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Effect of chloroquine on the expression of genes involved in the mosquito immune response to *Plasmodium* infection

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Abstract

Chloroquine has been described to increase *Plasmodium* infectivity to the mosquito vector and is known to affect the vertebrate host immune response including during malarial infection. Although knowledge of the mosquito immune response has recently improved, nothing is known about the impact of chloroquine on mosquito immunity. In order to characterize the influence of chloroquine on the mosquito immune system, we have analyzed the effect of chloroquine on *Anopheles gambiae* (i) serine proteases and (ii) antimicrobial peptide gene expression, in uninfected and *Plasmodium berghei* infected mosquitoes, using real-time PCR. We have demonstrated for the first time that mosquitoes fed on chloroquine-treated mice showed a significant down regulation of some immune-related genes. This effect was independent of midgut bacterial burden. These results suggest that chloroquine might act on the *Anopheles* serine proteases cascade, interfering with signal transduction pathways and at a transcriptional activation level.

Keywords: Chloroquine; *Anopheles gambiae*; Immune response; Real-time PCR; *Plasmodium berghei*

1. Introduction

Malaria is a vector-borne disease that still remains, to our days, as one of the major causes of mortality worldwide. The *Plasmodium* parasite life cycle is complex and involves two hosts: a vertebrate host and a mosquito vector. In Africa, where most deaths occur, malaria is transmitted mainly by members of the *Anopheles gambiae* complex. Strategies aimed at interrupting parasite transmission by the mosquito vector are thus an essential component of malaria control.

During its passage through the mosquito, the parasite triggers a robust innate immune response against malaria infection (Dimopoulos et al., 2001), that in some cases is capable of controlling parasite development.

The innate immune response of the mosquito consists of humoral and cellular defense mechanisms. Humoral immune responses involve the recognition of microorganisms by pattern recognition receptors (PRRs) molecules, resulting in the activation of serine protease cascades that in turn can activate defense reactions such as melanotic encapsulation or initiate intracellular immune signaling pathways which regulate the transcription of antimicrobial peptide (AMP) genes (Dimopoulos, 2003). An increasing number of serine proteases and antimicrobial peptides have been isolated and characterized in *A. gambiae*. Changes in their transcript abundance in response to bacterial and *Plasmodium* infection have been demonstrated (Richman et al., 1997; Dimopoulos et al., 1997, 1998; Gorman et al., 2000; Vizioli et al., 2000, 2001). Components of the blood meal are also known to interfere with parasite development in the mosquito vector. Presence of the antimalarial drug chloroquine has been associated with an increase in infectivity of *Plasmodium* parasites to the

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mosquito. Mosquitoes fed with *Plasmodium falciparum* and *P. berghei* gametocytes in the presence of serum from chloroquine-treated human volunteers showed a 4–5-fold increase in the mean oocyst number (Hogh et al., 1998). The same effect was also observed in infections with *P. yoelii nigeriensis* N67, which displayed enhanced infectivity to mosquitoes when mice were treated with chloroquine 12 h before feeding (Ichimori et al., 1990). In these studies, enhanced infectivity to the mosquito was not associated with increased gametocyte number, suggesting that it was caused by the interference of chloroquine with the capability of the mosquito to respond to the infection. Several studies have also reported the effect of chloroquine on the vertebrate host immune response during malaria infection (e.g. Rosa et al., 1999). Moreover chloroquine is known to (i) inhibit intralysosomal degradation of proteins (Schultz and Gilman, 1997); (ii) enhance fungal protease activity (Staszczak et al., 2000); (iii) block the nuclear factor-kappaB (NF-kB) induction by Interleukin-1β in epithelial cells [IL-1 receptor belongs to super family TLR (toll like receptor) (Bonizzi et al., 1997)], (iv) inhibit tumor necrosis factor α (TNF-α) release from human and murine cells (Jeong and Jue, 1997), (v) inhibit nitric oxide synthase expression in murine macrophages (Park et al., 1999) and (vi) inhibit pH-dependent steps of the replication of several viruses (Savarino et al., 2003).

In this study, we have characterized, for the first time, the effect of chloroquine on the mosquito immune response at two levels: (i) serine proteases and (ii) antimicrobial peptide gene expression. For that, seven *A. gambiae* gene products were analyzed: four serine proteases (*Sp14D2*, *AgSP24D*, *ISPL5* and *1644280*), and three antimicrobial peptides (*gambicin*, *defensin* and *cecropin*).

2. Materials and Methods

2.1. Mosquitoes

A. gambiae s.s. (Suakoko strain) mosquitoes were reared at 25 °C and 75% humidity with a 12-h light/dark cycle. Adult mosquitoes were maintained on a 10% glucose solution.

2.2. Chloroquine treatment

Female BALB/c mice (*Mus musculus*) were injected intraperitoneally (i.p.) with two doses of chloroquine (10 and 50 mg/kg), 17 h prior mosquito blood feeding. Female mosquitoes, 5–6 days post-emergence and deprived from sugar for 12 h prior feeding, were fed on chloroquine treated and untreated mice for 2 h. Unfed mosquitoes were discarded.

Mosquitoes were collected 24 h post-blood feeding (p.bf.). Batches of 35 midguts were dissected in cold DEPC-treated phosphate-buffered saline (PBS) and processed for RNA preparation. A set of unfed female mosquitoes was used as control. Six independent experiments were performed.

2.3. Midgut bacterial quantification

In order to confirm that chloroquine had no bactericidal effect on midgut bacteria, one dose (50 mg/kg) of chloroquine was administered i.p. in mice and the same procedure of chloroquine treatment was followed.

Batches of 50 treated and untreated mosquito midguts were externally sterilized in 70% ethanol for 5 min, rinsed in PBS and dissected under sterile conditions. Pools of five midguts were homogenized in 50 μl of PBS. Several dilutions were made and 10 μl of each dilution was streaked on Columbia III Agar with 5% Sheep Blood (Becton Dickinson, MD, USA). Plates were incubated at 25 °C under aerobic conditions. Bacterial counts (colony-forming units [cfu]/midgut) were performed after 48 h in three independent experiments.

2.4. Antibiotic treatment

The large spectrum sulfonamide antibiotic Sulfutrim® (Trimethoprim/Dimerazol) from HELSINN was used to test the effect of midgut bacterial reduction by antibiotic treatment in the immune response of *A. gambiae*. One dose T/D (2.5/12.5 mg K g^{-1}) was administered i.p. in mice and the same procedure as for chloroquine treatment was followed. Three independent experiments were performed.

2.5. Plasmodium berghei infection and chloroquine treatment

Female BALB/c mice were inoculated intraperitonally with 10^7 *P. berghei* Anka parasitized red blood cells per mililiter. The course of infection was determined by Giemsa-stained blood films prepared from tail blood. When the parasitaemia reached 5%, two doses of chloroquine (10 and 50 mg/kg), were administered intraperitonally, as previously described.

Seventeen hours after chloroquine treatment, gametocitaemia and parasite exflagellation were confirmed and 5–6 days post-emergence female mosquitoes, were naturally fed on chloroquine treated and untreated infected mice. Unfed females were used as control. Mosquito were collected 24 h post-blood feeding and batches of 35 midguts were dissected in cold DEPC-treated PBS.

The remaining mosquitoes were maintained thereafter at 19/21 °C to allow *P. berghei* development. Three independent experiments were performed.

2.6. RNA extraction and cDNA synthesis

Total RNA from each group of mosquitoes, was extracted with TRIzol ® (Invitrogen- Life Technologies, Barcelona, Spain) following the manufacturer's instructions. For cDNA synthesis, 1 µg of total RNA was treated with DNAse I (Invitrogen- Life Technologies) and reverse transcribed with M-MLV-RT (Invitrogen-Life Technologies) in the presence of oligo(dt)15 primer (Roche Molecular Biochemicals).

2.7. Quantitative real-time PCR analysis

Quantitative real-time PCR was performed to analyze relative levels of *A. gambiae* immune response gene transcripts after chloroquine treatment. Sequences from insect serine proteases (*Sp14D2*, *AgSP24D*, *ISPL5*, *and 1644280*) and antimicrobial peptides (*gambicin*, *defensin* and *cecropin*) previously described to be involved in immune response against *Plasmodium* infection (Dimopoulos et al., 1997; Gorman et al., 2000, Vizioli et al., 2000, 2001) were obtained from GenBank (accession numbers: AF117749, U21917, AJ000675, Z69978, AJ237664, AF063402 and AF200686, respectively) and used to generate specific primers. The sequences of the primers used in the experiments were: Sp14D2-F (5′-TGGGGCCAGACGGAAAACAGT-3′) and Sp14 D2-R (5′-CCGCGGCACGAGTCCTTACCC-3′); AgSP 24D-F (5′-TGGCCCGAGTAATAACGCACGAG-3′) and AgSP24D-R (5′-TACATACGCCCCAGCCCGAG ATAA-3′); ISPL5-F (5′-CTTAACAACATTGCCGTGC TGGAG-3′) and ISPL5-R (5′- ATATCTGCGTCGGTG GTGCGTTCT-3′); 1644280-F (5′-GCCGGCGAGCAC GACTTCAG-3′) and 1644280-R (5′-CGGTTCCGGCA GCGAGAC-3′); gambicin-F (5′-GCATCGGGGCACG CTACTGT-3′) and gambicin-R (5′-GGTCCTGCCGAT GATGGT TCC-3′); defensin-F (5′-GTACCATTGCCG TTGTGCTG -3′) and defensin-R (5′- GATAGCGGC GAGCGATACAG-3′); cecropin-F (5′-CAACCCAGA GACCAACCAACCAC-3′) and cecropin-R (5′-ACTGC CAGCACGACAAAGATGAAG-3′).

Quantitative analysis of the expression of these genes was done by real-time PCR with qPCR core kit for SYBR Green (Eurogentec, S.A., Seraing, Belgium) using a Gene Amp 5700 Sequence Detector (Applied Biosystems). Final concentrations of reagents were 1 × reaction buffer, 3.5 mM $Mgcl_2$, 200 µM dNTP's, 0.3 µM of primer concentrations, 0.025 U/µl of Hot GoldStar enzyme and 1/66,000 of Sybr green for a final volume of 20 µl. One microliter (ca.0.02%) of cDNA was used as template. Cycle conditions were: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min.

Ribosomal protein *S7* gene (Salazar et al., 1993) was used for data normalization (S7-F: 5′-CCTGGAGCTG-GAGATGAACT-3′ and S7-R: 5′-CGGCGCTCGGCA ATGAACAC-3′).

2.8. Confirmation of P. berghei parasites on A. gambiae midgut

In order to confirm the presence of *P. berghei* parasites committed to sporogonic development, we analyzed the expression of the ookinete surface protein Pbs25 (Tsuboi et al., 1997) in *A. gambiae* midguts dissected at 24 h post-feeding by real-time PCR. For this purpose, two specific primers were designed (Pbs25-F: 5′-TATAACGCAGCAATTTCACCAA-3′ and Pbs25-R: 5′-ATTTTTCATTACATCGGCATTCT-3′). Real-time PCR conditions followed those used for the mosquito genes.

The remaining mosquitoes were maintained to allow *P. berghei* development, oocysts detection and quantification on day 10 post-blood feeding.

2.9. Statistical analysis

Data analysis was performed using SPSS v 9.0 for Windows (SPSS, Inc.). The Student's *t*-test was used to compare the mean levels of expression measured in each mosquito treatment: unfed vs. untreated, chloroquine treated (10 mg/kg) vs. untreated, chloroquine treated (50 mg/kg) vs. untreated and chloroquine treated with 10 vs. 50 mg/kg. The Wilcoxon signed rank test was used to compare the bacterial counts per midgut in chloroquine-treated and untreated mosquitoes and the number of oocysts per midgut on day 10 post-blood feeding in each mosquito group: chloroquine treated (10 mg/kg) vs. untreated, chloroquine treated (50 mg/kg) vs. untreated and chloroquine treated with 10 vs. 50 mg/kg. Statistically significant differences were considered for a $p < 0.05$.

3. Results

3.1. Expression analysis of A. gambiae transcripts after chloroquine treatment

The levels of expression of seven immune-related genes (*Sp14D2*, *AgSP24D*, *ISPL5*, *1644280*, *gambicin*, *defensin* and *cecropin*) were measured in the midguts of *A. gambiae* mosquitoes. Two factors were considered when measuring levels of gene expression: the effect of blood feeding and the presence of chloroquine in the blood meal. There was an overall increase of expression

associated with blood feeding, which was suppressed by the presence of chloroquine (Fig. 1).

The increase of expression observed in blood fed mosquitoes, compared to unfed mosquitoes, ranged 3–20 times in the genes studied. When mosquitoes were fed on mice that were treated with chloroquine, we observed a 1.5–12.1-fold decrease in gene expression when compared with untreated mosquitoes. In some cases, expression levels were set back to values approximately similar to those observed in unfed mosquitoes. *Gambicin*, *defensin* and *Sp14D2* expression levels were significantly lower in mosquitoes fed on the higher dose of chloroquine (50 mg/kg) when compared to mosquitoes fed on a blood meal without chloroquine.

Antimicrobial peptides showed a more pronounced reduction of expression in the chloroquine-treated mosquitoes than the serine proteases analyzed.

3.2. Midgut bacterial quantification

Although the amounts of chloroquine used in our study, are considerably lower than those described to have antibacterial effect, we decided to test if the suppression observed with chloroquine treatment could be related to a decrease in mosquito midgut bacterial number. We observed considerable individual variation in bacterial counts within each group of mosquitoes (treated and untreated), ranging from 3×10^4 to 1.24×10^6 cfu/midgut in chloroquine treated, and 1×10^4 to 2.21×10^6 cfu/midgut in untreated mosquitoes. No significant differences were observed between bacterial counts in the chloroquine treated (median = $5.1\text{x}10^5$ cfu/midgut) and untreated (median = 4.1×10^5 cfu/midgut) mosquitoes.

3.3. Expression analysis of A. gambiae transcripts after antibiotic treatment

In order to confirm that effect of chloroquine treatment on the immune system was not mediated through antimicrobial activity, mosquitoes were fed on BALB/c mice treated with a large spectrum antibiotic recommended for veterinary treatment.

Once again, an increment of gene expression induced by blood ingestion was observed (Fig. 2) thereby reinforcing the data obtained with the chloroquine experiment (Fig 1). Real-time PCR results revealed that the antibiotic treatment did not significantly reduce the expression of the transcripts analyzed. The same effect was observed in an extra experiment in which a 2-fold higher dose ($5.0/25.0\,\text{mg K g}^{-1}$) of antibiotic was used (data not shown). These data, together with the observations on midgut bacterial counts of chloroquine-treated and untreated mosquitoes indicate that the effect of chloroquine on the immune response was independent of antimicrobial activity.

3.4. Expression analysis of A. gambiae transcripts following P. berghei infection and chloroquine treatment

This part of the work, aimed at investigating the effect of *P. berghei* infection in the chloroquine modulation of

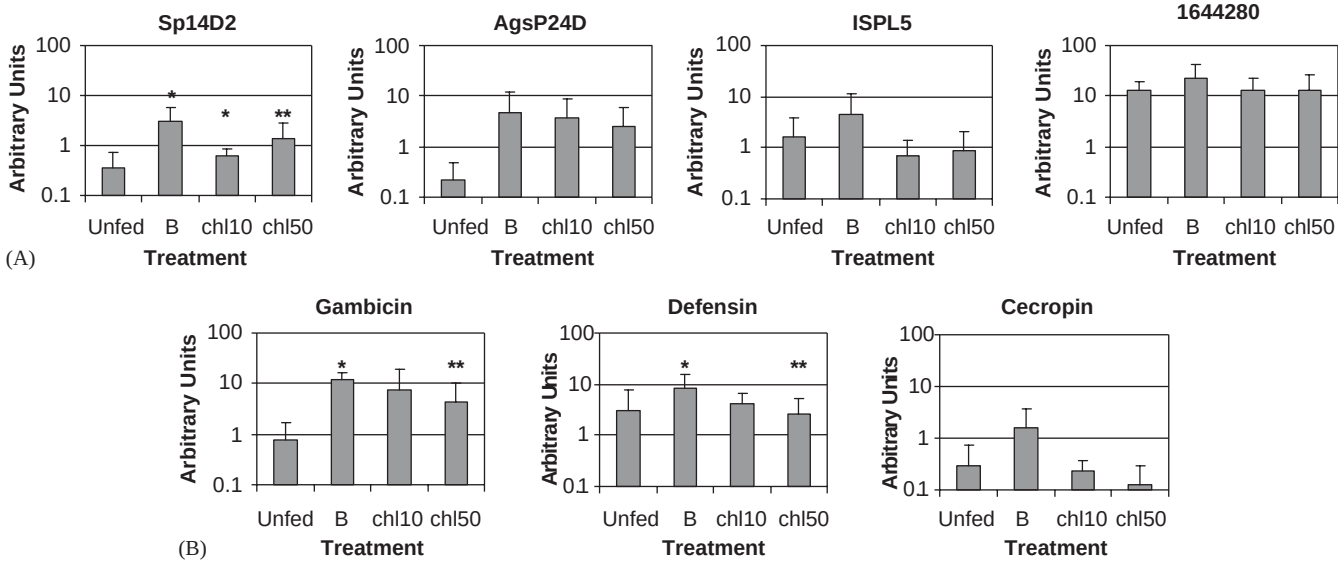

Fig. 1. Effect of chloroquine on the transcript abundance of (A) serine proteases—*Sp14D2, AgSP24D, ISPL5 and 1644280* and (B) antimicrobial peptides—*gambicin, defensin* and *cecropin* in mosquito midguts 24 h after blood feeding. Unfed, non-blood fed control; B, blood fed on untreated mice; chl10, blood fed on mice previously (17 h) treated with 10 mg/kg of chloroquine; chl50, blood fed on mice treated with 50 mg/kg of chloroquine. Data was normalized using *A. gambiae* ribosomal protein S7 gene expression levels. Values represent the mean and standard deviation of six independent experiments (significant differences $p < 0.05$ between *Unfed and B, and **B and chl10 or chl50).

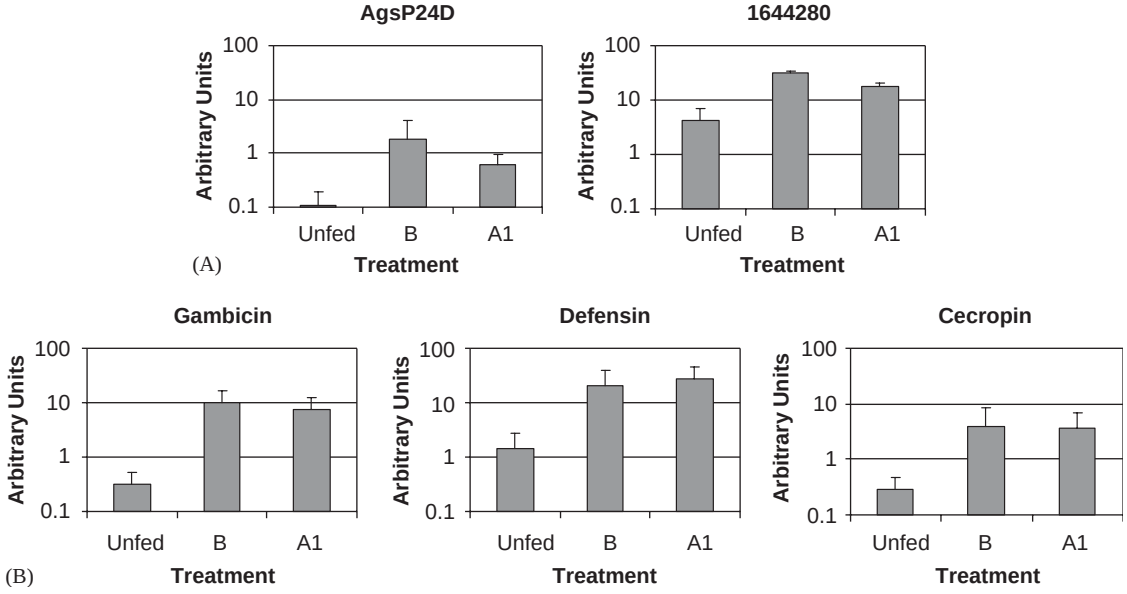

Fig. 2. Effect of large spectrum antibiotic on the transcript abundance of (A) serine proteases—*AgSP24D and 1644280* and of (B) antimicrobial peptides—*gambicin, defensin* and *cecropin*, at day one (24 h post-blood feeding). Unfed, non-blood fed control; B, blood fed on untreated mice; A1, blood fed on mice previously (17 h) treated with 2.5/12.5 mgKg^{-1} trimethoprim/dimerazol. Data was normalized using *A. gambiae* ribosomal protein S7 gene expression levels. Values represent the mean and standard deviation of three independent experiments.

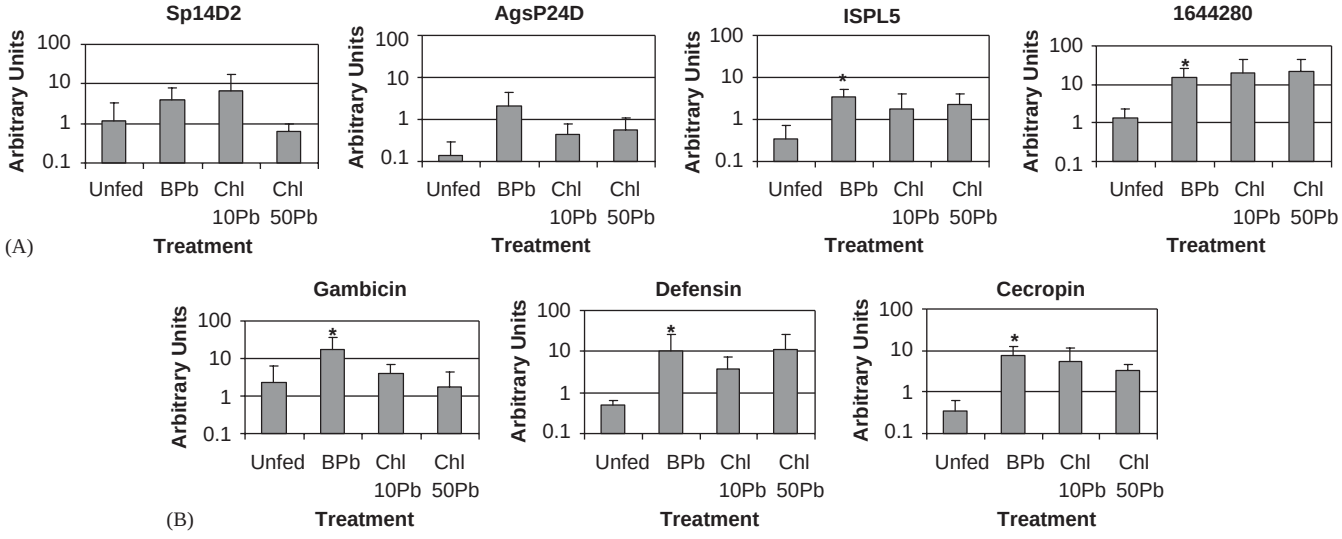

Fig. 3. Effect of chloroquine on the transcript abundance of (A) serine proteases—*Sp14D2, AgSP24D, ISPL5 and 1644280* and of (B) antimicrobial peptides—*gambicin, defensin and cecropin on Plasmodium berghei* infected mosquitoes, at day one (24 h post-blood feeding). Unfed, non-blood fed control; BPb, blood fed with *P. berghei* parasites; chl 10Pb, blood fed on *P. berghei* infected mice previously (17 h) treated with 10 mg/kg of chloroquine; chl 50Pb, blood fed on P. berghei infected mice previously (17 h) treated with 50 mg/kg of chloroquine. Data was normalized using *A. gambiae* ribosomal protein S7 gene expression levels. Values represent the mean and standard deviation of three independent experiments (significant differences $p < 0.05$ between *Unfed and BPb).

the mosquito immune system observed in uninfected mosquitoes.

A general increase (4–22-fold) in the expression levels of the serine proteases and the antimicrobial peptides monitored in this study was again associated with blood feeding. Chloroquine treatment was once again responsible for a general 1.5–9.8-fold decrease of gene expression (Fig. 3). The only exceptions were seen in the expression of *Sp14D2* at low dose treatment (10 mg/kg), *defensin* at high dose treatment (50 mg/kg) and *1644280* at both doses treatment.

When we compared the effect of chloroquine on infected and uninfected mosquitoes (Figs. 1 and 3), a less pronounced down-regulation in infected chloroquine-treated mosquitoes was observed, suggesting that the response triggered by *Plasmodium* infection can to some extent bypass the suppression effect exerted by chloroquine.

3.5. Confirmation of *P. berghei* parasites presence in *A. gambiae* midgut

In order to guarantee that all *P. berghei* life stages were normally developing in all the experiments, several stages of the parasite were monitored.

Presence of gametocytes and parasite exflagellation in treated and untreated mice was confirmed by optical microscopy prior to mosquito blood meal. *P. berghei* chloroquine-treated mice presented lower gametocitaemia than untreated mice. The geometric mean of gametocitaemia varied from 0.91% for untreated, 0.08% for 10 mg/kg chloroquine and 0.08% for 50 mg/kg chloroquine-treated mosquitoes.

The presence of *P. berghei* that were committed to sporogonic development was confirmed 24 h post-feeding by the analysis of the transcripts that code for the ookinete's surface protein *Pbs25*. As expected, expression was detected in *P. berghei* blood fed mosquitoes but no transcripts were observed in unfed mosquitoes or in mosquitoes fed on uninfected blood (data not shown). No significant differences were observed between *Pbs25* expression levels on *P. berghei* chloroquine-treated and untreated mosquitoes. The oocyst burden in the midguts was measured 10 days post-blood feeding (Table 1). Mosquitoes treated with the lower dose of chloroquine (10 mg/kg) presented the highest median intensity of infection when compared with the other two groups, which had similar oocyst counts.

Table 1
Effect of chloroquine treatment on *P. berghei* oocyst formation

Mosquitoes (n)[a]	# oocysts/midgut[b] (min.–max.)
Blood fed on untreated mice ($n = 45$)	5.5 (1–116)
Blood fed on mice treated with 10 mg/kg chloroquine ($n = 33$)	9.5 (1–132)[c]
Blood fed on mice treated with 50 mg/kg chloroquine ($n = 46$)	4.0 (1–33)

Treated (10 and 50 mg/kg) and untreated mosquitoes were dissected on day 10 post-blood feeding and the number of oocysts per midgut determined.

[a]Number of infected mosquitoes used for determination of oocyst counts.

[b]Values represent the median, the minimum and the maximum number of oocysts per midgut.

[c]Significant differences ($p < 0.05$) between 10 mg/kg chloroquine treatment and 50 mg/kg chloroquine treatment.

4. Discussion

This is the first report on the effect of the antimalarial drug chloroquine, a 4-aminoquinoline, on the immune response of *A. gambiae*. The levels of expression of seven immune-related genes (*Sp14D2, AgSP24D, ISPL5*, *1644280, gambicin*, *defensin and cecropin*) previously described as being differentially transcribed during *Plasmodium* infection, were analyzed in the midguts of *A. gambiae* by real-time PCR. We observed an overall increase of gene expression associated with blood feeding, which was suppressed by the presence of chloroquine.

The transcriptional activation after a blood meal, probably associated with the metabolic needs of the blood meal digestion, is consistent with previous transcription studies carried out for most serine proteases and antimicrobial peptides (Müller et al., 1993; Ribeiro, 2003).

Activation of immune-related genes could also be a consequence of an increase in midgut bacterial numbers, as blood meals boost midgut flora (Pumpuni et al., 1996). These authors also observed that midgut bacteria at high concentration can inhibit sporogonic development and suggested that bacteria could trigger defense mechanisms. Therefore alterations in midgut bacterial numbers could induce differential immune-related gene transcription. The presence of chloroquine in the blood meal abrogated the transcriptional activation observed with blood meal intake, which could be a consequence of chloroquine antimicrobial activity. In fact it is known that high concentrations of chloroquine can exert a bactericidal effect (Ciak and Hahn, 1966). However, a bactericidal effect of chloroquine in our experiments was ruled out, since we observed similar midgut bacterial counts in treated and untreated mosquitoes and no significant impact of antibiotic activity on serine proteases and antimicrobial peptides expression was detected with the antibiotic doses tested. Higher doses than those used here would probably have similar effect. These results suggest that the down-regulation of gene expression seen after chloroquine treatment was independent of antimicrobial activity indicating a direct effect of chloroquine on the mosquito immune system.

Taken together, our results suggest that chloroquine might act directly on *A. gambiae* serine proteases cascade, interfering at signal transduction pathways and transcriptional activation level and thus leading to the down-regulation of serine proteases and antimicrobial peptides.

Although chloroquine mechanisms of action have been largely studied, many questions still exist and

nothing or very little is known about the chloroquine mechanisms of action in insects.

Most of the inhibition effects related to chloroquine treatment are attributed to a perturbation of intravesicles acidification. Seyama et al. (2003) observed this effect at the serine proteases level. Indeed, they reported that chloroquine pretreatment completely blocked the intracellular generation of trypsinogen activation peptide (TAP) in cerulein-induced pancreatitis, mediated by neutralization of acidic subcellular compartments. In *A. gambiae* at least seven trypsinogens have been described (Müller et al., 1993). In our study, all serine proteases are trypsin-like, and were described to be involved in insect innate immune response (Hoffmann et al., 1999; Dimopoulos, 2003), mainly as prophenoloxidase (PPO)-activating enzymes.

Considering the above result, we think that interference with endosomes pH, mediated by chloroquine, represents one possible explanation for the down-regulation of mosquito serine proteases and antimicrobial peptides expression observed in our study. Perturbation of endosomal pH can interfere with TAP activation (or other factor activation peptide), and consequently with trypsin synthesis. This will have consequences in the activation of intracellular signaling pathways and the subsequent transcription of effector genes, such as antimicrobial peptides.

Other inhibition effects related to chloroquine treatment appear to be independent of a chloroquine-lysosomotropic mechanism of action. In human peripheral mononuclear blood cells, chloroquine inhibits TNF-α gene expression involving a nonlysosomotropic mechanism (Weber and Levitz, 2000). In addition, chloroquine blocks the activation of NF-κB induced by IL-1β in epithelial cells, by a mechanism involving the acid sphingomyelinase pathway (Bonizzi et al., 1997).

Given that chloroquine affects NF-κB activity, a transcription factor that is rapidly inducible in most cell types following a large variety of stimuli (Bonizzi et al., 1997), and that NF-κB is a pivotal transcription factor of innate immune responses (Delhase et al., 2000; Sacks and Sher, 2002), we can thus presume that chloroquine interference at NF-κB activity can constitute another possible explanation for the reduction of expression observed. Interference of chloroquine at NF-κB transactivators (or at other nuclear factor(s) binding activity) could have blocked the activation of mosquito-specific signaling transduction pathways (Toll, Imd and STAT), and in the end, affected the activation of antimicrobial peptides, such as the ones studied (*gambicin*, *defensin and cecropin*).

The fact that *1644280* expression was not affected by chloroquine treatment, and that its sequence is closer to that of mammalian serine protease families than that of insect families (Dimopoulos et al., 1996) suggests that *1644280* is located upstream of the chloroquine affected pathway or that a different pathway (not affected in the same way by chloroquine) is regulating the expression of this serine protease.

Differences between oocyst counts were similar between treated and untreated mosquitoes ($p > 0.05$), but gametocyte counts were one log magnitude different between them. This might have implications on the outcome of midgut infection. Therefore, if we divide the oocyst counts by gametocitaemia we will obtain a ratio that might give a better view of the parasite infection success in the mosquitoes. These ratios varied from 6.04 in untreated mosquitoes to 50 or 118.75 in treated mosquitoes, showing that infection success was higher in treated mosquitoes.

The effect of the higher dose of chloroquine (50 mg/kg) on mosquito's infectivity was interesting, as we would expect that the dose-dependent down-regulation observed in the majority of the genes studied would correspond to an increase in oocyst burden with the higher dose of chloroquine (as seen with the lower dose).

However, if we take into account the proportion of parasites ingested by the mosquito (less gametocytes ingested by the higher dose chloroquine-treated mosquitoes than by untreated mosquitoes) and those that successfully developed into oocysts, we can presume that, despite apparently similar, mosquitoes treated with the 50 mg/kg dose of chloroquine had higher infectivity than untreated mosquitoes.

The significant difference of mosquito infectivity observed between the two chloroquine doses had a parallel on the *defensin* expression trend. *Defensin* constituted an exception and presented higher levels of expression in the higher dose of chloroquine when compared with the lower dose (Fig. 3). The antimicrobial peptide *defensin* has been shown to be induced upon *Plasmodium* infection (Richman et al., 1997) and might be induced by the presence of gametocytes in the blood meal (Tahar et al., 2002). Furthermore it seems to exert its activity differentially in different life cycle stages of the parasite (Dimopoulos et al., 1997). In vitro parasiticidal effects of *defensin* have also been reported (Shahabuddin et al., 1998). Nevertheless, we believe that this cannot be the only mechanism to justify the differences in infectivity observed, as silencing of the *defensin* gene has little impact on oocyst formation (Blandin et al., 2002).

Sp14D2 is another gene that seems to have an opposite pattern of expression in the two dosages. Lower chloroquine dose increased gene expression in *P. berghei* infected mosquitoes, while at a higher dose a decrease in expression levels was observed (even not significantly different). As described in the literature, *Sp14D2* is slightly up-regulated after *Plasmodium* infection, it has features of a PPO activating enzyme and is probably involved in melanization (Gorman et al., 2000). Other effector genes not covered by our

analysis might also be affected differentially by the higher dose of chloroquine in the same way as *defensin* and *Sp14D2* thus contributing to the infectivity results observed by us and others (Ramkaran and Peters, 1969).

In conclusion, our work provides the first description of the effect of chloroquine on the immune response of *A. gambiae*. We have shown that chloroquine down-regulates the expression of immune-related serine proteases and antimicrobial peptides independently of midgut bacterial numbers. These results suggest that chloroquine might act on *Anopheles* serine proteases cascade, interfering at signal transduction pathways and at the transcriptional activation level. Understanding the mechanisms of chloroquine action in mosquito vector and vertebrate hosts will reveal biological details that can be fruitful for novel malaria control strategies such as those based in transmission-blocking vaccines.

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