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Antimalarial drug resistance and combination chemotherapy

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Antimarial drug resistance develops when spontaneously occurring parasite mutants with reduced susceptibility are selected, and are then transmitted. Drugs for which a single point mutation confers a marked reduction in susceptibility are particularly vulnerable. Low clearance and a shallow concentration–effect relationship increase the chance of selection. Use of combinations of antimalarials that do not share the same resistance mechanisms will reduce the chance of selection because the chance of a resistant mutant surviving is the product of the per parasite mutation rates for the individual drugs, multiplied by the number of parasites in an infection that are exposed to the drugs. Artemisinin derivatives are particularly effective combination partners because (i) they are very active antimalarials, producing up to 10 000-fold reductions in parasite biomass per asexual cycle; (ii) they reduce malaria transmissibility; and (iii) no resistance to these drugs has been reported yet. There are good arguments for no longer using antimalarial drugs alone in treatment, and instead always using a combination with artemisinin or one of its derivatives.

Keywords: antimalarials; resistance; combinations; artemisinin

1. INTRODUCTION

Malaria, the most important parasitic infection of man, affects *ca.* 5% of the world's population. In tropical countries, malaria is one of the most common causes of fever, and kills somewhere between 0.5 and 2.5 million people each year. The widespread availability of cheap and effective antimalarial drugs, particularly chloroquine and pyrimethamine–sulphadoxine, has undoubtedly limited both morbidity and mortality, but it has also encouraged the development and spread of resistance. This is a catastrophe for poor tropical countries, which cannot afford more expensive alternative antimalarial drugs. Mortality is already rising (Trape *et al*. 1998). This paper reviews current understanding and explores the possibility that the use of drug combinations in antimalarial chemotherapy can prevent or delay the development of drug resistance.

Antimalarial drug resistance usually arises when spontaneously arising mutants are selected by antimalarial drug concentrations that provide differential inhibition to distinct genetic parasite types: i.e. the drug concentrations are sufficient to reduce the susceptible parasite population, but inhibit less or do not inhibit multiplication of the mutants (Peters 1990). Antimalarial drug resistance is usually a result either of changes in drug accumulation or efflux (chloroquine, amodiaquine, quinine, mefloquine, halofantrine resistance) or reduced affinity of the drug target resulting from point mutations in the respective genes encoding the target (pyrimethamine, cycloguanil, sulphonamide, atovoquone resistance) (Ward *et al*. 1995; Foote & Cowman 1994). Other mechanisms of drug resistance found in bacteria such as transferable resistance genes, the production of drug-destroying enzymes, or the activation of accessory metabolic pathways, do not appear to be involved in antimalarial resistance.

2. DRUG RESISTANCE

(a) *Chloroquine resistance*

Chloroquine is still the most widely used antimalarial drug in the world. Between 200 and 400 million doses are taken annually. It is still the drug of choice for nearly all *Plasmodium vivax*, *P. malariae*, and *P. ovale* malaria, and it is still used extensively to treat falciparum malaria despite widespread resistance. Resistance in *P. falciparum* developed independently in South-East Asia and South America in the late 1950s, and was first reported in *P. vivax* ten years ago in Papua New Guinea (Baird *et al*. 1991; White 1992*a*; Rieckmann *et al*. 1989). In western Cambodia (one of the two original foci), resistance first to pyrimethamine, and then to chloroquine may have resulted from the general distribution of sea salt impregnated with the drugs as a malaria control measure.

Chloroquine resistance results from a reduced parasite accumulation of the drug, although the precise molecular mechanisms responsible have not been elucidated fully. The intra-erythrocytic malaria parasite consumes haemoglobin, detoxifying haem by polymerization to haemozoin or malaria pigment. This process is inhibited by chloroquine and related arylamino alcohols (Dorn *et al*. 1995; Slater 1993). Resistance to chloroquine is

associated with reduced concentrations of the drug in the parasites' food vacuole. Both reduced ingress and increased efflux have been reported, although the current balance of evidence favours reduced accumulation. *In vitro* chloroquine resistance can be reversed by a number of different drugs that interfere with the accumulation–efflux mechanism. Resistance can be induced experimentally by repeated exposure of parasite populations to sub-inhibitory concentrations of the drug (Peters 1990). Chloroquine resistance is difficult to induce experimentally, although once reduced sensitivity has been obtained further reductions in sensitivity (i.e. increases in resistance) are relatively easy to induce. It has been thought that choroquine resistance is a multigenic process (Rosario *et al*. 1978). Natural resistance is associated with polymorphisms in a 36 kb segment of the parasite's chromosome 7. This contains *cg*2, a polymorphic gene encoding a unique 330 kDa protein, which may have a transporter function (Su *et al*. 1998). Chloroquine resistance appears to have spread from its two original foci as an expanding pandemic, which now involves most of the malarious tropics. Amodiaquine, the biologically active metabolite desethylamodiaquine, and the related compound amopyraquine are more active than chloroquine against chloroquine-resistant *P. falciparum*, although there is cross-resistance (Olliaro *et al*. 1996), and amodiaquine is not effective clinically against highly chloroquine-resistant parasites.

(b) *Mefloquine resistance*

Mefloquine is used for the oral treatment of uncomplicated multi-drug-resistant falciparum malaria. It is being prescribed increasingly in South-East Asia and some parts of South America. Mefloquine resistance is more easy than chloroquine resistance to induce experimentally. The multi-drug-resistant parasites prevalent in South-East Asia show reduced sensitivity to all the aryl aminoalcohols and also to other structurally unrelated drugs (Rathod *et al*. 1997). Mefloquine resistance correlates well with resistance to halofantrine and quinine, but within an area there is generally an inverse relationship between mefloquine resistance and chloroquine resistance (Wernsdorfer *et al*. 1994). Similar reciprocity occurs in laboratory isolates selected for mefloquine resisitance (Peel *et al*. 1993). Mefloquine resistance is often associated with mutations in or amplification of the ATP-dependent P-glycoprotein pump homologue, encoded by the *MDR* gene family (Wilson *et al*. 1993; Cowman *et al*. 1994). The *MDR* genes are part of the gene superfamily encoding ATP-binding structures. Resistance to mefloquine has developed relatively rapidly in comparison with chloroquine. For example, in Thailand, the country where mefloquine was first deployed, significant resistance emerged within six years of its deployment despite careful restriction of use only to slide-positive falciparum malaria (Nosten *et al*. 1991).

(c) *Resistance to the dihydrofolate reductase and dihydropteroate synthase inhibitors*

These drugs are not now used alone to treat malaria, although proguanil is still used in antimalarial prophylaxis. Combinations of pyrimethamine with sulphadoxine or sulphalene, or of chlorproguanil with dapsone are used to treat chloroquine-resistant falciparum malaria (Watkins *et al*. 1988). Proguanil is an oral pro-drug for the active triazine metabolite cycloguanil, and chlorproguanil is the corresponding precursor of chlorcycloguanil. Pyrimethamine, trimethoprim, cycloguanil, and chlorcycloguanil are all competitive inhibitors of plasmodial dihydrofolate reductase (DHFR). The sulphonamides and sulphones inhibit dihydropteroate synthase (DHPS) (Wang *et al*. 1997). Combinations of the two classes therefore provide sequential inhibition of folate biosynthesis, and show marked synergy in antimalarial activity. This synergy is very important for the efficacy of the drug. Following a standard dose, plasma concentrations provide effective antimalarial synergy against fully sensitive *P. falciparum* for about 50 days (W. Watkins, personal communication). Resistance to these drugs is easy to induce experimentally and develops relatively rapidly in wild parasites. Within four years of their initial deployment as single agents, high levels of resistance were documented in both *P. falciparum* and *P. vivax*. Resistance is caused by single point mutations in the genes encoding the target enzymes. For the DHFR inhibitors the initial mutation conferring resistance is usually at position 108 (SER-ASN) in the gene encoding DHFR (Peterson *et al*. 1988). This confers pyrimethamine resistance, but only slightly reduced sensitivity to cycloguanil (Foote & Cowman 1994; Foote *et al*. 1990). Conversely, a serine to threonine mutation at position 108, plus an alanine to valine mutation at position 16 confers cycloguanil but not pyrimethamine resistance. Additional mutations confer further reductions in susceptibility, usually to both drugs in parallel. Parasites with multiple mutations at positions 51, 59, and 108 are relatively resistant to pyrimethamine–sulphadoxine, but can still be treated with this drug as the plasma concentrations achieved in most patients are still in the range giving antimalarial synergy (Watkins *et al*. 1997). Such parasites are now increasingly prevalent in East Africa (W. Watkins, personal communication). Acquisition of a mutation at position 164 (found in many South-East Asian isolates) reduces sensitivity such that antimalarial synergy cannot be obtained at current doses, and the infections are highly resistant *in vivo* to pyrimethamine and cycloguanil, alone or in combination. Resistant *P. falciparum* often contain mutations in the genes encoding both DHFR and DHPS (Reeder *et al*. 1996). Point mutations in DHPS confer variable reductions in susceptibility to sulphas, although their precise contribution to synergy and thus pyrimethamine–sulpha resistance remains to be characterized (Curtis *et al*. 1998; Brooks *et al*. 1994). The different effects of the individual mutations on susceptibility, and the important role of synergy, result in a wide spectrum of overall susceptibility to the combination drugs.

(d) *Atovaquone resistance*

The hydroxynaphthaquinone atovaquone is a newly introduced antimalarial with a unique mode of action inhibiting cytochrome electron transport. Atovaquone is not used alone for the treatment of malaria; it is formulated in a fixed combination with proguanil. The two drugs are synergistic (Canfield *et al*. 1995). Interestingly, proguanil is not acting in this combination as a pro-drug for the DHFR inhibitor cycloguanil. It is the parent compound that synergizes, through an as yet undefined

mechanism of action. This is important as 3–5% of Africans and Caucasians, but 13–23% of Orientals, cannot convert proguanil to cycloguanil adequately because they possess a polymorphism in the gene encoding cytochrome P_{450} 2C19 (the main metabolizing enzyme). Thus, atovaquone–proguanil is highly effective in all populations, although it is not yet widely available. Unfortunately, single point mutations in the cytochrome *b* gene of *P. falciparum* confer a marked reduction in susceptibility to atovaquone. Resistance is easy to induce experimentally and develops rapidly if the drug is used alone. Although the combination with proguanil reduces the chance that such mutants will survive, this protection is limited because proguanil itself is intrinsically a very weak antimalarial.

(e) *Artemisinin resistance*

Artemisinin and its derivatives are the most rapidly acting of all antimalarials. They are sesquiterpene lactone peroxides derived from the plant *Artemesia annua*, and they are effective even against multi-drug-resistant falciparum malaria. These drugs are being used increasingly throughout the tropics. The artemisinin compounds act by the haem-catalysed intraparasitic production of highly reactive carbon-centred free radicals (Meshnick *et al*. 1996; Kamchonwongpaisan *et al*. 1996). Certain haemoglobinopathies are associated with reduced antimalarial activity of artemisinin, possibly because of reduced intraerythrocytic availability of iron to catalyse opening of the peroxide bridge. Stable resistance to artemisinin or its derivatives has not occurred in clinical practice, and cannot yet be induced in the laboratory. Parasites reported as showing reduced susceptibility may simply represent the tail-end of a normal distribution of drug susceptibility (Gay *et al*. 1994)—and remain well within the range of plasma concentrations obtained with clinical use of the drugs. It would be unwise to consider that resistance to these compounds cannot occur, although the information available to date is reassuring, and suggests that resistance will not emerge rapidly with appropriate prescribing in clinical practice. But this too depends on drug availability and use outside the medical system; for example, in some countries, misguided entrepreneurs have incorporated extracts of *A. annua* in herbal tonics and confectionery, providing a generally available and thus potentially powerful selective pressure for the emergence of resistance.

(f) *Global situation*

Currently, chloroquine-resistant *P. falciparum* are prevalent in most of the tropics, and in many areas resistance is high grade (i.e. potentially dangerous, with early treatment failures occurring). The situation is particularly serious in Africa where chloroquine is still the first-line treatment in most of the continent. Amodiaquine is used in some areas with low-grade chloroquine resistance as it is more effective. *P. vivax* has also developed significant resistance to chloroquine in some parts of South-east Asia and Oceania. *P. falciparum* resistance to pyrimethamine–sulphadoxine is widespread in East Asia and South America, and is increasing in Africa. Reduced sensitivity to quinine has been noted for many years, but there is no well-documented high-grade resistance. Mefloquine has been deployed in Thailand since 1984. Resistance was noted first in 1990, and since then, on the Western and Eastern borders, there has been a rapid decline in sensitivity. The artemisinin derivatives have been used extensively in Vietnam and Thailand over the past five years without change in the clinical or parasitological responses.

(g) *Assessment of drug responses*

Antimalarial drug susceptibility is assessed *in vivo* from rates of clinical response; coma recovery in cerebral malaria, resolution of vital organ dysfunction, fever clearance in uncomplicated malaria metabolic changes (e.g. resolution of acid–base disturbance, recovery of renal function) or parasite clearance, or from cure rates in uncomplicated malaria (White & Krishna 1989). *In vitro*, the ability of antimalarial drugs to inhibit the uptake of amino acids, labelled purines, the production of lactate or parasite-specific lactate dehydrogenase, can all be assayed, or growth inhibition can be assessed morphologically. The concentration–effect relationship is characteristically sigmoid in shape, and drug activity is usually expressed as the concentration required to produce 50%, 90% or 99% inhibition. A right shift in the sigmoid curve or a reduction in the maximum effect (E_{max}) reflects resistance. Point mutations in the genes encoding target enzymes may result in abrupt changes with very large changes in susceptibility, whereas changes in uptake or efflux mechanisms give gradual increases in resistance.

In vivo resistance manifests itself initially as an increase in the rate of recrudescence (reappearance of the parasites that caused the initial infection) in non-immunes with uncomplicated malaria. In endemic areas this manifests first in young children, who have less 'immunity' and often greater parasite burdens, and to a lesser degree in pregnant women. As resistance increases, treatment failure rates increase, and the median time to recrudescence shortens. Eventually, patients are encountered whose parasitaemia does not clear, although they may improve symptomatically. These patients are also usually young children in endemic areas. Ultimately, with high levels of resistance, there is no clinical or parasitological response at all to treatment (figure 1). In high-transmission areas an increasing prevalence of anaemia may be the first sign of worsening drug resistance (Bloland *et al*. 1998; Zucker *et al*. 1996). As host factors also contribute significantly to the therapeutic response in malaria, there tends to be a poor correlation between *in vitro* and *in vivo* antimalarial drug susceptibility responses.

(h) *Antimalarial pharmacokinetics*

As pathological processes in malaria result exclusively from the blood-stage infection, blood concentrations of the antimalarial drug determine the therapeutic effects directly, and it is not necessary to consider extravascular distribution and tissue penetration in assessing antimalarial drug reponses. Nevertheless, many of the antimalarial drugs are distributed extensively in the body, because of binding to tissues, leading to a large apparent volume of distribution, and they are cleared slowly. As a result residence times are long, and blood levels, exceeding those required to inhibit parasite multiplication, may persist for weeks or months following a course

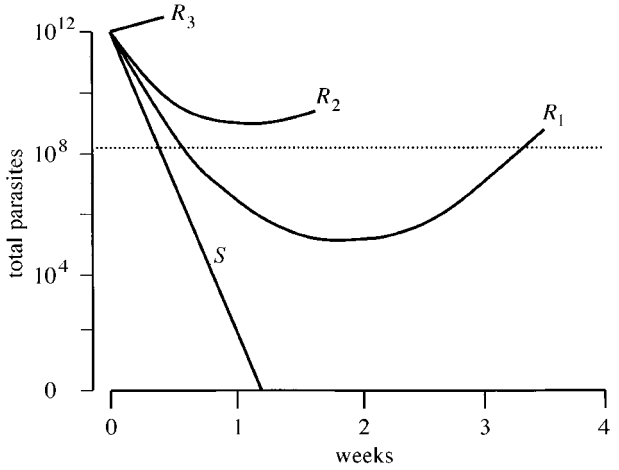

Figure 1. Conventional classification of aminoquinoline resistance based on parasitological responses to a standard treatment regimen. The total number of asexual malaria parasites in the body of an adult with 1–2% parasitaemia is shown.

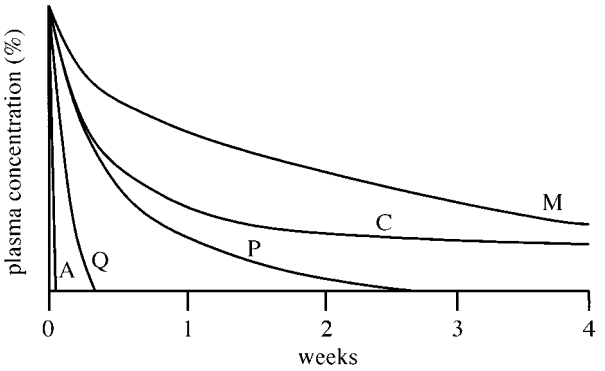

Figure 2. Plasma concentration profiles of the artemisinin derivatives (A), quinine (Q), pyrimethamine (P), chloroquine (C), and mefloquine (M) as a fraction of the maximum concentration following a single dose.

of treatment. The terminal elimination half-lives ($t_{\frac{1}{2}}$) of chloroquine (one to two months), mefloquine (two to three weeks), halofantrine (one to three days), lumefantrine (three to four days), pyrimethamine (three days), sulphadoxine (ten days), and atovaquone (one to three days), are such that either a single dose or a short course (one to three days) of the drug provides a complete antimalarial treatment (figure 2), whereas the considerably shorter lasting quinine ($t_{\frac{1}{2}}$, 16 h) and artemisinin derivatives (1 h) must be given at least once daily for seven days for complete treatment (White 1997; White 1992*a*). Although the malarial parasites are intra-erythrocytic, the concentration of antimalarial to which they are exposed corresponds closest with the free (unbound) plasma concentration rather than the red-cell concentration (derived from measurements mainly in uninfected erythrocytes). Acute malaria may (i) affect the absorption of drugs because of reduced food intake, vomiting, or reduced visceral perfusion, and (ii) change the apparent volume of distribution by altering plasma and tissue binding, and also reduce clearance by impairment of liver and renal function.

(i) *Antimalarial pharmacodynamics*

Antimalarials differ in their inhibitory effects on malaria parasite asexual stage development. The antifolates (pyrimethamine and cycloguanil) act late in maturation at the mature trophozoite to early schizont stage, whereas the quinoline and acridine antimalarials (quinine, mefloquine, chloroquine, mepacrine and amodiaquine) have the greatest amount of activity slightly earlier at the mid- to late-trophozoite stage. The artemisinin group has a very broad stage specificity of action, extending from young rings to schizonts (ter Kuile *et al.* 1993). Very young rings and mature schizonts are relatively resistant to all the drugs. The clinical and parasitological responses to drugs with earlier stage specificity are faster; for example, chloroquine acts more rapidly than pyrimethamine–sulphadoxine, and the artemisinin group produces the fastest therapeutic responses of all drugs. Stage specificity is important in severe malaria, but is of less significance in uncomplicated malaria. However, the relative lack of activity of all antimalarials against the very young ring forms may explain the persistence in low numbers of refractory young forms for days after drug exposure. These are viable and can lead to recrudescence of an apparently sensitive infection following treatment with rapidly eliminated drugs (artemisinin, quinine).

The principal effect of antimalarial drugs is to inhibit parasite multiplication (by arresting development). In theory, the untreated infection can multiply at a maximum rate given by the average number of viable merozoites per mature schizont (100% efficiency). In non-immunes, multiplication is often relatively efficient, with multiplication rates of 6–20 per cycle (30–90% efficiency). Antimalarials exerting their maximum effects (E_{max}) will convert this to a negative figure, reducing parasite numbers by between 100- and 10 000-fold per cycle (White 1997). The E_{max} represent the top of the sigmoid dose–response or concentration–effect relationship. Drugs differ in the E_{max} they can achieve; for example, the artemisinins often produce a 10 000-fold reduction per asexual cycle, whereas antimalarial antibiotics such as tetracycline or clindamycin may achieve at most a tenfold parasite reduction per cycle (figure 3). The lowest blood or plasma concentration of antimalarial drug that results in E_{max} can be considered a minimum parasiticidal concentration (MPC). Parasite reduction appears to be a first-order process throughout. Thus a fixed fraction of the population is removed each successive asexual cycle provided that the MPC is exceeded.

Resistance causes treatment failure when, because of reduced susceptibility, drug levels that would normally eliminate the infection can no longer do so. However, fully drug-sensitive parasites will still cause a recrudescent infection if the plasma concentrations of the drug are insufficient. Poor compliance, insufficient dosing, malabsorption, an expanded apparent volume of distribution, or increased clearance may all cause low drug levels. An infection will be eradicated, and the patient cured, if the plasma concentration of the free antimalarial drug still exceeds the concentrations required to maintain the parasite multiplication rate below one (the minimum inhibitory concentration), when either the last parasite has been removed (figure 4), or the number of parasites in the blood has fallen to a level at which the host's

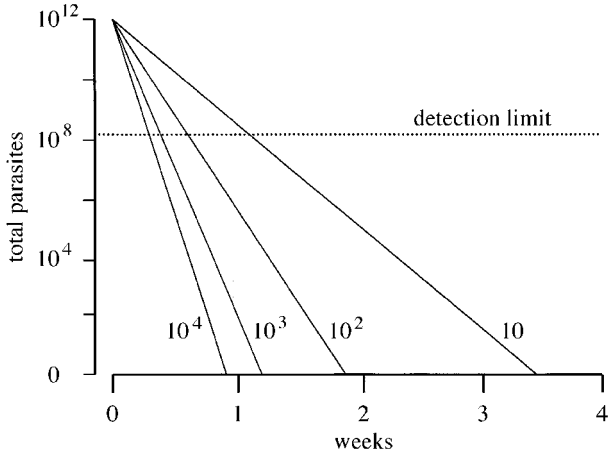

Figure 3. The effect of different parasite reduction ratios, or fractional killing, on the clearance of asexual parasites from the body. (Adapted from White (1997) with permission.)

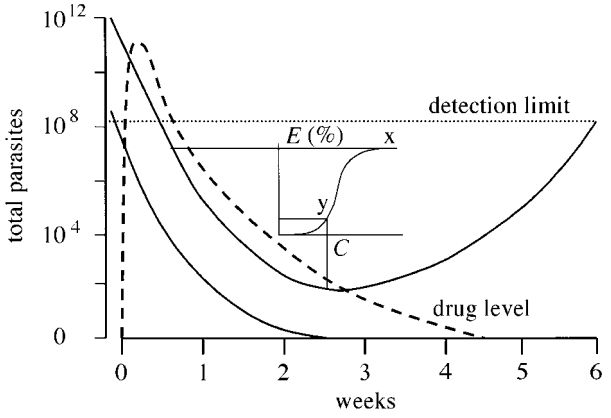

Figure 4. The effect of the initial parasite biomass in determining the therapeutic response with reduced drug sensitivity. In the upper profile (corresponding to approximately 1–2% parasitaemia in an adult), drug levels (shown as a dotted line) decline below the concentration (C), giving maximum effect (E) (this corresponds to point 'x' on the inset concentration–effect relationship) about five days after single-dose treatment. At this point the parasite reduction ratio begins to decline, and reaches a value of one when levels decline to point 'y'. The parasite reduction ratio (PRR) falls below one, and the biomass begins to expand again, reaching the level of detection six weeks after starting treatment. This provides considerable opportunity for the selection of mutant parasites, with a further reduction in drug susceptibility. In the lower profile, which starts at a low parasitaemia, the same drug levels are sufficient to eradicate the infection. (Adapted from White (1998) with permission.)

defences can remove the residuum. In endemic areas 'immunity' allies with the drug and treatment responses are always better in immune compared with non-immune patients. Rapidly eliminated drugs must be given until parasite eradication (which corresponds with four asexual cycles or seven days' treatment in non-immunes). For slowly eliminated drugs, dose, pharmacokinetic properties, and parasite susceptibility all contribute to the probability of cure.

(j) *The emergence of resistance*

(i) *Selection*

In certain cases highly drug-resistant mutants may arise spontaneously and multiply through 'therapeutic' concentrations of the antimalarial drug. This is best illustrated by the example of atovaquone resistance. When atovaquone alone was studied initially, one in three patients had a recrudescence of the infection (Looareesuwan *et al*. 1996). This was independent of the duration of treatment, and was usually associated with the emergence of a highly atovaquone-resistant mutant containing a single point mutation in the cytochrome *b* gene. This suggests that one in three patients already harboured at least one parasite with this mutation before treatment started (a frequency of approximately 1 in 10^{13} parasites). Recrudescence occurred approximately three weeks later, consistent with untrammelled multiplication of a single starting parasite.

More usually, the exposure of a parasite population to sub-inhibitory antimalarial drug concentrations provides the selective pressure to resistance. In bacteria, antibiotic concentrations exerting between 20% and 80% of maximum inhibitory activity provide the most selective pressure (Bonhoeffer *et al*. 1997; Lipsitch & Levin 1997). One or more genetic mutations may be required for a reduction in susceptibility. These mutations occur spontaneously and rarely. As they exist infrequently in parasite populations not exposed to antimalarial drugs, these mutations must be acquired and lost at a similar rate to maintain equilibrium (Mackinnon & Hastings 1998). Some mutational events may be lethal, and others may so reduce the fitness of the parasites that the advantages of drug resistance, even with drug selection, are still insufficient for their survival (Koella 1998). Thus the chance of a drug-resistant mutant malaria parasite being selected by that drug depends on the number of parasites, the mutation frequencies, the elimination profile of the drug, and the drug susceptibility and fitness of the mutants.

Obviously, a resistant mutant is more likely to occur in infections with a large number of parasites. Large biomass infections are more likely in non-immune patients. Host-defence mechanisms are also less likely to remove viable mutant parasites in non-immunes. In acute malaria, in an adult, there are usually between 10^9 and 10^{13} asexual parasites in the body. This corresponds to parasitaemias between 0.001% and 10%. Parasitaemias below 0.001% are increasingly likely to be asymptomatic, and are more common in malaria-endemic areas in semi-immune or immune subjects. Assuming a random distribution of mutants, a patient with 1% parasitaemia is therefore 1000 times more likely to harbour a drug-resistant mutant than a patient with 0.001% parasitaemia. But this patient is less likely to have significant immunity (which would clear the mutant parasites), and is more likely to receive antimalarial drug treatment (because they are more likely to be ill), so the chance that a drug-resistant parasite will be selected is even greater than 1000-fold more than that for a patient with the lower parasitaemia.

The conditions under which selection takes place depend on several factors, including the preceding level (distribution) of resistance, and the pattern of antimalarial drug use. Whereas a highly resistant mutant (relative to the distribution of susceptibilities in 'sensitive' parasites) may survive maximum blood concentrations of

the antimalarial drug and thus emerge from the initial biomass, mutants with lesser reductions in susceptibility will still be eliminated. They will only be selected if the initial treatment is inadequate and blood levels are low, or if they arise in subsequent generations. But as the number of parasites in each succeeding generation after drug treatment starts is orders of magnitudes lower than that in the preceding generation, this is an infrequent process. For example, in an adult with 1% parasitaemia who is treated with mefloquine, and whose infection recrudesces (at a parasitaemia of 0.01%) 28 days later, and who then receives effective treatment with artesunate, 97% of the parasites ever exposed to mefloquine are in the initial cycle. For a second infection newly acquired during the elimination phase of an antimalarial given to treat a previous infection, parasite numbers, initially, immediately following hepatic schizogony, are orders of magnitudes lower than during the fully developed infection. The considerably greater chance of selecting resistance in symptomatic infections emphasizes the importance of poor compliance and inadequate dosing as a powerful selective pressure for resistance to antimalarial drugs, particularly where resistance is multigenic, and develops in small increments. Mefloquine resistance may be a case in point. If selection from the initial parasite biomass is more likely than from second infections newly acquired during the elimination phase, then it may have been better to introduce mefloquine at the maximum dose ($25\,mg\,kg^{-1}$) rather than allowing selection to take place at a lower dose ($15\,mg\,kg^{-1}$) before increasing to the maximum tolerated dose (the current practice in some endemic areas).

(ii) *Spread*

Drug resistance will spread, and therefore increase, only if the resistant mutant parasites are transmitted to other hosts. This requires (i) transmission of the sexual stages (gametocytes), derived from the mutants to the anopheline mosquito vector; (ii) no loss of the resistance gene or genes during meoisis; and (iii) survival of the vector to enable its transmission to a new receptive host. The rate at which resistance spreads will depend on the relative reproductive rates of the drug-sensitive versus the drug-resistant parasites. This depends on the following:

(i) the relative risk of drug-resistant parasites transmitting viable gametocytes compared to drug-sensitive parasites. This in turn is a function of the duration for which patent gametocytaemia is present, and the infectiousness to mosquitoes of these gametocytes;
(ii) the intensity of transmission;
(iii) the immunity of the human population;
(iv) the pattern of antimalarial drug use.

As mentioned already, several factors favour the selection and spread of drug resistance in low-transmission areas where antimalarial drugs are available (Wernsdorfer *et al*. 1994; Paul *et al*. 1995).

(i) High-biomass infections are more likely.
(ii) There is less host defence (antibodies, cytokines and phagocytic cells) directed either against the asexual stages, which might remove a drug-resistant mutant, or the sexual stages, which could reduce transmissibility (Peel *et al*. 1993).
(iii) Most or all patients are symptomatic and therefore treated, so there is no 'competition' for transmission from infections that are not under selective drug pressure.
(iv) During sexual reproduction in the midgut of the anopheline mosquito male and female gametes will usually derive from the same original infection (selfing), whereas in high-transmission areas it becomes increasingly likely that multiple infections will occur and that the mosquito will feed sequentially on more than one infected person (Hill *et al*. 1995). This leads to mixing of gametocytes and sexual reproduction between the parasites of different infections, and it is possible that resistance will be lost by recombination breakdown between the two or more genetic loci encoding the resistance mechanism ('outbreeding') (Hill *et al*. 1995; Hastings 1997).

Balanced against these various factors, higher transmission results in increased gene traffic through the population, and the higher number of parasite clones per infection results in the transmission of resistance genes in increased copy numbers. Mackinnon & Hastings (1998) have argued recently that for these reasons high malaria transmission rates promote rather than hinder the spread of drug resistance. Indeed, since the original models proposed by Curtis & Otoo (1986), there has been considerable interest in modelling the evolution of antimalarial drug resistance in malaria parasites. In particular, the importance of 'outbreeding' has been examined. Analytical models suggest that outbreeding slows the spread of resistance only if the individual resistance genes are rare and do not confer significant resistance on their own, and where selection pressure is low (Hastings 1997; Dye & Williams 1997; Dye 1991). Although a single polymorphic gene *cg*2, of as yet undefined function, is strongly associated with chloroquine resistance, it is generally considered that multiple genes contribute to reduced susceptibility. The rate at which multigenic drug resistance develops depends on whether or not the effects of the individual mutations are independent (i.e. additive effects), or all the resistance mutations are required for any resistance (epistatic effect). Resistance mediated by genes acting additively develops more rapidly than resistance mediated by genes acting epistatically (Mackinnon & Hastings 1998).

Historically, drug resistance has always started in areas of low or intermediate malaria transmission. This is probably because the pattern of drug use is the overriding determinant of the rate at which drug resistance develops. Obviously, if malaria transmission is very low, then the the spread of resistance will also be low. As transmission increases, the rate at which resistance spreads increases, until premunition is acquired, and an increasing proportion of transmission derives from people with asymptomatic infections that are not under drug pressure. The resulting relationship between transmission intensity and rate of emergence of drug resistance is an asymmetrical parabola, with a probable maximum of between one and three infections per year.

(k) *Preventing antimalarial drug resistance*

Resistance may be inevitable, but the rate at which it develops can be altered considerably. The importance of treating an infection adequately and minimizing selective pressures cannot be overemphasized. Experimentally, drug resistance can be induced best by repeatedly exposing a large biomass infection *in vivo* or *in vitro* to an antimalarial drug treatment that is insufficient to eradicate it. A similar situation occurs *in vivo* with inadequately treated malarial infections either as a result of inappropriate prescribing, poor compliance or, occasionally, because of unusual pharmacokinetic properties of the drug. Ideally, antimalarial drugs should be used only to treat malaria in endemic areas, and if they are used then a full treatment course should be given. In many countries the majority of antimalarial drug use is through purchase and self-medication without microscopic diagnosis. Although this over-the-counter prescribing results in overtreatment, it nevertheless makes an important contribution to limiting malaria morbidity and mortality (Snow *et al*. 1992). Stopping it could do more harm than good. Education of dispensers and of patients can help. For example, short-acting drugs such as artemisinin and its derivatives, or quinine, need to be present at therapeutic (MPC) concentrations for at least four asexual cycles (a treatment course of seven days), to ensure eradication of all the parasites (figure 3). But drugs that persist for weeks or months at sub-therapeutic levels in the blood cannot be protected completely no matter how well prescribed.

(l) *Pharmacokinetic and pharmacodynamic factors contributing to the evolution of drug resistance*

Watkins & Mosobo (1993) have provided persuasive evidence that the terminal elimination half-life is an important determinant of the propensity to develop antimalarial drug resistance. Drugs with a long half-life will persist at concentrations in blood that are insufficient to eliminate the infection (figure 2). Selection can be considered in two phases. The first is when a newly acquired infection encounters residual sub-maximal blood concentrations of the antimalarial drug from an earlier treatment. These concentrations may be sufficient to eradicate a sensitive infection, but will be insufficient to eradicate a resistant one. This acts as a selective filter for resistant parasites acquired from elsewhere. The longer the half-life of the drug, the more likely will be such exposure. In some tropical communities the majority of the community has detectable antimalarial blood concentrations at any time. However, the chance of a resistant mutant arising *de novo* and surviving in the 10 000 to one million asexual parasites in the first cycle after liver schizogony, is obviously low. The second phase of selection is when the primary infection is not eradicated following treatment. If the infection is partially susceptible, or contains a mutant sub-population that is partially susceptible, then many asexual cycles will be exposed to progressively lower blood concentrations. The total number of parasites exposed to the drug is therefore increased. This offers a greater opportunity for resistant mutants arising *de novo* and surviving.

Drugs with short-elimination half-lives such as artemisinin and its derivatives, or quinine, are only present in the circulation for hours, or just over one day, respectively. Thus partially inhibitory concentrations are present only during the asexual cycle in which maximum concentrations were present previously, i.e. provided dosing is correct, parasites will encounter either maximum 'therapeutic' concentrations, or no drug. This provides considerably less selection. Both pyrimethamine and sulphadoxine are slowly eliminated (half-lives of four and ten days, repectively), whereas chlorproguanil and dapsone are both eliminated rapidly, with half-lives of less than two days, and may be less likely to induce DHFR and DHPS inhibitor resistance (Curtis *et al*. 1998; Watkins & Mosobo 1993). Short half-life drugs are therefore less likely to induce resistance, but they do require multiple dosing, and patients may not comply with this.

The slope of the antimalarial concentration–effect relationship is also relevant. If the slope is steep, then only a narrow range of drug concentrations provide partial inhibition, whereas a flatter slope offers a broad range of concentrations. The propensity to select resistance is therefore a function of the reciprocal of the first-order terminal elimination rate constant and the reciprocal of the slope of the linear portion of the sigmoid concentration–effect relationship. A rapidly eliminated drug with a steep slope (artemisinin) is much less vulnerable compared with a slowly eliminated drug with a flat slope (mefloquine).

(m) *Resistance and transmission potential*

The threshold for successful transmission of malaria is around six viable gametocytes in a 3 µl blood meal. This is slightly below the level of detection with routine microscopy (*ca*. $20\,\mu l^{-1}$) (Jeffery & Eyles 1955). As gametocytogenesis takes considerably longer than asexual reproduction, the wave of gametocytaemia usually follows that of the asexual parasites by seven to ten days. Gametocytaemia can be reduced by early treatment of the infection, or the use of gametocytocidal drugs (Sinden *et al*. 1996). For *P. falciparum*, drugs acting on the asexual parasites will kill stage I–III gametocytes, whereas stage IV and stage V gametocytes are resistant. The artemisinin derivatives reduce gametocytaemia by their marked effect on the asexual precursors, and also because of a broader stage specificity on developing sexual stages. The 8-aminoquinolines are specifically gametocytocidal (Butcher 1997; Jeffery *et al*. 1956). In contrast, the gametocytes of *P. vivax*, *P. malariae*, and *P. ovale* are considered just as sensitive to antimalarial drugs as the asexual stages. If the parasite population is 'stressed', as with the use of partially effective drugs, gametocytogenesis increases (Carter & Miller 1979) and gametocytaemia becomes more likely. Gametocytes formed following treatment of a resistant infection may have reduced transmission potential (Hogh *et al*. 1998). Recrudescent infections have been present in the body for longer than primary infections, and the parasite population has been under 'stress' from a partially effective treatment. These infections are more likely to comprise resistant parasites, and they are more likely to carry gametocytes. If these gametocytes are viable, then the increased transmissibility provides a powerful drive to the spread of resistance.

(n) *Drug combinations*

The concept that resistance could be delayed or prevented by combining drugs with different targets was pioneered first in the treatment of tuberculosis, and has been adopted for the treatment of cancer, and HIV infection. The use of combinations in antimalarial treatment and the concept that drug combinations could delay antimalarial drug resistance are not new. Peters has argued for decades that this strategy will prolong the useful life of existing drugs (Peters 1990). However, the rationale for most of the combinations in current use is to exploit synergy (chlorproguanil plus sulphones, pyrimethamine plus sulphonamides, atovaquone plus proguanil). In 1984, a triple combination of mefloquine plus pyrimethamine plus sulphadoxine was introduced for the treatment of multidrug resistance falciparum malaria in Thailand. This was intended specifically to delay the onset of resistance to mefloquine (Peters & Robinson 1984), but it did not work because *P. falciparum* in Thailand was already highly resistant to pyrimethamine–sulphadoxine. More recently, artemisinin and its derivatives have been combined with other antimalarials, notably mefloquine, and have accelerated recoveries, increased cure rates, reduced transmissibility, and appear also to have delayed the development of further resistance and reduced the incidence of disease (Price *et al*. 1997). The rationale behind antimalarial combinations, and suggestions for future use of this strategy to treat all malaria are presented below.

(o) *Rationale*

If resistance results from spontaneous genetic mutations, then the chance of a parasite developing resistance simultaneously to two drugs with unrelated modes of action (i.e. drug targets) is the product of the individual per parasite mutation frequencies multiplied by the total number of parasites exposed to the drugs. Put simply, if one in 10^6 parasites are resistant to drug A, and one in 10^6 are resistant to drug B, then one in 10^{12} will be simultaneously resistant to A and B. If all of these mutants are fit, then the chance of selecting a drug-resistant mutant is reduced a million-fold by using drugs A and B together. Sequential deployment of the drugs (a strategy used for insecticides, and more recently antibiotics) is much less effective as it does not exploit the multiplicative reduction in selection risk. Thus combinations will considerably delay the selection of multiple drug resistance. They also interrupt the spread and further increase of established resistance. For example, if a patient acquires an infection that is already resistant to drug A, then drug B should still be effective on its own, and vice versa. Obviously, it is much more effective to deploy the two drugs together from the outset rather than waiting for resistance to develop to one or other of the components. It should be noted that although various mathematical models have been applied to the evolution of drug resistance, and that somewhat different predictions have resulted, all agree that the use of antimalarial combinations will delay the onset and slow the rate of spread of resistance, especially when the drug-resistance mutant alleles are rare (Hastings 1997).

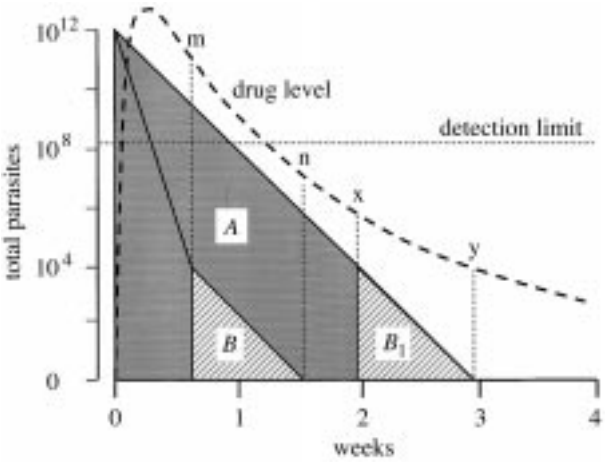

Figure 5. The addition of an artemisinin to a more slowly eliminated and less active drug (e.g. mefloquine) results in rapid reduction in the parasite biomass. The large shaded triangle (A) represents the total parasite numbers exposed to the less active drug. If a three-day course, an artemisinin derivative is added, then parasite numbers are reduced considerably and only B parasites are exposed to the less active drug alone. Furthermore these parasites 'see' much higher concentrations of the less active drug (from 'm' to 'n') compared with those 'seen' by the same residue (B_1) if no artemisinin derivative is given, i.e. from 'x' to 'y'. This provides considerably less selection opportunity for resistance. (Adapted from White (1998) with permission.)

(p) *Use of artemisinin and its derivatives in combinations*

These compounds have several advantages over other antimalarial drugs for use in combinations (Chawira *et al*. 1987; White 1997, 1998). These are detailed as follows.

(i) They are very active, reducing parasite numbers by more than the other antimalarials (by approximately 10^4 per asexual cycle). When combined with a slower-acting, and more slowly eliminated compound, such as mefloquine, relatively few parasites (usually less than 100 000 after a three-day course of artemisinin) remain to be exposed to mefloquine alone, and of course none are exposed to the artemisinin derivative alone (figure 5). Even if a single dose of artemisinin is given, the total number of parasites ever exposed to mefloquine alone (assuming relative resistance and a parasite reduction ratio (PRR) of only 10^2 thereafter) is still 0.01% of those present originally. Furthermore, this residuum is exposed to maximum blood mefloquine concentrations. These may be sufficient to drive to extinction even a partially resistant infection, whereas without artemisinin, the infection may not have been reduced to zero before blood levels have fallen below the minimum inhibitory concentration value. This latter advantage obtains only for drugs such as mefloquine, where there is a continuum of sensitivity (i.e. the concentration–effect slope moves gradually to the right with resistance). It would not obtain with a drug such as atovaquone–proguanil, where a single point mutation confers such a high level of resistance that even maximum drug levels have no effect. Nevertheless, an atovaquone-resistant mutant parasite should be killed by a co-administered

artemisinin derivative, and resistance prevented by such a combination.

(ii) They reduce considerably gametocyte carriage and thus transmissibility. In an area of mefloquine resistance they reduced gametocyte carriage ninefold. As recrudescent infections (i.e. treatment failures) are more likely to carry gametocytes, this selective transmission advantage to resistant parasites is reduced both because cure rates are increased (therefore there are less recrudescent infections), and the infections that do recrudesce are prevented from developing gametocytes after combination treatment has started. Reduced transmissibility translates into reduced transmission if the treated patients are the main reservoir of gametocytes (as they may be in low-transmission areas where effective antimalarial drugs are available). Reduced transmission results in reduced incidence of malaria, reduced drug use, and thus a further slowing in the rate of evolution of drug resistance.

(iii) Stable resistance to this group of drugs has not yet been recorded, either in therapeutic use or in experimental systems.

(iv) These drugs are very rapidly eliminated and thus provide no opportunity for parasites to be exposed to sub-therapeutic concentrations if the dosage is correct.

(vi) They have operational advantages: they produce a rapid clinical response, which encourages acceptance, and they have an excellent safety and side-effect profile that encourages compliance.

Ideally, combination partners should have similar pharmacokinetic properties so that no drug is left 'unprotected' by the other. In most combinations with artemisinin derivatives the partner drug is eliminated much more slowly, and is not 'protected' when a newly acquired infection is contracted during or after the episode being treated. If hepatic schizogony occurs after clearance of the artemisinin drug, only the residual levels of the more slowly eliminated drug are present. However, as mentioned previously, in comparison with the symptomatic infection the number of parasites involved is relatively small. Even with an unusually large viable sporozoite inoculum of 100, a maximum of around five million asexual parasites would be generated in the first cycle after hepatic schizogony. A more likely figure, compatible with volunteer and malaria therapy experience, is approximately 500 000. If this infection already comprises a single highly resistant parasite genotype, then it will probably be unaffected by residual drug levels and thus no selection will take place. If it contains several genotypes, then there is the opportunity for differential selection. In contrast, a new mutation is much less likely to arise from this starting biomass, compared to that in the fully developed symptomatic infection of between 10^9 and 10^{12} parasites, but the parasite biomass will never reach these numbers unless the drug has declined to levels that are ineffective and therefore relatively non-selective. In summary, newly acquired infections emerging from the liver and encountering residual drug levels are several orders of magnitude less 'efficient' in producing *de novo* resistant mutants than partially treated, established, large-biomass infections—but they do act as a selective filter in mixed-genotype infections, encouraging the spread of drug resistance.

(q) *Drug interactions*

Although the use of antimalarial combinations may improve therapeutic efficacy and delay resistance, the combination of two or more drugs may also result in pharmacological interactions between the components leading to altered disposition, or toxicity. Formal pharmacokinetic interaction studies need to be conducted, and adverse effect profiles determined in large clinical trials, before a combination can be recommended generally. Artesunate hydrolyses spontaneously in plasma, whereas artemether is metabolized predominantly by cytochrome (CY) P_{450} $3A_4$. Their common metabolite dihydroartemesinin is glucuronidated. They have not been shown to interact significantly with other antimalarials. It has been claimed that the artemisinin derivatives alter mefloquine disposition, but this is much more likely to represent an indirect pharmacodynamic effect by accelerating clinical recovery, and thus altering the apparent volume of distribution of mefloquine. In any case the effects are small. No significant pharmacokinetic interaction occurs between artemether and lumefantrine (a combination shortly to be introduced) or artesunate and atovaquone plus proguanil (another potential combination). Artemisinin, but not artesunate, artemether or dihydroartemisinin, appears to induce its own metabolism (Hassan Alin *et al*. 1996), possibly via CYP $2C_{19}$, and this could theoretically alter the disposition of other drugs biotransformed by this enzyme (e.g. proguanil). Quinine is commonly combined with antibiotics that possess antimalarial activity, such as tetracycline or clindamycin. No significant interactions have been reported with these drugs. Quinine metabolism, also mediated initially by CYP $3A_4$, is induced by rifampicin, but this combination is not recommended.

(r) *Cost*

Combinations obviously cost more than individual single drug treatments, but should make considerable savings over the longer term by delaying the onset of resistance. After chloroquine, pyrimethamine, sulphadoxine, and perhaps amodiaquine, the next effective antimalarials (quinine, mefloquine, halofantrine, etc.) cost at least ten times as much per unit treatment. Resistance itself has a price too through increased morbidity and mortality. In low-transmission areas, there may be additional benefits from artemisinin combinations in terms of reduced incidence of malaria. For example, the use of the artesunate–mefloquine combination on the western border of Thailand has been associated with a significant reduction in the incidence of malaria, which has more than offset the doubling in price of individual treatments.

(s) *What to do?*

A crisis is brewing in Africa, where it is estimated that 90% of the annual 0.5–2.5 million deaths from malaria occur (Marsh 1998). Significant levels of chloroquine resistance are now prevalent throughout the continent. This is resulting in increased morbidity, and in some

areas higher mortality. Amodiaquine is more effective than chloroquine, but is not widely available, and it is not effective against highly chloroquine-resistant infections. The only affordable alternatives are antifolate sulphonamide or sulphone combinations, but resistance to these develops easily. Already *P. falciparum* parasites with three point mutations in the DHFR gene are being isolated in East African countries that have changed their first-line recommendations from chloroquine to pyrimethamine–sulphadoxine. The health and economic consequences of losing these drugs to resistance would be disastrous. Many of these countries have an annual per capita health expenditure of less than US$10 per person, and simply cannot afford expensive drugs. The use of antimalarial combinations, particularly artemisinin derivatives together with the existing drugs, will significantly increase the cost of antimalarial treatment (an increase of around US 25 cents to treat a 15 kg child), but this may be a worthwhile investment if lives are saved and resistance is delayed for five to ten years. Further studies of the economics and health impact of this strategy are required, but it may be necessary to make operational decisions on the use of these drugs with incomplete information as the situation is worsening rapidly, and the benefits are greater the earlier in the evolution of drug resistance that the combination is deployed.

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