Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle

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Six gene markers have been used to map the progress of the innate immune response of the mosquito vector, *Anopheles gambiae*, upon infection by the malaria parasite, *Plasmodium berghei*. In addition to four previously reported genes, the set of markers included *NOS* (a nitric oxide synthase gene fragment) and *ICHIT* (a gene encoding two putative chitin-binding domains separated by a polythreonine-rich mucin region). In the midgut, a robust response occurs at 24 h post-infection, at a time when malaria ookinetes traverse the midgut epithelium, but subsides at later phases of malaria development. In contrast, the salivary glands show no significant response at 24 h, but are activated in a prolonged late phase when sporozoites are released from the midgut into the haemolymph and invade the glands, between 10 and 25 days after blood feeding. Furthermore, the abdomen of the mosquito minus the midgut shows significant activation of immune markers, with complex kinetics that are distinct from those of both midgut and salivary glands. The parasite evidently elicits immune responses in multiple tissues of the mosquito, two of which are epithelia that the parasite must traverse to complete its development. The mechanisms of these responses and their significance for malaria transmission are discussed.

Keywords: epithelial barriers/immune responses/malaria parasite/mosquito vector

Introduction

The life cycle of the rodent malaria parasite *Plasmodium berghei* in the mosquito vector *Anopheles gambiae* begins with the ingestion of sexual stage gametocytes during blood feeding on an infected rodent host. In the mosquito midgut lumen, the gametocytes release male and female gametes which fuse to produce zygotes that in turn develop into motile ookinetes. The ookinetes traverse the midgut epithelium and develop into oocysts lodged extracellularly between epithelium and basal lamina. After ~9 days of maturation, oocysts start to rupture, releasing thousands of motile sporozoites that disperse throughout the mosquito hemocoel. From there, some sporozoites reach and selectively invade the distal lateral and median lobes of the salivary glands, where they complete their time-dependent maturation and can be transmitted to the next host (Touray *et al.*, 1992).

For the successful completion of the life cycle in the mosquito, the parasites must traverse the epithelial barriers of both the midgut and the salivary gland, and evade any immune responses that may be mounted by the mosquito (Dimopoulos *et al.*, 1997). Significant parasite losses occur repeatedly during the life cycle, even in mosquitoes which are susceptible to the parasite (Vaughan *et al.*, 1994; Beier, 1998). Loss in numbers of two orders of magnitude have been recorded between the ookinete and oocyst stages, and only a fraction of the released sporozoites successfully invade the salivary glands after release from the oocysts, while the haemolymph is quickly cleared of those that fail to reach the glands. The losses are extreme in species or strains of mosquitoes that are refractory to the particular parasite, for example because they lyse the ookinetes (Vernick *et al.*, 1995) or melanotically encapsulate the early oocysts (Collins *et al.*, 1986). The molecular mechanisms underlying parasite loss in the mosquito are still unknown. Studies in other organisms suggest that microbial and macroparasitic surface antigens such as lipopolysaccharides, peptidoglycans or β -1,3-glucans may bind to specific pattern recognition receptors, and thereby elicit immune responses such as phagocytosis, secretion of defence molecules or activation of prophenoloxidase leading to melanization (Hoffmann *et al.*, 1996; Söderhäll and Cerenius, 1998).

Malaria infection has been reported to exert both physiological and behavioural effects on the mosquito, such as increased mortality, decreased fecundity (Hogg and Hurd, 1997) and shorter probing times during feeding (Rossignol *et al.*, 1984). However, few studies have addressed the effect of the parasite at a molecular level. Recently it was shown that *A.gambiae* mounts an immune response against the *P.berghei* parasite during midgut invasion (Dimopoulos *et al.*, 1997), as manifested by transcriptional activation of the infection-responsive genes *ISP13* (a putative serine protease), *ISPL5* (serine protease-like), *GNBP* (a putative β -1,3-glucan-binding protein), the antimicrobial defensin gene and *IGALE20* (a putative gal-lectin). Transcriptional activation occurs both locally in the midgut and systemically elsewhere in the body, indicating the possible presence of an inter-tissue immune signalling mechanism. Defensin production has also been documented in the midgut of the fly *Stomoxys calcitrans* (Lehane *et al.*, 1997). It was also shown recently (Luckart *et al.*, 1998) that a nitric oxide synthase (*NOS*) gene in *A.stephensi* is transcriptionally activated at a modest level after malaria infection; the early induction (1.7- to 2.1-fold at 1–3 days post-feeding) partly occurs in the midgut, but the origin of late induction (1.1- to 1.4-fold at 9–18 days post-feeding) has not been characterized. In *Drosophila melanogaster*, an antifungal peptide promoter can be activated in the salivary gland (Ferrandon *et al.*, 1998), raising the possibility that this epithelial organ in the mosquito may also mount an immune reaction to the invading malaria parasites.

To expand the number of infection-responsive markers, we identified two new *A.gambiae* genes, *NOS* and *ICHIT*, and demonstrated that they are transcriptionally activated both by bacteria and by the malaria parasite. We have used both these and previously characterized infection-responsive markers to document that the malaria parasite elicits temporally and spatially distinct immune responses in the salivary glands, the abdomen and the midgut during its life cycle in the mosquito. Such responses may be important for the vectorial capacity of the mosquito.

Results

Cloning and immune responsiveness of ICHIT and AgNOS

ICHIT was isolated by differential display as a PCR fragment specifically amplified from midgut RNA. Using this fragment as a probe, we screened a cDNA library constructed from adult female abdomens including the midgut, and obtained a 1371 bp cDNA encoding a 374 amino acid complete open reading frame (Figure 1A). The sequence (Figure 1B) begins with a signal peptide-like region and bears a central 174 residue threonine-rich mucin-like domain. This domain is flanked by two partial repeat regions (Figure 1C) that show substantial similarity (including six invariant cysteine residues) to the chitin-binding domains of mosquito chitinases from *A.gambiae* (Shen and Jacobs-Lorena 1998) and *Aedes aegypti* (DDBJ/EMBL/GenBank accession No. AF026492), an intestinal mucin from the cabbage-looper *Trichoplusia ni* (Wang and Granados, 1997) and the antimicrobial peptide tachycitin from the horseshoe crab *Tachypleus tridentatus* (Kawabata *et al.*, 1996).

A 432 bp fragment of the *A.gambiae NOS* gene (AgNOS) was cloned using a pair of degenerate primers which were designed from the conserved cofactor-binding domain of NOS sequences from the human inducible and constitutive genes (DDBJ/EMBL/GenBank accession Nos AF068236 and L26914), *D.melanogaster* (Regulsky and Tully, 1995) and *Rhodnius prolixus* (Yuda *et al.*, 1996). The amplified sequence encodes a 142 residue region (Figure 1D) that is 44–85% identical at the amino acid level to the corresponding region of these known NOS sequences, as well as 95% identical to the recently isolated *A.stephensi* NOS sequence (Luckhart *et al.*, 1998).

Reverse transcription–PCR (RT–PCR) experiments established that both *ICHIT* and *NOS* are immune inducible (Figure 1E), as shown by significant increases in transcript levels ~1 day following pricking of larvae and adult females with a needle dipped in a mixture of *Escherichia coli* and *Micrococcus luteus*. These and subsequent experiments were normalized using as standard the S7 ribosomal protein transcript. *ICHIT* and *NOS* are also induced at 22 h after feeding the mosquitoes on a malaria-infected mouse, a time when an infection-dependent immune response has been documented using previously known immune-responsive markers (Dimopoulos *et al.*, 1997).

Expression profiles of immune-responsive genes during development and in various body parts

The developmental expression profiles of *ICHIT* and *NOS* were analysed by RT–PCR in parallel with *ISLP5* and *GNBP* (Dimopoulos *et al.*, 1997). *ICHIT* is essentially

limited to pupal and adult stages. *GNBP* and *ISLP5* are expressed throughout life, but the latter is especially enriched in pupae and adults. *NOS* is also expressed throughout life, albeit somewhat variably (Figure 2). As previously reported, defensin is expressed at all stages but mainly in pupae and adults, and *IGALE20* transcripts are present predominantly in larvae (Dimopoulos *et al.*, 1996; Richman *et al.*, 1996).

The tissue specificity of expression in the adult, in the absence of immune challenge, was documented for these six markers, using quantitative radioactive RT–PCR (Figure 3A). The levels of expression of a given marker in different tissue samples were measured by a phosphorimager and normalized by the level of S7 transcript in the same sample. They are presented in Figure 3A on a relative scale, with 100% corresponding to the level in the body part that is most enriched in each particular marker. In these experiments, we determined the expression levels in the two organs that are crucial for malaria transmission, the midgut and the salivary glands (G and S, respectively). We also assessed the levels in the rest of the abdomen which includes the body wall, Malpighian tubules and ovaries, and in the thorax which contains a considerable part of the fat body, the major immune organ (A and T, respectively).

RT–PCR reveals different tissue specificity profiles for each gene probe. The two best characterized immune markers, the defensin and *GNBP* transcripts, are maximally enriched in the thorax, presumably reflecting association with the fat body. Surprisingly, these markers are also present at high levels in the salivary glands; in the case of *GNBP*, the relative concentration in the salivary glands is equal to that in the thorax, while for defensin it is comparable with the level in another immune organ, the midgut. The four other markers are also present in the salivary gland, with the level of *IGALE20* being particularly notable. The midgut is very enriched in *IGALE20*, as well as in the two new markers, *ICHIT* and *NOS*.

To confirm association with the salivary glands, we performed whole mount *in situ* hybridization experiments with the two markers that are expressed at the highest absolute level, defensin and *GNBP* (Figure 3B). Both antisense RNA probes clearly hybridized with the glands. Interestingly, the defensin signal was confined largely to the median lobe and the proximal regions of the lateral lobes, and was minimal in the distal regions of the lateral lobes. The *GNBP* probe stained the proximal lateral lobes strongly and gave a weak signal in the median lobe. The male salivary glands are smaller and seem to contain a single cell type similar to the proximal regions of the female lateral lobes (data not shown).

Induction profiles following malaria infection

The presence of all six immune markers in the salivary glands raises the possibility that in anopheline mosquitoes the glands are immune organs that may respond to invasion by malaria sporozoites late in infection. As a first step in testing this possibility, we examined the immune response of the mosquito as a whole, late in infection (Figure 4). Under our conditions, *P.berghei* oocysts grow but remain intact until day 9 post-infection, and sporozoites begin to be released at approximately day 10. No significant induction was detected in infected mosquitoes relative to

Fig. 1. ICHIT and AgNOS sequences. (A) Graphic presentation of the ICHIT putative protein structure. (B) Amino acid sequence of ICHIT.

Polythreonine repeats are boxed, the putative mucin domain is underlined and the regions shown in (C) are outlined by arrows. (C) Alignment of ICHIT putative chitin-binding sequence motifs with *Tachypleus tridentatus* (T.t.) antimicrobial peptide tachycitin, chitin-binding domains of *A. aegypti* (A.a.) and *A. gambiae* (A.g.) chitinases and the cysteine-rich domains of *Trichoplusia ni* (T.n.) intestinal mucin. Conserved cysteines in the chitin-binding domains are marked with asterisks. Homology between the two putative chitin-binding domains of ICHIT extends beyond the aligned sequences (not shown). (D) Alignment of the AgNOS fragment with the homologous sequences of *A. stephensi* (A.s.), *D. melanogaster* (D.m.), *Rhodnius prolixus* (R.p.), human inducible (H.s.) and mouse constitutive (M.m.) NOS. Conserved regions corresponding to the cofactor-binding sites of NADPH ribose, NADPH adenine and NADPH are overlined. (E) Agarose gel showing RT-PCR-assayed transcriptional induction of ICHIT and NOS in bacterially challenged larvae (I) 24 h after infection compared with naive larvae (N), bacterially challenged adults 30 h after infection (I) compared with naive adults (N), and *P. berghei*-infected (I) G3 strain mosquitoes at 24 h after feeding on an infected mouse as compared with mosquitoes fed on a naive mouse (N). All cDNA templates were normalized for equal yield of ribosomal protein S7 RT-PCR product.

mosquitoes fed on naive (uninfected) blood for any of the markers at day 5 (separate experiment, data not shown). On day 9 (Figure 4A), minor induction was detected only for NOS, whereas the other markers were slightly under-represented in the infected mosquitoes (possibly because of lower nutritional quality of the infected blood). However, by day 11, defensin, ICHIT and NOS were induced, and in pooled mosquitoes between days 13 and 21 post-infection all the markers showed significant induction. In

these experiments, the developmental progression of the parasite could be monitored by the relative ratios of transcripts for two *Plasmodium* surface proteins, the circumsporozoite protein (CS), which is thought to be synthesized by all sporozoite stages, and the thrombospondin-related adhesion protein (TRAP), which is thought to be synthesized mostly after entry in the salivary gland (Beier, 1998). Although no one to date has determined the levels of TRAP and CS transcripts during development, it is

Fig. 2. Agarose gel showing RT-PCR-assayed developmental expression profiles of ISPL5, GGBP, ICHIT and NOS in 18 h embryos (E1), 42 h embryos (E2), first instar larvae (L1), second instar larvae (L2), third instar larvae (L3), fourth instar larvae (L4), pupae (P) and adult females (A) of the 4A *r/r* mosquito strain. ISPL5 has the highest expression levels in late larvae, pupae and adults; GGBP is expressed at all stages at various levels. ICHIT is expressed exclusively in pupae and adults, and NOS is expressed mainly in embryonic, early larvae, pupa and adult stages. Templates were normalized using the ribosomal protein S7 gene-specific primers.

known that only 2% of the haemolymph sporozoites but >95% of salivary gland sporozoites are positive for TRAP protein, whereas all stages of sporozoite development are positive for CS protein (Robson *et al.*, 1996).

In a second experiment, salivary glands and abdomens (minus the midgut) were dissected from infected and naive mosquitoes, at 15 and 20 days post-feeding. Induction of GGBP and defensin was observed at both stages and in both tissues (Figure 4B).

Induction of each immune marker was monitored quantitatively in a third experiment that assayed the midgut, salivary glands and the remaining abdomen between 24 h and 25 days post-infection (in these labour-intensive experiments, the thorax was not analysed because of its damage during dissection). Data are summarized in Figure 5. In this experiment, both infected and control mosquitoes were given a second naive blood meal at day 5 to ensure equal nutrition levels. As expected from our previous

Fig. 3. (A) Expression levels of marker RNAs in naive unfed G3 strain female salivary glands (S), thorax (T), midgut (G) and abdominal wall containing ovaries and Malpighian tubules (A). Transcript levels were assayed by radioactive RT-PCR on templates which had been normalized using the ribosomal protein S7 primers, products were quantified by phosphorimager and the normalized expression level in a given tissue was plotted, with the most abundant marker in that tissue set at 100. Defensin and GGBP are highly expressed in the thorax as well as in the salivary glands, ISPL5 is expressed mainly in the thorax and abdominal tissues, while ICHIT, IGAL20 and NOS are most abundant in the gut. (B) *In situ* hybridization of GGBP and defensin digoxigenin-labelled antisense RNA probes to whole mount salivary gland tissues. The GGBP probe hybridizes strongly to the proximal lateral (pl) lobes and more weakly to the median (ml) lobe. The defensin (DEF) probe stains the same salivary gland regions, but more equally. Weak staining of the distal lateral lobes was sometimes detected for both both GGBP and defensin (not shown).

Fig. 4. (A) Autoradiograms showing radioactive RT-PCR-assayed transcriptional induction of six infection-responsive markers in malaria-infected 4a r/r strain mosquitoes (I) compared with mosquitoes fed on a naive mouse (N). The assayed templates correspond to 9, 11 and 21 days, as well as a pool containing equal amounts of cDNA from 13, 15, 17 and 19 days after feeding. NOS shows induction already at day 9, and at day 11 defensin and ICHIT also begin to be induced. All markers are induced in the pooled 13–19 day sample and at 21 days after feeding on an infected mouse. Expression of the *Pberghei* sporozoite surface antigen genes CS (circumsporozoite) and TRAP (thrombospondin-related adhesion protein) was assayed by RT-PCR on the same templates. CS is amplified at all time points with a peak at day 13 (not shown), and TRAP appears at day 13 with a peak at day 19 (not shown). (B) Autoradiograph showing transcriptional induction of GNBP and defensin in infected (I) versus naive (N) salivary glands (SG) and abdomens (AB) at 15 and 20 days post-infection. Templates were prepared in an experiment different from that in (A), from 4a r/r strain mosquitoes, and normalized using specific primers for the ribosomal protein S7 gene.

work (Dimopoulos *et al.*, 1997), the midgut showed a strong early immune response at 24 h, but was essentially quiescent during the late phase of infection; the opposite was true of the salivary glands, while the abdomen showed immune induction of some markers at both early and late times.

The induction of the immune markers was non-coordinated, presumably reflecting differences in the triggering stimuli or the regulation of markers in different tissues. In the midgut, five markers were induced in parallel 24 h after blood feeding. In contrast, the salivary glands showed no induction at 24 h and responded later (at 10–25 days), with the markers showing diverse expression profiles. In particular, induction began at 10 days for only three markers, GNBP, defensin and NOS. Subsequently, the first two of these markers showed sustained induction in the salivary glands, peaking at 20 days; however, the induction of NOS was only transient and the transcript was substantially repressed at 20–25 days. Induction of ISPL5 and especially IGALE20 and ICHIT occurred later than for the other three markers. In the multi-organ abdominal compartment, the induction profiles of different markers were even more diverse. In this case, NOS induction was seen at all stages and was especially high both early (24 h)

Fig. 5. Phosphorimager-estimated radioactive RT-PCR-assayed induction levels of immune markers in salivary glands (SG), midguts (MG) and abdominal wall tissues (containing ovaries and Malpighian tubules) (AB) of malaria-infected 4a r/r strain mosquitoes at 24 h, 10, 15, 20 and 25 days after feeding on an infected mouse as compared with mosquitoes fed on naive mice. Ribosomal protein S7 gene-specific primers were used to normalize the cDNA amounts of each sample. Induction ratios (infected/naive expression levels) are shown, with the naive level set at N and induction or repression plotted above or below that level respectively. For analysis of the induction profiles, see the text.

and late (20–25 days); ISPL5 and IGALE20 also showed both early and late induction peaks. ICHIT was induced primarily in a narrow late phase (20 days). The induction period of defensin and GNBP was broad.

Discussion

Two new *A.gambiae* infection-responsive genes

ICHIT has a mosaic structure consisting of a mucin domain flanked by two putative chitin-binding domains. Chitin-binding domains have been found in plant (Nielsen *et al.*, 1997) and arthropod (Kawabata *et al.*, 1996) defence molecules and are believed to be involved in binding to microbial carbohydrate surfaces. Mucin domains are highly *O*-glycosylated polythreonine segments which may

function as adhesive domains (for lectins), or repulsive domains because of charged sugars (Shimizu and Shaw, 1993). Mucins are connected to other peptide domains, usually of an adhesive nature, potentially resulting in combinatorial effects. Some vertebrate mucins are involved in trafficking of leukocytes and neutrophils towards inflammation sites through binding to selectins (Imai *et al.*, 1997), and invertebrate proteins containing mucin domains also have been proposed to be involved in defence mechanisms. Examples are the *Drosophila* scavenger receptor CI (dSR-CI) (Pearson *et al.*, 1995) and haemomucin (Theopold *et al.*, 1996), both believed to be involved in triggering immune responses upon microbial challenge. Demonstration that ICHIT is transcriptionally induced by both bacteria and malaria parasites, and the availability of its full-length cDNA, opens up the possibility of generating antibodies to test whether the protein is secreted (as the sequence suggests), and whether it may have functions such as immobilization and opsonization of microorganisms or sealing of wounds in the midgut or other tissues. ICHIT may also prove to be associated with the peritrophic matrix, a chitinous sac separating the blood meal from the midgut epithelial cells.

The enzyme NOS is responsible for the formation of nitric oxide which, in vertebrates, is involved in many physiological processes such as vasodilation, neurotransmission and defence killing of bacteria and macroparasites. Our knowledge of NOS function in invertebrates is still limited (Martinez, 1995), and the gene has only been cloned from a few insect species (Regulski and Tully, 1995; Yuda *et al.*, 1996; Luckhart *et al.*, 1998). Modest transcriptional induction of *A.stephensi* NOS (Luckhart *et al.*, 1998) was reported in whole mosquito extracts after malaria infection, both relatively early (1–3 days) and late (9–18 days) post-feeding. Induction was also measured at the level of enzyme activity, and detectable increases of nitrite/nitrate levels were observed in the hemolymph. The most interesting observation was that a dietary NOS inhibitor increases oocyst formation, while the NO precursor L-arginine depresses oocyst numbers. Diaphorase staining suggested that NOS activity is present in the midgut, and is partially altered after malaria infection (Luckhart *et al.*, 1998).

Insect NOS activities are found in multiple tissues, and have been associated with multiple functions such as neurotransmission in brain (Regulski and Tully, 1995) and vasodilator activity in saliva (Ribeiro and Nussenzweig, 1993). It is not clear whether these functions are served by the same or different enzymes. Three types of NOS have been described in vertebrates; two of them are constitutive in neuronal (nNOS) and endothelial (eNOS) tissues, and one is inducible in macrophages (iNOS). The insect enzymes are reported to be closest to the vertebrate nNOS in sequence (Luckhart *et al.*, 1998), or intermediate between constitutive and inducible vertebrate forms (Regulski and Tully, 1995). The available AgNOS sequence does not illuminate this question and the possibility of multiple NOS forms in the mosquito cannot be excluded at this stage.

Expression profiles of the markers and induction by malaria

Both new and previously identified immune markers are expressed in adult mosquitoes, and thus are suited for

monitoring induction by the malaria parasite. The markers show distinct and in some cases complex developmental expression profiles, which may reflect potential non-defence-related functions. Developmental and immune-related processes often share the same components: examples are regulatory factors such as Dif, Dorsal and Relish in *D.melanogaster* (Dushay *et al.*, 1996; Hoffmann *et al.*, 1996), or effectors such as the *Sarcophaga* antibacterial defence proteins sapecin, sapecin B and cathepsin L (Natori and Kubo, 1996). Some of the *A.gambiae* infection-responsive markers, GGBP, IGALE20 and ICHIT, encode adhesive motifs (Dimopoulos *et al.*, 1996, 1997), and could be involved both in binding to microbial substances in defence and in the removal of apoptotic cells and tissue remodelling during development. NOS is known to have multiple functions in a variety of physiological processes. The serine protease-like gene *ISPL5* has sequence features (Dimopoulos *et al.*, 1997) similar to the *masquerade* gene of *D.melanogaster* (Murugasu-Oei *et al.*, 1995), and may also be involved in development.

We have determined the relative concentrations of marker RNAs in dissected tissues. Of our present set of six immune markers, four are expressed strongly in adult midgut and are well suited to analysis of the immune response of this major entry point of infectious microorganisms (Dunn *et al.*, 1994). Interestingly, all the markers are also expressed in the salivary glands, either at high (defensin, GGBP and IGALE20) or at moderate (*ISPL5*, ICHIT and NOS) relative levels, again permitting analysis of immune responses in this second epithelial organ which is crucial for malaria transmission. Previously, little was known about the expression of immune factors in insect salivary glands. A bacteriolytic lysozyme activity has been detected in the *A.aegypti* salivary glands (Rossignol and Lueders, 1986), and the *D.melanogaster* salivary glands show constitutive expression of lysozyme P (Kylsten *et al.*, 1992) and have been reported to express the antifungal gene drosomycin, in the distal lobes (Ferrandon *et al.*, 1998). The apparently induced expression of drosomycin in this tissue was found to be independent of the spaetzle–Toll signalling pathway, and was proposed to be activated locally by pattern recognition receptors (Ferrandon *et al.*, 1998). Expression of defence molecules in the mosquito salivary glands may minimize microbial proliferation in the saliva and, in conjunction with salivation during feeding, it may promote sterility of the nectar in the crop, of the host wound during blood feeding and of the ingested blood meal. In addition to its potential vasodilator activity, the enzymatic product of NOS could also exert an antimicrobial effect in the saliva. *GGBP* and defensin, the most abundant infection-responsive genes in the salivary glands, are expressed in the proximal lateral lobes and in the median lobe; the ratios of intensities at these two locations differ for the two markers. The largest number of parasites are found after heavy infections.

In addition to the local epithelial immune responses, systemic responses accompany the development of the parasite. This is best exemplified by the robust induction of markers in the midgut-free abdominal compartment at 24 h, at a time when the parasite is confined within the midgut by an intact basal lamina. The subsequent temporal complexity and marker specificity of the abdominal response strongly suggests that multiple organs might be

induced asynchronously, probably including the fat body and the hemocytes, and possibly additional organs. It remains to be determined whether these responses are triggered by metabolic by-products of the parasite or by specific inter-tissue signalling processes.

The levels and kinetics of induction show some variability between experiments, especially in the prolonged late phase of oocyst growth, sporozoite release and salivary gland invasion (this phase lasts ~3 weeks, as opposed to 1 day for midgut invasion). The variability is not surprising in view of the complexity of the system. Variations in parasitaemia and associated differences in the nutritional value of the blood meal, variations in signalling constituents of the blood meal (e.g. xanthurenic acid; Billker *et al.*, 1998) and, presumably, polymorphisms within the mosquito population could easily lead to variations in mosquito infection levels and rates. However, the described main features of marker induction are reproducible, as documented by the consistency of the results from three different experiments shown in Figures 4A and B, and 5. These features include the contrast in timing of the responses in the midgut and salivary glands (early and late, respectively), the marker specificity of responses in different tissues and at different times, and the complexity of the abdominal responses.

The levels and fluctuations of NOS expression during parasite development are noteworthy. First, in the salivary gland, NOS induction is transient at ~10 days, and is followed by substantial repression. Secondly, NOS is the marker that shows the highest level of inducibility, in particular in the abdomen at both early and very late stages (24 h and 20–25 days, respectively). In view of the multiple physiological roles of NO, it is quite possible that the effects on NOS will prove to be related to the success of parasite infection (Luckhart *et al.*, 1998), as well as to diverse effects of the parasite on the mosquito (Rossignol *et al.*, 1984; Hogg and Hurd, 1997). The same may be true for the other markers.

Concluding remarks

The multiplicity of our markers has been important for comparing and contrasting convincingly the immune responses in different tissues. Notably, the midgut shows only an early response, when it is being invaded by ookinetes. During later stages of the parasite life cycle, when sporozoites are released gradually in the hemolymph and invade the salivary glands, the glands show a correspondingly late, prolonged and non-coordinate immune reaction. Thus, *P.berghei* locally activates a variety of immune markers during its critical epithelial transition stages.

We suspect that the immune responses that we have documented may be pertinent to some of the massive losses of parasites that occur in the susceptible and refractory strains alike, rather than to more specialized refractoriness mechanisms such as melanotic encapsulation. Considering the prevalence of these general losses (Beier, 1998), it is not surprising that we have observed immune responses in both susceptible and refractory strains (see also Dimopoulos *et al.*, 1997). The levels of the responses may vary and be consequential, but their precise quantification is beyond the capabilities of our presently available tools.

The work to date has established a number of informative markers and characterized how they respond at the RNA level to the development of the parasite. Future studies, involving antibodies to the encoded proteins, will be necessary in the next phase, to increase the resolution of our analysis, to define the cellular basis of the immune responses and to begin to unravel their functional significance in mosquito–parasite interactions. In-depth functional analysis of these interactions may lead to the development of novel transmission-blocking strategies.

Materials and methods

Mosquito rearing and infections

The *A.gambiae* strains G3 and 4A r/r were raised at 28°C, 75% humidity, under a 12 h light/dark cycle, and maintained on a 10% sucrose solution during adult stages. Female mosquitoes were blood-fed on anaesthetized Balb/c mice. Fourth instar G3 larvae and adult females were pricked with a needle previously dipped in a solution of *E.coli* and *M.luteus* for bacterial infections; naive controls were not pricked. RNA for RT-PCR analysis was extracted from surviving larvae at 24 h and from adults at 30 h post-bacterial challenge. For malaria infections, 4-day-old female mosquitoes were fed on anaesthetized Balb/c mice which had been infected with *P.berghei*, and were assayed for high levels of parasitaemia and the presence of gametocyte-stage parasites capable of exflagellation, as described (Sinden, 1979). The mosquitoes were maintained thereafter at 19°C prior to dissection and RNA extraction.

Dissections and RNA extraction

Tissues were dissected in Aedes saline solution (Hagedorn *et al.*, 1997) (0.6 mM MgCl₂, 4 mM KCl, 1.8 mM NaHCO₃, 150 mM NaCl, 25 mM HEPES, 1.7 mM CaCl₂, pH 7) and were frozen immediately on dry ice. Total RNA was prepared from dissected tissues and intact animals using the RNaid PLUS kit (bio 101) according to the manufacturer's instructions.

Cloning of ICHIT by differential display, and of NOS by homology

Differential RNA display comparing female midgut and carcass, and cloning of a differentially expressed sequence was performed as previously described (Dimopoulos *et al.*, 1996; Dimopoulos and Louis, 1997) using the 10mer primer L3 (5'-CCAGCAGCTT-3'; Operon Technologies, Alameda, CA). The product and cDNA clone to which it hybridized were sequenced, leading to identification of ICHIT. A pair of degenerate primers: NOS1d [5'-GCICCTT(T/C)AG(A/G)(A/T)(C/G)ITT(T/C)TG-GCA-3'] and NOS2d [5'-CC(A/G)AA(G/A/T)AT(A/G)TC(C/T)TC(G/A)TG(A/G)TG(A/G)TAICG-3'] were used to amplify a 432 bp fragment of AgNOS from an *A.gambiae* G3 strain cDNA using the following PCR cycling programme: 5 min denaturation at 94°C; five 30 s steps at 94, 65 and 72°C; five 30 s steps at 94, 60 and 72°C; five 30 s steps at 94, 55 and 72°C; five 30 s steps at 94, 50 and 72°C; 45 1 min steps at 94, 65 and 72°C; and a final 10 min extension at 72°C. The product was cloned and sequenced, and was confirmed as an NOS fragment by sequence comparison.

Library construction, screening, sequencing and sequence analysis

Poly(A)⁺ RNA was prepared from abdominal tissues including the midgut, from 4-day-old female Suakoko strain *A.gambiae* (1000 mosquitoes), using the Oligotex Direct mRNA Kit (Qiagen). The preparation (7 µg) was used for library construction based on the ZAP Express™ system according to the manufacturer's instructions (Stratagene). Sequencing was performed using the USB Sequenase Kit according to the manufacturer's instructions. Sequence comparisons were performed using BLAST with the GCG software (Devereux *et al.*, 1984).

Expression analysis by RT-PCR

RT-PCR expression analysis was performed as previously described in Dimopoulos *et al.* (1997). For the estimation of expression levels in tissues and infected mosquitoes, radioactive RT-PCRs were performed by adding 0.05 µl of radiolabelled [α -³²P]dCTP to each PCR. The amplification products were visualized by autoradiography on X-ray film (Kodak) after electrophoresis on 6% acrylamide gels. Quantification of

the radioactive amplification products was performed using a phosphoimager. The ribosomal protein S7 gene (Salazar *et al.*, 1993) sequence was used for normalization of compared templates. In each experiment, the PCR cycle numbers were chosen empirically to avoid saturation and attain comparable PCR product amounts of the different markers. The PCR cycle number in a given experiment was constant for a particular sequence in the multiple samples analysed. The levels and kinetics of induction showed some variability between experiments, probably because of parameters which are difficult to control such as differences in parasitaemia in the mouse blood, differences in the age of the mosquitoes or their level of infection. However, the main features as described above are reproducible. The primers used were as follows: S7A, 5'-GGCGATCATCTCTACGT-3', and S7B, 5'-GTAGCTGCTGCAAACTTCGG-3'; ISPL5A, 5'-AAAGACCTTGTGATGGAGATG-3', and ISPL5B, 5'-ATGTTGTACGTTTTATTGAAG-3'; GNBPA, 5'-GCAACGAGAATCTGTACC-3', and GNBPB, 5'-TAACCACAGCAACAGG-3'; DEFA, 5'-CTGTGCCTTCTAGAGCAT-3', and DEFB, 5'-CACACCCTCTTCCCAGGAT-3'; IGALE20A, 5'-CCTGTCCAGAGAAGTCC-3', and IGALE20B, 5'-TAGATGTGAATGACATGG-3'; ICHITA, 5'-GACAACAGTAGGATAGATCC-3' and ICHITB, 5'-TGTTGTGACGTACATCGATGCC-3'; AgNOSU, 5'-GAAAACATCCAAAGACCT-3', and AgNOSL, 5'-GCCGAAAATGTCCTCGTG-3'; PbCSA, 5'-ATGACCCAGCACCACCACAAGG-3', and PbCSB, 5'-GTTACGTTACATTGAGACCATTC-3'; and PbTRAPA, 5'-ATCTGACTCACAAGTAGAATCC-3', and PbTRAPB, 5'-TTCATCATCTGCCATTACATCTTC-3'.

Whole-mount *in situ* hybridization

Dissected salivary glands were fixed for 30 min in phosphate-buffered saline (PBS) containing 67 mM EGTA and 4% formaldehyde; dehydrated by successive washes in 25, 50 75 and 100% methanol and kept in ethanol overnight at -20°C; rehydrated in PBS by successive washes in 75, 50, 25% ethanol and subsequently fixed for 30 min in PBS containing 5% formaldehyde; treated with proteinase K (33 µg/ml) for 10 min and fixed for 30 min in PBS containing 5% formaldehyde. Hybridizations were performed in a formamide hybridization solution with digoxigenin-labelled (Boehringer Mannheim) RNA probes overnight at 55°C. Hybridization signals were detected using the *in situ* hybridization and detection system kit (Gibco-BRL) as described by the manufacturer.

Accession numbers

The DDBJ/EMBL/GenBank accession number for ICHIT is AJ010903 and for NOS it is AJ010904.

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