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The *de novo* selection of drug-resistant malaria parasites

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Antimalarial drug resistance emerges *de novo* predominantly in areas of low malaria transmission. Because of the logarithmic distribution of parasite numbers in human malaria infections, inadequately treated high biomass infections are a major source of *de novo* antimalarial resistance, whereas use of antimalarial prophylaxis provides a low resistance selection risk. Slowly eliminated antimalarials encourage resistance largely by providing a selective filter for resistant parasites acquired from others, and not by selecting resistance *de novo*. The *de novo* emergence of resistance can be prevented by use of antimalarial combinations. Artemisinin derivative combinations are particularly effective. Ensuring adequate treatment of the relatively few heavily infected patients would slow the emergence of resistance.

Keywords: resistance; antimalarials; *Plasmodium falciparum*; malaria

1. INTRODUCTION

Since the wide-scale deployment of antimalarial drugs in the latter half of the 20th century, human malaria parasites have been under tremendous selection pressure to evolve mechanisms of resistance. The most extensively used antimalarials (chloroquine and sulphadoxine–pyrimethamine) are inexpensive, widely available, and are eliminated slowly from the body. Together with antipyretics, antimalarials are among the most commonly used medications in tropical areas of the world. Misuse is widespread. This extensive use has resulted in the emergence of resistance, particularly in *Plasmodium falciparum*, and contributed to a global resurgence of malaria in the past three decades. The loss of affordable antimalarial drugs, particularly in Africa, which harbours the majority of the world's malaria, is a humanitarian disaster (Marsh 1998). The effects of resistance on morbidity and mortality are usually underestimated (Trape *et al.* 1998; White 1999*a*). Despite increased global awareness, the situation appears to be getting worse and mortality is rising. Predicting what will happen to antimalarial drugs in the future is necessary for planning malaria control and instituting strategies which might delay the emergence of resistance (Hastings & D'Alessandro 2000). Resistance has already developed to all the antimalarial drug classes with one notable exception: the artemisinins. These drugs are already an essential component of treatments for multidrug-resistant *falciparum* malaria. Loss of the artemisinins to resistance would raise again the spectre of untreatable malaria. The emergence of resistance can be considered in two parts: first the initial genetic event which produces the resistant mutant, and second the subsequent selection process in which the survival advantage in the presence of the drug leads to preferential transmission and the spread of resistance. The factors affecting the probability of the first event, the *de novo* selection of resistance, are examined here.

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2. MALARIA PARASITE NUMBERS AND SELECTION PROBABILITIES

(a) *Distribution of numbers in the parasite life cycle*

Malaria parasites are eukaryotes. Meiosis occurs following the ingestion of viable gametocytes in an anopheline mosquito's blood meal. All the other 10^8 to 10^{13} cell divisions in the complete life cycle are mitotic. Although the distribution is skewed, usually less than 10 sporozoite parasites are inoculated by a mosquito to start the malaria infection (Rosenburg & Wirtz 1990; Ponnudurai *et al.* 1991; figure 1).

Each infected hepatocyte liberates *ca.* 30 000 merozoites after 5–6 days of pre-erythrocytic schizogony which suggests that, following successful sporozoite invasion of liver cells, mitotic division must occur approximately three times daily. Thus in *P. falciparum* malaria *ca.* 100 000 to 300 000 merozoites are liberated into the blood stream to begin the 48 h asexual reproduction cycle. The density of parasites in the blood at which symptoms and fever occur (the pyrogenic density), and thus the stage at which appropriate antimalarial treatment could be given, varies considerably (James *et al.* 1932; Fairley 1947; Kitchen 1949). In non-immunes, non-specific symptoms often occur a day or two before parasites are detectable on the blood smear (*ca.* 50 parasites μl^{-1} of blood). This density corresponds to a total of between 10^8 and 10^9 asexual parasites in an adult. In endemic areas parasitaemias considerably above this level may be tolerated without symptoms, although densities over 10 000 μl^{-1} (between 10^{10} and 10^{11} parasites in the body of an adult, and correspondingly less in children) are usually symptomatic, even in very high transmission settings (Smith *et al.* 1994). Median or geometric mean parasite counts in malariometric surveys are usually below this value. Although sequestration of *P. falciparum*-infected erythrocytes in the microvasculature confounds the relationship between peripheral parasitaemia and disease severity, it is generally accepted that severe malaria is associated with a large parasite biomass

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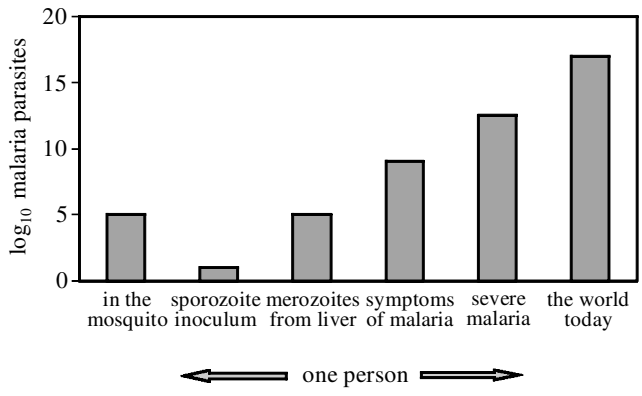

Figure 1. The distribution of malaria parasite numbers at different stages of the life cycle in the vector mosquito and in man (logarithmic scale). The total number of malaria parasites in the world on any day is estimated to be between 10^{16} and 10^{18}. Thus relatively few high-biomass infections (more than 10^{12} parasites per person) contribute a significant proportion of the entire world's parasites.

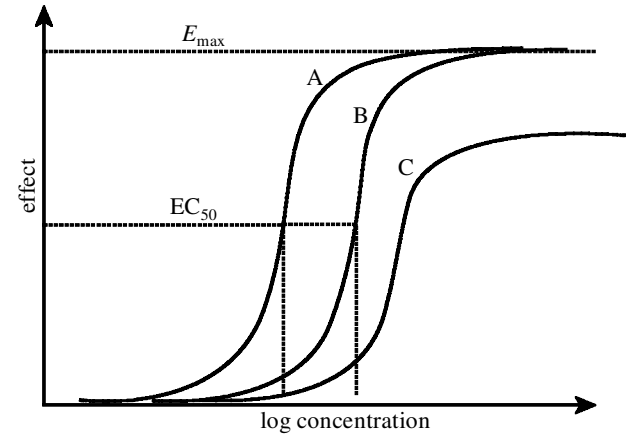

Figure 2. Resistance is a rightward shift in the concentration–effect relationship for a particular parasite population. A is the concentration–effect relationship for sensitive parasites and B and C represent different patterns of resistance. This may be a parallel shift (B) or, in some circumstances, the slope changes, and/or the maximum achievable effect is reduced (C). The E_{max} is the maximum effect produced by the drug, and the EC_{50} is the concentration which produces 50% of the maximum effect.

(Field 1949). A total biomass over 10^{12} malaria parasites in adults is probably a prerequisite for lethal malaria, and a fatal outcome becomes increasingly likely with body numbers exceeding 10^{13} malaria parasites. Parasite burdens much over 10^{14} (i.e. 10% parasitaemia) are physically impossible.

(b) *Distribution of parasite numbers in the world*

It is estimated that *ca.* 300 000 000 people in the world now have malaria parasites in their blood. The global distribution of parasite numbers is not known but it must be skewed towards lower numbers, with a relatively small proportion of the infected people in the world being ill on any day, and much fewer still harbouring parasite burdens over 10^{12}. Most of these asymptomatic infected people are older children and adults living in malaria endemic areas and most have parasite densities below the level of blood smear detection (i.e. less than 10^{8} in an adult; Snow *et al.* 1999). Thus the total number of parasites in the world's asymptomatic carriers must be less than $3 \times 10^{8} \times 10^{8}$; i.e. less than 3×10^{16} parasites. The true number could be orders of magnitude lower.

Geometric mean or median admission parasitaemias in clinical studies of *falciparum* malaria usually lie between 5000 and 50 000 μl^{-1}. If this reflects the true geometric mean parasitaemia at the time of treatment seeking in all the *symptomatic P. falciparum*-infected people in the world (the majority of whom are children with lower blood volumes than adults), then an approximate estimate of the total number of asexual parasites in these people can be made as follows.

Assuming an average blood volume of 2 l, for example an 8–10-year-old African child (range across human populations 0.25–8 l), then the average symptomatic individual 'contains' between 10^{10} and 10^{11} malaria parasites.

If between five and 50 million people are symptomatic in any two-day period, these people therefore would 'contain' between 5×10^{16} and 5×10^{18} malaria parasites.

This compares with less than 3×10^{16} parasites in asymptomatic infected people.

Thus on any day, although the majority of malaria-infected people are asymptomatic, the majority of malaria parasites in the world are probably in people who are ill (figure 1).

Assuming that the probability of a *de novo* resistance mutation arising is distributed evenly among these parasites, then, because of their logarithmic distribution, those patients with high parasitaemias who survive their infection to transmit viable gametocytes carry a significant proportion of all the world's 'potentially transmissible' malaria parasites. They therefore are an important potential source of resistance (White 1999*a*). For example, a single adult with 10% parasitaemia (i.e. 10% of the red cells contain at least one parasite) 'contains' approximately the same number of malaria parasites as 100 000 to one million adults with asymptomatic infections. Considering a country such as Cambodia, with predominantly low and unstable seasonal transmission, and *ca.* 10^{6} people living in malaria endemic areas, such a single hyperparasitaemic adult would provide approximately the same probability (in 48 h) of generating a drug-resistant mutant parasite as the entire country's asymptomatic malaria-infected population. But, should he or she survive, the chance of *selection* and preferential survival of such a drug-resistant mutant from this one hyperparasitaemic individual is even greater, as this patient probably has little immunity (otherwise a 10% parasitaemia would not have developed), will receive antimalarial drug treatment (whereas nearly all the asymptomatic patients will not), and has a higher chance of failing treatment than patients with lower parasite numbers (ter Kuile *et al.* 1995). Thus if the probabilities of *de novo* selection of a resistant parasite are equally distributed, the importance of high-biomass infections which receive inadequate treatment as a source of resistance is evident.

3. RESISTANCE

Drug resistance to an anti-infective compound is defined by a rightward shift in the concentration–effect (dose-response) relationship (figure 2). The principal drug

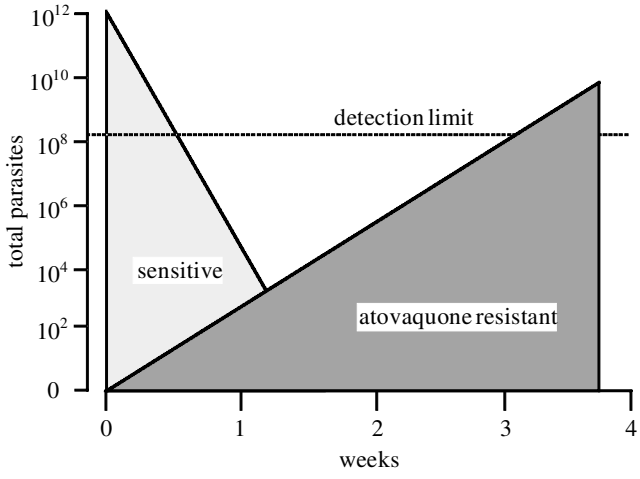

Figure 3. The most extreme example of selecting antimalarial drug resistance. The total number of parasites in an infected patient is shown in a logarithmic scale on the *y*-axis. Parasite killing is a first-order process. Following atovaquone treatment of uncomplicated *falciparum* malaria one in three treated patients had a recrudescence of their infection, usually with a highly resistant parasite (Looareesuwan *et al.* 1996). This suggests that only a single parasite was present in the initial biomass but that this grew exponentially (i.e. normally), unconstrained by host defences or drug effects (Simpson *et al.* 2000). The decline in the drug-sensitive parasites (light grey triangle) occurs while the drug-resistant population expands (dark grey triangle).

effect of antimalarial drugs is inhibition of parasite multiplication. This is a first-order process leading to a log–linear reduction in parasite numbers with time (Day *et al.* 1996). Uninhibited blood-stage multiplication at 100% efficiency results in a parasite multiplication rate (PMR) equal to the median number of viable merozoites liberated by rupturing schizonts. *In vivo* efficiencies may exceed 50% in non-immune patients, giving PMRs of *ca.* 10 per asexual cycle (Simpson *et al.* 2002). Antimalarial drugs convert this to a negative value, resulting in PMRs which range between 10^{-1} and 10^{-4} per cycle. These are also termed parasite killing rates or parasite reduction ratios (White 1997).

The genetic events which confer antimalarial drug resistance (whilst retaining parasite viability) are spontaneous and rare. They are thought to be independent of the drug. These can be mutations in or changes in the copy number of genes relating to the drug's target (e.g. *Pfdhfr*) or pumps which affect intraparasitic concentrations of the drug (e.g. *PfMDR*). A single genetic event may be all that is required, or multiple unlinked events may be necessary (epistasis). *P. falciparum* parasites from South East Asia seem constitutionally to have an increased propensity to develop drug resistance (Rathod *et al.* 1997). The mutation frequencies derived from *in vitro* studies are often much higher than those derived from observations *in vivo* (Paget-McNicol & Saul 2001). The highest rates of emergence of resistance *in vivo* are for pyrimethamine and atovaquone (Peters 1987; Looareesuwan *et al.* 1996). In the case of atovaquone it has been estimated that one in three patients with symptomatic *falciparum* malaria 'contained' a spontaneously arising highly atovaquone-resistant mutant parasite (White 1999*a*). This translates into a per-parasite frequency of 1 in 10^{12} , whereas *in vitro* the frequency is as high as 1 in 10^{5} (Rathod *et al.* 1997). When the antifols pyrimethamine and proguanil were deployed alone in the late 1940s and 1950s resistance developed very rapidly. Pyrimethamine resistance could also be selected readily in human volunteers (Peters 1987). These data suggest relatively high mutation frequencies (table 1). Resistance to sulphadoxine–pyrimethamine appears to arise *de novo* much less frequently (T. Anderson, personal communication), although it spreads very rapidly (Watkins *et al.* 1999), probably because gametocyte production is stimulated by the drug. By contrast, for drugs such as chloroquine or artemisinin, the genetic events conferring resistance are much rarer (they may have happened only a few times in the case of chloroquine, and significant resistance has not yet been detected for artemisinin). Assuming an equal distribution of probabilities throughout the life cycle, the genetic event is likely to take place in only a single parasite at the peak of infection. These genetic events may result in moderate changes in drug susceptibility, such that the drug still remains effective (e.g. treatment of infections carrying the 108*Asn*DHFR mutation with sulphadoxine–pyrimethamine), or, less commonly, very large reductions in susceptibility such that achievable concentrations of the drug are completely ineffective (e.g. the cytochrome *b* mutations giving atovaquone resistance; Cowman *et al.* 1994; Looareesuwan *et al.* 1996; Korsinczky *et al.* 2000; Reed *et al.* 2000; figure 3).

Table 1. Approximate per-parasite frequencies for genetic events (mutations or gene amplifications) which lead to the emergence of clinically significant drug resistance of *Plasmodium falciparum in vivo*.
(If the resistance mechanism is multigenic then this represents the frequency of the parasite becoming resistant and thus it is the product of the individual mutation frequencies. The estimate for pyrimethamine is for the drug alone. When it is combined together with a sulphonamide the frequency appears to be significantly lower. The estimate for mefloquine is in already chloroquine-resistant parasites. The estimates for chloroquine and artemisinin are speculative. In the former case, this assumes two events in 10 years of use with exposure of 10% of the world's *falciparum* malaria (Burgess & Young 1959; Martin & Arnold 1968; Looareesuwan *et al.* 1996; Su *et al.* 1997; Nosten *et al.* 2000).)

drug	per-parasite resistance mutation frequency
pyrimethamine	1 in 10^{11}
atovaquone	1 in 10^{12}
mefloquine	1 in 10^{14}
chloroquine	1 in 10^{19}
artemisinin	$<$1 in 10^{18}

4. *DE NOVO* SELECTION OF RESISTANCE

The probability of *de novo* selection depends on several external factors:

(i) the number of parasites exposed to the drug (figures 1 and 4*a*,*b*);
(ii) the concentrations of drug to which these parasites are exposed;

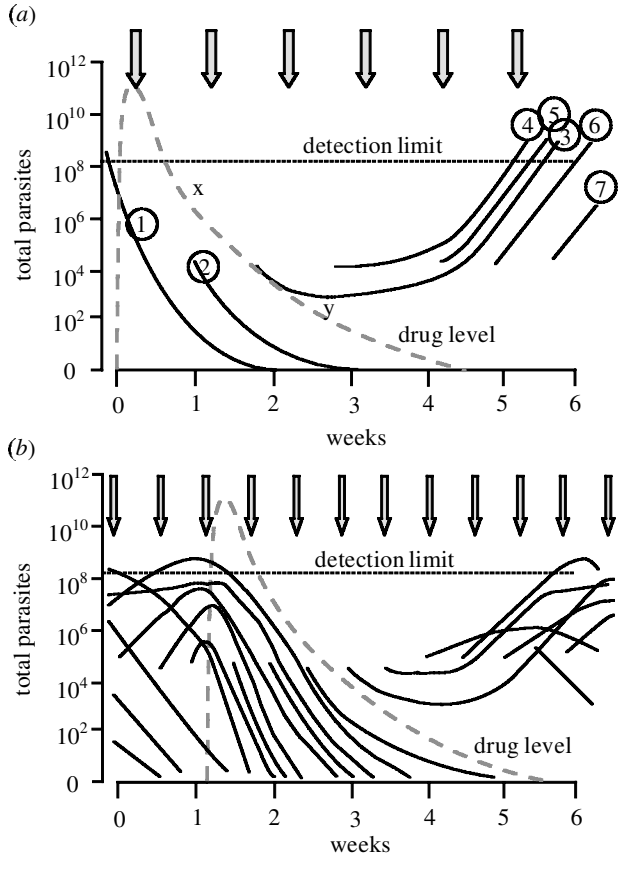

Figure 4. Selection opportunity in high-transmission settings. (*a*) This shows the fate of different infections (continuous lines) in a patient living in an area with an entomological inoculation rate of 50 yr^{-1} who is treated with mefloquine, which has a terminal elimination half-life of approximately two weeks in malaria. Multiple infections are exposed to mefloquine. Each newly acquired infection emerges from the liver after 5–7 days at a total starting parasitaemia of *ca.* 100 000. The arrows represent infected bites and sporozoite inoculations. Lines 2–7 represent new infections following mefloquine treatment of a symptomatic malaria episode (line 1). The axes are the same as in figure 3, and the blood concentrations of mefloquine are shown as a dotted line. Point x on the mefloquine concentration slope corresponds to the minimum parasiticidal concentration for the infections, namely the lowest concentration giving maximum parasite killing. Below this concentration the rate of fall in parasite numbers slows, until the MIC (point y) is reached. At the MIC the PMR is transiently one, and below the MIC the parasite population can expand. In this example the original symptomatic infection (line 1) is cured—the parasite numbers fall to zero in two weeks. The next infection (line 2) to emerge from the liver is also cured, but infections 3–5 survive even without the development of resistance. Infections 1–5 are under drug selection and offer the possibility of generating a resistant mutant. However, in the context of an EIR of 50, immunity is acquired rapidly and this will tend to remove parasites independently of resistance. The chance that a single resistant mutant parasite will survive host defence and generate sufficient parasites to be transmitted is lowered as a consequence. (*b*) In this example the entomological inoculation rate is 100 yr^{-1} and there is exposure to mefloquine (e.g. self treatment of a fever which was not caused by malaria). The dotted line represents the drug concentrations. Each continuous line represents a new infection. There is considerable background immunity, which limits the density of acquired infections and prevents most of them transmitting. The chance that a single resistant mutant parasite will survive host defence and generate sufficient parasites to be transmitted is small.

(iii) the pharmacokinetic and pharmacodynamic properties of the antimalarial drug or drugs;
(iv) the degree of resistance (the shift in the concentration–effect relationship) that results from the genetic changes (figure 2);
(v) the level of host defence (non-specific and specific immunity); and
(vi) the simultaneous presence of other antimalarial drugs or substances in the blood to which the parasite is not resistant.

Resistance to one drug may be selected by another for which the mechanism of resistance is similar (cross resistance). There are many parallels with antibiotic resistance (Bonhoeffer *et al.* 1997; Lipsitch & Levin 1997; Austin & Anderson 1999), particularly antituberculous drug resistance where, as for malaria, transferable resistance genes are not involved in the emergence of resistance. In experimental models, drug-resistance mutations can be selected without mosquito passage (i.e. without meiotic recombination) by exposure of large numbers of malaria parasites (either *in vitro*, in animals, or—in the past—in volunteers) to sub-therapeutic drug concentrations (Peters 1987).

5. TRANSMISSION INTENSITY AND THE SELECTION OF RESISTANCE

The recrudescence and subsequent transmission of an infection which generated *de novo* resistant malaria parasites is necessary for resistance to be propagated (White 1999*a*). Killing the transmissible sexual stages (gametocytes) during the primary infection does *not* affect the *de novo* emergence of resistance because these gametocytes derive from drug-sensitive parasites. Gametocytes carrying the resistance genes will not reach transmissible densities until the resistant parasite population has expanded to numbers close to those producing illness (more than 10^7 parasites; Jeffery & Eyles 1955). Thus to prevent resistance spreading, gametocyte production from the *recrudescent* resistant infection must be prevented.

In low-transmission areas the majority of malaria is symptomatic and selection therefore takes place in the context of treatment. Relatively large numbers of parasites in an individual encounter antimalarial drugs. In higher-transmission areas the majority of infections are asymptomatic and these are acquired repeatedly throughout life (see Appendix A). Symptomatic and sometimes fatal disease occurs in the first years of life, but thereafter malaria becomes increasingly likely to be asymptomatic. This reflects a state of imperfect immunity (premunition), where the infection is controlled, usually at levels below those causing symptoms. The rate at which premunition is acquired depends on the intensity of transmission. In the context of intense malaria transmission, people still receive antimalarial treatments throughout their lives

(often inappropriately for other febrile infections), but these 'treatments' are largely unrelated to the peaks of parasitaemia, thereby reducing the resistance selection probability. Immunity also considerably reduces the emergence of resistance (White 1999*b*). Host defence contributes a major anti-parasitic effect, and any spontaneously generated drug-resistant mutant malaria parasite must contend not only with the antimalarial drug concentrations that are present, but also with host immunity. This further reduces the individual survival probability. Even if the resistant mutant does survive the initial drug treatment, and multiplies, the chance that this will result in sufficient gametocytes for transmission is likely to be reduced as a result of both asexual-stage immunity (which reduces the multiplication rate and lowers the density at which the infection is controlled) and transmission-blocking immunity. Furthermore, other parasite genotypes are likely to be present, competing with the resistant parasites for red cells and increasing the possibility of outbreeding of multigenic resistance mechanisms or competition in the feeding anopheline mosquito (Dye & Williams 1997). These factors reducing the probability of selecting and transmitting resistance in high transmission settings are balanced against the increased frequency of vector biting, and thus the increased probability that a feeding anopheline will encounter the resistance-bearing gametocytes. Even if the resistance-bearing parasites do establish themselves in the anopheline mosquito, they must still be transmitted to a susceptible recipient for resistance to spread. As the majority of the population is immune, the individual probability of propagation is likely to be reduced as inoculation in a subsequent mosquito feed often does not result in an infection capable of being transmitted onwards. In high-transmission areas only the young children develop significant symptoms, and so the chance of a drug encountering large numbers of parasites in a semi-immune host is confined to the first few years of life. Depending on the population structure, as little as 20% of the population (with blood volumes in infants 5–20 times lower than in adults, and therefore fewer total parasites for any given density) contribute the scenario conducive to the *de novo* selection of resistance. Even in these young children in endemic areas there is evidence of immunity. Parasite clearance times in young children in high-transmission areas following antimalarial drug treatment are nearly always faster than corresponding values in non-immune children and adults in low-transmission areas. The net result is considerable reduction in the probability of *de novo* selection and subsequent transmission of a resistant parasite mutant in high-transmission compared with low-transmission areas (figure 4*a*,*b*). Historically, chloroquine resistance emerged in low-transmission areas and antifol resistance increased more rapidly in low-transmission compared with high-transmission areas.

6. ANTIMALARIAL PHARMACOKINETICS AND THE *DE NOVO* SELECTION OF RESISTANCE

(a) *Absorption and disposition*

The probability of selecting a *de novo* resistance mutation during the initial phase of treatment depends on the per-parasite frequency of the genetic event, the number of parasites present, 'immunity' and the relationship between the drug levels achieved and the degree of resistance conferred by the mutant parasite. Obviously if the range of blood concentrations achieved in the patient exceeds considerably the IC_{90} values (concentrations giving 90% inhibition of multiplication) for the most resistant mutant (IC^{R}_{90}), then resistance cannot be selected in the acute phase of treatment as even the resistant mutants are prevented from multiplying. It should be noted that the relationship between the IC_{90} concentrations *in vivo* and the IC_{90} concentrations derived indirectly from *in vitro* susceptibility tests has not been determined precisely. Conversely, if the degree of resistance provided by the genetic event is very small, the window of opportunity for selection may be negligible. But provided that there is such a window, then the broader the range of peak antimalarial concentrations, and the closer the median blood concentration value approaches IC^{R}_{90}, the greater the probability of selecting a resistant mutant in a patient. Peak drug concentrations are determined by absorption, distribution volume and dose taken. Several antimalarial drugs (notably lumefantrine, halofantrine, atovaquone and to a lesser extent mefloquine) are lipophilic, hydrophobic and very variably absorbed (inter-individual variation in bioavailability up to 20-fold; White 1992; White *et al.* 1999). Inter-individual variability in distribution volumes tends to be lower (usually less than fivefold) but, taken together, the product is considerable inter-individual variation in peak concentrations in blood. Unless the genetic change confers very high levels of resistance (as for cytochrome *b* mutations and atovaquinone resistance) then *de novo* resistance is more likely to arise in those patients with lower drug levels. The main source of under-dosing globally is incorrect self-medication, either because of poor adherence to the correctly prescribed drug regimen, poor-quality drugs, uncontrolled drug availability and purchase of incorrect dose regimens or sub-standard drugs in shops or the market place, or incorrect administration in the home. As the acute infection is the principal source of *de novo* resistance selection, this emphasizes the pivotal roles of quality-assured drugs, education, correct prescribing, good adherence, and optimized packaging and formulations in preventing the emergence of antimalarial high bioavailability drug resistance.

(b) *Drug elimination rates*

In some areas of the world, transmission intensities may reach as high as three infectious bites per person per day. Everyone there has malaria all the time, and each person harbours many different parasite genotypes (although many are below the level of PCR detection). In this context a person who takes antimalarial treatment for symptomatic malaria exposes not only the parasites causing that infection to the drug, but also any newly acquired infections which emerge from the liver during the drug's elimination phase (figure 4*a*,*b*). The longer the terminal elimination half-life, the greater is the exposure. Watkins and colleagues have shown that the length of the terminal elimination half-life is an important determinant of the propensity for an antimalarial drug to fall to resistance (Watkins & Mosobo 1993; Nzila *et al.* 2000; Hastings *et al.* 2002). The terminal elimination half-life, particularly if that elimination phase traverses the steep part of the concentration–effect relationship for the prevalent malaria

parasites, determines the probability that any newly acquired parasite will encounter a partially effective (i.e. selective) drug concentration. Some rapidly eliminated antimalarial drugs (e.g. the artemisinin derivatives) never present an intermediate drug concentration to infecting malaria parasites because they are eliminated completely within the two-day life cycle of the asexual parasite. Other drugs (e.g. mefloquine, chloroquine) have elimination half-lives of weeks or months and present a lengthy selection opportunity. The selection probability can be considered as a function of the slope of the concentration–effect relationship (a sigmoid E_{max} model is usually fitted of the general form: $E(C) = E_{max}\, C^N/(C^N + EC_{50}^N)$, where the antimalarial effect is E at concentration C, N is a parameter affecting the steepness of the slope and EC_{50} is the concentration producing 50% of the E_{max} or maximum effect), and the first-order terminal elimination rate constant (k_e) for the drug. The relative importance of the elimination phase, where reinfection can take place in the presence of declining blood levels, in determining *de novo* resistance selection depends on two ratios:

(i) the ratio between the total number of parasites exposed to sub-therapeutic concentrations of the drug in treatment of the acute infection versus the total number of newly acquired infections exposed during the elimination phase; and
(ii) the relative probabilities of this *de novo* resistant mutant surviving and being transmitted in these two contexts.

The probability of providing a selective drug concentration, and thus preferential survival of a resistant parasite, during the elimination phase depends the degree of right shift in the concentration–effect relationship, its slope and the terminal elimination half-life of the drug (White 1999*a*). The probability of transmission depends on immunity, subsequent drug exposure, parasite multiplication capacity (which must take into account any fitness disadvantage conferred by the resistance mechanism), the reduction in susceptibility conferred by the resistance mechanism, and intra-host competition from drug-sensitive parasites. There is then a variable 'window of opportunity' for the selection of *de novo* resistance from an infection newly acquired during the elimination phase (figure 5*a*). The window opens when the concentration of antimalarial drug falls below the IC_{90}^R (when the resistant mutant PMR = 1). The window closes when the drug concentration falls below that at which the multiplication of the usually single drug-resistant mutant can outstrip the growth of the *ca.* 100 000 sensitive sibling parasites emerging from the liver to generate sufficient gametocytes for transmission. The resistant mutant is therefore in a 'race' with its siblings to attain parasite densities sufficient to transmit. Once these densities are reached, multiplication of all parasites slows and the opportunity for a small sub-population of parasites to reach transmissible densities is lost. This 'start' or numerical advantage of the sensitive sibling parasites in the race to produce gametocytes creates a boundary condition for elimination half-life, relative to the degree of resistance induced (both slope and shift in the concentration–effect relationship), below which *de novo* selection during the elimination phase *cannot* take place. Asynchronous hepatic schizogony further tips the balance in favour of the majority sensitive parasites. For drugs with short elimination half-lives (less than 4 days), a small increment in drug resistance cannot lead to selection because, by the time drug levels have fallen to the IC_{90}^S (the IC_{90} for the sibling-sensitive parasites) there still remain more sensitive than resistant parasites in the body (figure 5*b*).

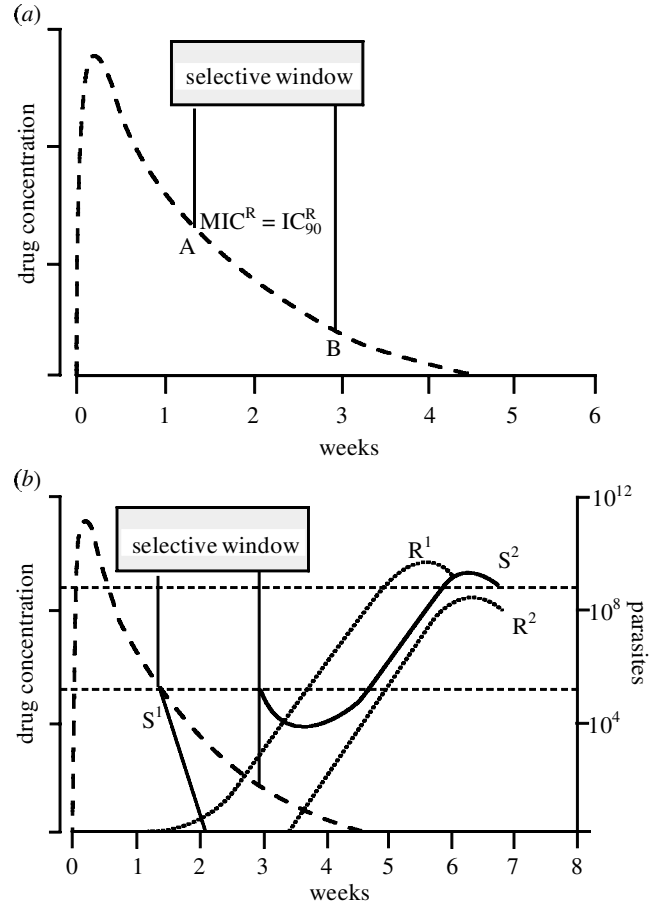

Figure 5. The opportunity for selection of *de novo* resistant malaria parasites during the elimination phase. (*a*) The selection of resistance from exposure of a newly acquired infection to residual antimalarial drug concentrations of a slowly eliminated drug. The window of selection of resistance opens at A when the blood concentration (*y*-axis) falls below the IC_{90} for the resistant mutant (IC_{90}^R, which corresponds approximately to the concentration producing a multiplication rate of one) and closes at B when the net growth of the 100 000 drug-sensitive parasites which emerge from the liver outstrips that of the single drug-resistant mutant to reach the density required to produce transmissible numbers of gametocytes. (*b*) If an infection emerges from the liver (there are *ca.* 10^5 malaria parasites at this stage) when the selection window opens, a resistant parasite R^1 (dotted line) will survive and multiply to produce an infection capable of reaching the parasite densities (*ca.* 10^9 malaria parasites) needed for transmission, whereas the remaining sensitive parasites S^1 will be eliminated. If an infection emerges from the liver just after the selection window closes, then the growth of the sensitive parasites S^2 outstrips that of the resistant parasites R^2 (dotted line). When these reach detectable levels population growth stops. The resistant parasites do not attain transmissible densities, and there is no selection of resistance.

With the exception of the artemisinin derivatives, maximum antimalarial parasite reduction ratios (kill rates) do not exceed 1000-fold per cycle (White 1997). Following hepatic schizogony exposure of at least two asexual cycles (4 days) to therapeutic drug concentrations is therefore required to eradicate the blood-stage parasites emerging from the liver. Even with maximum kill rates in the sensitive parasites and maximum growth rates in the resistant parasites, the resistant parasites only 'overtake' the sensitive parasites in the third asexual cycle. Thus rapidly eliminated drugs (such as the artemisinin derivatives or quinine) *cannot* select for *de novo* resistance during the elimination phase. Obviously the greater the degree of resistance conferred by the resistance mutation (i.e. the higher the IC^{R}_{90} relative to IC^{S}_{90}) the wider is the window of selection opportunity.

Patent gametocytaemia is more likely in recrudescent than primary infections. Therefore, if *de novo* resistance arose in an acute symptomatic treated infection, the transmission probability from the subsequent *recrudescent* infection (bearing the new resistance genes) would be higher than from a *primary* infection arising from selection of resistance in an infection newly acquired during the elimination phase of the antimalarial drug given for a previous infection, even if it attained the same parasite densities (Price *et al.* 1999).

It has been suggested that the repeated exposure of parasite populations to residual drug concentrations of slowly eliminated drugs in areas of frequent infection is an important source of resistance (Bloland *et al.* 2000). But what is the probability that resistance can be selected *de novo* from residual antimalarial drug levels in this way? Approximately 10^5 merozoites leave the liver after hepatic schizogony. This is the total number of parasites from which a resistant mutant can arise, and if resistance does develop, it is most likely that only one parasite is resistant. Very rarely a *de novo* mutation will have occurred early during intra-hepatic development and some, or all, of these parasites will be resistant. Assuming an equal probability of mutations among blood-stage parasites, the probability of resistance arising during the first asexual cycle following emergence from liver (10^5 parasites) is therefore between 1000 and 10^7 times lower than in a symptomatic infection. In the earlier example, in which the per-parasite mutation frequency for resistance was 1 in 10^{12}, the probability of there being one resistant parasite in a symptomatic infection with 10^{11} parasites at the peak of infection is therefore 10%, and for 10 or more resistant parasites is 1.11%: but resistance would arise immediately following emergence from the liver in only 1 in 10^7 malaria infections. To survive, this usually single resistant parasite must encounter the selective antimalarial blood concentrations described above.

Selection from an infection which emerges from the liver during the elimination phase of antimalarial treatment given previously (or during prophylaxis) usually takes place in the *first* generation of blood-stage malaria parasites (*ca.* 10^5 parasites). It is unlikely to arise later because, for a resistant mutant to arise and survive from a larger number of parasites in generations *subsequent to the first following hepatic schizogony*, the antimalarial blood concentrations must have fallen below the minimum inhibitory concentration (MIC) *for the sensitive parasites* (otherwise their numbers would not have increased). If antimalarial concentrations exceed the sensitive parasites' MIC, then total parasite numbers will fall, and the chance of resistance selection in subsequent generations falls in parallel. In bacteria the maximum differential selection for antibiotic resistance occurs at concentrations which exert between 20% and 80% of the maximum effect for the sensitive organisms (Lipsitch & Levin 1997). The MIC for most antimalarials corresponds to a concentration exerting less than 20% of maximum effect: PMR1/PMR10–20.

Taken together the balance strongly favours the acute symptomatic infection as a source of *de novo* resistance because vastly more parasites are present then. But the long elimination phase of the antimalarial does provide a very efficient selective filter for resistant infections *acquired from elsewhere*, as it allows resistant infections to develop but suppresses sensitive infections. This selectively amplifies resistance. Thus, although it is a very unlikely source of *de novo* resistance, the duration of the antimalarial drug's elimination phase *is* a very important determinant of the spread of antimalarial drug resistance (Watkins & Mosobo 1993; Hastings *et al.* 2002).

In summary, the opportunity for differential selection of *de novo* resistance by exposure of *newly acquired infections to residual antimalarial drug levels* is greatest within the *ca.* 10^5 parasites in the first generation after emergence from the liver, providing a per-infection probability some *five million times* lower than that in a fully developed symptomatic infection (1% parasitaemia) in an adult, or a child with 5% parasitaemia. In up to 90% of cases (a proportion determined by the PMR) only a single resistant organism confronts the antimalarial drug. Put another way, if an individual acquires 20 symptomatic and potentially transmissible infections per year for 50 years, then the selection probability from residual drug exposure to newly acquired infections in that half-century would be 1% of that in a single symptomatic infection of 10^{12} parasites.

7. IMPLICATIONS FOR ANTIMALARIAL PROPHYLAXIS

The use of antimalarial prophylaxis by travellers has been considered an important potential source of resistance. This is unlikely to be true for the following reasons.

(i) If an infection is acquired when effective antimalarial drug concentrations are already present, the number of parasites exposed (after emergence from the liver) and therefore the selection probability is orders of magnitude lower than during the acute infection. For example if a person took antimalarial prophylaxis in an area of very intense transmission (Entomological Inoculation Rate 100), then 10 years of continuous prophylactic drug use would provide the same *de novo* resistance selection probability as a single mild infection (10^8 parasites). In a low-transmission setting (EIR 1), 1000 person years of prophylaxis would provide this risk.

(ii) The parasites which emerge from the liver usually encounter therapeutic concentrations of the antimalarial, and even if adherence (compliance) is poor, the probability of the infection reaching the densities required for gametocytogenesis and then transmitting is relatively low.

(iii) Travellers often leave the endemic area before they become ill, and if they do not, they are usually nursed in a setting where transmission risk is lower.

8. PREVENTION OF RESISTANCE BY COMBINATIONS

The theory underlying combination treatment of tuberculosis, leprosy and HIV infection is well known, and has recently been applied to malaria (Peters 1969, 1987, 1990; Curtis & Otoo 1986; Chawira *et al.* 1987; White 1997, 1999*b*; White *et al.* 1999). If two drugs are used with different modes of action, and therefore different resistance mechanisms, then the per-parasite probability of developing resistance to both drugs is the product of their individual per-parasite probabilities. For example, if the per-parasite probabilities of developing resistance to drug A and drug B are both 1 in 10^{12}, then a simultaneously resistant mutant will arise spontaneously every 1 in 10^{24} parasites. As there are *ca.* 10^{17} parasites in the entire world, and a cumulative total of less than 10^{20} in 1 year, such a simultaneously resistant parasite would arise spontaneously roughly once every 10 000 years, provided the drugs always confronted the parasites in combination. Thus the lower the *de novo* per-parasite probability of developing resistance, the greater the delay in the emergence of resistance.

Stable resistance to the artemisinin derivatives has not yet been identified, and cannot be induced yet in the laboratory, which indicates that it may be very rare indeed. *De novo* resistance to chloroquine is also very rare, and appears to have arisen and spread only twice in the world during the first decade of intensive use in the 1950s, and only four times in total (Su *et al.* 1997). However, antifol and atovaquone resistance arises relatively frequently (e.g. antifol resistance rose to high levels within 2 years of the initial deployment of proguanil in peninsular Malaya in 1947) and can be induced readily in experimental models (Peters 1990; Looareesuwan *et al.* 1996). Sulphadoxine–pyrimethamine resistance has emerged rapidly, but much less rapidly than would be predicted for pyrimethamine alone, which suggests 'protection' by the antimalarial activity of the sulphonamide component. On a background of chloroquine resistance, mefloquine resistance arose over a 6-year period on the northwest border of Thailand (suggesting a starting frequency for significant mefloquine resistance of *ca.* 1 in 10^{14} parasites in this context; Nosten *et al.* 1991, 2000; table 1).

Artemisinin derivatives are particularly effective in combinations with other antimalarials because of their very high killing rates (parasite reduction ratio *ca.* 10 000-fold per cycle), lack of adverse effects and absence of significant resistance (White 1999*b*). The ideal pharmacokinetic properties for an antimalarial drug have been much debated. Rapid elimination ensures that the residual concentrations do not provide a selective filter for resistant parasites, but these drugs (if used alone) must be given for 7 days to ensure cure, and adherence to 7-day regimens is poor. In order to be effective in a 3-day regimen, elimination half-lives usually need to exceed 24 h. Combinations of artemisinin derivatives (which are eliminated very rapidly) given for 3 days, with a slowly eliminated drug such as mefloquine, provide complete protection for the artemisinin derivatives if adherence is good (i.e. no parasite 'sees' artemisinin during one asexual cycle without mefloquine being present), but they do leave the slowly eliminated 'tail' of mefloquine unprotected (White 1997). Perhaps resistance could arise within the residual parasites which have not yet been killed by the artemisinin derivative? However, the number of parasites exposed to mefloquine alone is a tiny fraction (less than 0.000 01%) of those in the acute symptomatic infection. Furthermore these residual parasites 'see' relatively high levels of mefloquine and, even if susceptibility was reduced, these levels may be sufficient to eradicate the infection. The long mefloquine tail does, however, provide a selective filter for resistant parasites acquired from elsewhere, and therefore contributes to the spread of resistance once it has developed. But on the northwestern border of Thailand, an area of low transmission where mefloquine resistance had developed already, systematic deployment of the artesunate–mefloquine combination was dramatically effective, both in stopping resistance and also in reducing the incidence of malaria (Nosten *et al.* 2000, Brockman *et al.* 2000). This strategy would be expected to be effective at preventing the *de novo* emergence of resistance at higher levels of transmission, where the high-biomass infections still constitute the major source of *de novo* resistance.

The main obstacles to the success of combination treatment in preventing the emergence of resistance if they are deployed will be incomplete coverage or inadequate treatment and, as for antituberculous drugs, use of one of the combination partners alone. Unfortunately, policy makers are often reluctant to deploy antimalarial combinations until resistance has already emerged to one of the compounds. Sub-standard drugs are common in tropical areas of the world, adherence to antimalarial treatment regimens is often incomplete, and antimalarials are available widely in the market place. Resistance to the artemisinins may not have happened yet. If it does arise it would be most likely to arise in a hyperparasitaemic patient who received an inadequate dose, and no other antimalarial drug. Patients with high parasitaemias and those with severe malaria require more treatment than patients with uncomplicated malaria to prevent subsequent recrudescence (Price *et al.* 1998), so ensuring that such patients receive a full course of treatment with two drugs would be an efficient method of preventing the *de novo* emergence of resistance.

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APPENDIX A: WHO SELECTS RESISTANCE IN A HIGH-TRANSMISSION SETTING?

(a) *Example*

For simplicity, in an area of intense malaria transmission the population is divided into non-immunes (ages 0–4) who comprise 20% of the population and immunes (age 5 years or more) who make up the other 80%. The EIR is 100 yr^{-1}. Chloroquine is the available treatment.

(i) *Young children*

Thirty-four infections per child per year reach densities capable of transmitting: 20 infections produce 10^9; 10 infections produce 10^{10}; 3 infections produce 10^{11}; and 1 infection produces 10^{12} parasites. On average 10 are treated, but, because the drug is eliminated slowly, all infections are drug exposed. Note that because of the logarithmic distribution of parasite numbers the single high-biomass infection contributes more than all the remaining infections combined.

Total number of 'potentially resistance developing and transmissible' parasites yr^{-1} per child = 1.4×10^{12}.

(ii) *Older children and adults*

The number of infections averaged across the age groups reaching densities capable of transmitting is 11 per person per year: 5 infections produce 10^9; 5 infections produce 10^{10}; and 1 infection produces 10^{11} parasites. These are not symptomatic and therefore not treated, but drug exposure occurs in half the cases because misuse of drugs is common.

Total number of 'potentially resistance developing and transmissible' parasites yr^{-1} per person = 1.55×10^{11}.

Young children comprise 20% of the population, so in this simple example the relative contributions of young children to 'potentially resistance developing and transmissible' infections overall in the community is $1.4 \times 10^{12}/[4(1.55 \times 10^{11}) + 1.4 \times 10^{12}]$ or 69%, of which a single infection per child per year contributed half the total.

In this hypothetical example 15 transmissible infections per 100 people per year generated an EIR of 100, which is approximately one-sixth of transmissible infections resulted in a new infection.

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