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The genetics of drug resistance in malaria parasites*

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The available experimental data on the genetics of drug resistance in malaria parasites are reviewed. Seven possible mechanisms for the origin of drug resistance are considered, and it is pointed out that spontaneous gene mutation is probably the most important. Experiments on the production of pyrimethamine-resistant and chloroquine-resistant strains of rodent Plasmodium *species, and on the inheritance of such drug resistance, are reviewed. Relevant biochemical data are also considered in relation to the genetics of drug resistance. Studies on competition between drug-sensitive and drug-resistant parasites in mixed populations of rodent plasmodia are described. The implications of these findings for drug resistance in* P. falciparum *are discussed.*

At the present time it is not possible to carry out genetic experiments directly with human malaria parasites, but the *in vitro* culture technique for *Plasmodium falciparum* (*1*, *2*), which at present is limited to the erythrocytic stages, may eventually be extended to the sexual and pre-erythrocytic stages, thus making genetic experiments by classical Mendelian methods technically feasible. In addition, molecular methods, involving recombinant DNA and gene cloning in plasmids, will probably soon be applied to *P. falciparum*, thus producing rapid advances in our knowledge of the genetics of this species. At present, however, we are limited to genetic studies on rodent plasmodia (*P. berghei* and related forms) by the classical methods of hybridization and progeny analysis. The results of this work, which are summarized here, can be used, with some caution, to interpret some characteristics of *P. falciparum*.

A number of different mechanisms can be postulated to account for changes in organisms affecting their ability to grow in the presence of drugs, including:

(1) non-genetic and probably temporary changes causing physiological adaptations of the cells to the drugs;

(2) selection of previously existing drug-resistant cells from mixed sensitive and resistant populations, under the influence of drug pressure;

(3) spontaneous mutations in the nuclei of one or more cells and subsequent selection of the drug-resistant mutants;

(4) induced gene mutation, as a result of the action of mutagenic drugs;

(5) mutation of extranuclear genes, e.g., mitochondrial genes;

(6) changes in gene expression caused by alterations in cytoplasmic or environmental factors; and

(7) introduction into cells of resistance-transfer factors or other plasmids.

These mechanisms have been shown to operate in various other organisms, especially in bacteria and to some extent in free-living protozoa, e.g., *Paramecium* (*3*, *4*, *5*). Present indications are that, as regards drug resistance in *Plasmodium*, mechanism 3 (spontaneous gene mutation) is probably the most important, though others are also possible. Mechanism 2 (selection from mixed populations) could operate, since it is known from studies of enzyme variation (*6*) that populations of parasites in individual vertebrate hosts sometimes comprise mixtures of genetically diverse

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organisms. Mechanism 1 (physiological adaptation) also operates to some extent, as shown by the occurrence of unstable changes in laboratory selection experiments (*7*). However, this is unlikely to be important in the long run because of the temporary nature of the changes produced. Mechanism 5 (extra-nuclear mutations) can probably be neglected in plasmodia, in view of the minor metabolic role played by mitochondria in the blood stages of the parasites. Mechanism 7 (resistance-transfer factors) has so far not been demonstrated to occur in *Plasmodium*. If such a mechanism were to exist, as it does in bacteria, one would expect the dissemination of drug resistance about the world to be even more rapid than that already seen.

EXPERIMENTAL METHODS

Among the rodent *Plasmodium* species, drug resistance has been studied in *P. berghei*, *P. yoelii*, *P. chabaudi*, and *P. vinckei*, but genetic experiments have been carried out only with *P. yoelii* and *P. chabaudi*. Some preliminary work was also done on pyrimethamine resistance in *P. gallinaceum* (*8*, *9*), but it has not been followed up and will not be further discussed here. Relevant work with *P. falciparum* has consisted only of comparisons of drug resistance, *in vivo* and *in vitro*, in different strains, without any genetic analysis.

The methods used involve the production of lines of parasites with increased drug tolerance by exposure to a drug in one of two ways: (1) a 'single' treatment (usually lasting 4 successive days) with a high dose, which eliminates most of the parasites and allows only the rare resistant ones to persist; or (2) a long-lasting series of treatments, covering many passages, starting at a low concentration of the drug, with periodic increases.

After parasites with increased drug tolerance have been obtained, the newly resistant line must be tested for its stability following successive blood and mosquito passages. If the resistance is found to be stable, the genetic basis is determined by making a series of crosses between resistant and sensitive lines. The method of carrying out such crosses has been described in detail elsewhere (*6*). In brief, mosquitos are fed on mouse blood containing a mixture of the two types of parasite, and then allowed to infect other mice with the sporozoites thus produced. To ensure that the progeny parasites tested result from cross-fertilization, genetic markers (enzyme variants) are included in the parental strains, and recombinants involving the markers are selected in the progeny.

Cloning is an essential part of the technique, since the initial strains may contain several distinct types of parasite (possibly even different species), and drug pressure may result in the selection of one at the expense of another. The resistant lines might then belong to a species or strain which was a minority constituent of the original strain. Since the resistant parasites might differ from the sensitives in a number of characters unrelated to resistance, comparisons between the two groups could give very misleading results. Cloning must also be done with the progeny parasites derived from crosses. The technique of cloning of malaria parasites has been described by Demidowa (*10*), Bishop (*11*), and others. In current work with rodent plasmodia, a dilution technique has been adopted.

The resistance of rodent malaria parasites to drugs is measured by inoculating samples of parasites into mice and treating the mice with known concentrations of drug (expressed as milligrams of drug per kilogram of mouse body weight), usually on 4 successive days. The drugs are administered either orally or by intraperitoneal injection. Presence or absence of parasites in the blood is then recorded after various time intervals. These procedures are subject to several errors, and the effect of the drug cannot be measured precisely. *In vitro* methods (*12*, *13*, *14*) are now available for assessment of drug resistance of *P. falciparum* and should give more precise results, but so far they have not been widely used with rodent plasmodia.

The genetic work done so far concerns pyrimethamine and chloroquine resistance, and to a lesser extent sulfadiazine and mefloquine resistance.

EXPERIMENTAL FINDINGS

Pyrimethamine resistance

Pyrimethamine-resistant parasites have been readily obtained from initially sensitive strains of various *Plasmodium* species, by both low- and high-pressure selection techniques (*15*, *16*). Moreover, it is well known that clinical treatment of human malaria patients with pyrimethamine leads to the rapid development of drug-resistant parasites (*17*). In this review, attention will be confined to cases where some genetic analysis has been done.

With *P. yoelii*, Morgan (*18*) used a clone of sensitive parasites as starting material and found that when mice containing about 10^9 parasites were treated with a high dose (e.g., 50 mg/kg) of pyrimethamine, approximately 1 in 50 developed drug resistant parasites. Similar results have been obtained with *P. chabaudi* (*19*, *20*). The 'mutation rate' of sensitive parasites to pyrimethamine resistance has been estimated as being approximately 1 in 10^{10}. This is a very

low frequency in comparison with other mutations in genetically well-studied organisms, and it seems likely that the procedures used with rodent malaria parasites succeeded in selecting only a small fraction of the mutations that actually occurred. In any case, only those mutants giving resistance to a high dose of pyrimethamine, in a single step, were selected in these experiments. It is likely that more mutations would be obtained if a lower concentration of drug were used.

The pyrimethamine-resistant mutants that have been obtained are of various types in regard to cross-reaction with sulfadiazine and requirement for 4-aminobenzoic acid (PABA). Earlier workers (e.g., Jacobs) had found that pyrimethamine resistance was usually accompanied by an increased PABA requirement (*21*). Morgan (*18*) reported obtaining eight pyrimethamine-resistant mutants of *P. yoelii* with an increased PABA requirement, and one with a reduced PABA requirement. Macleod (*22*), working with *P. chabaudi*, isolated one mutant with a 20-fold increase in resistance to pyrimethamine accompanied by a 27-fold increase in sensitivity to sulfadiazine and associated with an increased PABA requirement. The same worker obtained another type of mutant with only a 3-fold increase in pyrimethamine resistance but a 5-fold increase in resistance to sulfadiazine, and reduced PABA requirement.

It should also be mentioned that pyrimethamine resistance has often been found to be associated with resistance to other antifolate drugs, such as proguanil (*23*) and cycloguanil.

To illustrate the stability of pyrimethamine-resistant mutants, it may be mentioned that Morgan (*18*) obtained a resistant clone of *P. yoelii* whose resistance remained unaltered after 55 blood passages, 18 mosquito transmissions, and 5 months' storage in liquid nitrogen.

Following hybridization between pyrimethamine-resistant and pyrimethamine-sensitive parasite lines, and isolation of progeny clones, segregation and recombination have been shown to take place (*19*). Table 1 gives some typical results, which show that a substantial proportion of recombinant clones were found. Similar results have been found with *P. chabaudi* (*22*).

These and many other similar results show that, following crosses between pyrimethamine-resistant and sensitive lines, segregation and recombination involving the genes concerned take place in mosquitos, presumably at meiosis. Recombinants have not been found when mixtures of blood forms, or of sporozoites, are made, and the parasites cloned after a period of asexual growth only. The earlier claim of Yoeli et al. (*24*) that genetic exchange may occur among populations of trophozoites, has not been confirmed (*25*).

Taking together the findings that abrupt changes from sensitivity to resistance occur in single clones of parasites, that the resistance thus obtained is stably inherited, and that segregation and recombination only occur during mosquito passage, it is concluded that nuclear gene mutation is the cause of pyrimethamine resistance. There is no evidence that pyrimethamine is mutagenic, though this point has not been studied specifically. It is therefore assumed that the mutations are spontaneous.

Table 1. Crosses between pyrimethamine-resistant and sensitive lines of *P. yoelii* (after Walliker et al. (*19*))

Parents	(1)	pyr-r GPI-1	x	(2)	pyr-s GPI-2	
Progeny clones	Non-recombinants			Recombinants		
		pyr-r GPI-1	21 clones		pyr-r GPI-2	13 clones
		pyr-s GPI-2	30 clones		pyr-s GPI-1	7 clones

pyr-r — pyrimethamine resistant; pyr-s — pyrimethamine sensitive.
GPI-1, 2 — electrophoretic variants of glucose phosphate isomerase.

In view of the phenotypic diversity of different pyrimethamine-resistant mutants, it is possible that mutation occurs at more than one chromosomal locus, but the detailed genetic studies needed to prove this have not been carried out.

Chloroquine resistance

In comparison with their apparently uniform sensitivity to pyrimethamine, different species of rodent malaria parasites display considerable variation in their response to chloroquine. *P. yoelii* is innately resistant to high doses (e.g., 50 mg per kg of body weight on four successive days) (*26*), while *P. chabaudi*, *P. berghei*, and *P. vinckei* normally tolerate only small doses (2–5 mg per kg) (*16*).

Many workers have reported that it is more difficult to raise the resistance of malaria parasites to chloroquine, than to pyrimethamine, under laboratory conditions (*23*, *16*). However, stably resistant lines of *P. chabaudi* (*27*) and *P. vinckei* (*28*) have been produced by low-level drug treatments, usually lasting over many blood passages, with gradual increases in drug concentration. In this way resistance of *P. vinckei* to chloroquine was raised from 5 mg/kg to 200 mg/kg of body weight (the maximum dose tolerated by mice) (*28*); the resistance of *P. chabaudi*, starting with cloned material, was raised from 2 mg/kg to 30 mg/kg of body weight (*27*). In both species the resistance finally obtained was stable after repeated passaging.

Several reports of the development of lines of

P. berghei resistant to chloroquine have been summarized (*16*), and in one case, Peters (*7*) found that chloroquine resistance was unstable in the absence of the drug. In another study, the same worker (*29*) recorded the occurrence of a dramatic rise in the chloroquine resistance of *P. berghei* (strain NS) following only a short course of chloroquine treatment; however this was from uncloned material which probably contained a mixture of sensitive *P. berghei* and resistant *P. yoelii*, the latter being selected by the drug treatment. It is possible that some of the earlier reports of chloroquine resistance in *P. berghei* have a similar explanation. Recent attempts to obtain mutations to a high level of chloroquine resistance in a single step, by treating cloned material with a single drug dose at a high concentration, have been unsuccessful.

Genetic work on chloroquine resistance has so far been carried out only with *P. chabaudi* (*27*). Following crosses between parasites resistant to 3 mg/kg and others resistant to only 2 mg/kg, segregation of the two classes occurred, and recombinations with other genetic characters (pyrimethamine resistance and two types of enzyme variant) were also seen. Padua (personal communication, 1979) made crosses between sensitive parasites and parasites from a highly resistant (30 mg/kg) line, and obtained in the progeny mainly sensitive and low-level resistant clones, although a small number of high-level resistant recombinants could also be detected by special selective techniques. These results are consistent with the hypothesis that high-level resistance is due to the combined action of several mutant genes at different chromosomal loci. As with pyrimethamine resistance, no recombination involving chloroquine-resistant genes was found among asexual parasites.

Resistance to other drugs

Little or no knowledge is available concerning the genetics of resistance to other drugs. Ramakrishnan et al. (*30, 31*) obtained sulfadiazine-resistant strains of *P. berghei* by selection in the presence of the drug and also by selection in mice fed on a PABA-deficient diet. Sulfadiazine-resistant mutants of *P. chabaudi* have also been obtained (*22*), which, after crossing with sensitive strains, produced parasite clones in which segregation of resistant and sensitive genotypes had occurred. There was some evidence indicating independent segregation of sulfadiazine resistance and pyrimethamine resistance. Further study is needed to clarify the interrelations of the genes controlling these characters, however. Mefloquine-resistant lines of *P. berghei* (*32*) and *P. chabaudi* (Padua, personal communication, 1979) have also been obtained, but their genetics has not yet been investigated.

Competition between drug-sensitive and drug-resistant parasites in mixed populations

When vertebrate hosts are treated with a drug there is naturally a selection of drug-resistant parasites from mixtures of sensitive and resistant parasite populations. It is, however, interesting to ask what are the selective advantages or disadvantages of resistant parasites in the absence of drugs. Some preliminary experiments have been carried out to examine this question by setting up populations containing mixtures of sensitive and resistant parasites in various proportions, carrying out a series of passages in the absence of drugs, and determining the proportions of sensitive and resistant parasites that survive (*33*). With regard to low-level chloroquine resistance in *P. chabaudi*, a surprising result was obtained indicating that there was a selective advantage of resistant over sensitive parasites (Table 2). It should be stressed, however, that the numbers of clones tested were small, and further experiments of this type are needed. If chloroquine resistance is selectively favoured in *P. chabaudi*, it is difficult to understand why the wild strains of this species have not all become resistant. Presumably environmental conditions in nature somehow compensate for those producing a predominance of resistant parasites in the laboratory.

As regards pyrimethamine resistance, Rosario et al. set up a competition experiment starting with a population of *P. chabaudi* containing 50% resistant and 50% sensitive parasites. After 30 days in the blood of mice not exposed to pyrimethamine, 56 sensitive and 11 resistant clones were isolated. It is concluded that pyrimethamine resistance is neutral, or possibly selectively disadvantageous by comparison with sensitivity.

Table 2. Drug responses of clones derived from mixed infections of chloroquine-resistant and sensitive lines of *Plasmodium chabaudi* (after Rosario et al. (*33*))

Initial inoculum (10^6 blood forms)	Blood induced infections at 30 days		
	Total clones	Sensitive	Resistant
100% resistant	23	0	23
50% resistant 50% sensitive	9	0	9
90% resistant 10% sensitive	8	0	8
10% resistant 90% sensitive	4	0	4
100% sensitive	13	13	0

DISCUSSION

The results of genetic experiments with rodent *Plasmodium* species show that resistance to both pyrimethamine and chloroquine arises as a result of gene mutation and selection of resistant mutants under drug pressure. There are, however, some differences in the genetic mechanisms affecting resistance to the two drugs. With pyrimethamine, resistance to a high concentration of the drug may arise by a single step. Moreover, Bishop (*15*) reported that treatment even with a low dose could result in the appearance of mutants resistant to a high dose. With chloroquine, however, convincing evidence of single step mutations to a high level of resistance has so far not been presented, and a series of mutations, each having a small effect, is required. Hence, assuming that the situation in *P. falciparum* is similar to that in rodent and bird plasmodia, treatment with either low or high doses of pyrimethamine will result in selection of high-level resistant strains, but treatment with large doses of chloroquine should theoretically not yield highly resistant mutants.

Some of the genetic characteristics of pyrimethamine and chloroquine resistance can be explained, at least in part, by reference to the biochemical steps affected. In the case of pyrimethamine, it has been found that the enzyme dihydrofolate reductase (EC. 1.5.1.4) is altered in drug-resistant mutants. According to Ferone (*34*, *35*) the enzyme in resistant mutants has reduced substrate- and drug-binding properties, and is produced in larger amounts than in sensitive parasites. This would account for the increased resistance of mutants to a given dose of pyrimethamine, and also for the accompanying increase in sensitivity to sulfadiazine and in requirement for PABA. However, the class of mutants resistant to both pyrimethamine and sulfadiazine cannot be explained in this way. Further biochemical and genetic studies are required to clarify this situation.

At present we are also not certain of the cause of the extraordinarily wide range of concentrations of pyrimethamine tolerated by different strains of *P. falciparum*. For example, the author, in collaboration with S. Thaithong, has tested a strain of *P. falciparum* from Thailand with a resistance to pyrimethamine some 10 000 times greater than that of some other strains. Conceivably these differences may be explained on the basis of amplification of the gene locus concerned with production of dihydrofolate reductase, similar to that demonstrated in the somewhat analogous case of methotrexate resistance in cultured mouse cells (*36*). This hypothesis however requires experimental proof.

With regard to chloroquine resistance, nothing is known with certainty about the biochemical steps that are altered in resistant parasites, though it is noteworthy that the latter are known to have a decreased capacity to concentrate the drug (*37*). Since chloroquine is known to react with many different cellular constituents, it is likely that drug resistance is controlled by a number of unrelated metabolic processes, which are themselves controlled by different genes.

Finally, it is worth pointing out that the three classes of drug resistance in malaria parasites, exemplified by pyrimethamine, sulfadiazine, and chloroquine, illustrate three different types of mechanism by which drugs act selectively on a parasite in a host cell (in this case both parasite and host being eukaryotic organisms): (1) pyrimethamine acts on an enzyme (dihydrofolate reductase) that is essential for both host and parasite, but binding of the drug to the parasite enzyme is much stronger than to the host enzyme; (2) sulfadiazine and other sulfa drugs act on another enzyme (dihydropteroate synthetase (EC. 2.5.1.15)) which is thought to be essential for *Plasmodium*, but is absent from vertebrate cells (*35*); and (3) chloroquine acts more powerfully on the parasites than on the host cells because of the selective uptake of the drug in parasitized cells. The genetic peculiarities of drug resistance no doubt reflect these biochemical features of drug action. Further biochemical and genetic studies are needed to give a more complete understanding of these mechanisms, which would then help in combating the growing problem of increasing drug resistance of malaria parasites.

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RÉSUMÉ

GÉNÉTIQUE DE LA PHARMACORÉSISTANCE DES PARASITES DU PALUDISME

Le présent article passe en revue les données d'expérience disponibles sur la génétique de la résistance qu'opposent aux médicaments les parasites du paludisme. Plusieurs mécanismes pouvant être à l'origine de l'apparition de la résistance sont évoqués, parmi lesquels une mutation génique spontanée—qui peut se faire en une seule étape à l'égard de la pyriméthamine—est sans doute le plus important. L'auteur décrit les méthodes utilisées pour la production, parmi des espèces de *Plasmodium* des rongeurs, de souches résistantes à la pyriméthamine et à la chloroquine, et expose les constatations faites sur l'acquisition par la descendance de cette résistance. Les caractéristiques biochimiques du mode d'action des médicaments, qui peuvent jouer un rôle dans l'acquisition génétique de la pharmacorésistance, sont aussi examinées. L'auteur rapporte également les résultats d'études sur la compétition entre parasites sensibles aux médicaments et résistants dans des populations mixtes de plasmodies infectant les rongeurs, compétition qui peut s'exercer aussi bien sous la pression des médicaments qu'en l'absence de traitement. Les conclusions à tirer des résultats de ces études en ce qui concerne la résistance aux médicaments de *P. falciparum* sont discutées.

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