CLEARANCE OF DRUG-RESISTANT PARASITES AS A MODEL FOR PROTECTIVE IMMUNITY IN *PLASMODIUM FALcipARUM* MALARIA

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Abstract. Residents of malaria-endemic areas sometimes spontaneously clear *Plasmodium falciparum* infection without drug treatment, implying an important role for host factors such as immunity in this clearance. Host factors may also contribute to clearance of parasites resistant to a treatment drug. Chloroquine resistance is caused by point mutations in *P. falciparum* chloroquine resistance transporter (*pfCRT*) gene. We investigated the clearance of malaria parasites carrying the key chloroquine resistance-conferring PICRT mutation K76T in patients treated with chloroquine. We found that the ability to clear these resistant parasites is strongly dependent on age (the best surrogate for protective immunity in endemic areas), suggesting that host immunity plays a critical role in the clearance of resistant *P. falciparum* infections. Age-adjusted comparison of subjects able to clear resistant parasites and those unable to do so provides a new phenotype for identifying host immune and genetic factors responsible for protective immunity against malaria.

INTRODUCTION

Many studies in malaria-endemic areas have shown that the prevalence of malaria infection and the incidence of clinical malaria episodes differ among age groups. This relationship is complex, being determined by multiple factors that vary among locations, but in general older subjects show increased protection that has long been thought to be due to acquired immunity. Various immune responses increase with age in inhabitants of malaria-endemic areas, and the challenge is to differentiate those that are critical for protective immunity from those that are simply markers of age and/or of exposure to infection. Part of the problem is to determine what types of host effector mechanisms are truly protective. For example, it has been proposed that the ability of monocytes to kill intra-cellular parasites through antibody-dependent mechanisms is critical and this may explain various correlations observed between immunoglobulin isotype and infection intensity.1,2 The other major issue confronting vaccine developers is to identify parasite antigens that are critical immune targets, and to develop assays that accurately measure the immunogenicity of these antigens when they are formulated as vaccine candidates. For example, antibodies to the 19-kD C-terminal fragment of merozoite surface protein-1 have been associated with protection against clinical episodes of *Plasmodium falciparum* malaria.3 Similarly, variant-specific antibodies to *P. falciparum* erythrocyte membrane protein-1 appear to reduce the risk of clinical attacks due to the homologous strain of *P. falciparum*.4

A limited number of epidemiologic models are available to identify immune correlates of clinical protection. Case-control studies may be used to define correlates of susceptibility or resistance to severe malaria but do not distinguish factors that suppress infection in the absence of drug treatment from those that determine disease severity once infection has occurred. These studies also provide only a single time-point for comparison, and may miss important factors by comparing severe malaria cases to uncomplicated malaria controls that are different from the cases only with respect to their stage in the natural history of the disease when they come to medical attention. Longitudinal studies or large cross-sectional studies can explore immunologic correlates of infection intensity or of clinical attack rates, but since both measures may vary greatly with time in a single individual, significant inaccuracy arises from practical and ethical constraints on sampling frequency. Moreover, such studies do not distinguish between factors that prevent infection and those that clear parasites after a clinically significant infection has occurred. Many malaria infections are cleared without treatment after variable periods of time, but for obvious ethical reasons it is not feasible to study this phenomenon and its immune determinants in untreated individuals.5,6 This paper explores ethically acceptable ways of identifying and validating immune correlates of clinical protection against malaria in individuals receiving standard malarial treatment.

In Mali, West Africa, approximately 15–20% of *P. falciparum* infections fail to respond to chloroquine treatment. The public health policy, based on World Health Organization recommendations, is to treat uncomplicated malaria episodes with chloroquine as the first-line drug, and to reserve sulfadoxine-pyrimethamine as the second-line drug for those individuals who fail chloroquine treatment. This study arose from the recent characterization of a molecular determinant of chloroquine resistance. Through a combination of genetic mapping studies, population surveys, and mutational analysis by parasite transfection, a specific lysine to threonine mutation (*P. falciparum* chloroquine resistance transporter [*pfCRT*] K76T gene) has been identified as the determinant of chloroquine resistance in laboratory strains of *P. falciparum*.7,8 This marker has also been shown to be predictive of rates of in vivo chloroquine resistance among different settings when adjusted for age.9 Although mutations in another candidate chloroquine resistance gene (*P. falciparum* multiple drug resistance 1 [*pfdmR 1*] gene) have been shown to modulate the

* Deceased.
level of in vitro chloroquine resistance in clones that were already resistant, these mutations did not confer resistance directly and no evidence has been found that this modulation affects treatment outcomes in vivo, nor has an interaction between pfcr and pfmdr1 been found.11

Extensive epidemiologic studies have shown that the pfcr76T mutation is the most predictive marker of chloroquine resistance in the field.8,11 In this study, we show that following a clinical malaria episode treated with chloroquine, a significant proportion of individuals clear parasites carrying the chloroquine resistance mutation. We examine the epidemiologic correlates of this clearance phenomenon, and explore its possible utility as a phenotypic marker of protective immunity in immunologic and genetic studies.

MATERIALS AND METHODS

Study sites. Prospective chloroquine efficacy studies were conducted in 1997 in Mopti (population = 60,000) in the river delta of central Mali with a highly mixed ethnic composition but a Fulani majority; in 1997 and 1998 in Bandiagara (population = 12,500) on the relatively arid escarpment east of Mopti with a predominantly Dogon population; and in 1998 and 1999 in Kolle (population = 2,500) a rural village in southern Mali with a nearly exclusively Malinké population. Malaria is endemic in all three sites with seasonal peaks of transmission. Rates of malaria transmission vary, with 40.9 infected bites per person per month at the peak of the malaria season in Mopti, 5.2 in Kolle, and 4.0 in Bandiagara.9

All protocols were reviewed and approved by the Institutional Review Board (IRB) of University of Mali. In addition, the studies in Mopti 1997 and Bandiagara 1997 and 1998 were approved by IRBs of University of Maryland and the U.S. National Institute of Allergy and Infectious Diseases. Informed consent was obtained from each patient or, in case of minors, from parents or guardians.

Chloroquine efficacy. Patients presenting with uncomplicated P. falciparum malaria were recruited. After obtaining informed consent, in vivo parasitologic resistance to chloroquine was measured as described.12 Briefly, resistance was defined as follows: RIII = persistent parasitemia with reduction to > 25% of initial parasitemia (or no reduction in parasitemia) by day 3; RII = persistent parasitemia with reduction to < 25% of initial parasitemia; RI = initial clearance of parasites, with subsequent parasitemia by day 14; sensitive = clearance of parasites with no recurrence of parasitemia by day 14. Therapeutic efficacy was measured according to the World Health Organization 1996 protocol.13

Inclusion criteria in Mopti and Bandiagara 1997 were an age ≥ 2 years; a positive thick or thin blood smear for monospecific P. falciparum asexual forms and treatment seeking for symptoms consistent with malaria (fever, chills, headache, body aches); no history of allergy to chloroquine; no signs of concurrent non-malarial febrile illness, severe malnutrition, or other significant medical illness; and no signs of severe malaria (coma, obtundation, seizures, prostration, respiratory distress, shock, protracted vomiting, parasitemia greater than 100,000/mm3, hematocrit < 15%). The same inclusion criteria were used in Bandiagara in 1998 and in Kolle in 1998 and 1999 with the exception of age. In the later studies, children ≥ 6 months old were included. Chloroquine phosphate was administered directly by study staff at doses of 10 mg/kg on day 0, 0 mg/kg on day 1, and 5 mg/kg on day 2. Subjects were observed after each dose, and a full dose was repeated if vomiting occurred within 30 minutes and a half dose was repeated for vomiting within one hour. Subjects were followed up for 14 days as described.12 Briefly, on post-treatment days 1 and 2, symptoms, other medications, temperature, and results of a physical examination were recorded, but microscopy was not routinely performed. The same clinical information was collected and thick and thin blood smears for microscopic diagnosis of malaria were prepared on days 3, 7, and 14. Whenever microscopy was performed, finger prick blood was also blotted onto filter paper strips (3MM; Whatman, Hillsboro, OR) for extraction of DNA and subsequent molecular biology analyses. Asexual forms were counted at 1,000× magnification on Giemsa-stained thick smears against 300 leukocytes as described.14 Smears were considered to be negative only after the entire thick smear was read. Parasitologic chloroquine treatment outcome were categorized as sensitive and resistance levels as RI, RII, or RIII according to a modification of World Health Organization protocols.12 Therapeutic efficacy was classified as an adequate clinical response (ACR), an early treatment failure (ETF), and a late treatment failure (LTF) according to the World Health Organization protocols.

Molecular analysis. DNA was extracted from finger prick blood blotted onto filter paper and the presence or absence of the chloroquine resistance-conferring mutation pfcr76T was determined as described.11 Briefly, a nested mutation-specific polymerase chain reaction (PCR) and/or nested PCR followed by restriction endonuclease digestion were used to determine the pfcr codon 76 genotype. Any samples with restriction digestion results suggesting mixed infections, i.e., infections with both the wild type K76 and the mutant form 76T were repeated and subjected to repeated mutation-specific PCR and/or direct DNA sequencing. Detailed protocols for these methods are available on the Internet at http://medschool.umd.edu/CVD/iplowe.html.

Data analysis and statistics. Samples were analyzed from all pre-treatment infections for which the chloroquine treatment outcome was known. To best assess genetically determined parasite phenotypes, only classic parasitologic definitions of resistance, rather than therapeutic efficacy, were used for data analysis and interpretation. Cases of RI, RII, and RIII resistance were grouped for analysis. Only those samples that carried the pfcr76T mutant were included in the analysis. For analysis purposes, mixed infections were categorized as mutant pfcr76T. Chi-square or Fisher's exact tests for two-tailed significance at P = 0.05 were used for univariate frequency comparisons. Multiple logistic regression analysis was performed using Stata (Stata Corp., College Station, TX).

RESULTS

A total of 1,487 patients were enrolled into the study, of whom 1,401 (94.2%) had a treatment outcome that could be evaluated. The median age of the study population was seven years, and 48.8% of patients were male. Hematocrits were available for 1,226 patients and hemoglobin type was determined in 574 patients from Bandiagara.

The prevalence of parasitologic resistance was 22.6% (316 of 1,401). Most chloroquine-resistant infections were the low level RI type (Figure 1). Analysis of therapeutic efficacy showed that 90.1% (1,284 of 1,425), 4.3% (62 of 1,425), and
5.6% (79 of 1,425) were ACR, ETF, and LTF, respectively. The \textit{pfcrt} codon 76 genotype prior to chloroquine treatment was determined in 1,326 of these infections. The mutant allele \textit{pfcrt} 76T was found in 803 of 1,326 (60.6%). Among those subjects, 246 of 803 (30.6%) did not clear their mutant parasites (i.e., had a resistant \textit{in vivo} outcome) while 557 of 803 (69.3%) successfully cleared their \textit{pfcrt} mutant parasites (i.e., had a sensitive \textit{in vivo} treatment outcome). Because \textit{pfcrt} 76T mutant parasites are always resistant to chloroquine \textit{in vitro}, we hypothesized that host factors contributed to their clearance \textit{in vivo}.\textsuperscript{8}

**Association of \textit{pfcrt} 76T parasite clearance with age, hematocrit and ethnic background.** The role of host characteristics in the ability to clear \textit{pfcrt} 76T mutant parasites was assessed by analyzing the association of clearance rates with age, sex, parasitemia, axillary temperature, residence, ethnic background, hematocrit, and hemoglobin type. Univariate analysis showed that the ability to clear chloroquine-resistant parasites was strongly associated with age \((P < 0.0001)\), hematocrit \((P < 0.001)\), and ethnic background \((P = 0.006)\) (Table 1). There was a trend towards an association between hemoglobin AS/SS and the clearance phenotype, but this did not reach statistical significance (odds ratio \(\text{OR} = 5.2, 95\% \text{ confidence interval [CI]} = 0.69–108.58, P = 0.1\)). Outside of this trend, the clearance phenotype was not dependent on sex, residence, fever, parasitemia, and hemoglobin type.

These results were confirmed by logistic regression with a single dependent variable adjusted for age, temperature, and parasitemia, which showed that age and hematocrit were independently associated with the clearance phenotype \((P < 0.0001 \text{ and } P = 0.02, \text{ respectively})\), and that Fulani subjects were approximately half as likely to clear resistant parasites as were Dogon subjects \(\text{OR} = 0.45, 95\% \text{ CI} = 0.24–0.84, P = 0.013\). Dogon and Malinké subjects had similar resistant parasite clearance rates (Table 2).

**Age and resistant parasite clearance.** Among children 6–11 months old, 77.8\% \((n = 9)\) carrying chloroquine-resistant parasites were able to clear these resistant parasites versus 30.0\% \((n = 50)\) in children 12–23 months old \((P < 0.001)\) and 40.4\% \((n = 52)\) and 45.0\% \((n = 60)\) of children 24–35 and 36–47 months old, respectively. After four years of age, there was a dramatic increase in the ability to clear resistant parasites, with 72.0\% \((n = 68)\), 72.5\% \((n = 51)\), 74.4\% \((n = 43)\), 80\% \((n = 40)\), 66.7\% \((n = 18)\), and 74.0\% \((n = 27)\) of children 4, 5, 6, 7, 8, and 9 years old, respectively, clearing their \textit{pfcrt} 76T mutant parasites (Figure 2). A minimum of 75\% of children \(\geq 10\) years old and at least 93\% of those \(\geq 13\) years old were able to clear their \textit{pfcrt} 76T mutant parasites following chloroquine treatment (Figure 2). The ability to clear chloroquine-resistant parasites was highly dependent on age. Children more than one year of age but less than five years of age were much less likely to clear resistant parasites than younger or older children and adults.

**DISCUSSION**

The ability to clear chloroquine-resistant parasites was strongly associated with age, which is the most consistent correlate of protective immunity in areas endemic for \textit{P. falciparum} malaria. This suggests that acquired immunity due to...
repeated exposure is a dominant factor in clearing malaria parasites. The data indicate that at this site protection in infancy is followed by high vulnerability in those 1–3 years old, then a large increase in protection at four years of age. This is consistent with studies in asymptomatic \textit{P. falciparum} carriers, which showed that subjects living in endemic countries do clear some of their malaria parasites without any anti-malarial treatment in an age dependent manner.\cite{5}

The rate of clearance in infants, which is significantly higher than rates of clearance in young children, is the first demonstration that the widely reported infant protection is at least in part attributable to differences in the ability to clear drug-resistant parasites.\cite{15,16} Although age-related immunologic changes could be involved in parasite clearance,\cite{17,18} high prevalence of fetal hemoglobin, presence of circulating maternal antibodies, or other environmental factors have all been proposed but not proven to be responsible for infant protection in malaria.\cite{19−22}

The resistant parasite clearance phenotype was independently associated with ethnic background. Fulani subjects were less likely to clear their resistant parasites than Dogon or Malinké subjects. These results suggest that there may be genetic characteristics that are important in the host’s ability to mount a protective immune response to malaria infection. Other reports have found differences in malaria morbidity between the Fulani and other ethnic groups in west Africa.\cite{23} However, in those studies, the Fulani were more protected than the other ethnic groups, suggesting that the immune mechanisms involved in clearing resistant parasites after chloroquine treatment may be different from those involved in the measures of protection reported in previous studies.

Subjects capable of clearing their resistant parasites had a significantly higher hematocrit levels than patients who did not clear resistant infections. This suggests that persistent parasitemia with or without previous chloroquine treatment in the later group has led to an increased prevalence of anemia, as shown in another report.\cite{24} The data support the view that the ability to clear resistant parasites does confer protection against malaria-associated anemia.

The rates of chloroquine-resistant parasite clearance found in this study are significantly higher than rates of spontaneous parasite clearance by patients living in malaria-endemic areas.\cite{25} Although the other known \textit{pfcrt} mutations were not investigated in this study, our previous report found no significant difference in the prevalence of \textit{pfcrt} 74I, 75E, 220S, 271E, 326S, 356T, or 371I mutations in \textit{P. falciparum} infections that cleared after chloroquine treatment and those that did not clear after chloroquine treatment.\cite{11} Several reports show that the addition of \textit{pfmdr1} mutations to \textit{pfcrt} 76T does not improve the predictive value of \textit{pfcrt} 76T alone, suggesting that mutations in \textit{pfmdr1} are not likely to explain these differences in the ability to clear \textit{pfcrt} 76T parasites.\cite{11,26,27} Other host-related factors such as differences in diet or in rate of chloroquine absorption or metabolism could contribute to clearance but have not been investigated here.\cite{28}

Recent studies suggest that some anti-malarial drugs act by increasing the rate of parasite removal by the host spleen.\cite{29} We also note that chloroquine treatment has been associated with higher production of host cytokines involved in parasite killing.\cite{30−32} Taken together, these results lead us to hypothesize that chloroquine treatment may be boosting the host’s ability to eliminate the infection even when the drug is not directly or fully effective against resistant parasites.\cite{33}

In areas of moderate levels of chloroquine resistance, the great majority of patients ≥ 4 years old and nine of ten of those ≥ 13 years old were capable of clearing chloroquine...
resistant parasites following treatment with chloroquine. These findings are consistent with those in earlier reports, which showed that even in areas of widespread chloroquine resistance, older children respond better to treatment than younger ones.34–38 These results have potential public health implications in malaria-endemic-sub-Saharan African countries. They suggest that treatment policy changes aimed at switching first-line treatment from chloroquine to other more expensive antimalarial drugs could be initially targeted to children less than five years old, especially when the cost of the second-line drug is an obstacle to implementing a complete policy change.38

Subjects living in malaria-endemic settings are known to clear some of their infections spontaneous,6 However, because it is not ethical to leave clinically symptomatic infections untreated, the immunologic mechanisms involved in the clearance of these infections have not been investigated. Because a molecular marker of chloroquine resistance provides a precise measurement of the clearance of drug-resistant parasites, this phenomenon is a potentially valuable phenotypic marker of protective immunity in immunologic and genetic studies. Similar approaches may be possible with other drugs for which molecular markers for resistance have been identified, such as sulfadoxine-pyrimethamine.39 Once adjusted for age, comparing characteristics of resistant parasite clearers and non-clearers represents an ethically acceptable method that may shed light on the immunologic and genetic determinants of the clearance phenotype. These determinants may lead to the discovery of immune correlates of malaria protection that would serve as valuable tools for evaluating antimalarial vaccine candidates.

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