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Combination Therapy Counteracts the Enhanced Transmission of Drug-Resistant Malaria Parasites to Mosquitoes

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Malaria parasites carrying genes conferring resistance to antimalarials are thought to have a selective advantage which leads to higher rates of transmissibility from the drug-treated host. This is a likely mechanism for the increasing prevalence of parasites with resistance to chloroquine (CQ) and sulfadoxine-pyrimethamine in sub-Saharan Africa. Combination therapy is the key strategy being implemented to reduce the impact of resistance, but its effect on the transmission of genetically resistant parasites from treated patients to mosquito vectors has not been measured directly. In a trial comparing CQ monotherapy to the combination CQ plus artesunate (AS) in Gambian children with uncomplicated falciparum malaria, we measured transmissibility by feeding *Anopheles gambiae* mosquitoes with blood from 43 gametocyte-positive patients through a membrane. In the CQ-treated group, gametocytes from patients carrying parasites with the CQ resistance-associated allele *pfcrt*-76T prior to treatment produced infected mosquitoes with 38 times higher *Plasmodium falciparum* oocyst burdens than mosquitoes fed on gametocytes from patients infected with sensitive parasites ($P < 0.001$). Gametocytes from parasites carrying the resistance-associated allele *pfmdr1*-86Y produced 14-fold higher oocyst burdens than gametocytes from patients infected with sensitive parasites ($P = 0.011$). However, parasites carrying either of these resistance-associated alleles pretreatment were not associated with higher mosquito oocyst burdens in the CQ-AS-treated group. Thus, combination therapy overcomes the transmission advantage enjoyed by drug-resistant parasites.

Chloroquine (CQ) remains the first-line treatment for malaria in 29 of the 42 countries in sub-Saharan Africa (13), despite the significant reduction in its clinical efficacy over recent years. This has led to calls for the widespread introduction of effective combination therapy for falciparum malaria as a matter of urgency (12). In addition to the therapeutic benefits, substantial public health gains will be enjoyed if combination therapy also brings about a significant reduction in parasite transmission to *Anopheles* mosquitoes, particularly parasites carrying genes conferring drug resistance (2, 8). This requires that the carriage of gametocytes, the parasite's transmissible stage, be minimized in treated patients (2). Antimalarials such as artesunate (AS) act against immature gametocytes during the period of sequestration (~7 days) that precedes emergence into the peripheral circulation as mature infectious gametocytes and can thus be used to reduce gametocyte carriage (2, 10).

In 1998, we observed a significant rate of failure of CQ monotherapy in The Gambia and showed that this was linked to mutations in two genes of *Plasmodium falciparum*: *pfcrt* and *pfmdr1* (8). The combination CQ-AS was evaluated in 2000 in the hope that satisfactory efficacy would be exhibited, leaving sulfadoxine-pyrimethamine and other affordable antimalarial drugs in reserve as treatments for recrudescent infections (9). Both the efficacy of treatment with this combination and its ability to decrease the rate of transmission of CQ-resistant parasites from treated individuals have been reported previously (2, 9).

Parasitological and entomological data suggest that CQ-treated patients with persisting drug-resistant parasites carry higher densities of mature gametocytes and are more infectious to mosquitoes than successfully treated patients (2, 4, 6, 7). However, these experiments were carried out without molecular confirmation that the resistance-associated alleles *pfcrt* and *pfmdr1* were present in infections with higher rates of transmission success. In this study we present new evidence that, under CQ monotherapy, genetically resistant parasites are significantly more transmissible to *Anopheles* mosquitoes than sensitive parasites. Under combination therapy with CQ and AS, however, enhancement of transmission of resistant parasites does not occur.

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MATERIALS AND METHODS

Ethical approval. Ethical approval for the study was obtained from the Joint Gambian Government-Medical Research Council Ethics Committee (Project 838) and the London School of Hygiene and Tropical Medicine Ethics Committee.

Study subjects. The clinical treatment trial held in Farafenni, The Gambia, in 2000 and the associated experiments of feeding through a membrane that provided the samples for this study are described in detail elsewhere (2, 8). Briefly, 536 children with mild malaria were recruited and treated either with CQ alone ($n = 136$; 25 mg of oral CQ base [Alkaloida Ltd., Tiszavasvári, Hungary] per kg of body weight over 3 days) or with a combination of CQ and three doses of AS ($n = 400$; 4 mg of AS [Guilin Pharmaceutical Works, supplied by Sanofi, Paris, France] per kg with each dose of CQ). Eligible children were aged 1 to 9 years and had a body weight >5 kg, a history of fever, and asexual parasitemia at levels of >500 parasites/μl of blood. Exclusion criteria included anemia (packed cell volume [PCV], <20%), any other signs or symptoms of severe malaria, an inability to take drugs orally, treatment with any antimalarial within the past 2 weeks, and any evidence of chronic disease or other acute infection. Field assistants visited each child at home on postrecruitment days 3, 14, and 28. On each occasion a finger-prick blood sample was taken and used to make a thick film for microscopy and a filter paper blood spot for DNA extraction. On day 7, the patients were brought to the Medical Research Council laboratory in Farafenni, where samples for thick blood film and PCV estimation were obtained.

Mosquito infections. Blood from children who were gametocytemic on the day 7 follow-up visit (limit of detection, 5 gametocytes/μl) and who had a PCV >20% was selected for the membrane feeding experiments. Venous blood samples (2 to 3 ml) were taken from consenting gametocyte carriers and fed through a membrane to female *Anopheles gambiae* mosquitoes in cages, with approximately 50 mosquitoes per cage, as described previously (2, 10). If the number of gametocyte-positive patients was greater than the number of cages of mosquitoes available for feeding on a particular day, consenting gametocyte carriers were selected in the order in which the finger-prick blood samples were obtained. The carriers were selected independently of the treatment group. The midguts of the blood-fed mosquitoes were dissected 7 to 8 days after they were fed, and the number of *P. falciparum* oocysts was counted and used as the primary transmission end point.

Parasite genotyping. DNA was extracted from the blood spots on filter paper obtained on day 0 and from pellets of venous blood obtained on day 7. Oocyst DNA was obtained from the pooled midguts of oocyst-positive mosquitoes from a single cage of fed mosquitoes. Two mutations in key parasite genes, a threonine (T) encoded by codon 76 of *pfcrt* and a tyrosine (Y) encoded by codon 86 of *pfmdr1*, are strongly associated with CQ treatment failure in this population (8). These amino acid substitutions result from single nucleotide substitutions that were detected by a sequence-specific oligonucleotide probing assay, as described previously (1, 8). Infections harboring mixtures of CQ-sensitive and CQ-resistant parasites were scored as resistant to reflect the expected phenotype of the infection.

Data analysis. To compare midgut oocyst burdens between groups of mosquitoes fed through a membrane, the ratio of the arithmetic means was fitted to the negative binomial distribution, with compensation for within-patient clustering, as described previously (2). Adjustment for confounding between the *pfcrt* and *pfmdr1* loci was made. The difference between two proportions was tested by using the χ^2 statistic. Gametocyte density was also fitted to the negative binomial for comparison of (unadjusted) means between groups. Directional selection within hosts was evaluated by McNemar's test.

RESULTS AND DISCUSSION

During the 2000 transmission season, the rates of prevalence of *pfcrt*-76T and *pfmdr1*-86Y among children presenting with confirmed clinical malaria were 61.8% ($n = 89$) and 49.5% ($n = 91$), respectively (R. Ord and C. J. Sutherland, unpublished data). The two alleles exhibit strong linkage disequilibrium ($P = 0.002$) in this population, probably reflecting coselection by CQ (8). We determined the genotypes of these two loci for the pretreatment parasite population from each of 43 gametocyte donors, 18 of whom had received combination therapy. As neither drug regimen affects transmission in cases in which mature gametocytes are already circulating prior to treatment (2), the relationship between the resistance genotype, the treatment received, and the oocyst burden in blood-fed mosquitoes was explored only for those patients without gametocytes at presentation. As a nonrandom association between *pfcrt* and *pfmdr1* has previously been observed in this population (8), the ratio of the means for each locus is presented after adjustment for confounding between the loci (Table 1).

TABLE 1. Relationship between pretreatment parasite genotype at *pfcrt*-76 and *pfmdr1*-86 and oocyst numbers in midguts from mosquitoes fed on gametocyte-positive patient blood at day 7

Genotype prior to treatment and drug treatment group	No. of mosquitoes analyzed[a]	Ratio of mean oocyst no. (95% CI)[b]	*P*
pfcrt-76T			
CQ	592	38.29 (12.18–120.3)	<0.001
CQ/AS	456	0.987 (0.349–2.794)	0.981
pfmdr1-86Y			
CQ	619	14.58 (1.831–116.2)	0.011
CQ/AS	494	0.266 (0.076–0.928)	0.038

[a] Mosquitoes were fed on blood from gametocyte-positive patients at day 7 posttreatment who were not carrying gametocytes at the time of treatment ($n = 25$ and $n = 18$ in the CQ and CQ-AS groups, respectively). Mosquitoes were excluded from the analysis for three carriers (one and two carriers in the CQ and CQ-AS groups, respectively) for which genotype data for either or both loci were missing for parasites obtained on day 0. This accounts for the different numbers analyzed in the top and bottom halves of the table.

[b] Counts were compared by calculating the ratio of the means; significance was calculated by fitting the oocyst counts to the negative binomial distribution, after compensation for within-patient clustering and adjustment for confounding between the two loci.

Among patients treated with CQ monotherapy, the day 7 gametocytes from those carrying *pfcrt*-76T parasites prior to treatment produced 38-fold more midgut oocysts than did those from patients carrying the wild-type allele, *pfcrt*-76K. Similarly, mosquitoes fed on day 7 gametocytes from CQ-treated children carrying *pfmdr1*-86Y parasites prior to treatment carried approximately 14-fold more midgut oocysts than mosquitoes fed on blood from patients carrying the wild-type allele (Table 1). This confirms that the *pfmdr1*-86 genotype has an incremental effect on parasite transmission separate from that of *pfcrt*-76. Therefore, as predicted by parasitological and entomological data, treatment of human hosts with CQ monotherapy imparts to the parasites carrying CQ resistance genes a considerable transmission advantage compared with that for sensitive parasites.

What effect does combination therapy have on this transmission benefit? Among children receiving the CQ-AS combination, the parasites carrying *pfcrt*-76T enjoyed no transmission advantage (Table 1); the ratio of the adjusted mean oocyst burdens was 2.58% of the ratio observed under CQ monotherapy (95% confidence interval [CI], 0.6 to 11.8%; $P < 0.001$). Under CQ-AS therapy, the *pfmdr1*-86Y genotype was associated with a statistically significant fourfold lower oocyst burden. There is thus a transmission deficit among those carrying *pfmdr1*-86Y rather than *pfmdr1*-86N under CQ-AS treatment. The adjusted mean ratio for these children was 1.82% of the ratio for those receiving CQ (95% CI, 0.16 to 21%; $P = 0.001$). Therefore, the addition of AS to CQ monotherapy nullifies the greater transmissibility of CQ-resistant infections, supporting the notion that artemisinin-based antimalarial combination therapy can reduce the transmission of drug-resistant malaria parasites from treated patients (5, 8, 12).

In order to understand the mechanism of enhanced transmission, we identified changes in the *pfcrt*-76 or *pfmdr1*-86 genotype occurring between samples obtained on day 0 and those obtained on day 7 posttreatment from all gametocyte

TABLE 2. Changes in CQ resistance genotype at day 7 posttreatment among donors whose venous blood successfully transmitted infections to mosquitoes[a]

Day and locus	Genotype	Oocyst genotype	No. of infections
Day 0			
pfcrt-76	Wild type	Resistant	5
	Resistant	Wild type	0
pfmdr1-86	Wild type	Resistant	3
	Resistant	Wild type	5
Day 7			
pfcrt-76	Wild type	Resistant	2
	Resistant	Wild type	0
pfmdr1-86	Wild type	Resistant	4
	Resistant	Wild type	3

[a] Blood from 29 donors was tested.

TABLE 3. Relationship between pretreatment parasite genotype at the *pfcrt*-76 or *pfmdr1*-86 locus and gametocyte density in patients who were treated with CQ or CQ-AS and who were gametocyte donors at day 7

Genotype prior to treatment and drug treatment group[a]	No. of patients[b]	Ratio of mean gametocyte density (95% CI)[c]	P^d
pfcrt-76, T vs K			
CQ	23	28.09 (2.21–35.68)	0.01
CQ-AS	16	0.286 (0.041–1.99)	0.21
pfmdr1-86, Y vs N			
CQ	24	4.63 (1.01–21.20)	0.049
CQ-AS	17	0.35 (0.045–2.70)	0.314

[a] A mixed genotype at either locus (i.e., KT and NY) was scored as resistant (T and Y, respectively) to reflect the phenotype expected under drug selection.
[b] Patients who were not carrying gametocytes at the time of treatment ($n = 25$ and $n = 18$ in the CQ and CQ-AS groups, respectively).
[c] Densities were compared by calculation of the ratio of the unadjusted arithmetic means.
[d] Significance was calculated by fitting of gametocyte densities to the negative binomial distribution.

donors, irrespective of whether the patient presented with gametocytemia ($n = 69$). Changes in the *pfcrt*-76 genotype were noted in eight infections, seven of which went from the wild type to the resistant genotype ($P = 0.0339$), suggesting that after 7 days of drug pressure, the selection of gametocytes carrying *pfcrt*-76T was occurring within the human host. Evidence of this directional selection was also apparent when we compared pretreatment parasites with midgut oocysts from patients with infections that were successfully transmitted to mosquitoes; five of five changes were from *pfcrt*-76K to *pfcrt*-76T (Table 2) ($P = 0.025$). Both of the changes observed between the parasite genotype on day 7 posttreatment and the genotype of the resulting oocysts were also from *pfcrt*-K to *pfcrt*-76T (Table 2) ($P = 0.157$), suggesting that additional directional selection may occur in the mosquito midgut. However, a larger sample would be required to test this. CQ treatment pressure contributed to selection in both treatment groups (data not shown). No evidence of directional selection for *pfmdr1*-86N/Y was seen at any stage, as genotype changes were observed in both directions (Table 2). Our interpretation of these findings is that drug selection for *pfcrt*-76T, but not *pfmdr1*-86Y, does occur among gametocytes in treated patients and that the CQ resistance genotype of the transmitted parasites in infected mosquitoes largely reflects the genotypes of the circulating gametocytes from which they are descended.

The enhanced transmission of CQ-resistant parasites that we have observed may be a direct effect of higher gametocyte numbers in infections caused by resistant parasites (2, 4, 6–8, 10). We therefore examined the relationship between the pretreatment resistance genotype and the gametocyte density on day 7 posttreatment in both drug treatment groups (Table 3). As predicted, those children in the CQ treatment group harboring parasites with either resistance allele prior to treatment carried significantly higher gametocyte densities on day 7 posttreatment. This was not seen in the CQ-AS treatment group.

Thus, artemisinin-based combination therapy for acute uncomplicated falciparum malaria in African children can overcome the transmission advantage of parasites genetically resistant to the primary drug. Under CQ monotherapy, resistant parasites enjoyed a 14- to 38-fold higher intensity of transmission to mosquitoes, which was ablated in the combination group. Exposure to AS rendered parasites carrying the *pfmdr1*-86Y allele less infectious than wild-type parasites, consistent with predictions from in vitro studies that *pfmdr1* mutants are more sensitive to artemisinins than parasites with wild-type *pfmdr1* (3). This provides an explanation for the apparent lack of directional selection for the *pfmdr1*-86Y allele among the gametocyte donors (Table 2).

Gametocytes arising from resistant parasites had a selective advantage in the CQ-treated host, even if their asexual progenitors were present at levels below the PCR detection threshold at the time of treatment. This is why in a small number of infections, apparently wild-type parasites gave rise to resistant gametocytes at day 7. We cannot say whether the gametes (and ookinetes) arising from these gametocytes enjoyed a further selective advantage in the mosquito host. We can say that a key parameter of the transmission success of resistant parasites is the production of greater numbers of mature circulating gametocytes, which are present over an extended period of time after treatment (2, 4, 6, 7, 10). Our findings are that CQ-AS reduces the infectivity of resistant parasites at day 7. However, children in the CQ-AS treatment group frequently became gametocyte positive in the third and fourth weeks after treatment and carried gametocytes at relatively high densities (2), which shows that this particular combination would not provide a sustainable long-term benefit in reducing the spread of drug-resistant parasites. Other AS combinations tested with the same population, such as sulfadoxine-pyrimethamine plus AS or co-artemether (10, 11), give more sustained reductions in the levels of gametocyte carriage, as the level of resistance to the accompanying drug(s) remains low.

This work supports a major rationale for combination theory: that its implementation can reduce the spread of drug-resistant parasites in areas of endemicity. Thus, the concerted use of fully effective artemisinin-containing antimalarial combinations in sub-Saharan Africa should counteract the enhanced transmission of drug-resistant parasites. Our study also illustrates the importance of analysis of the molecular genetics

of the parasite in both the human and the mosquito hosts for evaluation of the effectiveness of combination therapy interventions. Such studies are needed to inform the critical choice as to which combination should be deployed in a particular setting.

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