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Resistance of *Plasmodium falciparum*

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Introduction

Resistance of *Plasmodium falciparum* to antimalarial drugs is emerging as one of the two most important technical factors obstructing the worldwide effort to eradicate, or at least control the disease. The other major factor is entomological – the diminishing efficacy of residual insecticidal spraying of dwellings. There are two reasons for the diminishing impact of spraying on vector populations: physiological resistance of certain vectors to insecticides and strain differences or adaptive mechanisms in some anophelines resulting in the avoidance of sprayed surfaces.

Depending heavily on anti-vector techniques, the Malaria Eradication Programmes established in the late 1950s did not generally employ antimalarial drugs as attack measures, using them strategically instead for elimination of parasite reservoirs. In certain countries, however, drug administration alone may be the only method available for any degree of malaria control.

Antimalarial drug action

In a discussion of the development of parasite resistance to chemotherapeutic agents, it may be useful to review the sites at which drugs act to interrupt the life cycle of plasmodia. Antimalarial drugs may be divided into the following categories:

1) causal prophylactic agents (acting against primary tissue stages);
2) anti-relapse drugs (acting against latent tissue stages);
3) blood schizontocides;
4) gametocytocides;
5) sporontocides.

Drugs to which activity against primary tissue (liver) stages of the parasite has been attributed include the antifolates proguanil and pyrimethamine, possibly the sulfas, tetracycline (although adequate field testing of this application is far from complete), and primaquine, although it is thought that doses of this drug required for prophylactic activity would be toxic.

Primaquine and some of its more toxic analogues are the only available agents which act against the latent tissue stages (hypnozoites) of the relapsing malarias, *P. vivax* and *P. ovale*.

The list of blood schizontocides is headed by the oldest of western antimalarials–quinine. The 4-aminoquinolines chloroquine and amodiaquine also act against erythrocytic schizonts, as does the obsolete agent mepacrine. Mefloquine, the most promising of the new drugs, acts exclusively as a blood schizontocide. Pyrimethamine and proguanil are both active, but slowly, against blood schizonts. The sulfas are only moderately active when given alone but highly effective when administered in synergistic combination with the antifolate pyrimethamine. Primaquine also has schizontocidal activity, but only at toxic doses.

The gametocytes of *P. vivax, P. malariae,* and presumably *P. ovale,* are killed by usual doses of the effective blood schizontocides, but mature gametocytes of *P. falciparum* are not affected. Primaquine, which kills gametocytes of all species, is particularly useful for interrupting transmission of *P. falciparum*.

Primaquine, as well as pyrimethamine and proguanil (against susceptible strains), is known to prevent development of oocysts and sporozoites in the vector mosquito.

Mechanisms of drug action

Surprisingly little is known with certainty about the mechanisms of antimalarial drug activity. Research in this area has been especially difficult due to the obligate intracellular nature of the parasites, and, until recent years, the inability to work in vitro or in animal models. However, a large body of information is now being accumulated on the distribution of various substances in the life stages of the plasmodia, parasite nutritional requirements, and the metabolic pathways utilized.

It is known that nucleic acid metabolism in plasmodia is similar to that in other eukaryotic organisms, and that both DNA and RNA are synthesized during nuclear growth and division. It is clear that antifols and sulfonamides exert their antimalarial action through inhibition of parasite enzymes involved in the synthesis of folate cofactors. In experimental avian malaria, nuclear division is seen to be interrupted at metaphase. The antifols (such as pyrimethamine) act by binding dihydrofolate reductase and the sulfonamides by inhibiting dihydropteroate synthetase. Potentiation occurs when the two classes of drugs are used simultaneously.

Important information is being collected concerning the mode of action of the 4-aminoquinolines. It is known that a number of blood schizontocides, including 4-aminoquinolines, mepacrine, quinoline methanols such as mefloquine, and phenanthrene methanols are concentrated in infected red cells through a saturable, energy-dependent process involving high-affinity binding sites. Red cells harboring chloroquine-resistant parasites concentrate this drug to a much lesser extent obviously due to a deficiency in the binding mechanism[14].

Fitch and his coworkers, in work of major importance[13], have identified a product of hemoglobin degradation, ferriprotoporphyrin IX (FP), as a high-affinity receptor for chloroquine. It is hypothesized that chloroquine acts by diverting FP from non-toxic complexes with haem binders to a toxic chloroquine-FP complex, resulting in membrane damage, ion-gradient alteration,

and eventual cell lysis. These investigators theorize that resistant parasites may contain haem binders which preferentially bind FP either because of increased affinity or amount of these binders, or that FP sequestration in pigment may be increased in resistant parasites. This work appears to be corroborated by the results of Jearnpipatkul[19] who also demonstrated binding of quinacrine and mefloquine as well as chloroquine to haemozoin and protoporphyrin IX. Yuthavong[42] has demonstrated a difference in the distribution of binding sites between infected and uninfected cells and showed that most of the binding occurring in infected cells takes place in the parasites, further supporting this hypothesis for the mechanism of drug effect.

The possibility that 4-aminoquinolines (and quinine) intercalate with parasite DNA is still disputed, although, in any case, this is not considered to be a major factor in their parasitocidal action.

Possible mechanisms for the antimalarial activity of antibiotics are being explored. A recent report[15] suggests that tetracycline and erythromycin inhibit *P. falciparum* through actions on mitochondrial synthesis or function. These workers also provide supporting information for the clinically observed slowness of action of tetracycline in falciparum infections. In their in vitro system, parasites exposed to tetracyclines (or to erythromycin) for 48 h were killed by the drug only at concentrations somewhat higher than those observed therapeutically. When exposure was extended to 96 h, lethal effects were observed at concentrations within the therapeutic range.

History and current status of resistance

The earliest reports of antimalarial drug resistance came from Brazil in 1910[23], when failures of quinine were reported. Whether this situation reflected naturally low levels of sensitivity of the local parasite population to this drug or true resistance selected by large scale suppressive use is unclear. It is known that strains of *P. falciparum* encountered in Southeast Asia, the Pacific, and the Americas are by nature relatively less susceptible to quinine than strains found in India and westward including the African continent. On the other hand, experimental evidence indicates that resistance can be selected through serial passage of falciparum isolates under drug pressure. Solid evidence of clinical refractoriness to quinine is accumulting from Thailand, where usual 7–10-day courses of quinine produce low rates of radical cure of infections acquired at the Kampuchean border[29]. Studies of malaria in Thai children also indicate changing patterns of susceptibility[5]. The Thai antimalaria program is documenting evidence of increasing resistance using in vitro test systems of Rieckmann (Laksami Suebsaeng, personal communication). Although quinine, at least in Thailand, is gradually losing its ability to provide radical cure of falciparum infections, it is still a dependable agent for producing relatively rapid initial reduction in parasitemia, retaining its life-saving value in cerebral and other complicated or severe manifestations of falciparum malaria.

The earliest of the synthetic blood schizontocides, Atebrin (now known as mepacrine or quinacrine), was developed in the early 1930s. This is a toxic drug, and is now obsolete, having been replaced by a related series of compounds, the 4-aminoquinolines, particularly chloroquine and amodiaquine. Although chloroquine was initially synthesized in Germany in 1934, its importance was not fully appreciated until 10 years later when clinical studies proved it to be an excellent antimalarial. Amodiaquine was shown to have similar efficacy and tolerance.

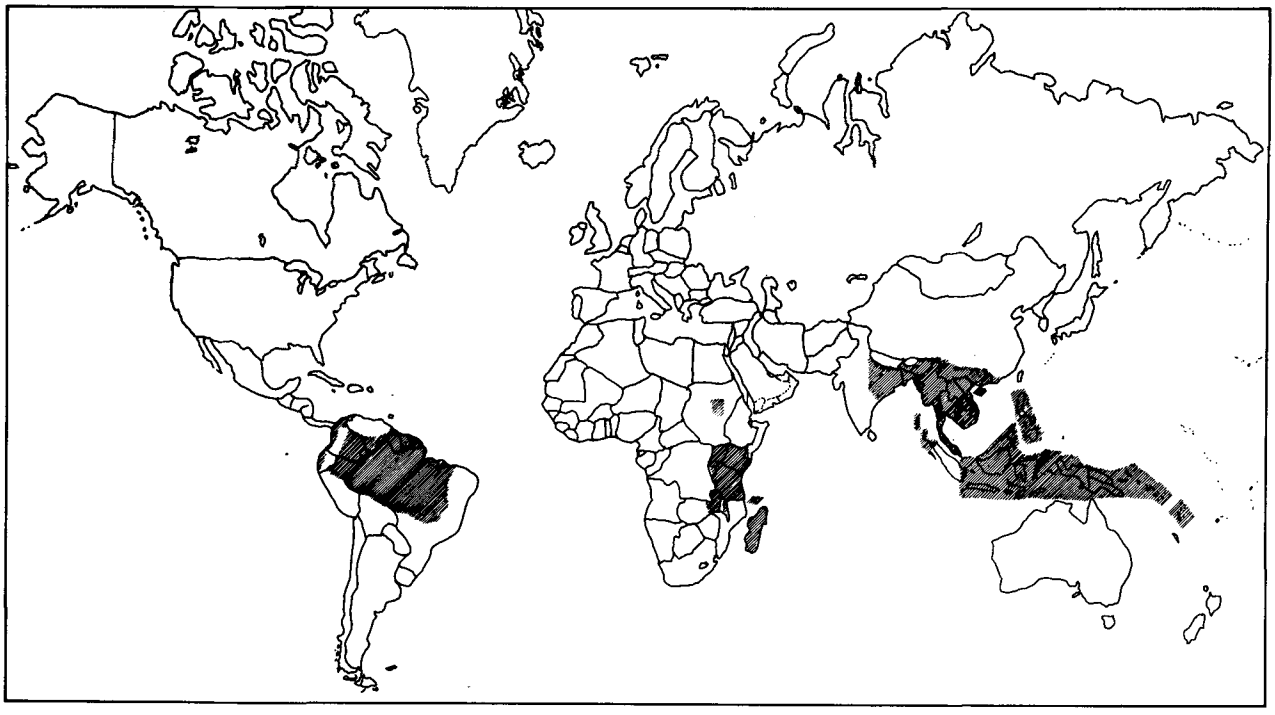
Chloroquine became the mainstay of antimalarial chemotherapy, and is still the most widely used schizontocidal drug. However, in the late 1950s, clinical treatment failures were noted in Thailand[17]. At about the same time, two reports from South America[21,41] indicated resistance in falciparum infections acquired in Colombia. Since that time, resistance has spread widely to involve most malaria-endemic regions of the world (fig.). In Asia, the most seriously-affected regions include Thailand, Burma, Vietnam, Kampuchea, Laos, and southern China. Also involved are India (originally limited to the east coast, but now widely reported), Bangladesh, Malaysia, Indonesia, the Philippines, Papua-New Guinea, the Solomon Islands and Vanuatu (formerly New Hebrides).

In most areas of South America where transmission of *P. falciparum* continues, resistance to chloroquine is prevalent, although the problem has not increased in severity or distribution to the extent seen in Asia during the same period of time. Especially affected are parts of Brazil, Colombia, French Guiana, Guyana, and Surinam. Resistance is present but focal in Bolivia, Equador, Venezuela and southern Panama.

In Africa, resistance to the 4-aminoquinolines has been well documented in cases acquired in Kenya, Tanzania, the Comoro Islands, Uganda, Madagascar, the Sudan, Zambia, and Malawi. There are reports of cases, though not fully confirmed, from Rwanda, Burundi, Ethiopia, Somalia, Nigeria, and Zaire. Although most cases have been reported in non-immune travellers to the continent, resistance has now been detected in presumably semi-immune indigenous populations in Tanzania and Zanzibar[20,24,34].

The antifolate drugs proguanil and pyrimethamine were developed in 1945 and 1951, respectively. At the end of the Second World War, proguanil was widely used as an effective and well-tolerated causal prophylactic drug, although it was never a potent therapeutic agent. The development of pyrimethamine was considered to be an important advance, since it was more potent than proguanil, had a longer half-life in man and a wide margin of safety between effective and toxic doses. However, resistance to pyrimethamine was found to develop extremely rapidly – within a year or two of its introduction – and crossresistance between proguanil and pyrimethamine was demonstrated[1]. Currently, resistance to pyrimethamine by both *P. falciparum* and *P. vivax* is widely distributed throughout nearly all endemic regions. There is an apparent association with prior application of mass drug distribution techniques (e.g. medicated salt).

An unresolved question concerns the efficacy of antifolates as causal prophylactic agents in the face of resistance of asexual blood forms. It has been assumed



Distribution of chloroquine-resistant *P. falciparum*.

on very little evidence that these drugs would be ineffective in preventing falciparum malaria in areas where blood forms are known to be resistant. However, an early study from Malaya[40] indicated that proguanil remained useful in prophylaxis despite erythrocytic schizont resistance: other studies have appeared to corroborate this finding[10]. There is a need for a well-controlled field evaluation of the prophylactic value of proguanil in an area of falciparum resistance to the schizontocidal action of antifolates.

The synergistic combination of pyrimethamine with various sulfonamides and sulfones became important as chloroquine resistance spread. Beginning in the 1960s, several such compounds were tried, the most important of which was pyrimethamine-sulfadoxine. The two drugs in this combination have similar half-lives in man, and were demonstrated to be highly effective, combined in a single dose, against chloroquine-resistant malaria[12]. However, repeating the pattern set by chloroquine, by the mid-1970s, resistance to the pyrimethamine-sulfadoxine combination was observed at the Thai-Kampuchean border. By the end of the decade, resistance was well established in much of Thailand, both in Kampuchean refugees and the Thai population[18,27], in Burma[36], in Vietnam, as well as in Columbia and Brazil.

A short course of quinine given in association with a dose of sulfadoxine-pyrimethamine provided a remarkably high cure rate and fast control of parasitemia and symptoms in Thailand in 1973–1974[16]. Currently in Thailand, this association is much less successful, and there is apparently cross-resistance between the antifol-sulfa combinations pyrimethamine-sulfadoxine and trimethoprim-sulfamethoxazole (table).

The regimen which has replaced sulfadoxine-pyrimethamine in areas where resistance to this combination is problematic includes tetracycline, a highly effective but slow blood schizontocide, in association with a short course of quinine. Early work establishing the efficacy of the tetracyclines given for 7 (but not for 5) days was provided by Clyde et al.[6]. These investigators also stressed the fact that parasite densities generally

Quinine association for treatment of *P. falciparum* in Thailand

Date	Reference	No. doses quinine	Drug given in association	No. patients	Radical cure rate	Comments
1972	Colwell et al.[9]	9	Tetracycline 10 days	59	98%	
1972	Colwell et al.[7]	3	Tetracycline 7 days	32	84%	Late recrudescences (may have been reinfections)
1973	Colwell et al.[8]	3	Cotrimoxazole 5 days	31	81%	
1975	Hall et al.[16]	1–4	Sulfadoxine-pyrimethamine	89	98%	
1982	Pinichpongse et al.[27]	9	Tetracycline 7 days	90	95%	
1982	Doberstyn (unpublished)	3	Sulfadoxine-pyrimethamine	28	54%	
1982	Doberstyn (unpublished)	3	Cotrimoxazole	29	41%	
1982	Doberstyn (unpublished)	9	Cotrimoxazole	27	67%	
1982	Doberstyn (unpublished)	3	Tetracycline 7 days	30	97%	Slow parasite clearance

increase during the first three days of therapy with tetracycline alone, declining abruptly thereafter. Quinine is a reliable agent for initial reduction of parasitemia and control of symptoms, and the tetracycline component of the regimen can generally be depended upon to eliminate the infection. 9 doses (3 days) of quinine given together with a 7–10-day course of tetracycline usually produces radical cure[29,27], and is well tolerated by patients. However, in a certain group of patients treated at the Hospital for Tropical Diseases in Bangkok, who had acquired their infections in Kampuchea, the cure rate for this regimen has fallen to 57%, raising the possibility that resistance to tetracycline is also being selected in that area (Danai Bunnag, personal communication). In this patient population, extending the quinine course to 7 days results in nearly 100% cure, however.

The combined course of quinine and tetracycline, though generally effective, is difficult to supervise, and because of the side effects of quinine, compliance in outpatients is poor. This prevalence of partial treatment may help to explain the apparent emergence of resistance in the area concerned.

Of the new antimalarial drugs, the most promising potential replacement for sulfadoxine-pyrimethamine and quinine-tetracycline is mefloquine, a 4-quinoline methanol developed by the U.S. Army Antimalarial Drug Development Program with further development supported by the World Health Organization. A single 750-mg dose of this drug has been shown to cure over 95% of patients treated. The reported failures have generally been of the 'R I' late recrudescence type, although two cases of 'R II' type failures have been reported (persistence of diminished parasitemia). One of these may have been the result of inadequate dosing due to vomiting, and the other may have been related to the host factor of unusually fast metabolism[3]. In a chloroquine resistant strain of rodent malaria, mefloquine resistance has been seen to begin after only one passage under drug pressure[25], although it has been extremely difficult in vitro to produce resistant *P. falciparum*. An extraordinarily well-documented human infection demonstrating increasing resistance in a recrudescing infection has recently been reported[4]. In order to delay the selection of parasites resistant to mefloquine, this drug will most likely be used operationally in combination with sulfadoxine-pyrimethamine.

Genetics of resistance

There is little direct information about the genetics of resistance of human malaria parasites because of the inability to carry out direct experimentation. However, a great deal may be inferred from the relative wealth of information obtained from the study of *P. berghei* and other rodent malarias using the classical methods of hybridization and analysis of progeny. Much of the work on this subject has been carried out at the Institute of Animal Genetics in Edinburgh.

Beale[2] suggested seven possible mechanisms which could account for adaptive changes in plasmodia, allowing them to develop in the presence of antimalarial drugs:

1) physiologic adaptations, non-genetic and probably temporary:
2) the selection, from a mixed population of sensitive and resistant organisms, of a resistant line, under the influence of drug pressure;
3) spontaneous nuclear mutations followed by selection of resistant mutants;
4) gene mutation caused by the action of mutagenic drugs;
5) extranuclear gene mutation (e.g., in mitochondria);
6) changes in gene expression caused by alterations in cytoplasmic or environmental factors; and
7) resistance-transfer factors or other plasmids.

The most likely mechanism is spontaneous mutation and selection, although, as Beale points out, others are also possible. For example, it is known from enzyme marker studies that many infections consist of a mixture of diverse organisms and, therefore, selection of pre-existing resistant cells under drug pressure may occur in the field. Extremely important work currently underway in Thailand lends added support to this alternative. Thaithong[35] has demonstrated that two isolates of *P. falciparum* from Thailand, which were shown to be resistant to chloroquine, actually contained parasite clones of varying susceptibility, ranging from resistance to complete sensitivity to the drug.

R-factors have not been demonstrated in plasmodia, and it is likely that if such a mechanism were active, the spread of resistance would have been much faster than has been observed.

Factors influencing the spread of resistance in the field

Geographical spread of chloroquine resistance is favored by the apparent biological advantage of resistant parasites over sensitive strains. Evidence of this situation was first obtained when it was observed that statistically larger numbers of oocysts developed when the vector, *An. stephensi,* had fed on chloroquine-resistant *P. berghei*-infected mice which had received chloroquine, when compared with untreated mice[28]. This finding was confirmed in humans when *An. balabacensis* s.l. were fed on patients with naturally-acquired chloroquine-resistant infections[39]. Further confirmation of the advantage of resistant over sensitive parasites was supplied by Rosario et al.[33], when it was shown that in a mixture of chloroquine-sensitive and chloroquine-resistant lines of *P. chabaudi,* the resistant parasites overgrew the sensitive ones. Work with cloned parasites[35] showed that a chloroquine-sensitive clone grew at a slower rate than did resistant clones. Peters[25] suggests that the large-scale use of chloroquine in resistant infections may preferentially enhance the dissemination of such parasites.

Unlike the situation with chloroqine, resistance to pyrimethamine does not appear to confer biological advantage. Although resistant parasites become predominant over susceptible strains during the period of drug pressure, they apparently subside gradually and become submerged in the susceptible population when pressure is removed. This finding has been confirmed in experimental malaria by Rosario et al.[33]. Even in the absence of operational use of pyrimethamine, however, anti-

folate drug pressure can continue in malaria-endemic areas through large-scale use of antibacterials, e.g. trimethoprimsulfamethoxazole, which belong to the same class of drugs.

Transmission patterns and the immune status of the population also undoubtedly affect the speed with which resistance becomes established. In much of Southeast Asia, infection is often sporadic, related to occupational exposure, particularly to forest vectors. Such patterns are not likely to result in high levels of immunity, especially in children. In endemic areas of Africa, on the other hand, village transmission is the rule, and individuals surviving childhood (approximately a million children die yearly in the attempt) may be presumed to have a high degree of acquired immunity. When a resistant strain is introduced, either through mutation and selection, or through importation, it may be expected to advance more slowly through a highly immune population, in the absence of drug pressure. This may also help to explain the difference between the speed of the spread of antifol resistance (a large proportion of the parasite population adapts even after a single exposure) and the late appearance and relatively slow spread of chloroquine resistance.

Techniques for the recognition and monitoring of resistance

The global collaborative studies for the assessment and monitoring of the drug response of malaria parasites sponsored by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR) are providing a great deal of information about the appearance and progression of 4-aminoquinoline resistance. This program has been reviewed by Wernsdorfer and Kouznetzov[37]. Initiated using the Rieckmann 'macro' technique[30], the collaborative studies are now primarily advocating use of the 'micro' system[31]. Both of these systems are based upon observation of the degree of development of falciparum ring forms to the schizont stage in short-term in vitro culture in the presence of increasing concentrations of antimalarial drug. Aside from chloroquine, the techniques may be used for determination of susceptibility to amodiaquine, mefloquine, and quinine.

A modification of the microtechnique has been developed[32] which permits direct visual interpretation of the susceptibility of the isolate based on the precipitation of pigment from developing schizonts. This technique has been proven effective in cultured parasite lines, but it remains to be seen whether this system can be applied in the field, using specimens from naturally-acquired infections.

In the face of the development of resistance to antifol-sulfa combinations, the need for an in vitro system for the assessment of susceptibility to this combination is acute. A technique developed by Nguyen-Dinh and Payne[22] is useful for the determination of pyrimethamine susceptibility, but standardization of a system for the testing of sulfas and the pyrimethamine-sulfa combinations in the field is still required. Following a recent meeting of investigators interested in this issue, there is reason to be optimistic.

Desjardins and coworkers[11] described a sophisticated technique for the rapid screening of antimalarial drugs in the laboratory. This is an automated, computerized system based on the Trager-Jensen technique of in vitro cultivation, in which the incorporation of a radiolabelled essential amino acid (tritiated hypoxanthine) into parasitized blood cells is measured in relation to the presence or absence of drug. Although specimens collected in the field for use in this technique are transportable, a certain minimum parasitemia is required and a strong central laboratory capable of supporting continuous culture is necessary. A further disadvantage exists in that culture adaptation may modify characteristics of the original isolate.

In vitro techniques are particularly useful for the determination of the average drug susceptibility of the general population of parasites in an area, and for monitoring changes over time. In vitro test results do not require the follow-up of patients, are not affected by compliance with drug regimens, and are not significantly influenced by the immune status of the host. However, for the country-based planning of antimalarial drug policy, in vivo tests are generally preferred. Field trials are normally conducted according to the procedure originally proposed by the World Health Organization for the evaluation of chloroquine response[43]. Two tests are commonly used, requiring either 7 or 28 days of follow-up. These systems have some shortcomings and are generally modified in the field. Obviously, the 7-day test is not able to detect delayed parasite recrudescence and is, therefore, of very limited value in areas where resistance is only beginning to appear. For drugs with long metabolic half-lives, e.g. pyrimethamine-sulfadoxine and mefloquine, a suppressive blood level may persist beyond the 28-day limit, and for use with such drugs, the follow-up period should be extended to a total of at least 6 weeks.

Research required

Research priorities in the control of drug-resistant falciparum malaria have recently been reviewed[38]. A list of topics for research leading to a better understanding of practical chemotherapy and drug resistance might include the following:

1) Clarification – through continued elucidation of parasite metabolic processes – of mechanisms of drug action, aimed at the rational development of new antimalarial agents. The empirical approach, whereby literally hundreds of thousands of compounds have been screened for antimalarial activity has been productive but prohibitively expensive.

2) More information must be gathered on the clinical pharmacology, metabolism, and pharmacokinetics of the standard antimalarial drugs including quinine, chloroquine, amodiaquine, the antifols and sulfas, particularly in infected patients.

3) Genetic experimentation with malaria parasites, both in vivo using animal models and in vitro using human parasites in culture, should be expanded. Questions of the stability of resistance and the modes of inheritance need to be clarified further.

4) Further development and simplification of field in vitro techniques for resistance screening is of very high priority. Adaptation of the Rieckmann systems for easier use by field staff and the development of a standard field technique for the study of pyrimethamine and sulfonamide susceptibility are important requirements.
5) Socio-economic factors relating to the spread of resistant parasites, particularly the influence of population migration, require study. A more thorough understanding of patient attitudes towards illness and factors leading to better compliance with drug regimens will assist in limiting the extension of resistance.
6) Characterization of parasite strains using, for example, biological markers including determination of their drug sensitivity patterns may be useful epidemiologically in predicting and monitoring resistance in the field. Further development of cloning technology will refine this work.
7) The possibility both that chloroquine use may actually enhance the transmission of chloroquine-resistant parasites through enhanced sporogony, and that sulfonamides may increase transmission because of their stimulation of gametocyte production, should be further studied.
8) Individual and possible ethnic differences in regard to the activity of antimalerial drugs should be further investigated. It is will documented that repeated doses of primaquine may be dangerous in patients with G-6-PD deficiency. The prevalence and variants of this enzyme deficiency should be determined in populations requiring therapy for *P. vivax* infections. It has been further suggested that certain races, e.g. Thais, may metabolize this drug faster, than others, accounting for the high failure rate of standard courses of *P. vivax* antirelapse therapy.
9) Information should be gathered on the influence of administrative drug policy on the spread of resistance. Factors which should be considered both retrospectively and prospectively, include drug types and formulations, sources, cost, methods of distribution, and governmental control policies.

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