

Immune response of *Anopheles gambiae* to the early sporogonic stages of the human malaria parasite *Plasmodium falciparum*

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Deciphering molecular interactions between the malaria parasite and its mosquito vector is an emerging area of research that will be greatly facilitated by the recent sequencing of the genomes of *Anopheles gambiae* mosquito and of various *Plasmodium* species. So far, most such studies have focused on *Plasmodium berghei*, a parasite species that infects rodents and is more amenable to studies. Here, we analysed the expression pattern of nine *An.gambiae* genes involved in immune surveillance during development of the human malaria parasite *P.falciparum* in mosquitoes fed on parasite-containing blood from patients in Cameroon. We found that *P.falciparum* ingestion triggers a midgut-associated, as well as a systemic, response in the mosquito, with three genes, *NOS*, *defensin* and *GNBP*, being regulated by ingestion of gametocytes, the infectious stage of the parasite. Surprisingly, we found a different pattern of expression of these genes in the *An.gambiae*–*P.berghei* model. Therefore, differences in mosquito reaction against various *Plasmodium* species may exist, which stresses the need to validate the main conclusions suggested by the *P.berghei*–*An.gambiae* model in the *P.falciparum*–*An.gambiae* system.

Keywords: *Anopheles gambiae*/immune response/interaction/*Plasmodium berghei*/*Plasmodium falciparum*

Introduction

Malaria is a devastating disease in many tropical and sub-tropical regions that results in the death of ~2.7 million people each year. Malaria is essentially due to *Plasmodium falciparum*, one of the four malaria species that specifically infect humans. *Plasmodium falciparum* is exclusively transmitted by *Anopheles* mosquitoes, mainly from members of the *Anopheles gambiae* complex in Africa. This host–parasite combination is one of the most effective to maintain transmission of *P.falciparum* to human beings. Measures to control malaria have been hampered by the spread of malaria parasites resistant to

anti-malarial drugs and of insecticide-resistant mosquitoes. To combat malaria, a better understanding of the biology of both parasite and mosquito, as well as of the interactions between *P.falciparum* and *Anopheles* vectors, is needed. The recent sequencing of the genomes of *An.gambiae* mosquito (Holt *et al.*, 2002) and of various *Plasmodium* species will be very helpful towards this aim (Carlton *et al.*, 2002; Gardner *et al.*, 2002).

When ingested by a female mosquito during a blood meal, *Plasmodium* gametocytes differentiate into male and female gametes, which fuse to form zygotes. Each zygote elongates to become a motile ookinete in the blood bolus and crosses the peritrophic matrix and the midgut epithelium to reach the haemocoel gut side. The ookinete becomes attached to the midgut wall and transforms into an oocyst, which undergoes divisions to form sporozoites. Sporozoites are released into the haemolymph and invade salivary glands, where they attain maturity and can be injected into a new host during the next blood meal.

During these crucial sporogonic developmental steps in the mosquito midgut lumen, the midgut epithelium and the haemolymph, parasites face a hostile environment, leading to a considerable reduction in the number of parasites reaching the oocyst stage (Vaughan *et al.*, 1994; Beier, 1998; Gouagna *et al.*, 1998). The exact biological processes implicated in the mosquito response to the presence of *Plasmodium* parasites are still largely unknown. Recent studies using the rodent malaria parasite *P.berghei* have provided evidence that several mosquito genes presumably involved in immune surveillance are activated transcriptionally in infected mosquitoes (Dimopoulos *et al.*, 1997, 1998; Richman *et al.*, 1997; Oduol *et al.*, 2000). In addition, nitric oxide synthase (NOS) was found to play an important role in controlling the rate and intensity of infection by *Plasmodium* parasites of *An.stephensi* (Luckhart *et al.*, 1998).

Anopheles gambiae and *An.stephensi* support the development of *P.berghei* in the laboratory, but these host–parasite systems never occur in nature. The laboratory conditions used for *P.berghei* development inside mosquitoes involve large numbers of gametocytes and usually lead to the formation of more than fifty oocysts on the mosquito midgut wall. In contrast, in the field, only a handful of oocysts can be seen in the midgut of *Anopheles* infected by *P.falciparum* (Pringle, 1966; Collins *et al.*, 1984). In this latter case, even if a mosquito ingests a large number of gametocytes, only a few will ultimately develop to the oocyst stage (Sinden and Billingsley, 2001). On the other hand, as few as 10 gametocytes per microlitre of ingested blood can be sufficient for establishing infection in *An.gambiae* (Gouagna *et al.*, 1998; Bonnet *et al.*, 2001). These observations suggest that *P.falciparum* development depends on a fine balance between the ability of the mosquito to build a strong defence response to the

Table I. *Anopheles gambiae* infections by *P.falciparum*

Infection	Number of parasites/ μ l		Mosquito infection				Mean intensity of infections ^c
	Gametocytes	Asexual stages	Ratio of infected mosquitoes				
			14 h ^a	24 h ^a	48 h ^a	7 days ^b	
KOU	2325	0	10/10	9/10	10/10	30/60 (50%)	3.76
ES64	62	106	10/10	10/10	8/15	22/30 (73%)	12.36
AKU	19	191	10/10	10/10	6/15	24/30 (80%)	7

^aNumber of Pfs25 mRNA positive mosquitoes at 14, 24 and 48 h PBM.

^bNumber and percentage (in parentheses) of infected mosquitoes harbouring oocysts at day 7 PBM.

^cMean number of oocysts per positive midgut observed at 7 days.

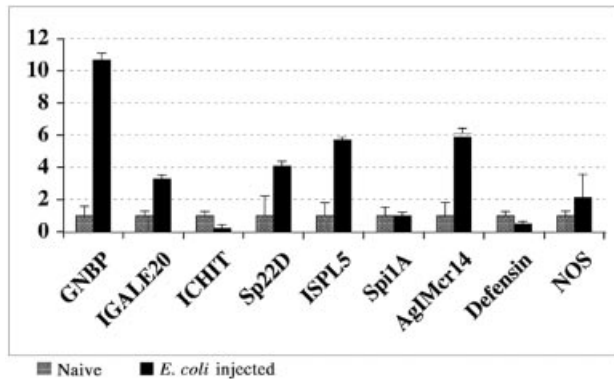


Fig. 1. Quantitative expression of *An.gambiae* immune-related genes by real-time PCR. mRNA was extracted from a pool of naive or *E.coli*-wounded larvae and adult mosquitoes, reversed transcribed and amplified by real-time PCR. The ribosomal protein S7 mRNA was used to normalize the data expressed as the relative expression level in *E.coli*-wounded mosquitoes (black) to the naive ones (grey). Bars indicate standard deviation from three PCR experiments.

presence of the parasite and that of the parasite to evade the immune response of its host.

Here, we analysed the expression of nine *An.gambiae* genes (*GNBP*, Dimopoulos *et al.*, 1997; *ICHIT*, Dimopoulos *et al.*, 1998; *IGALE20*, Dimopoulos *et al.*, 1996; *ISPL5*, Dimopoulos *et al.*, 1997; *Sp22D*, Gorman *et al.*, 2000; *Spi1A*, Danielli, DDBJ/EMBL/Genbank accession No. AJ271352; *AgIMcr14*, Oduol *et al.*, 2000; *defensin*, Richman *et al.*, 1996; and *NOS*, Dimopoulos *et al.*, 1998) involved in several steps of insect immunity pathways during the sporogonic development of *P.falciparum* in mosquitoes fed on blood of gametocyte carriers. Gene expression was quantified by real-time PCR. We also analysed the expression of several of these genes in *P.berghei*-infected mosquitoes, under the same experimental methodology. Our results indicate that *An.gambiae* immune reactions to *P.falciparum* and *P.berghei* infection are distinct.

Results

Mosquito infection

To investigate the interaction between *P.falciparum* and *An.gambiae* under natural conditions of transmission, mosquitoes were fed with the blood of three gametocyte

carrier volunteers harbouring different parasite loads. *Pfs25*, a well-characterized *Plasmodium* gene that is expressed during the sporogonic development from gametocyte to ookinete stage (Thompson and Sinden, 1994) was used to identify individual midguts containing these stages of *Plasmodium* parasites. The number of *Pfs25*-positive midguts at 14, 24 and 48 h post-blood meal (PBM), the proportion of mosquitoes harbouring oocysts, and the mean intensity of infection observed on day 7 PBM are shown in Table I. RT-PCR amplification of *Pfs25* mRNA revealed that all but one mosquito midguts contained sporogonic stages of *Plasmodium* parasites, presumably zygotes and ookinetes at 14 and 24 h PBM, respectively. At 48 h PBM, the proportion of *Pfs25*-positive midguts varied from 40 to 100%, depending on the initial gametocyte load in the ingested blood. The decrease in the number of mosquitoes with *Pfs25* mRNA between 14 and 48 h PBM in ES64 and AKU infection could be due to the reduction in parasite numbers during sporogonic development (Beier, 1998; Gouagna *et al.*, 1998). Alternatively, it could also be due to the difficulty in detecting *Pfs25* mRNA in late ookinetes or young oocysts by RT-PCR. This was probably the case for the AKU infection, as the proportion of infected mosquitoes on day 7 PBM was higher than that of *Pfs25*-positive mosquitoes at 48 h. On day 7 PBM, 50–80% of mosquitoes had oocysts on their midgut wall. It is noticeable that the high number of gametocytes taken up during KOU infection did not produce high number of oocysts. A similar plateau effect on the oocyst load was also observed with *in vitro*-produced *P.falciparum* gametocytes (Ponnudurai *et al.*, 1987, 1989).

Quantification of immune-related gene expression by real-time PCR

Our first objective was to investigate the insect immune response within the *An.gambiae* midgut during early sporogonic development of *P.falciparum* under semi-natural conditions of transmission. For this, we selected a set of genes involved in different pathways of insect innate immunity (Hoffmann *et al.*, 1996, 1999). Three genes (*GNBP*, *IGALE20* and *ICHIT*) belong to the 'Pattern Recognition Receptor' (PRR) gene family (Janeway, 1989). Two genes (*ISPL5* and *Sp22D*) encode serine proteases presumably involved in activation cascades, although *Sp22D* may also play a role as a PRR. Two other genes (*Spi1A* and *AgIMcr14*) encode serine protease inhibitors, while the two remaining genes encode an

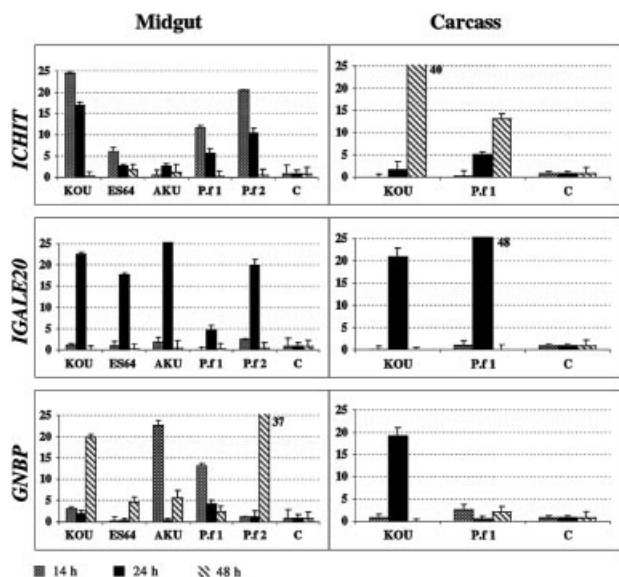


Fig. 2. Expression of *An.gambiae* PRR genes during the early sporogonic development of *P.falciparum*. Real-time RT-PCR induction ratio of *ICHIT*, *IGALE20* and *GNBP* genes at different time points PBM: 14 (dotted bars), 24 (black bars) and 48 h PBM (diagonal striped bars), in midguts and cognate carcasses of *An.gambiae* fed on gametocyte-containing blood (KOU, ES64, AKU) and asexual stage-containing blood (P.f1: 20 000 parasites; P.f2: 200 parasites). Results are represented as fold induction relative to mosquitoes fed on non-infected blood after normalization of the data to the expression level of the ribosomal protein S7 gene. Bars represent the standard deviation from three PCR experiments.

anti-microbial peptide (Defensin) and a molecule with a broader activity spectrum (NOS). The expression of all but one of these genes (*Spi1A*) has been documented in *Anopheles* mosquitoes infected either with bacteria or with the rodent malaria parasite *P.berghei* (Oduol *et al.*, 2000; Dimopoulos *et al.*, 2001). To validate our real-time PCR methodology and the selected primers, amplification of transcripts corresponding to each gene was performed using a mixture of RNAs isolated from *Escherichia coli*-wounded larvae and adults. As illustrated in Figure 1, transcripts were successfully amplified and each gene showed a typical and reproducible expression pattern after *E.coli* inoculation. This confirms that real-time PCR is a sensitive and reliable method for assessing gene expression in our system. In addition, our data showed that expression of the studied genes (except *ICHIT*, *Spi1A* and *defensin*) was induced after a Gram-negative bacteria injection. Expression of these genes was also induced by inoculation of a mixture of a Gram-positive bacteria (*Micrococcus luteus*) and *E.coli* (Dimopoulos *et al.*, 1997, 1998; Oduol *et al.*, 2000). As *ICHIT* expression was found to be stimulated after inoculation of this latter bacterial mixture (Dimopoulos *et al.*, 1998), our result suggests that *ICHIT* is induced in response to Gram-positive bacteria only. We did not observe induction of *defensin* expression although expression of this gene has been reported after *E.coli* injection (Blandin *et al.*, 2002). Such a variation in *defensin* expression after *E.coli* injection may be dependent upon the bacterial strain used. Indeed, the defensin peptide was shown to be active against particular *E.coli* strains only (Vizioli *et al.*, 2001).

Expression of selected *An.gambiae* immune-related genes during early sporogonic development of *P.falciparum*

We then analysed expression of the nine selected genes after mosquito ingestion of *P.falciparum* gametocytes (Figures 2–4). Gene expression was monitored at 14, 24 and 48 h PBM, corresponding to the transformation of zygotes into ookinetes, to the interaction of ookinetes with the peritrophic matrix and midgut cells, and to the migration and early differentiation of ookinetes into oocysts, respectively. At each time point, gene expression was assessed on RNA pools from Pfs25-positive midguts. For analysis of gene expression in mosquito carcasses, we restricted our analysis to pools of the cognate carcasses from KOU infection. As this infection displayed the highest load of gametocytes and no asexual stages, we anticipated that KOU samples would provide a clear-cut expression profile. In some instances, RNAs pools of carcasses from ES64 and AKU infections were used to confirm gametocyte-specific regulation of gene expression. As controls, similar pools were made from RNAs of 10 midguts and 10 carcasses of mosquitoes fed on non-infected blood or on blood containing asexual stages of *P.falciparum* exclusively, which do not develop in mosquitoes (see Materials and methods).

PRR gene expression in *An.gambiae*

As shown in Figure 2, the expression of the three PRR genes was higher in midguts of mosquitoes fed on blood containing gametocytes (KOU, ES64 and AKU infections) or non-infective asexual stages (P.f1 and P.f2) than in control mosquitoes fed on non-infected blood (C). *ICHIT* and *IGALE20* showed a time-specific expression upon parasite (gametocyte and asexual stage) presence in the blood meal. *ICHIT* was overexpressed at 14 and 24 h PBM, except in AKU infection, with induction rates ranging from 6- to 24-fold. There was a gradual decrease in expression over time after parasite ingestion in midguts, whereas an opposite trend was observed in carcasses: gradual increase of expression from 14 to 48 h PBM. The expression of *IGALE 20* was stimulated strongly 24 h PBM (induction rates 5–62-fold) in both midguts and carcasses after ingestion of gametocytes or asexual parasites.

GNBP showed the most variable expression pattern in mosquito midguts. Its expression was stimulated in only two infections (AKU and P.f1) at 14 h PBM, and in all infections at 48 h PBM, with induction varying between 3- and 37-fold. At 24 h PBM, *GNBP* expression was either not stimulated (ES64, AKU and P.f2) or slightly stimulated (KOU and P.f1). In contrast, in carcasses, this gene was overexpressed strongly in KOU infection (Figure 2), as well as in ES64 and AKU infections (data not shown), and *GNBP* overexpression in carcasses occurred at 24 h PBM only. This result supports the conclusion that ingestion of *P.falciparum* gametocytes triggers a specific control of *GNBP* expression in *An.gambiae* carcasses.

Expression patterns of serine protease genes

The *Sp22D* gene, which can be classified in both the PRR and protease gene families (Gorman *et al.*, 2000), was overexpressed at 24 h PBM in midguts corresponding to two infections with gametocytes (Figure 3). However,

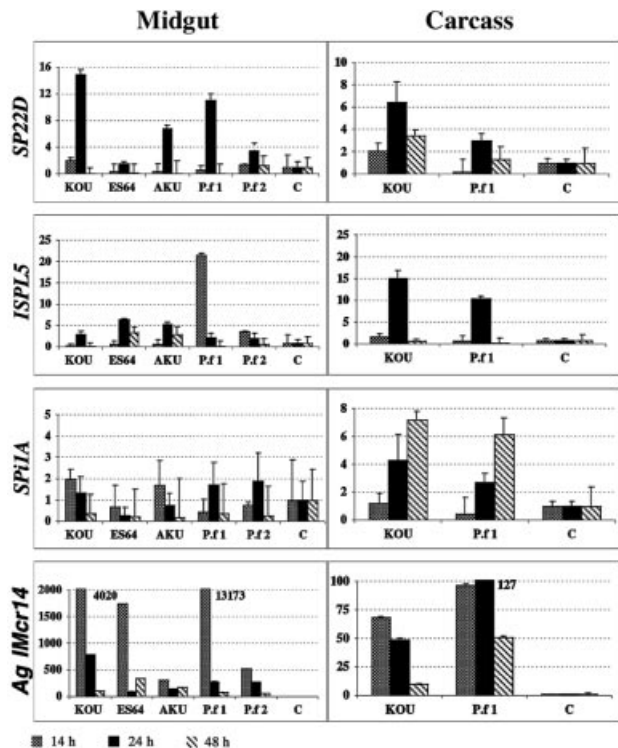


Fig. 3. Expression of *An.gambiae* genes encoding serine proteases and protease inhibitors during the early sporogonic development of *P.falciparum*. Real-time RT-PCR induction ratio of two serine protease genes (*Sp22D* and *ISPL5*) and two protease inhibitor genes (*Spi1A* and *AgIMcr14*) at different time points PBM. Legends are as in Figure 2. Note that the scale varies between graphs.

induction of *Sp22D* gene expression did not seem to be gametocyte specific, as increased gene expression was also detected in midguts from mosquitoes fed on asexual stages. In carcasses, increased *Sp22D* gene expression was also noticed at 24 h, but remained moderate compared with midgut expression.

Expression of *ISPL5* was only slightly modified in midguts of infected mosquitoes. The most noticeable effect was a 14 h-induced *ISPL5* expression in mosquitoes fed on blood that did not contain gametocytes, but high numbers of asexual parasites. In carcasses, ingestion of gametocytes or asexual blood stages induced *ISPL5* expression at 24 h PBM.

Expression patterns of protease inhibitor genes

No major difference was observed in the expression of *Spi1A* in mosquito midguts in various infection experiments (Figure 3). Nonetheless, a slight induction was observed in two gametocyte-containing infections at 14 h PBM and in the two asexual stage-containing infections at 24 h PBM. In contrast, the expression of this gene was upregulated at 24 and 48 h PBM in carcasses of mosquitoes fed on a large number of gametocytes or asexual parasites (KOU and P.f1 infections, respectively). This result illustrates that the presence of *P.falciparum* parasites in the blood meal does not lead to midgut overexpression of all immune-related genes.

AgIMcr14 showed an interesting pattern of expression. In midguts of mosquitoes fed on gametocyte- or asexual

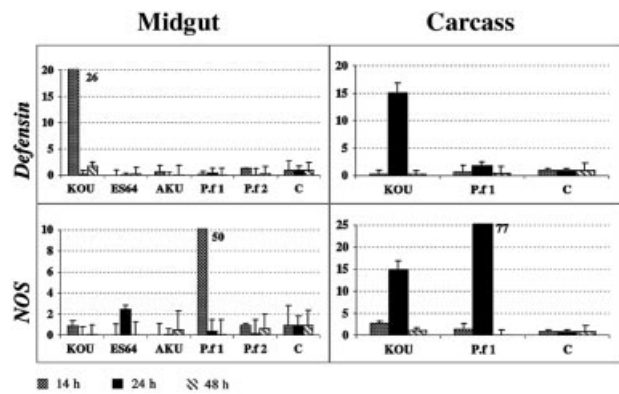


Fig. 4. *Defensin* and *NOS* expression in midguts and carcasses of *An.gambiae* during the early sporogonic development of *P.falciparum*. Real-time RT-PCR induction ratio of *defensin* and *NOS* genes at different time points after blood feeding. Legends are as in Figure 2.

stage-containing blood, a very high level of induction was observed at 14 h PBM. In addition, although *AgIMcr14* expression decreased over time, the level of expression was still 100-fold higher than in control mosquitoes at 24 or 48 h PBM. In carcasses, although *AgIMcr14* expression was higher in the two analysed infections than in the control, the expression level was considerably lower than in midguts. The strong expression of *AgIMcr14* in response to ingested parasites, in both midguts and carcasses, supports the conclusion that this molecule possibly plays a critical role in mosquito immunity.

Defensin and NOS expression

Midgut expression of *defensin* displayed a 26-fold induction at 14 h PBM in KOU infection, which contained a high load of gametocytes, whereas its expression was not induced in the two other gametocyte-containing infections, nor in the two asexual stage-containing infections (P.f1 and P.f2) (Figure 4). Interestingly, induction of *defensin* was observed in carcasses of mosquitoes from KOU, ES64 and AKU infections (5- to 15-fold induction, Figure 4; data not shown) at 24 h PBM, whereas asexual stages triggered a 2-fold induction only. This result strongly suggests that expression of *defensin* in *An.gambiae* carcasses is specifically regulated by the presence of gametocytes in the ingested blood.

The level of *NOS* midgut expression was equivalent to the control or slightly lower in all infections, except in one gametocyte infection (ES64) at 24 h PBM and in P.f1 infection at 14 h PBM (Figure 4). In this latter infection, a 50-fold induction of *NOS* was observed. However, in carcasses, *NOS* expression was increased at 24 h PBM, 15 or 75-fold in KOU and P.f1 infections, respectively. Absence, or low level, of *NOS* expression in midguts of mosquitoes fed on gametocyte-containing blood may reveal that early sporogonic stages of *P.falciparum* are able to repress *NOS* expression.

Specificity of the immune response

As mentioned earlier, *An.gambiae* is a natural host for *P.falciparum*, but not for rodent malaria parasites such as *P.berghei*. It can be anticipated that host-parasite interaction can vary depending on the species involved (Yoeli, 1973; Templeton *et al.*, 1998; Lecuit *et al.*, 2001; Bonas

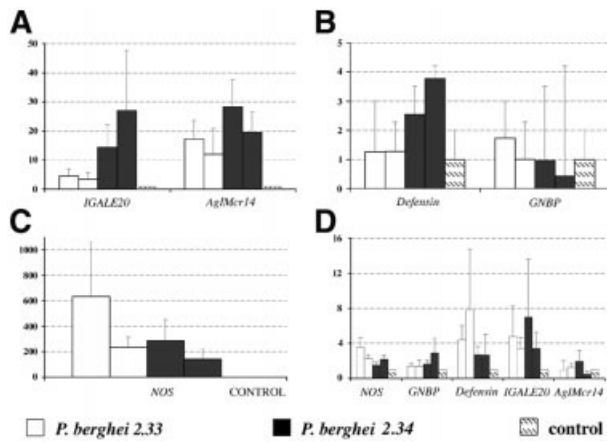


Fig. 5. Expression of immune responsive genes in *An.gambiae* 24 h after ingestion of *P.berghei*. (A–C) Midgut expression. (D) Carcass expression. Real-time RT-PCR induction ratio of *Igale20* and *AgIMCR14* (A), of *defensin* and *GGBP* (B) and of *NOS* (C). Results are represented as fold induction relative to mosquitoes fed on non-infected blood (control), after normalization of the data to the expression level of the ribosomal protein *S7* gene. Bars represent the standard deviation from triplicate PCR of at least two independent RT experiments.

and Lahaye, 2002). We thus tested whether the mosquito immune surveillance system was stimulated differently by *P.berghei* and *P.falciparum*, using real-time PCR to measure gene expression. It should be emphasized that in humans, gametocyte carriers harbour a moderate level of, and sometimes no detectable, asexual stages in peripheral blood circulation and that individuals having symptomatic malaria outburst usually carry a high density of asexual stages without gametocytes, whereas rodents infected with *P.berghei* harbour gametocytes and a high number of asexual stages. In addition, *P.falciparum* infections under field conditions of transmission lead to the formation of few oocysts per mosquito midgut, whereas *P.berghei* laboratory infections usually lead to the formation of at least 50 oocysts per midgut. To limit the number of parameters that vary between the two parasite systems as much as possible, we analysed *P.berghei* infections that led to a mean intensity of midgut infection similar to the *P.falciparum* situation that we analysed above. Furthermore, to distinguish the effects associated with *P.berghei* gametocytes from those associated with *P.berghei* asexual stages, we analysed the expression pattern of some of the previously studied genes in mosquitoes fed on mice infected with a *P.berghei* gametocyte-producing strain or a non-gametocyte producing one. We restricted our analysis to genes (*NOS*, *defensin*, *GGBP*, *IGALE20* and *AgIMcr14*) and a time point (24 h PBM) that showed the most significant regulation in *P.falciparum*-infected mosquitoes.

Results presented in Figure 5A and B indicate that *IGALE20*, *AgIMcr14* and *defensin* are overexpressed in the midgut of mosquitoes infected with the gametocyte producing-clone as compared with control mosquitoes and mosquitoes infected with the non-gametocyte-producing clone. In contrast, midgut *GGBP* expression remained at similar levels in mosquitoes infected with either parasite clones or in controls. Finally, *NOS* expression was increased in all infections, but overexpression did not

appear to be gametocyte-specific (Figure 5C). In mosquito carcasses (Figure 5D), even if *NOS* was overexpressed in *P.berghei*-fed mosquitoes as compared with control mosquitoes, the level of expression was considerably lower than in cognate midguts. Carcass expression of *defensin* and *IGALE20* was also higher in *P.berghei*-fed mosquitoes than in control mosquitoes; this induction was not dependent on the presence of gametocytes in the blood meal. Expression of *GGBP* and *AgIMcr14* remained almost unchanged.

Most of these results are in agreement with published results obtained with semi-quantitative analysis of the *P.berghei*–*An.gambiae* system (Richman *et al.*, 1997; Dimopoulos *et al.*, 1998). However, we did not observe the previously reported gametocyte-specific expression of *GGBP* (Richman *et al.*, 1997). Another discrepancy concerns the level of *NOS* expression in infected midguts, which was reported lower than expression in carcasses (Dimopoulos *et al.*, 1998). By using the two *P.berghei* strains, our data provided two novel observations: non-gametocyte-specific expression of *NOS* and gametocyte-specific induction of *IGALE20* in midguts of *P.berghei*-infected *An.gambiae*. When comparing these results with those obtained with *P.falciparum*-infected mosquitoes, all the studied genes, with the exception of midgut expression of *GGBP*, were regulated differently in the two systems.

Discussion

During development in mosquitoes, *P.falciparum* parasites suffer major population losses at two developmental stages: when gametocytes transform into migrating ookinetes, and when sporozoites invade salivary glands. Presumably, mosquito innate immunity contributes to these losses. Evidence has been provided that the *An.gambiae* immune response is indeed stimulated at different stages of *P.berghei* development, particularly after ingestion of gametocytes (Dimopoulos *et al.*, 2001; and references herein). However, *P.berghei* is not naturally transmitted by *An.gambiae*, and the number of developing *P.berghei* ookinetes in experimental infections exceeds the number of *P.falciparum* ookinetes under natural transmission conditions by far. Therefore, we asked here whether a similar immune response is triggered in *An.gambiae* upon infection with *P.falciparum* or *P.berghei*, under the same experimental design. The results described in this report support several important conclusions.

The first conclusion is that most genes whose expression is regulated by the presence of the *P.falciparum* parasite in the blood meal are not specifically regulated by the presence of gametocytes. The presence of asexual stages in two of the studied gametocyte infections may be the source of this confounding effect for some genes (i.e. *GGBP* midgut expression). Alternatively, parasite molecules shared by gametocytes and asexual stages, rendered accessible to the immune surveillance system as a consequence of the digestion process stimulated by blood feeding, could trigger these responses. In a few instances, however, gene expression was modified specifically after gametocyte ingestion: *NOS* was repressed in midguts, and *GGBP* and *defensin* were induced in carcasses (which contain the fat body cells that behave

as an important source of immune response in insects; Boman, 1995). Results obtained from the KOU infection, which harboured gametocytes only, clearly suggest that *P.falciparum* gametocytes specifically upregulate *GNBP* and *defensin* expression in mosquito carcasses, but not in mosquito midguts, at 24 h PBM. The timing of expression corresponds to the invasion of the mosquito midgut by ookinetes. As discussed by Richman and collaborators (Richman *et al.*, 1997), it is not known whether such an effect (in the carcasses) results from receptor-mediated immune recognition of developing parasites or from midgut injury (and possibly induced by bacteria contained in the blood bolus) associated with ookinete invasion. In *Glossina* flies, establishment of *Trypanosoma* parasites triggers a sustained *defensin* expression in the fat body over several days (Hao, 2001). The fact that in our system, *defensin* and *GNBP* expression was tightly upregulated at 24 h PBM and not expressed later on suggests that, once established in the mosquito midgut epithelium, *P.falciparum* ookinetes might be able to repress *defensin*, and possibly *GNBP*, expression. Alternatively, this tight regulation might be solely due to bacterial or ookinete components accessible to the immune system in a narrow window. It is intriguing that *defensin* was overexpressed in midguts of mosquitoes fed on KOU infection early in the infection process (14 h PBM). As expression of this gene was similar to the control in all other infections, it may be hypothesized that this overexpression is controlled by the large number of ingested gametocytes, which leads to the loss in parasites reaching the oocyst stage (Table I). It is also worth noting that not all genes were regulated by parasite ingestion in our system. Parasite presence had almost no effect on *Spi1A* midgut expression, and ingestion of gametocytes did not induce or even repress midgut expression of *NOS*. *Spi1A* was reported not to be regulated by bacterial infection in *An.gambiae* cell culture (Dimopoulos *et al.*, 2000), whereas *NOS* was found to be upregulated in *P.berghei*-infected *Anopheles* mosquitoes (Dimopoulos *et al.*, 1998; Luckhart *et al.*, 1998).

The second observation concerns the spatio-temporal regulation of the studied immune-related genes following ingestion of *P.falciparum*. Some genes were expressed at the same time in both midguts and carcasses (*IGALE 20*, *Sp22D* and *AgIMcr14*), others were expressed later in carcasses rather than in midguts (*ICHIT* and *defensin*). In addition, *NOS*, whose midgut expression was virtually unchanged after gametocyte ingestion, was clearly induced in carcasses by both asexual stages and gametocytes. A similar observation can be made for *Spi1A* after ingestion of gametocytes and asexual stages. Since tissue components of the carcasses were never directly in contact with parasites at any time point studied, these results would indicate that signals arising from the parasite midgut interaction reached these tissues, which might then behave as a second line of defence. As mentioned earlier, the nature of the signals could be molecules common to both gametocytes and asexual stages, released from the parasites through the action of the mosquito digestive enzymes or ookinete-specific molecules during midgut penetration. It might also come from a midgut injury response possibly involving midgut bacteria moving along with the migrating ookinetes. Of particular interest are the differential expression patterns of *AgIMcr14* and *ICHIT*.

Midgut expression of these two genes gradually decreased from 14 to 48 h PBM. Moreover, carcass expression of *ICHIT* gradually increased during that same time period. Such a regulation involving an early induction in the midgut and transferred (transposed) and sustained response in the carcasses suggests that this pathway may contribute efficiently to pathogen clearance. *ICHIT* encodes a protein with chitin-binding and mucin domains that are found also in several molecules involved in defence mechanisms (Dimopoulos *et al.*, 1998), and could be involved in opsonization of microorganisms. Such a function may similarly be associated with *AgIMcr14*. This gene has been recently characterized as the first insect member of the mammalian $\alpha 2$ -macroglobulin and C3 complement family and was shown to be specifically activated by *P.berghei* infection rather than by bacterial infection in whole mosquitoes (Oduol *et al.*, 2000). We show here its expression pattern in both midguts and carcasses. In midguts of *P.falciparum*-infected mosquitoes, *AgIMcr14* was strongly upregulated after ingestion of both gametocytes and asexual stages. Our data indicate that this immune pathway, which has recently been documented in an established *An.gambiae* cell line and in haemocytes (Levashina *et al.*, 2001), is also present in the mosquito midgut, clearly confirming the status of the midgut as an immune organ.

A third observation was made by comparing the regulation of immune-related genes in the natural *An.gambiae*-*P.falciparum* system and in the model *P.berghei*-*An.gambiae* system. Regulation of gene expression was compared at 24 h PBM, a time that corresponds to the initial interaction of ookinetes with the mosquito midgut. A global analysis of our results obtained with the two host-parasite systems showed that the *An.gambiae* immune surveillance behaves differently in the presence of *P.falciparum* or *P.berghei*. First, whereas no gene was specifically regulated by gametocyte in midguts of *P.falciparum*-infected mosquitoes, three genes (*IGALE20*, *AgIMcr14* and *defensin*) were upregulated upon ingestion of *P.berghei* gametocytes. Second, expression of *defensin* and *GNBP* was increased upon ingestion of gametocytes in carcasses of *P.falciparum*-infected mosquitoes, but not in carcasses of *P.berghei*-infected mosquitoes. Third, *P.berghei* gametocytes and asexual stages triggered *NOS* expression midguts, whereas *P.falciparum* gametocytes and asexual stages did not. On the contrary, both gametocytes and asexual stages of *P.falciparum* upregulated *NOS* expression in mosquito carcasses, while *P.berghei* gametocytes and asexual stages did not. Lastly, the level of *AgIMcr14* expression differed considerably between the two systems.

As we used infection conditions that were as similar as possible in the two parasite systems (see Results and Materials and methods), it is likely that the above differences are mainly due to the two different species involved. For example, the difference in the infection pathway by ookinetes of the two species may account in part for the differences in mosquito immune reaction. Indeed, the mode of ookinete migration across the midgut epithelium appears to be different in the two species, either via an intracellular (*P.berghei*) or an intercellular (*P.falciparum*) mode (Meis *et al.*, 1989). Previous work

confirmed the intracellular route followed by *P.berghei* ookinetes (Han *et al.*, 2000).

In conclusion, our work provides the first description of the immune response of *An.gambiae* against the human malaria parasite *P.falciparum*, during the interaction of ookinetes with the mosquito midgut barrier. The study of the immune response at different time points during this infection process emphasizes that mosquitoes are able to mount a finely tuned response against the parasite. Furthermore, our analysis shows that *An.gambiae* immune response to the presence of *P.falciparum* is different to that of *P.berghei*. Although *P.berghei* is more amenable to manipulation than *P.falciparum*, *P.falciparum* is the major human malaria pathogen. The differences reported here emphasize the need to validate the main conclusions suggested by the *P.berghei*–*An.gambiae* model in the *P.falciparum*–*An.gambiae* system, especially if one wants to exploit the mosquito immune system to produce mosquitoes refractory to the development of the human malaria parasite.

Materials and methods

Gametocyte carriers

Asymptomatic Cameroonian schoolchildren aged <10 years old were mass-screened to detect parasite carriers in four villages located ~100 km east of Yaoundé, the capital city of Cameroon, during the rainy season in May–June 2000. Thick blood smears from finger-pricked samples were stained with 10% Giemsa, and the number of *P.falciparum* gametocytes was determined by microscopy observation. Children with at least 10 gametocytes/μl of blood who had not taken any anti-malarial treatment within the previous two weeks were enrolled in our study. Children with lower gametocytaemia or mixed infections with *P.ovale* and/or *P.malariae* were excluded. Children with >1000 parasites/μl and symptoms associated with malaria were treated with sulfadoxine-pyrimethamine. All participants were volunteers and their parents' consent was obtained. The study was approved by the Cameroonian National Ethics Committee.

Mosquitoes and *P.falciparum* infection

Anopheles gambiae (Yaoundé strain, M cytotype) was reared under standard conditions, in the insectarium set up at OCEAC, Yaoundé. This strain originated from mosquitoes collected in Yaoundé city and was maintained under laboratory conditions for several generations (Tchuinkam *et al.*, 1993). Venous blood (10 ml) from gametocyte carrier volunteers was collected in a heparin-coated tube and immediately used for infecting mosquitoes. For each experiment, batches of 50 nulliparous females (5 days old) starved of sugar 24 h prior to blood feeding were fed on patient's blood for 20 min, using the artificial membrane feeding technique (Tchuinkam *et al.*, 1993). Fully engorged females were maintained in the insectarium until dissection. Three independent infections, referred to as AKU, ES64 and KOU, were performed by feeding mosquitoes on blood containing 19, 62 and 2325 gametocytes/μl, respectively. As gametocyte carriers usually harbour asexual parasites also, a set of experiments was performed with blood containing asexual parasites, without gametocytes, with parasitaemia of 20 000 parasites/μl or 200 parasites/μl. The latter sample was obtained by diluting the former in non-infected blood so that the asexual parasite density corresponds to that found in gametocyte carriers. A series of mosquitoes were also fed on uninfected blood. Mosquitoes were dissected in cold phosphate-buffered saline (PBS pH 7.2), at 14, 24 and 48 h PBM. Thus, 180 midguts and corresponding carcasses (remaining tissues) were individually isolated and conserved in RNA Later® (Ambion) for further RNA isolation. For each feeding on infected blood, oocyst detection was performed on day 7 PBM on the remaining mosquitoes ($n \geq 30$).

Plasmodium berghei infection

The same *An.gambiae* Yaoundé strain was reared at the Pasteur Institute and fed on *P.berghei* infected mice (Swiss). Two infections were performed with the ANKA gametocyte-producing strain 2.34 (parasitaemia: 7.25 and 5.9%) and two with the ANKA gametocyte-defective

strain 2.33 (parasitaemia: 4.1 and 5%). A series of mosquitoes was fed on an uninfected mouse. Mosquito midguts and carcasses were isolated at 24 h PBM as described above, and their RNA content isolated for real-time RT-PCR analysis. Mosquito infection was scored on day 11 PBM. Prevalence of infection and mean number of oocysts per positive midgut were 51.7% and 10.5, and 68.75% and 7.3, respectively, for *P.berghei* 2.34. No oocyst was detected on midguts from mosquitoes fed on *P.berghei* 2.33, as expected.

Bacterial infection

Larvae and adult females (*An.gambiae*, G3 strain) were wounded with a needle dipped into a concentrated *E.coli* suspension. Larvae and adults surviving after 24 h were used for RNA extraction.

RNA extraction and reverse transcription

Total RNAs from individual midguts and carcasses was extracted with Tri-Reagent™ following the manufacturer's instructions. After DNase I treatment (Ambion DNA-free), the pellet was suspended in a final volume of 20 μl of Tris-EDTA buffer. RNA (2 μl) was transcribed using a random hexamer mixture and MMLV reverse transcriptase (Gibco-BRL), in a final volume of 40 μl. The absence of contaminating genomic DNA was checked in each RNA sample by specific amplification of the actin gene (Salazar *et al.*, 1994).

Detection of Pfs25 transcripts in infected mosquitoes

In order to identify infected mosquitoes harbouring sporogonic stages of *P.falciparum*, the presence of Pfs25 transcripts in RNA sample from each mosquito midgut was assessed by RT-PCR. After reverse transcription, 5 μl of cDNAs were subjected to a series of nested PCR using the following primers: PCR1, Pfs25-1U (5'-ATGCGAAAGTTACCGTGGAT-3') and Pfs25-1L (5'-CAAGCGTATGAAACGGGATT-3'); and PCR2, Pfs25-2U (5'-ATAATGCGAAAGTTACCG-3') and Pfs25-2L (5'-CAGGTTTCATTTTCTTT-3'). cDNAs of Pfs25-positive midguts corresponding to one infection and to one time point after infection were pooled. The cDNAs of the cognate carcasses were pooled as well.

Quantitative real-time PCR analysis

Real-time PCR was performed using the dsDNA dye SyberGreen (MasterMix Perkin Elmer) and the iCycler from Bio-Rad. Primers were designed using the Oligo 5 software (NBI) in such a way that the amplification products fall in the 100 bp size range and that primers do not form unwanted dimers. PCR was performed in triplicate in a 25 μl final volume containing 900 nM of each forward and reverse primers, and 5 μl of a 1/5 dilution of the RT products. Signals were normalized to the ribosomal protein S7 mRNA. Normalized data were used to quantitate relative levels of a given mRNA in uninfected and infected samples according to the ΔΔCt analysis (Hooper *et al.*, 2001). Before proceeding to the relative quantification using this method, similarity in amplification efficiency of target and reference was verified (User Bulletin 2, ABI). The following primers were used for amplification of the S7mRNA and quantification of the expression of nine immune-related genes: S7-U, 5'-CACCGCCGTGTACGATGCCA-3'; S7-L, 5'-ATGGTGGTCTGCTGGTTCT-3'; GNBP-U, 5'-CGGAGCAGGAGTTTACGAAGA-3'; GNBP-L, 5'-ATCCTCCCCGTTGATGCTTA-3'; IGAL20-U, 5'-TTACCGAAGATTAGGATTC-3'; IGAL20-L, 5'-GGACAGGACAACCGCAAAC-3'; ICHIT-U, 5'-GACCGTTGCCGACTATTTT-3'; ICHIT-L, 5'-CCTCATGTGGCATTCCGTAGAAC-3'; ISPL5-U, 5'-CGTACCTTCCGCTTCCGTTTC-3'; ISPL5-L, 5'-GGTCGTGTTTCGCTACCTGC-3'; Spi-1A-U, 5'-CGGTGAACGTGGCGAACA-3'; Spi-1A-L, 5'-GCTGCCGCCGACTCTC-3'; AgIMcr14-U, 5'-AATATGTCGCTGCGTCAC-3'; AgIMcr14-L, 5'-TCACCCGATTCCAGATG-3'; Sp22D-U, 5'-TGCAATCCGGTCCACCTC-3'; and Sp22D-L, 5'-CGGGTCGATCTGCTCCACAT-3'; Defensin-U, 5'-TTGTGCTGGCGGCTACCCTG-3'; Defensin-L, 5'-GGCATGGTGCCTTCTCCTCG-3'; NOS-U, 5'-GTTCTCGATCGCGTGTCTTG-3'; NOS-L, 5'-TGCAGGATGAGCTCCGATGAT-3'.

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