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Research note

The effect of chloroquine treatment on the infectivity of *Plasmodium chabaudi* gametocytes

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Abstract

The antimalarial drug chloroquine has been reported to increase the infectivity of the forms of blood-stage malaria parasites (gametocytes) that are capable of infecting mosquito vectors. This effect has been demonstrated convincingly in the short term (12 h post treatment), although several authors have suggested infectivity enhancement a week or more after treatment. We carried out experiments to investigate the effects of chloroquine on the longer-term infectivity of gametocytes of the rodent malaria parasite, *Plasmodium chabaudi*, to *Anopheles stephensi* mosquitoes. Gametocytes of chloroquine-treated infections were significantly more infectious than untreated infections 6 and 7 days post-treatment, although not on days 8 and 9. However, this effect was most likely the result of a reduction in infectivity in untreated infections, caused by immune activity which was not so pronounced in chloroquine-treated infections. Gametocytaemia (gametocytes per r.b.c.) showed a strong positive and linear relationship with infectivity. Infectivity was not influenced by either asexual parasitaemia, asexual density or anaemia. Parsimonious interpretations of the effect of chloroquine on gametocyte infectivity are discussed.

Keywords: *Anopheles stephensi*; Chloroquine; Gametocyte; Infectivity; Malaria; *Plasmodium chabaudi*; Transmission

Antimalarial chemotherapy has frequently failed to significantly reduce malaria prevalence, and resistance to many antimalarial drugs has spread at an alarming rate [1]. This is partly because some of the commonly used antimalarial drugs have little parasite killing activity against the transmission stages of a *Plasmodium* infection, gametocytes [2, 3]. However, two other mechanisms by which chemotherapy might enhance transmission have been suggested. First, chemotherapy may induce greater gametocyte production, both through genotypic [4–6] and short-term phenotypic changes [7]. Given the generally positive correlation between gametocyte densities and infectivity to mosquitoes [8, 9], such changes are likely to increase transmission. Second, the antimalarial drug chloroquine (CQ) has been reported to increase the infectiousness

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of gametocytes, independent of gametocyte densities [1].

Data on infectivity enhancement of gametocytes by CQ are ambiguous. It is clear that CQ per se does not increase gametocyte infectivity: the addition of CQ to cultures prior to feeding mosquitoes did not increase either the proportions of mosquitoes infected, or the number of parasites (oocysts) that subsequently developed [1, 10, 11]. Enhanced gametocyte infectivity 12-h post sub-curative CQ treatment has been demonstrated in vivo for the rodent malaria parasites *Plasmodium yoelii* [12, 13] and *Plasmodium yoelii nigeriensis* [14]. However, there was no reported increase in the infectivity of *Plasmodium falciparum* gametocytes when mixed with sera collected 24 h after CQ had been taken, compared with sera before treatment [15]. Similarly, there was no infectivity enhancement of *Plasmodium vivax* in human volunteers between 0 and 72 h post-treatment [3].

There have been suggestions that CQ can enhance per gametocyte infectivity a week or more after treatment, but here the data are even more ambiguous. Curative CQ-treatment of *P. falciparum*-infected humans resulted in greater median parasite burdens in *Anopheles balabacensis* mosquitoes (but not in *Anopheles dirus*) fed 2 and 7 days after treatment, compared with that before treatment. However, mean oocyst burdens and the proportions of mosquitoes infected did not change [16]. Hogh et al. [17] recently reported that "serum from chloroquine-treated, uninfected, non-immune volunteers enhanced gametocyte infectivity with increasing efficiency for 21 days following treatment", using cultured gametocytes of *P. falciparum* and of the rodent malaria, *Plasmodium berghei*. They report a significant increase in infectivity through time following CQ treatment with *P. berghei* gametocytes, and a non-significant increase with *P. falciparum* gametocytes, although the validity of these analyses is questionable. Infectivity of gametocytes obtained from naturally infected CQ-treated volunteers was not significantly greater when mixed with the patients' own (CQ-containing) plasma compared with European control plasma [17]. A field study investigating factors influencing human infectivity to mosquitoes also found no *P. falciparum* infectivity-enhancing effects of CQ [18].

It is clearly important to determine whether CQ enhances gametocyte infectivity for more than a matter of hours. We therefore carried out experiments to investigate gametocyte infectivity of the rodent malaria, *Plasmodium chabaudi*, in vivo 6–9 days after CQ treatment, at the time of peak gametocyte production.

Details of methodology are given in Buckling et al. [7]. Male C57/BL/6J mice were infected by i.p. inoculation with 10^6 parasites on day 0. Parasite clones and sample sizes are shown in Table 1. On day 5 or 6 p.i. (1–2 days before the average peak asexual parasite density), half the mice were orally treated with a sub-curative dose of chloroquine sulphate (12 mg kg^{-1}); the others received distilled water (controls). In each experiment, half the control and half the CQ-treated

Table 1
Details of experiments

Experiment	Clone	Day post-infection of chloroquine treatment	No. of mice infected	
			Chloroquine	Control
1	CR	6	6	5[a]
2	CR	5	6	6
3	ER	5	6	6

[a] One mouse died from severe anaemia on day 8 post-infection.

mice were picked randomly and fed to ~40 4–5-day-old *Anopheles stephensi* on day 12 p.i. (except in the control group in experiment 1, where two animals out of five were fed to mosquitoes). The other mice were fed to mosquitoes on day 14 p.i. Days 12 and 14 p.i. are the days of peak gametocyte densities in CQ-treated and untreated infections, respectively [7]. Asexual parasites and gametocytes per r.b.c. and r.b.c. density were determined for each mouse on both feed days; parasite densities were calculated from the product of these variables. Approximately 25 mosquitoes per mouse were dissected 8–9 days after feeding and the number of parasites (oocysts) on their midguts determined.

All analyses were carried out using GLIM 4. Proportions of mosquitoes infected per mouse were analysed by logistic regression with a binomial error structure. Over-dispersion in the data was corrected by using a heterogeneity factor (HF) of 3.05, determined from the ratio of Pearson's χ^2 to the degrees of freedom in the minimal model [19]. Day p.i. of feed (12 or 14), treatment (CQ + , CQ–) and experiment (1, 2 or 3) were fitted as factors, and square-root arcsin-transformed gametocytes per r.b.c. (gametocytaemia) fitted as a covariate. The minimal model was determined by stepwise deletion, starting with the highest order interactions, with non-significant terms discarded [19]. The explanatory power of remaining terms was determined by deletion from the minimal model. Additional covariates [$\log_{10}$-transformed gametocyte densities, asexual densities and r.b.c. densities, and square-root arcsin-transformed asexuals per r.b.c. (asexual parasitaemia)] were substituted with gametocytaemia in the minimal model to determine if they explained a significant amount of additional deviance.

Further analyses were carried out in the same way within days (HF = 3, 3.6 for days 12 and 14 p.i., respectively) and treatments (HF = 1.8, 4.1 for CQ+ and CQ−, respectively), using only covariates that were significant in the previous model. Analyses of oocyst burdens were also carried out within days and treatments using a negative binomial error structure, with the over-dispersion parameter, k, estimated separately within each analysis [19, 20]. Data from individual mosquitoes were nested within mouse to avoid pseudo-replication. Non-significant interactions were combined with the error term to determine significance of main effects. Differences in ($\log_{10}$-transformed) gametocyte densities between days and treatments were determined by ANOVA, using stepwise deletion as above, with treatment, day and experiment fitted as factors.

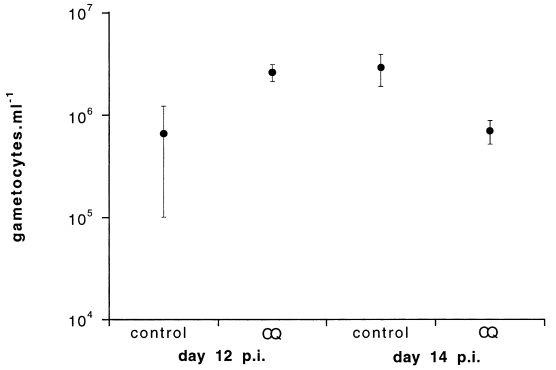

Fig. 1. Mean ± 1 S.E.M. gametocyte densities in chloroquine-treated and untreated infections on days 12 and 14 p.i.

Gametocyte densities were 3.8 times higher on day 12 p.i. than day 14 p.i. in CQ-treated infections, and 4.4 times higher on day 14 p.i. than day 12 p.i. in untreated infections (Fig. 1, day by treatment interaction: $F_{1,29} = 5.03$, $P < 0.05$). In the full logistic regression model, gametocytaemia explained 46% of the deviance associated with

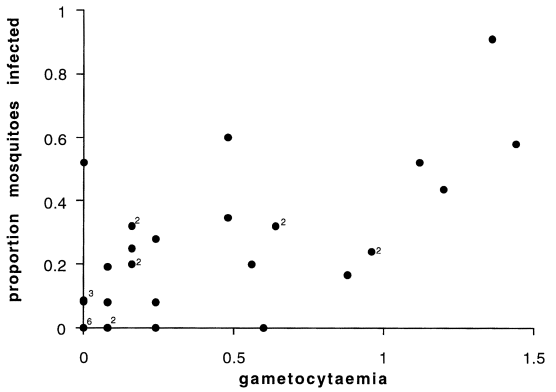

Fig. 2. Relationship between proportion of mosquitoes infected and gametocytaemia (gametocytes per 1000 r.b.c.) for all data. Small numbers in body of figure show multiple data points.

proportions of mosquitoes infected (Fig. 2, $\chi^2_1 = 25.12$, $P < 0.001$), which was significantly greater than the deviance explained by gametocyte density (difference between gametocytaemia and gametocyte density: $\chi^2_1 = 4.87$, $P < 0.05$). The relationship between (untransformed) gametocytaemia and proportion of mosquitoes infected was approximately linear (Fig. 2); allowing the best fit regression to curve, by the addition of the quadratic function, did not explain significantly more deviance ($P > 0.1$).

To determine the effect of CQ on per gametocyte infectivity, gametocytaemia had to be controlled for. When this was done, the effect of CQ-treatment on proportions of mosquitoes infected differed on days 12 and 14 p.i. (treatment by day interaction: $\chi^2_1 = 4.19$, $P < 0.05$). No main effects of the factors (day, experiment or treatment), covariates (gametocyte, asexual and r.b.c. densities, and asexual parasitaemia) or their other interactions explained a significant additional amount of deviance ($P > 0.05$, in all cases).

Because of the significant treatment by day interaction in the full model, data were analysed separately within days and treatments. When the data from day 12 p.i. alone were analysed, infectivity per gametocyte was 3.2 times greater (at the mean gametocytaemia) in CQ-treated than untreated infections (Fig. 3a, main effect of treatment: $\chi^2_1 = 4.3$, $P < 0.05$). Mean oocyst burdens (per gametocyte) were 3.1 times greater (at the mean gametocytaemia) in CQ-treated compared with untreated infections (main effect of treatment: $F_{1,10} = 12.12$, $P < 0.01$). The rates of increase of both proportion of mosquitoes infected and mean oocyst burdens with gametocytaemia did not differ between CQ-treated and untreated infections (treatment by gametocytaemia interaction: $P > 0.1$). Neither proportions of mosquitoes infected or mean oocyst burdens (per gametocyte) on day 14 p.i. differed between CQ-treated and untreated infections (Fig. 3b, $P > 0.1$ for main effects of treatment, and treatment by gametocytaemia interactions).

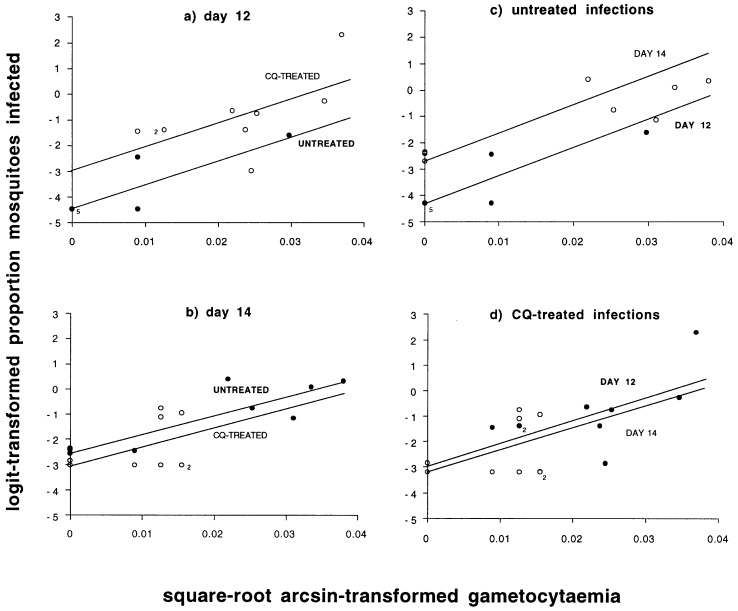

Fig. 3. Relationship between logit-transformed proportions of mosquitoes infected and square-root arcsin-transformed gametocytaemia. Lines show OLS regressions from statistical models. Labels in bold type correspond to solid symbols. Small numbers in body of figures represent multiple data points. (a) Data from day 12 p.i. (b) Data from day 14 p.i. (c) Data from all untreated infections. (d) Data from all chloroquine-treated infections.

When only untreated infections were considered, the proportion of mosquitoes infected and mean oocyst burdens (per gametocyte) were, at the mean gametocytaemia, respectively 3.4 and 2.9 times greater on day 14 p.i. than day 12 p.i. (Fig. 3c, main effects of day: $\chi^2_1 = 7.94$, $P < 0.01$, $F_{1,12} = 10.89$, $P < 0.01$, respectively; day by gametocytaemia interactions: $P > 0.1$, in both cases). However, within CQ-treated infections, gametocyte infectivity as measured by both proportions of mosquitoes infected and mean oocyst burdens, did not differ between days 12 and 14 p.i. (Fig. 3d, $P > 0.1$ for both day and day by gametocytaemia interaction, in both cases).

Gametocytaemia was the best predictor of infectiousness in this study, but did CQ enhance per gametocyte infectiousness? On day 12 p.i., gametocytes in CQ-treated mice were more infectious than those in untreated infections, both in terms of proportion of mosquitoes infected and mean oocyst burdens. Two days later, any effects of CQ on gametocyte infectivity were no longer detectable. These data can be interpreted in two ways. First, CQ enhanced infectivity on day 12 p.i., but the effect was lost by day 14 p.i. Second, infectivity was suppressed on day 12 p.i. in untreated infections, but not on day 14 p.i. That infectivity was greater on day 14 p.i. than day 12 p.i. in untreated infections, but there was no difference in infectivity between days in CQ-treated infections, leads to acceptance of the latter hypothesis. Thus, any effect of CQ on infectivity was not direct.

The reduction in infectivity in untreated infections relative to CQ-treated infections on day 12 p.i. is consistent with the infection dynamics. In untreated infections a phenomenon called "crisis" occurs. This is a rapid reduction in parasite numbers associated with low r.b.c. densities and strong immune activity [21], during which gametocyte infectivity is suppressed [22] (and see Carter et al. [23] for a related phenomenon in the human malaria parasite, *P. vivax*). Crisis occurred between days 8 and 10 p.i. in untreated infections in these experiments, and it is entirely plausible that gametocyte infectivity-suppressing "crisis factors" are still present by day 12 p.i., but not day 14 p.i. Crisis did not occur in CQ-treated infections because of the greatly reduced peak asexual parasite densities [7].

In untreated infections, reduced gametocyte infectivity on day 12 p.i. was also associated with low gametocyte densities. There was a similar pattern on day 14 p.i. in CQ-treated infections. The infectivity of individual gametocytes may therefore be a positive function of gametocyte density, as would be the case if the probability of gamete fusion in the mosquito midgut was considerably reduced at low gametocyte densities. There is, however, no evidence that per gametocyte infectiousness increased non-linearly at high gametocytaemias.

The results imply that if CQ does increase the infectiousness of *P. chabaudi* gametocytes, the effect is lost by 6–7 days post-treatment. This is before the vast majority of gametocytes were produced in both untreated and CQ-treated infections [7]. The plasma half-life of CQ in malaria-infected mice is approximately 7 h [24], and thus would be at very low concentrations after 6–7 days. The current results are therefore inconsistent with the suggestion that long-term metabolites of CQ are responsible for the reported infectivity-enhancing effects of CQ [1, 17]. Ultimately, our data are consistent with most previously published data: there is no strong evidence to suggest that CQ has long-term infectivity-enhancing effects on gametocytes.

Infectivity enhancement by CQ has been demonstrated convincingly only in the short term (12 h post-treatment), in controlled experiments using rodent malaria parasites in vivo [12–14]. A plausible explanation for these data is an immuno-suppressive effect of CQ. Chloroquine is known to inhibit antigen processing and presentation [25], and leukocyte function in general [26]. Such mechanisms could conceivably reduce anti-gametocyte immune activity, resulting in increased gametocyte infectivity in the short term. Infectivity-enhancing effects would be expected to last only as long as CQ (or the major antimalarial metabolite, desethyl-CQ) was at sufficient concentrations to have an immuno-suppressive effects. Infectivity enhancement would be expected to increase with CQ dose, up to a point where growing gametocytes were being

destroyed—mature gametocytes are unaffected by CQ [2]. This prediction is supported by intermediate doses of CQ having the greatest infectivity-enhancing effect [12]. Although a short-term immuno-suppressive effect of CQ could be of potential importance to malaria epidemiology and the spread of drug-resistant genotypes, longer-term effects, if they occur, would be of great significance. We find no evidence of such long-term effects in *P. chabaudi*. Further work on *P. falciparum* is required to resolve this important issue.

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