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# *In vitro* resistance patterns of *Plasmodium falciparum* to chloroquine—a reflection of strain-specific immunity?

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**Abstract**

Studies *in vitro* among children on the response of *Plasmodium falciparum* to chloroquine were conducted as part of the national long-term monitoring of drug resistance in a holo- to hyperendemic malarious area of Tanzania between 1983 and 1989. Overall, no significant increase in chloroquine resistance was observed. However, in children under 5 years old resistance increased during this period, whereas in schoolchildren resistance decreased from 1986 to 1989. A hypothesis based on antigenic differences between resistant and sensitive strains is proposed to explain this age-specific pattern. If immunity develops principally against the most frequent parasite strains, then as it develops the numbers of the most frequent strains will be reduced, whilst the rare strains may become predominant and thus be detected in the blood of immune patients. Thus, in an endemic area, the observed resistance pattern in non-immune infants will differ from that in immune schoolchildren, as was observed in the present study. These findings may have important implications for the control of malaria and the development of vaccines.

## Introduction

Chloroquine resistance of *Plasmodium falciparum* isolates from immune Tanzanians was first reported in 1982 (ONORI *et al.*, 1982; KIHAMIA & GILL, 1982). In spite of increasing resistance, which in some areas of East Africa may be as high as 71% *in vivo* and 66% *in vitro* (SPENCER, 1985), chloroquine remains the drug of choice for malaria treatment in Africa because of its low cost and ready availability. A comprehensive picture of chloroquine resistance is therefore relevant for the treatment and control of malaria (MOLINEAUX, 1986). The present study was undertaken as part of the national Tanzanian monitoring of drug resistance in different parts of the country to rationalize drug policy.

Current knowledge of the biology and epidemiology of resistance indicates that high drug pressure selects resistant parasites (PETERS, 1987), whilst reduced drug pressure selects sensitive parasites (THAITHONG *et al.*, 1988; JACQUIER *et al.*, 1985). However, nothing is known regarding possible modulation of this selection pressure by acquired immunity in endemic areas. The current study, which was undertaken in a holo- to hyperendemic area with increasing drug pressure, compared the evolution of chloroquine resistance in children under 5 years old and in schoolchildren in an attempt to examine the possible effect of acquired immunity on the observed expression of chloroquine resistance *in vitro*.

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## Materials and Methods

*Study area*

The study was carried out in the Ifakara division (Kilombero District, Morogoro Region), a rural area comprising some 70 000 individuals in south-eastern Tanzania. The rainfall varies between 1000 and 1600 mm annually with the heaviest rains from March to May. Malaria is hyper- to holoendemic with transmission peaks occurring in June and July. The most prevalent species, occurring in more than 90% of the infections, is *P. falciparum* (TANNER *et al.*, 1987b). The study area has recently been described in detail by TANNER *et al.* (1987a).

Studies *in vitro* were conducted at the Ifakara St Francis Designated District Hospital and at village health posts in rural communities between 1983 and 1985 (TANNER *et al.*, 1987c). Most of the patients (70–83%) involved in these studies were children under 5 years old; only such children were included in the analysis. Subsequent studies were conducted every April from 1986 to 1989 at Ifakara school involving pupils aged 8 to 16 years and from 1986 to 1988 at the Ifakara Mother and Child Health Clinic involving children aged 10 to 36 months.

In each of the studies thick blood films were made. Only children with a parasitaemia >800 parasites/μl, who had not taken chloroquine during the 21 d before the investigation, and with a negative Dill-Glazko test, entered the study. Subjects were residents of Kilombero District. All patients were subsequently treated with 25 mg/kg chloroquine base over 3 d. Drug ingestion was supervised at the school.

In vitro *tests*

Instructions for use of the *in vitro* micro-test kit supplied by the World Health Organization (WHO) were followed (WHO, 1987). Until 1986, whole blood samples were processed as described by TANNER *et al.* (1987a). Briefly, a blood sample of 100 μl was transferred into 900 μl RPMI 1640 medium buffered with 25 mmol/litre HEPES, 25 mmol/litre $NaHCO_3$, and 200 mmol/g L-glutamine. Starting in 1987, this medium was supplemented with a standardized packed cell volume (PCV) of 50 μl. 50 μl aliquots of the mixture were added to wells of pre-dosed microculture plates supplied by the WHO Production Centre (Manila, Philippines) to provide final chloroquine concentrations of 0 to 6·4 μmol/litre of blood. The plate batches were subsequently checked by WHO. The plates were incubated in a candle jar at 37–38°C

for 24–36 h. The number of schizonts per 200 asexual parasites was counted in each well. Cultures with less than 10% schizonts per control well were excluded.

*Statistical analysis*

The 50% and 90% effective doses (EC 50 and EC 90) of each isolate were estimated by a probit-analysis of the log/dose response (GRAB & WERNSDORFER, 1983). Because EC values were log-normally distributed, all subsequent analyses were performed with the logarithms of the values. Differences of the EC values between years and between age groups were evaluated with analyses of variance. Temporal trends were evaluated with linear regressions of EC values on year. The effect of using PCV was estimated for isolates from schoolchildren in 1988: an analysis of variance using the data of all cultured isolates and a paired $t$ test with the data of those isolates that could be cultured by both methods were performed.

## Results

The individual EC 50 values varied between 0·017 μmol/litre and 1·79 μmol/litre. The overall geometric mean was 0·287 μmol/litre, and the means within years ranged from 0·165 μmol/litre in 1987 to 0·424 μmol/litre in 1986.

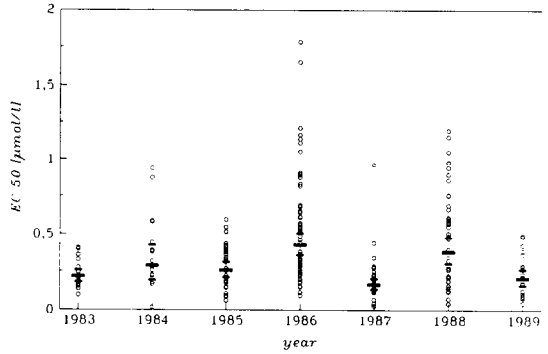



Fig. 1. The individual 50% effective concentration (EC 50) values from each test, presented by year of the study. The geometric mean and its 95% confidence interval are shown for each year by a long bar and short bars, respectively. The data from children under five years old and schoolchildren are pooled.

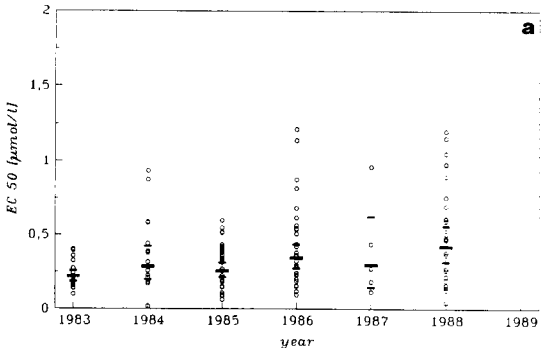
Fig. 1 shows the individual EC 50 values for each isolate, together with the geometric mean and its 95% confidence interval for each year. The data from children aged under 5 years and schoolchildren are pooled. Resistance varied significantly between years (EC 50: $F=10·72$, df [degrees of freedom]=6, $P<0·001$; EC 90: $F=13·39$, df=6, $P<0·001$), but no trend for either an increase or decrease over time was apparent (EC 50: $r^2=0·00005$, $P=0·91$; EC 90: $r^2=0·003$, $P=0·57$). Separate analysis of the resistance of isolates obtained from children under 5 years old (Fig. 2a) and from schoolchildren (Fig. 2b) showed a significant interaction of years and age (EC 50: $F=6·14$, df=2, $P=0·003$; EC 90: $F=9·96$, df=2, $P<0·001$). The resistance of isolates from children aged under five years increased with time (EC 50: $r^2=0·09$, $P<0·001$; EC 90: $r^2=0·17$, $P<0·001$). In contrast, EC 50 of isolates from schoolchildren decreased significantly with time ($r^2=0·11$, $P<0·001$) and EC 90 showed a slight, though not significant, downward trend ($r^2=0·02$, $P=0·11$). As a result, mean resistance of the isolates from schoolchildren was higher than that of those from children aged under 5 years in 1986, but was lower in 1987 and 1988 (Fig. 2). These results are summarized in the Table.

In 1988, the geometric mean EC 50 found using the original method was 0·309 μmol/litre, and the mean EC 50 using the supplemented PCV was 0·323 μmol/litre. The corresponding EC 90 values were 0·791 μmol/litre and 0·661 μmol/litre. The differences were not statistically significant (EC 50:

**Table. Linear trends of resistance between 1983 and 1989 in Ifakara, Tanzania**

| Age class | Trend | EC 50[a] | EC 90[a] |
|---|---|---|---|
| All children | None | $b=0·003$<br>$P=0·91$ | $b=0·028$<br>$P=0·37$ |
| Children under-five years old | Linear increase | $b=0·126$<br>$P<0·001$ | $b=0·197$<br>$P<0·001$ |
| School-children | Linear decrease | $b=-0·233$<br>$P<0·001$ | $b=-0·116$<br>$P=0·11$ |

[a]Abbreviations: $EC_{50}$ and $EC_{90}$=50% and 90% effective concentrations respectively; $b$=slope of log (resistance) over time (μmol/litre per year); $P$=significance level.



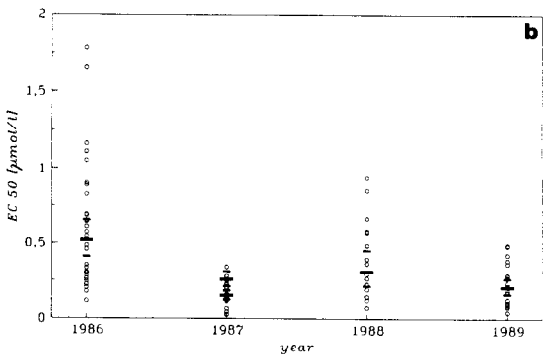




Fig. 2. The individual 50% effective concentration (EC 50) values from each test, presented by year of the study. The geometric mean and its 95% confidence interval are shown for each year by a long bar and short bars, respectively. The data from children under five years old (panel a) and schoolchildren (panel b) are shown separately.

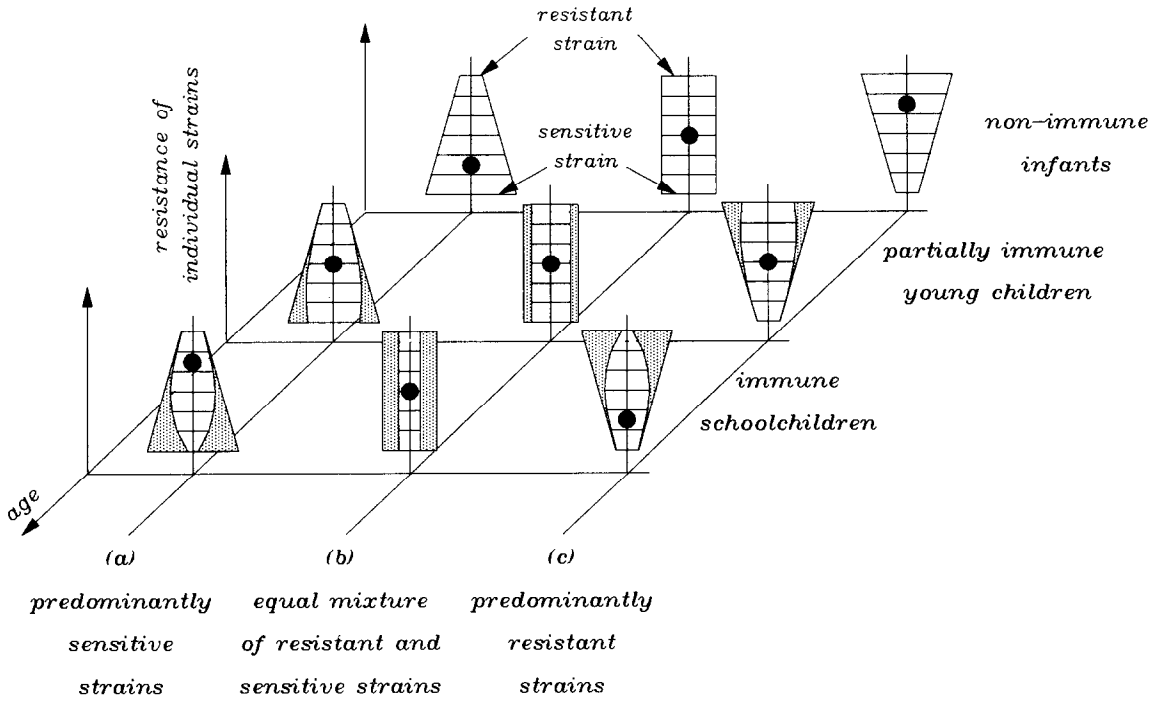



Fig. 3. Schematic presentation of the effect of antigenic differences between resistant and sensitive parasites on *in vitro* resistance observed in isolates. The age-specific changes of observed resistance are sketched for areas with low (a), intermediate (b), and high (c) frequencies of resistant parasites. Six parasite strains with varying resistance are shown. The relative frequency of each individual strain within isolates is indicated by the width of the horizontal bars, and the observed resistance of isolates from each age-class by a black circle (●). The parasites cleared by the immune system are shown as the stippled area.

(a) When most parasites are sensitive, non-immune infants harbour predominantly sensitive strains, and observed resistance is low. Immunity is stimulated almost exclusively by the sensitive strains, so that, with increasing age, patients clear an increasing proportion of the sensitive parasites. Resistant parasites are rare, thus stimulating immunity only slightly, and cannot be cleared. Therefore, parasites in isolates from immune schoolchildren are predominantly resistant, and observed resistance increases with age. (b) All strains are equally frequent, so that schoolchildren develop immunity against sensitive and resistant parasites. Immunity can clear all strains, so that the observed resistance in schoolchildren remains equal to that in non-immune infants. (c) Resistant strains predominate in the population. Immunity is developed principally against these resistant strains, so that predominantly resistant strains are cleared by the immune system. Therefore, observed resistance decreases with age.

$F=0{\cdot}03$, df=1, $P=0{\cdot}87$; EC 90: $F=0{\cdot}46$, df=1, $P=0{\cdot}50$), nor were the differences between these results and those of a paired test incorporating only the isolates that could be cultured with both methods (EC 50; $t=1{\cdot}81$, df=7, $P=0{\cdot}11$; EC 90; $t=1{\cdot}21$, df=7, $P=0{\cdot}26$).

## Discussion

Between 1983 and 1988 the isolates from the children under five years old showed an increase in chloroquine resistance. During the same period, due to the liberalization of the national trade policy, chloroquine became more readily available. Personal observations estimate an increased purchase from 350 000 to 500 000 chloroquine tablets (150 mg base) between 1986 and 1988. Available brands of chloroquine tablets were found to contain adequate drug levels. Following the pattern of resistance reported in other longitudinal studies, it was expected that chloroquine resistance would increase.

However, contrary to the expectation, between 1986 and 1989 resistance in isolates from schoolchildren decreased. This contrasting pattern in schoolchildren suggests that resistance is not affected by drug pressure alone, but by additional, age-dependent factors. Since in endemic areas immunity against malaria increases with age, such a factor may be an interaction of chloroquine resistance with the immune system.

Such an interaction is known to affect studies *in vivo* (MOLINEAUX, 1986; TARGETT, 1984) through the synergistic action of the drug and immunity. Chloroquine treatment can clear only sensitive strains of the parasite. The remaining, resistant parasites may be cleared by the host's immune system. Resistant parasites therefore may not be detected in immune hosts, but would appear in the blood of non-immune hosts. If this holds true, then resistance *in vivo* should decrease with age of the host, and this pattern has been indicated in studies carried out in Tanzania and Kenya (MUTABINGWA *et al.*, 1985; BRANDLING-BENNETT *et al.*, 1988).

Studies *in vitro* measure resistance of the isolate without the direct influence of the host's immunity. One hypothesis explaining the contrasting evolution of *in vitro* resistance in different age groups is based on the suggestion that chloroquine-resistant strains of *P. falciparum* differ antigenically from sensitive strains (PETERS, 1987). Thus resistant and sensitive parasites stimulate their host's immune system partly indepen-

dently. The effect of this hypothesis on observed resistance *in vitro* is shown in Fig. 3. When most parasites are sensitive (Fig. 3a), immunity is acquired principally against sensitive strains. Therefore, the immune system of schoolchildren can clear many of the sensitive parasites, whereas any resistant strain can break through the immunity. Thus most of the observed infections in immune schoolchildren are resistant. In contrast, the immune system of children under 5 years of age is not fully developed and cannot clear any of the parasites, so that the infections observed in non-immune infants reflect the level of resistance in the general parasite population. As the frequency of resistant strains in the population increases (Fig. 3b), so does the frequency of resistant infections in infants. Immunity is increasingly developed against resistant strains, schoolchildren can now clear resistant as well as sensitive strains, and the observed level of resistance in schoolchildren drops. As resistant parasites become predominant (Fig. 3c), the immune system can clear principally these resistant parasites. Now any sensitive strain can break through the immunity, and the observed resistance in schoolchildren drops below that observed in non-immune infants.

In summary, antigenic differences in immunogenic epitopes between resistant and sensitive strains lead to interactions between the patient's age and the parasite's *in vitro* resistance. The hypothesis leads to three predictions. First, as observed resistance in non-immune children increases, resistance in immune schoolchildren decreases. Secondly, in areas with low resistance, isolates from schoolchildren show higher resistance than isolates from infants, and vice versa in areas with high resistance. Thirdly, this pattern of interaction is found only in highly endemic areas where immunity against *P. falciparum* is developed. The first two of these predictions are confirmed by the results of the present study. The third prediction has, to our knowledge, not so far been evaluated.

It should be noted that our results were apparently not due to supplementing the culture medium with PCV after 1986. Isolates cultured by both the previous and the supplemented methods had similar resistances. Furthermore, although identical methods were used for all tests in any given year, the observed pattern differed between age groups. Thirdly, the trend observed in children under five years old before 1986 was continued after the method had been changed.

The pattern observed, and in particular the hypothesis that chloroquine-resistant strains of *P. falciparum* are antigenically distinct from sensitive ones (PETERS, 1987), requires confirmation. The most obvious consequence of the hypothesis is that the comparison of *in vitro* resistance between areas and between years is meaningful only if the immune status of the host is controlled for. Furthermore, the topic has great importance for strategies for the control of malaria in the community as well as for treatment in the individual. Perhaps most importantly, strain-specific immunity related to the level of chloroquine resistance should be fully appreciated during any development of future malaria vaccines.

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