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Plasmodium activates the innate immune response of *Anopheles gambiae* mosquitoes

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Innate immune-related gene expression in the major disease vector mosquito *Anopheles gambiae* has been analyzed following infection by the malaria parasite, *Plasmodium berghei*. Substantially increased levels of mRNAs encoding the antibacterial peptide defensin and a putative Gram-negative bacteria-binding protein (GNBP) are observed 20–30 h after ingestion of an infected blood-meal, at a time which indicates that this induction is a response to parasite invasion of the midgut epithelium. The induction is dependent upon the ingestion of infective, sexual-stage parasites, and is not due to opportunistic co-penetration of resident gut micro-organisms into the hemocoel. The response is activated following infection both locally (in the midgut) and systemically (in remaining tissues, presumably fat body and/or hemocytes). The observation that *Plasmodium* can trigger a molecularly defined immune response in the vector constitutes an important advance in our understanding of parasite–vector interactions that are potentially involved in malaria transmission, and extends knowledge of the innate immune system of insects to encompass responses to protozoan parasites.
Keywords: *Anopheles*/insect immunity/malaria/ *Plasmodium*

Introduction

The mosquito *Anopheles gambiae* is the principal vector of human malaria in Africa, where the disease is estimated to cause more than 1 million deaths per year (Collins and Paskewitz, 1995). The absence of effective malaria vaccines, and the spread of drug-resistant *Plasmodium* parasites as well as insecticide-resistance in vector populations, render the need for novel malaria control strategies increasingly acute. Historically, successful malaria control has been effected through management or control of vector mosquito populations, thereby breaking the cycle of human disease transmission (Collins and Besansky, 1994; Collins and Paskewitz, 1995). Advances in the molecular genetic manipulation of certain insect species have led to speculation that malaria could be controlled through genetic alteration of Anopheline vector mosquitoes rendered refractory to *Plasmodium* growth and differentiation (Collins, 1994; Curtis, 1994). Research efforts directed towards such a goal will greatly benefit from enhanced understanding at the molecular level of the interactions between vector insect and protozoan parasite. These interactions are also of broad biological interest, as they relate to the central paradox of metazoan parasitism: successful growth and differentiation of a parasite within a host endowed with elaborate defense mechanisms.

Plasmodium enters the female mosquito during blood-feeding upon an infected vertebrate host. Within the insect midgut, parasite gametocytes are rapidly activated to produce gametes. Fertilization follows, leading to formation of a motile ookinete which penetrates the epithelial cell monolayer surrounding the gut lumen. The ookinete ceases its migration upon reaching the basal lamina separating the midgut and hemocoel compartments. Here the ookinete undergoes differentiation into the oocyst form, which grows over a period of days and produces sporozoites; ultimately these are released into the hemolymph to colonize the salivary gland, where they become the only insect stage of the parasite that is again infective to vertebrates upon subsequent blood-feeding by the mosquito (Touray *et al.*, 1992). The great majority of *Plasmodium*–mosquito combinations are incompatible, and it is thought that multiple factors contribute to the inherent capacity of a particular Anopheline mosquito species to support growth and differentiation of a particular *Plasmodium* parasite (Warburg and Miller, 1991). At present these factors are not well understood at the molecular level, although laboratory studies have documented that several distinct types of incompatibility in some mosquito–*Plasmodium* combinations have a relatively simple genetic basis (Kilama and Craig, 1969; Vernick *et al.*, 1989, 1995; Zheng *et al.*, 1997). At the descriptive level, incompatibility might be ascribed to physiological or structural barriers, or to active innate defense (immune) responses of the insect, which result in killing specific stages of the parasite (Warburg and Miller, 1991).

It has long been recognized that insects possess highly effective innate defense mechanisms of both cellular and humoral nature (Boman and Hultmark, 1987; Lackie, 1988; Hoffmann *et al.*, 1996). The latter has received considerable recent attention and much is now known about the structure and activity of secreted antimicrobial peptides, as well as their regulated synthesis in model dipteran and lepidopteran species upon bacterial or fungal infection (Cociancich *et al.*, 1994; Hultmark, 1994; Hoffmann *et al.*, 1996). Studies of humoral immunity in medically important vector species are more limited and recent (Kaaya *et al.*, 1987; Chalk *et al.*, 1994; Ham *et al.*, 1994; Lowenberger *et al.*, 1995; Richman and Kafatos, 1996). In *A.gambiae* they include the cloning of sequences encoding the antimicrobial peptide defensin (Richman *et al.*, 1996), lysozyme protein (Kang *et al.*, 1996), a putative immune-related transcription factor (Barillas-

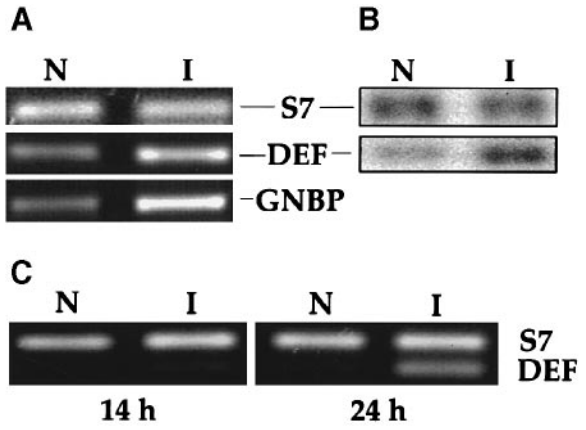

Fig. 1. Relative levels of immune-related mRNAs in *A.gambiae* mosquitoes fed on *P.berghei*-infected (I) and non-infected control mice (N). (**A**) RT–PCR analysis of gene expression for defensin (DEF, 28 cycles) and Gram-negative bacteria-binding protein (GNBP, 32 cycles) in 6-day-old female *A.gambiae* adults 24 h after feeding. Expression of the gene encoding ribosomal protein S7 (S7, 21 cycles) served as control. The *P.berghei* infected mouse displayed a parasitemia of 11% with an average of 6 exflagellation centers per field. (**B**) RNA blot analysis of defensin gene expression in the same samples using 12 mg of total RNA per lane. Defensin signal intensity, quantitated relative to S7 using a PhosphorImager (Molecular Dynamics), indicated a ~3-fold induction. (**C**) Time course of defensin gene expression in 4-day-old adult female *A.gambiae* (n = 25) at 14 and 24 h after feeding, assayed by RT–PCR (S7, 22 cycles; DEF, 27 cycles). In this experiment the infected mouse displayed a parasitemia of 5%, with an average of two exflagellation centers per field.

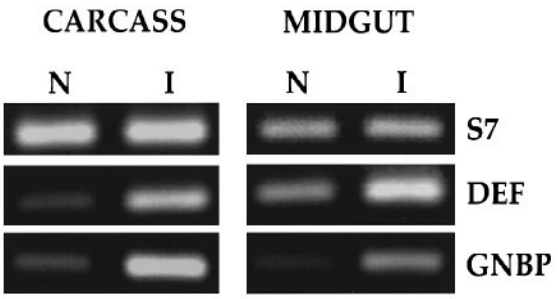

Fig. 2. Induction of defensin and GNBP RNA in midgut and carcass tissues. Five-day-old female mosquitoes were fed upon *P.berghei*-infected mice (~12% parasitemia; average of four exflagellation centers per field) (I) or upon non-infected, control mice (N). Twenty-four hours after feeding, midguts were dissected from remaining tissues (carcass) as described in Materials and methods. Total RNA preparation and RT-PCR analysis as in Figure 1A. The following cycle numbers were used: S7 (23 cycles, carcass and midgut); DEF (28 cycles carcass; 30 cycles midgut); GNBP (34 cycles carcass; 40 cycles midgut).

Mury *et al.*, 1996) and several mRNAs upregulated after bacterial infection (Dimopoulos *et al.*, 1996, 1997; and unpublished observations). The latter group includes the *A.gambiae* homolog (Dimopoulos *et al*., 1997) of a Gram-negative bacteria-binding protein (GNBP) identified originally in the silk moth *Bombyx mori* (Lee *et al.*, 1996). Exogenously administered antimicrobial peptides have been shown to be active against various insect stages of *Plasmodium* (Gwadz *et al.*, 1989; Rodriguez *et al.*, 1995). The availability of molecular markers, in particular defensin and GNBP, has permitted us to investigate the possibility that immune activation may be involved in insect–protozoan interactions.

Results

Defensin and GNBP RNA levels are induced locally and systemically during midgut tissue invasion

Figure 1A demonstrates elevated RNA levels for two immune markers in mosquitoes fed on *Plasmodium berghei*-infected as compared to naive mice. In this and subsequent experiments reverse transcription PCR (RT-PCR) was used as a convenient, sensitive, and semi-quantitative method to analyze changes in gene expression (Dimarcq *et al.*, 1994; Richman *et al.*, 1996); levels of immune marker RNAs were normalized relative to a control RNA encoding the ribosomal protein S7 (Salazar *et al.*, 1993). Figure 1B confirms this induction using quantitative RNA blot analysis of defensin RNA (the low abundance of GNBP mRNA renders blot analysis of this sequence impractical; data not shown). The expression of both defensin and GNBP is reproducibly induced 20 to 30 h after ingestion of infected blood. For defensin, induced levels are comparable to those observed in larval and adult stage mosquitoes following experimental infection with bacteria (Richman *et al.*, 1996). Defensin induction is not yet observed at 14 h after *P.berghei* infection, but is prominent at 24 h (Figure 1C). Thus, induction corresponds to the period when motile ookinete stage parasites are invading the epithelium of the posterior midgut (Meis *et al.*, 1989 and data not shown). Between 40 and 48 h post-feeding defensin induction is variable, possibly reflecting variable levels of late midgut invasion by a minority of ookinetes (data not shown). Induction is not due to ingestion of a normal blood-meal alone, as it is evident in comparison to mosquitoes fed upon uninfected mice.

While Figure 1 shows induction in intact mosquitoes, Figure 2 demonstrates that *P.berghei* infection induces defensin and GNBP RNA levels both locally in the midgut, and systemically in the remaining carcass. Carcass expression is presumably occurring primarily in cells of the fat body and also in some hemocyte cell types, as has been observed in model insect species challenged with bacteria (Hoffmann *et al.*, 1996).

Immune gene induction results from ingestion of infectious Plasmodium parasites, and is not elicited by vertebrate host factors or endogenous microorganisms

Rodent malaria elicits significant alterations in blood physiology of the vertebrate host, particularly the loss of red blood cells (Garnham, 1966). Levels of cytokines and acute phase response proteins may also be altered in the vertebrate, and opportunistic infectious microorganisms may be present. It is therefore conceivable that blood ingested from infected as opposed to naive mice may contain host factors or biological agents other than *Plasmodium*, which are themselves capable of eliciting mosquito immune induction. To control for this possibility, the mosquito responses to two different strains of *P.berghei* (Paton *et al.*, 1993) were compared: a strain (Anka 2.33) which undergoes normal asexual development in the mouse but does not produce any gametocyte stage parasites (and is thus non-infective for the mosquito), and a ‘wild type’ strain (Anka 2.34) which produces high numbers of

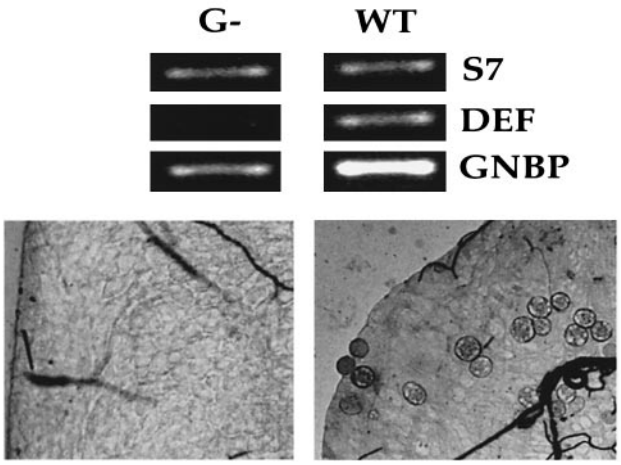

Fig. 3. Induction by wild type as compared with non-gametocyte producing parasite strains. Upper panels: 7-day-old female mosquitoes were fed on mice infected with either the non-gametocyte producing (G–) *P.berghei* Anka strain 2.33 (parasitemia 13%; $n = 40$ mosquitoes) or the wild type (WT) strain 2.34 (parasitemia 14%; $n = 21$ mosquitoes). RNA levels were analyzed in whole mosquito extracts by RT-PCR 24 h after feeding as described in the legend to Figure 1 (S7, 21 cycles; DEF, 27 cycles; GNBP 32 cycles). Lower panels: midguts (strain G3) 10 days after feeding, stained with mercurochrome (original magnification ×100). Abundant normal oocysts are seen on the midguts of 2.34 (WT)-infected mosquitoes (right), while no oocysts are produced after ingestion of the non-infective 2.33 (G–) strain.

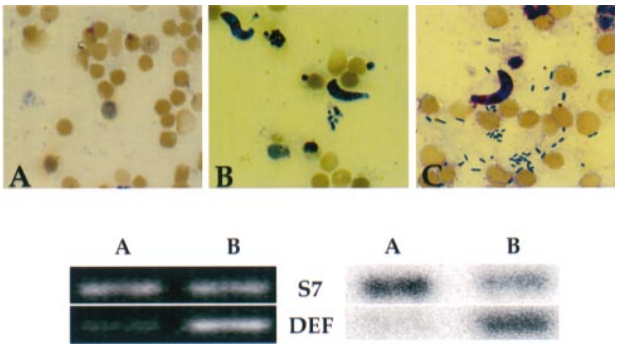

Fig. 4. Effect of antibiotic pre-treatment on endogenous midgut bacteria and induction of defensin gene expression in *P.berghei*-infected *A.gambiae*. Upper panels: (**A**) Blood-meal contents from an antibiotic pre-treated mosquito 18 h after feeding upon an uninfected mouse. No bacteria were observed. (**B**) Mosquitoes were pre-treated with antibiotics, then fed upon a *P.berghei*-infected mouse (21% parasitemia, average of 10 exflagellation centers per field), and midgut contents were examined. Typical banana-shaped ookinetes were observed in the complete absence of bacteria. (**C**) Mosquitoes reared under normal conditions (i.e. no antibiotic pre-treatment) fed on a *P.berghei*-infected mouse and analyzed as above. Note abundant rod-shaped microorganisms as well as an ookinete. Midgut preparations were visualized using phase contrast microscopy (original magnification ×1000). The lower panels compare the levels of defensin RNA in the corresponding antibiotic pre-treated mosquitoes, control (**A**) and *P.berghei*-infected (**B**), 26 h after feeding. RT–PCR analysis on the left (S7, 23 cycles; DEF, 27 cycles) and RNA blot on the right were performed as described in the legend to Figure 1. Quantitation of defensin signal intensity from the RNA blot indicates an ~12-fold induction.

gametocytes. Figure 3 compares the levels of gene activation of defensin and GNBP in *A.gambiae* challenged with these non-gametocyte producing (G–) and wild type (WT) strains of *P.berghei*, assayed at approximately equal levels of parasitemia. Both RNAs are substantially enhanced in mosquitoes infected with the wild type as compared with the non-infectious parasite strain. The difference in infectivity of the strains was confirmed by the ultimate appearance of oocysts in the midgut of mosquitoes fed on the WT but not the G– strain (Figure 3). In the WT strain, the vertebrate asexual stage parasites are preponderant relative to gametocytes (Janse and Waters, 1995). Taken together, the results comparing WT and G– strains, and the time course studies, indicate that the observed immune response is elicited by infectious ookinetes rather than vertebrate host factors, erythrocyte stage parasites, or compromised blood-meal quality (such as anemia).

Laboratory colonies of Anopheline mosquitoes have been reported to harbor endogenous bacteria (Seitz *et al.*, 1987). Conceivably, immune gene induction might be due to opportunistic bacterial co-penetration of the midgut during parasite invasion. Mosquitoes reared in the EMBL insectary were examined for the presence of microorganisms. Figure 4C shows ookinetes in the midgut lumen of fed mosquitoes reared under standard conditions: ookinetes are observed in association with rod-shaped microorganisms, presumably bacteria. Fungal infection was not observed (data not shown). Antibiotic treatment of mosquitoes prior to blood-feeding visibly eliminated gut bacteria (Figure 4A), without affecting development of ookinete stage parasites (Figure 4B). In the absence of bacteria, *P.berghei* infection strongly induced defensin RNA levels 26 h after infection (Figure 4, bottom panels), confirming that the response is due to the parasite. The relative induction of defensin by *Plasmodium* in antibiotic-treated mosquitoes was unusually high: ~12-fold as compared with the ~3-fold induction shown in Figure 1. This difference may be due in part to the effect of resident gut bacteria upon the background levels of defensin RNA expression (comparing the RNA blots in Figures 1 and 4, defensin RNA levels in antibiotic-treated control mosquitoes are ~4.5-fold lower than observed in untreated control animals). The relevance of precise quantitative comparison of induced defensin levels is further limited by possible differences in infectivity of the mice used in the experiments, reflected in differing parasitemias and average numbers of exflagellation centers (see legends to Figures 1 and 4). The important conclusion is that the response to *Plasmodium* is robust, as shown by the data presented here from five independent experiments.

Discussion

Mosquito–parasite interactions as related to innate immunity

The insect innate defense system represents a potentially formidable obstacle to the survival and growth of infecting microorganisms and eukaryotic parasites, in particular those which, like *Plasmodium*, undergo profound developmental changes associated with the invasion of multiple host tissues. *Plasmodium* transmission requires that the vector midgut provides an environment permissive for sexual reproduction and subsequent coordinated differentiation, translocation, and multiplication events leading to sporozoites: it also requires the survival of the sporozoites

as they traverse the hemocoel and specifically 'colonize' cells of the salivary gland, where they acquire vertebrate infectivity (Touray *et al.*, 1992). In principle, successful parasites may circumvent vector defense mechanisms during their developmental progression in a number of ways: by evading recognition, by suppressing the response, or through insensitivity to effector molecules (Loker, 1994). The inherent ease of triggering the defenses may also differ in different mosquitoes. The importance of potential insect defenses is highlighted by what happens to the parasite in non-permissive (refractory) mosquitoes. Two distinct types of refractoriness have been documented in *A.gambiae*: lysis of migrating ookinetes within midgut epithelial cells (Vernick *et al.*, 1995), and melanotic encapsulation of *Plasmodium* at a later stage as they initiate oocyst development (Collins *et al.*, 1986). These responses are genetically as well as morphologically distinct. Three genetic loci have been mapped recently, which cooperate to impart to the mosquito the capacity for semi-dominant melanotic encapsulation of *Plasmodium*; none of these loci are involved in prior killing of invading ookinetes (Zheng *et al.*, 1997). Studies in other insects have documented a variety of additional immune responses, both cellular and humoral: phagocytosis, nodule formation, cellular encapsulation and secretion of antimicrobial peptides (Boman and Hultmark, 1987; Lackie, 1988; Hoffmann and Hoffmann, 1990). Recent studies in *Drosophila melanogaster* have shown that the humoral response is itself regulated through multiple genetic pathways (Lemaitre *et al.*, 1995; Hoffmann *et al.*, 1996). In contrast to the dramatic refractory phenotypes, the nature of the specific molecular pathways that underlie them remains to be defined; similarly, the molecular mechanisms whereby parasites circumvent the immune mechanisms of susceptible mosquitoes are unknown.

Localized and systemic response of the mosquito to Plasmodium

We have documented that infection of the mosquito by the rodent malaria parasite *P.berghei* results in robust induction of RNAs that encode the known immune effector molecule defensin and the immune marker GNBP. In parallel experiments, this response has been detected in both encapsulation-refractory and susceptible mosquitoes (data not shown); although these parallel studies have not been quantitative, and none have been conducted at the protein as opposed to the RNA level, it appears likely that the encapsulation response is regulated independently of the particular immune pathway(s) that we have tested thus far. Be that as it may, our observations are important because they constitute the first molecular evidence that the *Plasmodium* parasite does not evade detection by the (poorly understood) immune surveillance mechanisms of the mosquito.

We have shown that the observed immune response is due to parasite infection, rather than blood-feeding *per se*, prior effects of the parasite on the vertebrate host, the presence of vertebrate stage parasites in the blood-meal, or opportunistic bacterial infections during *Plasmodium* invasion of the midgut. The full physiological significance and scope of the observed response remain to be determined in future studies. It will be of interest to examine the interaction between *A.gambiae* and *P.falciparum* (the major human pathogen) relative to the results reported here using a model rodent malaria system. Moreover, it is not known whether the induction of gene expression results from receptor-mediated immune recognition of *P.berghei* by the mosquito, or is a consequence of midgut injury associated with parasite invasion. Mechanical injury is associated with activation of humoral immunity in insects (Boman and Hultmark, 1987).

A second important conclusion of the present work is that mosquito midgut cells can themselves mount a robust immune response to parasite infection. While antimicrobial peptides have been reported in the alimentary systems of some insects (Kylsten *et al.*, 1992; Tryselius *et al.*, 1992; Dunn *et al.*, 1994) they have not been identified previously in the mosquito midgut. *In vitro* studies on midgut explants exposed to bacteria have provided further evidence that the *A.gambiae* midgut is an autonomous, immune-competent organ (data not shown). The alimentary tract is the first organ system that ingested parasites (indeed ingested microorganisms) encounter, and thus it is not surprising that it can also function as a first line of immune defense. This function clearly bears close analysis. It may well be related to the prominence of midgut-localized parasite refractoriness mechanisms whose molecular nature remains unknown.

Induction of defensin RNA, and indeed the presence of active defensin peptide (Richman *et al.*, 1996) in whole extracts of parasite-susceptible mosquitoes, may seem paradoxical in view of evidence that exogenously administered insect defensin can kill *Plasmodium* in the mosquito; interestingly, the pertinent studies show that sensitivity of the parasite is stage-specific and that only mature oocysts and sporozoites are killed (M.Shahabbudin, personal communication). It is worth recalling that the difference between susceptible and refractory mosquitoes is quantitative: although refractory mosquitoes can completely prevent parasite transmission, massive parasite losses occur at every stage of its development even within a susceptible mosquito (Vaughan *et al.*, 1994). It appears that in any mosquito, whether or not the ingested parasites can lead to malaria transmission depends on a balance between multiplication and differentiation of the parasite, and its elimination by physical barriers and active innate immune responses. Quantitative studies of parasite-induced endogenous defensin peptide levels in different mosquito strains, particularly in the midgut, will be needed to clarify the role of this factor in defense against *Plasmodium*. It will also be interesting to compare the repertoire of antimicrobial peptides in the midgut immune responses of different mosquitoes as related to the sensitivity of their co-adapted parasite species. It appears that defensin is the major antimicrobial peptide in adult mosquitoes (Lowenberger *et al.*, 1995; A.Richman, unpublished observations) and it is tempting to speculate that *Plasmodium* may have evolved mechanisms conferring specific insensitivity to defensin during the period of ookinete-induced expression in the midgut.

The third important observation in the present work is the marked 'systemic' immune response in the carcass, at a time when the parasite is confined within the midgut compartment by the basal lamina. The possibility of an immune-related signalling process between tissues, whether involving diffusible parasite products or specific

factors of insect origin, is worth considering. Cytokine-like activities have been described in some invertebrate species (Raftos *et al.*, 1991; Granath *et al.*, 1994), and it has been proposed that in *Drosophila*, expression of the gene encoding the antifungal factor drosomycin is mediated through soluble ligand binding and activation of a cell surface receptor (Lemaitre *et al.*, 1996).

The observed activation of two immune-responsive genes by a parasite entering via the natural route of infection for hematophagous insects clearly establishes a link between the better characterized invertebrate response to experimental bacterial infection, and the mosquito response to challenge by *Plasmodium*. In follow-up studies, isolation and utilization of additional markers representing various independent immune response pathways should prove revealing. In addition, high-resolution spatial analysis of gene expression patterns will be needed to identify the immune responding cells, both in the midgut and in the carcass, thus characterizing in molecular and cellular terms the multifaceted interactions between mosquito and parasite.

Materials and methods

Mosquito infections

Mosquito rearing and parasite infection were performed essentially as described (Sinden, 1997). The rodent malaria *P.berghei* provides a versatile, readily manipulated and safe model system for the study of *A.gambiae–Plasmodium* interactions (Janse and Waters, 1995). *P.berghei* strains Anka 2.33 and 2.34 were passaged in Balb/c mice. Experiments were performed with Anka 2.34 unless otherwise specified. All care and handling of experimental animals was in accordance with the guidelines of the European Molecular Biology Laboratory. The parasitemias of infected mice were determined from Giemsa-stained blood films prepared from tail blood. The average number of exflagellation centers per field was used as an index of mouse infectivity for mosquitoes. Infectivity of a given feed was further monitored by determining the incidence and density of oocysts in a sample of dissected midguts 8–10 days following infection. The melanotically encapsulating *A.gambiae* strain L3-5 was used throughout with one exception: strain G3 was used to demonstrate differential oocyst formation (see Figure 3, lower panel).

Antibiotic pretreatment and blood-meal analysis

Five- to eight-day-old female mosquitoes were maintained for 4 days on 10% sucrose plus penicillin (100 U/ml)/streptomycin (100 μg/ml) in phosphate buffered saline (PBS). 18 h after blood-feeding midguts were dissected out, the contents suspended in a small volume of PBS and 'smeared' on a glass slide, fixed in methanol, and Giemsa stained.

Midgut dissections

Twenty-four hours after blood-feeding, mosquitoes were anaesthetized on ice and dissected in ice cold PBS. Anterior and posterior midgut tissues were carefully separated from hindgut and malphigian tubules, rinsed in clean PBS, and immediately frozen in liquid nitrogen. All remaining tissues, designated 'carcass', were similarly frozen.

RNA analysis

RT–PCR procedures and sequences of defensin and S7-specific primers were as described (Richman *et al.*, 1996). Sequences of GNBP-specific primers were; 5′ GCAACGAGAATCTGTACC 3′ and 5′ TAACCACCA-GCAACGAGG 3′ (Dimopoulos *et al.*, 1997). Total RNA was extracted from whole or dissected mosquitoes at the indicated time points, using the RNaid PLUS kit (Bio 101). For RNA blot analysis, the electrophoresis, transfer to Hybond-N+ membrane (Amersham), probe preparation, and hybridization were performed as described (Sambrook *et al.*, 1989).

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