Chloroquine increases *Plasmodium falciparum* gametocytogenesis *in vitro*

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SUMMARY

Malaria parasites are capable of modulating the diversion of resources from asexual growth to the production of stages infective to mosquitoes (gametocytes). Increased rates of gametocytogenesis appear to be a general response to stress, both naturally encountered and novel. We have previously reported earlier and greater gametocytogenesis in response to subcurative antimalarial chemotherapy in the rodent malaria, *Plasmodium chabaudi*, *in vivo*. Using an immunofluorescent assay to detect parasites that had invaded red blood cell monolayers, we demonstrate a 5-fold increase in gametocytogenesis in the human malaria, *P. falciparum*, *in vitro*, in response to treatment with the antimalarial drug chloroquine. In all clones used, gametocytogenesis increased with increasing inhibition of asexual growth by chloroquine. Furthermore, there were clone differences in the relationship between stress and gametocyte production, implying the response was genetically variable. This was not, however, associated with chloroquine resistance. The epidemiological significance of these results is discussed.

Key words: *Plasmodium falciparum*, chloroquine, chemotherapy, gametocytogenesis.

INTRODUCTION

Malaria parasites (*Plasmodium* spp.) are capable of modulating the proportion of blood-stage asexual parasites that develop into non-replicating transmission stages (gametocytes). Both the probability of mosquito infection and the parasite burden per mosquito increase with gametocyte density (Carter & Graves, 1988; Taylor & Read, 1997; Taylor, Walliker & Read, 1997; Buckling *et al*. 1997). Conditions unfavourable for asexual replication, and thus future transmission opportunities, stimulate an increase in short-term gametocytogenesis (the proportion of asexual parasites that produce gametocytes; Mons, 1985; Carter & Graves, 1988; Sinden *et al*. 1996). Such compensatory increases in gametocytogenesis are likely to increase the total number of new hosts infected.

If increased gametocyte production is a general response to stress, it should occur following human-imposed stresses, such as antimalarial chemotherapy. Most antimalarial drugs kill asexual parasites. However, if some parasites survive (subcurative treatment is common in the field; Wernsdorfer, 1994), they will inevitably suffer some damage (the extent of which is likely to correlate with the drug-imposed mortality), and may be expected to have a higher probability of producing gametocytes.

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A number of clinical trials with *P. falciparum* in people have looked for drug-induced gametocytogenesis (reviewed by Buckling *et al*. 1997). However, for ethical reasons, there were no untreated control groups, and drugs were given in curative doses. Unambiguous detection of increased gametocytogenesis is impossible from such experiments. We have previously reported increased gametocyte production and infectivity to *Anopheles stephensi* mosquitoes following subcurative chemotherapy in the rodent malaria, *P. chabaudi*, *in vivo* (Buckling *et al*. 1997; Buckling, Crooks & Read, unpublished observations). In these experiments, a variety of drugs with different parasite-killing mechanisms were used against drug-naive parasite clones, suggesting the response is a general adaptation to stress, with the drugs mimicking naturally encountered stresses, such as host immunity. Precise assessment of gametocytogenesis is, however, difficult *in vivo*, given the possibility of different mortality rates and spatial distributions of asexual parasites and gametocytes. *In vitro* experiments overcome these problems as well as allowing the study of the human malaria parasite, *P. falciparum*.

We carried out experiments to determine whether the most commonly used antimalarial drug, chloroquine, increases gametocytogenesis in *P. falciparum* *in vitro*. Antimalarial drugs by definition kill asexual parasites, radically changing culture conditions, so that a simple comparison of the parasite dynamics between untreated and chloroquine-treated continuous cultures can not adequately test the hypothesis.

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Commitment of parasite progeny to development either as asexual parasites or gametocytes occurs prior to release from the parent schizont (Bruce *et al*. 1990). We were able to isolate the progeny of parasites exposed to short-term drug treatment by allowing controlled invasion of red blood cell (RBC) monolayers. The phenotypes of the progeny were then determined after 2 days using an immunofluorescence assay, rather than potentially ambiguous morphological characterization. Using these techniques we were also able to investigate the precise relationship between drug-induced stress and gametocytogenesis and possible differences in such responses between chloroquine-sensitive and -resistant parasite clones.

MATERIALS AND METHODS

Parasite culturing and chloroquine treatment

Parasites were cultured following a modification of the methods of Trager & Jensen (1976). Four *P. falciparum* clones were used in the study: chloroquine-sensitive 3D7A and HB3B, and chloroquine-resistant 7G8 and SUD124/8 (all from the WHO Registry of Standard Strains of Malaria Parasites, maintained at the University of Edinburgh). 3D7A is a clone (Walliker *et al*. 1987) made from isolate NF54 (Ponnundurai, Leeuwenberg & Meuwissen, 1981), which was obtained from a patient in the Netherlands. The geographical origin is unknown, although there is some suggestion that the parasite is from Africa (Collins *et al*. 1986). HB3B, 7G8 and SUD124/8 were cloned from the Honduran isolate H1 (Bhasin & Trager, 1984), the Brazilian isolate IMTM22 (Burkot, Williams & Schneider, 1984), and the Sudanese isolate SUD124 (Bayoumi *et al*. 1993), respectively.

Parasite cultures were prepared at 5 % haematocrit using freshly washed group O+, group RhD+human RBCs (Edinburgh and South East Scotland Blood Transfusion Service) in complete RPMI medium (RPMI 1640 supplemented with 25 mM HEPES, 80 mg/l gentamicin sulphate, 50 mg/l hypoxanthine, 0·2 % (w/v) $NaHCO_3$ and 10 % (v/v) pooled, heat-inactivated human serum). Cultures were established with an initial parasitaemia (parasites/RBC) of approximately 1 % in 5 ml volumes in 25 ml sterile polystyrene tissue culture flasks (Corning). Cultures were maintained at 37 °C and gassed daily with a mixture of 3 % CO_2, 1 % O_2 and 96 % N_2. The medium was also replaced daily, with fresh pre-warmed medium.

When a large proportion of the parasites were at the 'late ring' stage (less than 24 h old) and the culture parasitaemia was at least 4 %, two identical subcultures with parasitaemias ranging between 2 and 4 % were established from the original culture. One was treated with chloroquine sulphate (Nivaquine™; see Table 1 for doses) diluted in distilled water, and the other treated with an equal volume of distilled water (10–20 μl). The medium was replaced with untreated medium approximately 17 h later. Parasites were cultured for a further 48 h, at a volume of 4·5 ml because of removal of culture for RBC monolayer invasion (see below). From the day of the addition of chloroquine (day 0), daily thin blood smears were taken, Giemsa-stained and the number of asexual parasites counted per $1{\cdot}5 \times 10^3$ RBCs. One pair of cultures was called an experiment. Either 6 or 7 experiments were carried out for each clone (see Table 1); experiments were carried out by themselves or 2 at a time.

Preparation and infection of RBC monolayers

The method for creating RBC monolayers was based on that of Jackie Williams (Inselburg, 1983). Corning polystyrene tissue culture dishes (35 × 10 mm) were incubated with 1·5 ml of 10 μg/ml Concanavalin A (Sigma). Except when being manipulated, culture dishes were kept in a modular incubator at 37 °C at all times. The Concanavalin A was then removed and the dishes washed twice with incomplete RPMI medium (RPMI 1640 supplemented with 25 mM HEPES and 50 mg/l hypoxanthine). The dishes were incubated for a further 60 min with 1·5 ml of washed, uninfected RBCs at 0·5 % haematocrit suspended in incomplete RPMI medium. Unbound RBCs were carefully resuspended and removed by aspiration, and the monolayer washed twice more with incomplete RPMI medium to completely remove any unbound RBCs.

Shortly after removal of the chloroquine-treated medium, 0·5 ml of each culture was diluted in complete RPMI medium to 0·5 % haematocrit, and 1·5 ml of this diluted culture added to each monolayer (3 monolayers/culture). The modular incubator was gassed and the parasites kept at 37 °C for 18–22 h, to allow invasion of the monolayer. Unbound RBCs were resuspended in complete medium and removed. The monolayers were washed twice more with complete RPMI medium, to remove any unbound cells. This method allows only the progeny of the cohort of parasites that had been exposed to the experimental treatments to invade the monolayers. Because the progeny in the monolayer were never directly exposed to chloroquine, chloroquine-induced differential mortality between gametocytes and asexual parasites was controlled for. The culture dishes were gassed and left for a further 24 h. This prevented a further asexual cycle occurring in the monolayer, which would have resulted in parasites whose predecessors would not have been exposed to the appropriate treatments. The complete RPMI medium was then removed and the monolayers left to air dry. After determining whether parasite invasion was suitable for the subsequent assay by

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Table 1. Range of chloroquine (CQ) concentrations used with each clone (*n*, Number of pairs of cultures per clone.)

Clone	CQ concentration (μM)	*n*
3D7	0·06–0·067	6
HB3	0·06–0·07	6
7G8	0·075–0·4	7
124/8	0·2–0·275	7

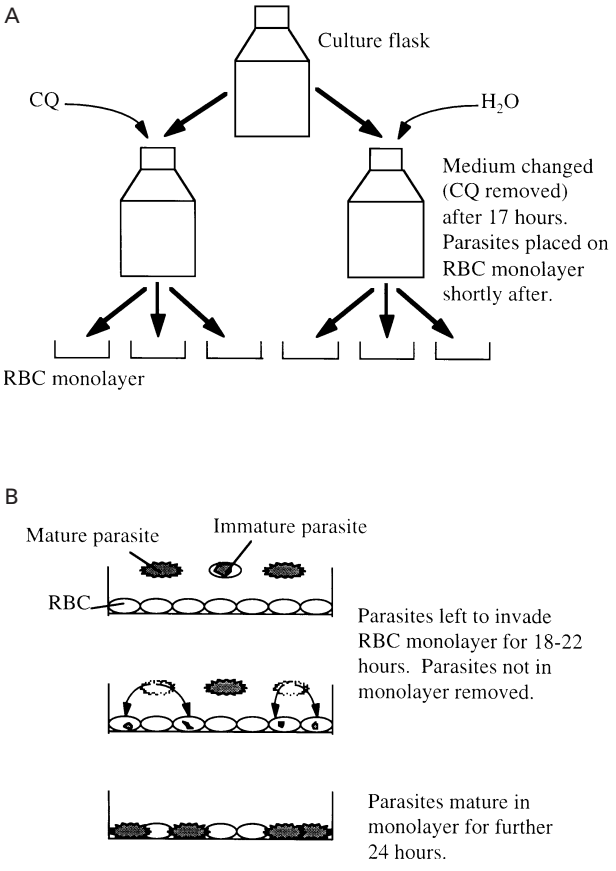

Fig. 1. Schematic of methods for basic experimental design (A), and events within RBC monolayers (B).

methanol-fixing and Giemsa-staining 1 of the 3 monolayers/culture, the remaining dishes were wrapped in foil and stored anhydrously at −70 °C until the immunofluorescence assay was carried out. A schematic of the basic methodology is shown in Fig. 1.

Immunofluorescence assay (*IFA*)

Monoclonal antibodies (mABs) used were mAB 93a3a2 (IgG1), which reacts to the gametocyte-specific Pfs16 antigen (Baker *et al*. 1994), and mAb 12.8.2.1 (IgG2b), which reacts to the 16 kDa segment of MSP2 (McBride & Heidrich, 1987), an asexual-specific antigen (Stanley, Howard & Reese, 1985). Both were mouse hybridoma culture supernatants. Pfs16 is expressed approximately 30 h after parasite invasion of the RBC (Bruce *et al*. 1994). The 16 kDa segment of MSP2 is present on both young ring stages and is expressed again in the developing schizont (from about 30 h post-invasion; McBride & Heidrich, 1987). The mAb 12.8.2.1 also showed limited cross-reactivity to young gametocytes.

In all cases, IFA of monolayers from paired chloroquine-treated and control cultures was carried out at the same time. After methanol-fixing of the monolayers, 1 ml of 'block' buffer (1 × PBS containing 1 % (w/v) Fraction V bovine serum albumin (Sigma), 0·05 % (w/v) NaAzide) was added to each dish, which were then left in a humidity chamber for 20 min at room temperature. The buffer was then aspirated off and the dishes incubated for 30 min in a humidity chamber at room temperature with 80 μl of block buffer with 1:50 and 1:300 dilutions of mAb 93a3a2 and mAb 12.8.2.1, respectively. Dishes were then washed 6 times with 1 × PBS and incubated for a further 20 min in a humidity chamber at room temperature with 80 μl of 1 × PBS containing 10 μg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma), 0·05 % (w/v) Na Azide and 1:40 dilutions of tetramethylrhodamine-conjugated goat anti-mouse IgG1 and fluorescein isothiocyanate-conjugated goat anti-mouse IgG2b (Southern Biotechnology Associates Inc.). The dishes were again washed 6 times with 1 × PBS and a drop of glycerol containing 2·5 mg/ml DABCO (1,4-diazabicyclo-(2,2,2)octane) to prevent fading of the fluorescence was added. A cover-slip was placed over the monolayer and sealed at the edges with nail polish, allowing the monolayers to be stored for a few days without drying out and fading of the fluorescence.

Fluorescence microscopy was carried out on the RBC monolayers using a Leitz Dialux 20 microscope with u.v. illumination. Parasites were observed under 3 different filters. Through Leitz filter blocks A, I2 and N2, the blue staining of parasite nuclei by DAPI, the red of rhodamine associated with asexuals and the green of fluorescein associated with gametocytes, respectively, could be observed. In each monolayer, between 50 and 400 parasites (based on DAPI-staining and morphology under white light) were counted. Only parasites that were old enough to contain haem pigment (detected by white light) were counted, to avoid inclusion of parasites that died at an early stage of their development. Parasites that did not contain haem frequently expressed the asexual-specific antigen but never the gametocyte-specific antigen, thus inclusion of young parasites would under-estimate the proportion of gametocytes if mortality in a particular monolayer was higher than normal. Included parasites were then observed under the other two filters to determine whether they were gametocytes or asexuals. Morphology under white light was used to confirm the phenotype where possible. Given the observed limited cross-reactivity of the asexual-specific mAb

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to gametocytes, but not vice versa with the gametocyte-specific mAb, on the occasions when both mAbs seemed to react strongly to a parasite, the parasites were determined to be gametocytes. The counting of parasites in a monolayer from a chloroquine-treated culture was carried out at the same sitting as a monolayer from the paired control culture, in a randomized order. The number of parasites counted in replicate monolayers were combined, to obtain an estimate of the proportion of gametocytes to total parasites for each culture. In 5 experimental culture pairs, only 1 monolayer/culture was assayed.

Statistical analysis

Where appropriate, all response variables were transformed to bring their distributions close to normal. All analyses were carried out using GLIM 4. Starting with the interactions, terms were removed from the full model by stepwise deletion (Crawley, 1993). Non-significant terms were pooled with the residual deviance to obtain the error deviance. Significance of terms was determined from F-ratios of the deviance explained by each term to the error deviance.

The effect of chloroquine on gametocyte production was analysed by logistic regression using a binomial error structure (heterogeneity factor = 3·2; Pearson's χ^2 divided by the residual degrees of freedom after all non-significant terms had been removed) with number of gametocytes used as the numerator and the sum of gametocytes and asexuals as the denominator (Crawley, 1993). The analysis was carried out using a split-plot design. TREATMENT (chloroquine+, chloroquine−) was fitted within-EXPERIMENT (pairs of cultures, 'plot', of which there were 6 or 7 per clone) and CLONE (3D7, HB3, 7G8 & SUD124/8) fitted as a between-EXPERIMENT factor. Between EXPERIMENT differences in the proportion of gametocytes to total parasites were very large. These differences are, however, of little biological interest and are not reported in this and subsequent analyses where TREATMENT was fitted within-experiment.

The effect of chloroquine on asexual growth was analysed by ANOVA of square-root arcsin-transformed asexual parasitaemia (asexual parasites/RBC) 2 days post-treatment, with TREATMENT fitted within-EXPERIMENT, and CLONE fitted between-EXPERIMENT. Asexual parasitaemia 2 days post-treatment represented the population of parasites after having undergone one more round of replication, and was thus the most appropriate time-point to measure inhibition of asexual growth.

In determining clone differences in the effect of chloroquine on gametocyte production, it is important to know whether clones differed in gametocytogenesis in the absence of chloroquine. This was determined by analysis of only untreated cultures. Proportion of gametocytes to total parasites were square-root arcsin-transformed, and analysed by ANCOVA with CLONE fitted as a factor and angular-transformed asexual parasitaemia on day 0 (when commitment of the progeny to gametocyte and asexual development likely took place) fitted as a covariate.

The effect of chloroquine-induced destruction and/or growth inhibition of asexual parasites on investment into gametocytes was analysed by ANCOVA, with the response variable being,

$$\frac{a-b}{(a+b)/2}, \tag{1}$$

the difference between proportion of gametocytes in chloroquine-treated (a) and the paired untreated culture (b) (standardized by dividing by the mean proportion of gametocytes within-pair, to control for the large differences in proportions of gametocytes between experiments). CLONE was fitted as a factor and the standardized difference between asexual parasitaemia (2 days post-treatment) of untreated (a) and chloroquine-treated cultures (b) (see equation 1) fitted as a covariate. Actual chloroquine concentrations were not used as the covariate, because the effect on asexual inhibition of a given concentration is dependent on overall asexual parasitaemia (Gluzman, Schlesinger & Krogstad, 1987).

Differences in successful binding of mAbs between chloroquine-treated and untreated cultures was analysed by logistic regression using a binomial error structure (heterogeneity factor = 4), with the number mAb- (and DAPI-) positive parasites used as the numerator and the number of DAPI-positive parasites as the denominator. TREATMENT was fitted within EXPERIMENT, and CLONE fitted between EXPERIMENT.

RESULTS

Two days after the addition of chloroquine to cultures, asexual parasitaemias were on average 40 % that of paired control cultures (Fig. 2, $F_{1,25} = 69{\cdot}2$, $P < 0{\cdot}0001$). The effect of chloroquine on asexual parasitaemia did not differ between clones (Fig. 2, CLONE by TREATMENT interaction: $F_{3,22} = 2{\cdot}56$, $P > 0{\cdot}05$).

The greater the asexual parasitaemia in untreated cultures prior to invasion of the monolayer (day 0), the greater the proportion of parasites that subsequently developed into gametocytes (Fig. 3, $F_{1,21} = 6{\cdot}12$, $P < 0{\cdot}05$). Neither the relationship between proportion of gametocytes and asexual parasitaemia, or proportion of gametocytes differed between clones (Fig. 3, Fig. 4, CLONE: $F_{3,21} = 0{\cdot}18$, $P > 0{\cdot}1$; CLONE by parasitaemia interaction: $F_{3,18} = 2{\cdot}77$, $P > 0{\cdot}05$).

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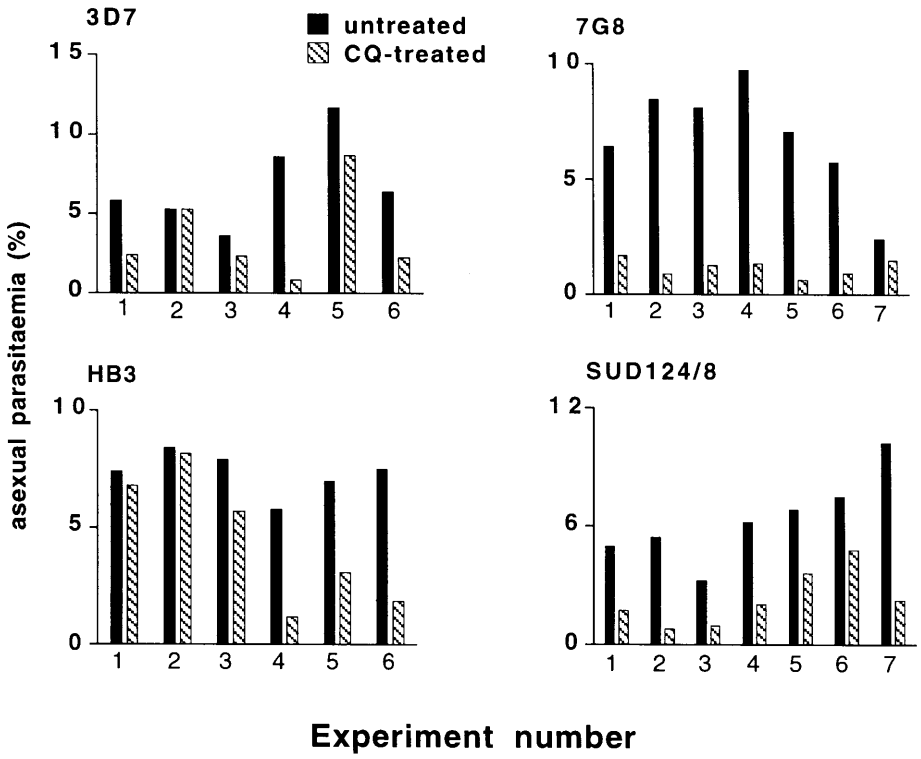

Fig. 2. Asexual parasitaemias 2 days post-treatment for all clones. Data are shown in culture pairs (experiments). CQ; chloroquine.

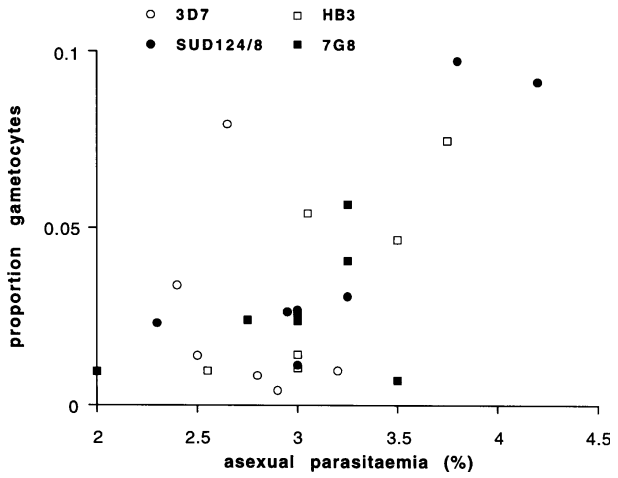

Fig. 3. The relationship between proportion of parasites that developed into gametocytes in the RBC monolayer and asexual parasitaemia on day 0 (time of commitment to producing gametocytes or asexual progeny) in untreated cultures.

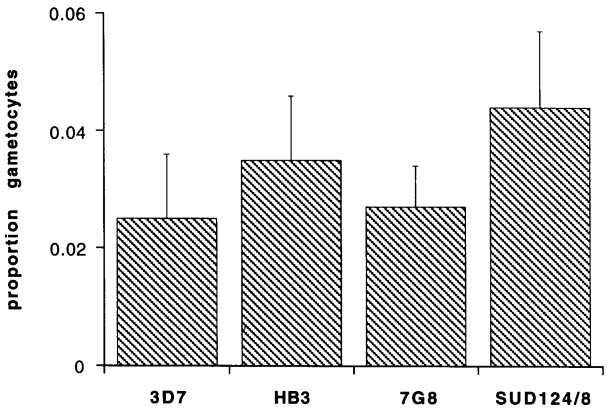

Fig. 4. Mean (+1 S.E.) proportion of parasites that developed into gametocytes for untreated clones.

In all experiments, the proportion of gametocytes to total parasites was on average 5-fold greater in chloroquine-treated compared to untreated cultures (Fig. 5, $F_{1,22} = 81{\cdot}29$, $P < 0{\cdot}0001$). The effect of chloroquine-treatment on proportion of gametocytes differed between clones (CLONE by TREATMENT interaction: $F_{3,22} = 4{\cdot}34$, $P < 0{\cdot}05$).

The level of gametocytogenesis induced by chloroquine treatment increased with the extent of chloroquine-induced asexual parasite destruction (Fig. 6, $F_{1,21} = 22{\cdot}89$, $P < 0{\cdot}001$). For a given value of relative asexual destruction, proportions of gametocytes in chloroquine-treated cultures relative to untreated cultures were more than 2 times greater in clones 3D7 and 124/8 than HB3 and 7G8 (2-level CLONE: $F_{1,23} = 11{\cdot}4$, $P < 0{\cdot}01$; CLONE could be reduced to a 2-level factor without a significant decrease in the deviance explained by the model).

Successful mAb detection (the percentage of DAPI-positive parasites that were also TRITC- or FITC-positive) were 79 % and 85 % for the progeny of chloroquine-treated and untreated cultures, respectively ($F_{1,25} = 10{\cdot}42$, $P < 0{\cdot}01$). Even if the additional 6 % of undetected parasites in

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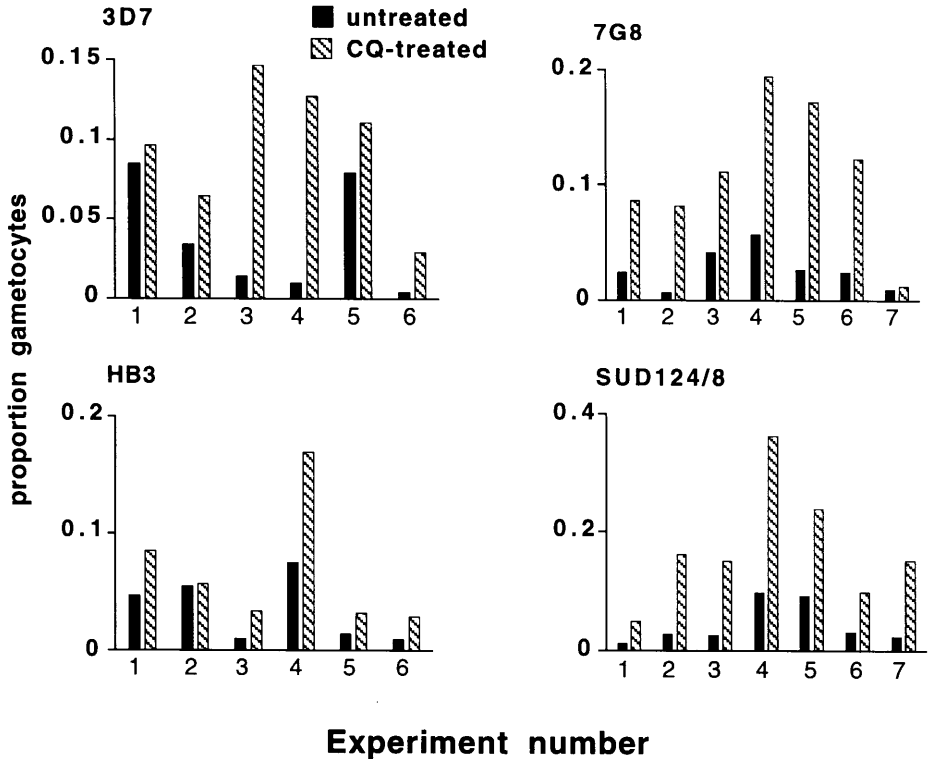

Fig. 5. Proportion of parasites that developed into gametocytes in the RBC monolayer for all clones. Data are shown in culture pairs (experiments). CQ; chloroquine.

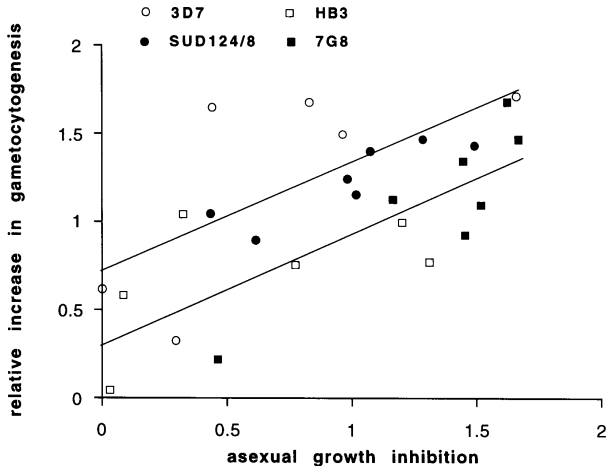

Fig. 6. The relationship between the relative increase in gametocytogenesis and inhibition of asexual growth following chloroquine treatment. Both measures are the difference between chloroquine-treated and control cultures, standardized by dividing by the within-culture pair mean (equation 1, see text). The upper line is the ordinary least-squares regression line for 3D7 and SUD124/8, the lower for HB3 and 7G8. CQ-sensitive and -resistant clones are shown by open and filled symbols, respectively.

chloroquine-treated cultures were all asexual parasites, this would not qualitatively change the results. Detection did not differ between clones ($F_{3,22} = 0{\cdot}48$, $P < 0{\cdot}1$), nor did the effect of treatment on detection differ between clones (TREATMENT by CLONE interaction: $F_{3,22} = 1{\cdot}83$, $P < 0{\cdot}1$).

DISCUSSION

The results demonstrate that subcurative chloroquine treatment increases gametocytogenesis in *P. falciparum*. Like *P. chabaudi* (Buckling *et al.* 1997) *P. falciparum* appears to increase its investment into gametocytes when conditions are unfavourable for asexual growth. Such a response is presumably adaptive in that it is likely to increase transmission from harsh environments.

If small amounts of chloroquine from the treated cultures were carried over to the RBC monolayers, the results might be explained by increased mortality of asexual parasites relative to gametocytes (Peters, 1987). It is, however, highly unlikely that any residual chloroquine would be at sufficient concentrations to damage the parasites in the monolayer.

In all clones, gametocytogenesis increased with increasing inhibition of asexual growth. There were genetic differences in this relationship, with 2 clones (3D7 and SUD124/8) showing a more than 2-fold increase in gametocytogenesis for a given level of drug efficacy than the other 2 clones (HB3 and 7G8). That gametocytogenesis did not differ between clones when untreated suggests that the observed clone differences in the relationship between chloroquine-imposed stress and gametocytogenesis represent differences in sensitivity to environmental stress.

These differences were independent of drug resistance: 3D7 and HB3 are chloroquine sensitive, and SUD124/8 and 7G8 are chloroquine resistant.

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Unlike previous field reports (Handunnetti *et al*. 1996; Robert, Molez & Trape, 1996), there was also no difference in gametocyte production of untreated chloroquine-resistant and -sensitive clones. It therefore appears that chloroquine-imposed selection *per se* has not modified *P. falciparum* gametocytogenesis, either intrinsically or in response to chloroquine. This supports the hypothesis that increased gametocyte production is a general response to retarded asexual growth in unfavourable conditions, with chloroquine treatment being an example of such. Increased resource competition, another form of stress for the parasite, also resulted in increased gametocytogenesis, consistent with previous data (Carter & Miller, 1979; Brockelman, 1982; Bruce *et al*. 1990).

Demonstration of chloroquine-enhanced gametocyte production *in vivo* in *P. chabaudi* (Buckling *et al*. 1997) and *in vitro* in *P. falciparum* strongly suggests that the phenomenon may be widespread. Given the positive relationship between gametocyte densities and infectivity to mosquitoes (Carter & Graves, 1988; Taylor & Read, 1997; Taylor *et al*. 1997; Buckling *et al*. 1997), such compensatory increases in gametocyte production may help to explain the relative ineffectiveness of chloroquine chemotherapy, which is frequently subcurative (Wernsdorfer, 1994), to reduce malaria transmission. That the same responses are observed in chloroquine-resistant parasites may also help explain the rapid spread of chloroquine resistance (Peters, 1987). Increased *P. falciparum* gametocyte production is induced by a wide variety of stresses (Mons, 1985; Carter & Graves, 1988; Sinden *et al*. 1996). It may be that any intervention strategy that inhibits asexual growth, including most methods of subcurative chemotherapy, as well as potential blood-stage vaccines, may induce compensatory increases in transmission stage production by the parasites.

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