoring the social epidemiology of AIDS cases in order to assess the strengths and weaknesses of particular estimates and projections.

We report on an effort to devise an independent estimate of the relative prevalence of AIDS across various population subgroups and geographic locations. Its rationale rests on the social network perspective as it is applied to randomly sampled population surveys (*5*). The strategy is to ask an individual with a known probability of selection from a well-defined population to scan his or her primary acquaintance network, defined to include all the persons he or she knows personally as kin, friends, neighbors, co-workers, and more casual and incidental acquaintances, in order to identify all those who possess a particular characteristic, such as a health condition like AIDS or being a victim of a homicide. For most people the size of such a network is fairly large (on the order of 2000 to 6000 persons) (*6*, *7*). However, it also is bounded imprecisely at the margins because of variations in social and personal definitions of who is included in various social relationships. Fully recognizing that different population subgroups may differ in the average sizes of their personal acquaintance networks, Bernard *et al.* (*7*) proposed measurement efforts to determine the size of such networks for the purposes of estimating hard to count populations (*8*).

Personal networks are known to vary greatly in size, social composition, levels of intimacy of mutual access, and density (*5*). All these network features are likely to affect the flow and extent of information about network members in a systematic fashion. However, we shall make the assumption that, on the average, these differences in network structure across individuals are not systematically organized by the social characteristics that are of special interest to us.

In the *1988 General Social Survey* (GSS) (*9*), conducted by the National Opinion Research Center, respondents were asked questions about their acquaintance with

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