

Mosquito–*Plasmodium* Interactions in Response to Immune Activation of the Vector

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*Animal Health and Biomedical Sciences, University of Wisconsin at Madison, 1655 Linden Drive, Madison, Wisconsin 53706, U.S.A.; †Department of Entomology, University of Wisconsin at Madison, 1630 Linden Drive, Madison, Wisconsin 53706, U.S.A.; and ‡Institut de Biologie Moleculaire et Cellulaire, UPR 9022 CNRS, 15 rue Rene Descartes, 67084 Strasbourg Cedex, France

Lowenberger, C. A., Kamal, S., Chiles, J., Paskewitz, S., Bulet, P., Hoffmann, J. A., and Christensen, B. M. 1999. Mosquito–*Plasmodium* interactions in response to immune activation of the vector. *Experimental Parasitology* 91, 59–69. During the development of *Plasmodium* sp. within the mosquito midgut, the parasite undergoes a series of developmental changes. The elongated ookinete migrates through the layers of the midgut where it forms the oocyst under the basal lamina. We demonstrate here that if *Aedes aegypti* or *Anopheles gambiae*, normally susceptible to *Plasmodium gallinaceum* and *P. berghei*, respectively, are immune activated by the injection of bacteria into the hemocoel, and subsequently are fed on an infectious bloodmeal, there is a significant reduction in the prevalence and mean intensity of infection of oocysts on the midgut. Only those mosquitoes immune activated prior to, or immediately after, parasite ingestion exhibit this reduction in parasite development. Mosquitoes immune activated 2–5 days after bloodfeeding show no differences in parasite burdens compared with naive controls. Northern analyses reveal that transcriptional activity for mosquito defensins is not detected in the whole bodies of *Ae. aegypti* from 4 h to 10 days after ingesting *P. gallinaceum*, suggesting that parasite ingestion, passage from the food bolus through the midgut, oocyst formation, and subsequent release of sporozoites into the hemolymph do not induce the production of defensin. However, reverse transcriptase-PCR of RNA isolated solely from the midguts of *Ae. aegypti* indicates that transcription of mosquito defensins occurs in the midguts of naive mosquitoes and those ingesting an infectious or noninfectious bloodmeal. Bacteria-challenged *Ae. aegypti* showed high levels of mature defensin in the hemolymph that correlate with a lower prevalence and mean intensity of infection with oocysts. Because few oocysts were found on the midgut of immune-activated mosquitoes, the data suggest that some factor, induced by bacterial challenge, kills the parasite at a preoocyst stage. © 1999 Academic Press

Index Descriptors and Abbreviations: *Aedes aegypti*; *Anopheles*

gambiae; *Plasmodium gallinaceum*; *Plasmodium berghei*; Diptera; Culicidae; mosquito; Liverpool; vector competence; refractoriness; insect immunity; defensin, malaria.

INTRODUCTION

Malaria, with 300–500 million new cases annually, continues to be one of the most devastating diseases affecting humans. The development of *Plasmodium* within the mosquito is a very complex process and represents a tightly coevolved system in which genetic features of both vector and parasite define the potential of the parasite to develop and be transmitted (Richman and Kafatos 1995). Parasite development within the insect may be interrupted at any step of a series of complex developmental stages from gametogenesis in the midgut to the penetration of the salivary glands by mature sporozoites (Richman and Kafatos 1995). Considerable research efforts currently are aimed at elucidating the genetic factors that define parasite resistance in mosquitoes, thereby providing an opportunity to manipulate genetically the susceptibility status of vectors.

Mosquitoes respond to metazoan parasites by a defense response involving the encapsulation of the parasite by hemocytes and/or deposition of protein–polyphenolic compounds (Christensen and Forton 1986) and these melanotic

capsules have been demonstrated to kill ookinetes of *Plasmodium* sp. in the mosquito midgut (Paskewitz *et al.* 1989). There is also a growing literature on the humoral immune response of insects to injected bacteria (Boman and Hultmark 1987; Cociancich *et al.* 1994; Lowenberger *et al.* 1995; Lowenberger 1996; Hoffmann *et al.* 1996). Insects produce a wide range of immune compounds to combat bacterial infections, including antibacterial peptides classified as defensins, cecropins, proline-rich and glycine-rich peptides (Cociancich *et al.* 1994; Hetru *et al.* 1994, 1998). Although these antibacterial peptides generally are considered inactive against eukaryotic organisms (Cociancich *et al.* 1994), the injection of synthetic cecropins and magainins was reported to reduce the prevalence and intensity of *Plasmodium* infections (Gwadz *et al.* 1989). Recently, Shahabuddin *et al.* (1998) reported that injecting insect defensins from *Phormia terranova* and *Aeshna cyanea* negatively affected the development of *P. gallinaceum* oocysts and sporozoites. In *Ae. aegypti* there is a very rapid production of three endogenous antibacterial insect defensins in response to inoculation with sterile saline or bacteria (Lowenberger *et al.* 1995, Chalk *et al.* 1995). We previously demonstrated that a generalized immune activation of susceptible *Ae. aegypti* with sterile saline or bacteria significantly reduced both the prevalence and the mean intensity of infection of *Brugia malayi* ingested in a blood meal (Lowenberger *et al.* 1996). These data suggest strongly that susceptible mosquitoes do have the ability to kill ingested parasites or limit their development, but that the compounds responsible for parasite killing are not expressed normally following ingestion of the parasites in a blood meal. Richman *et al.* (1997) and Dimopoulos *et al.* (1997) demonstrated an upregulation of defensin mRNA following an infectious bloodmeal in *An. gambiae*, and Lehane *et al.* (1997) reported a similar induction of insect defensin in the midguts of *Stomoxys calcitrans*. In light of these data, and our previous report (Lowenberger *et al.* 1996) demonstrating endogenous anti-parasite activity against *B. malayi* after immune activation, we wanted to determine if immune activation of mosquitoes would have a detrimental effect on ingested *Plasmodium*. We report here that a generalized immune activation of mosquitoes, by bacterial inoculation, has a detrimental effect on the development of *P. gallinaceum* in *Ae. aegypti* and *P. berghei* in *Anopheles gambiae* and that this effect is highly dependent on the timing of immune activation.

MATERIALS AND METHODS

Aedes aegypti Black-eyed Liverpool strain susceptible to *P. gallinaceum* and *An. gambiae* 4ARR susceptible to *P. berghei* were reared

as described by Christensen and Sutherland (1984) and Chun *et al.* (1995), respectively. White Leghorn chicks infected with *P. gallinaceum* were maintained as described by Thathy *et al.* (1994), and mice infected with *P. berghei* were maintained as described by Chun *et al.* (1995).

Immune activation. *Escherichia coli* K12 strain and *Micrococcus luteus* were grown in Luria-Bertani's rich nutrient medium overnight at 37°C. Following incubation, 0.50-ml samples of each bacterial culture were combined in a 1.5-ml microfuge tube and centrifuged. The supernatant was removed, leaving a moist pellet. Mosquitoes were kept cold-inactivated on wet ice and individual mosquitoes were held vertically in place by suction on a vacuum saddle as described previously (Beerntsen and Christensen 1990). A sterile stainless steel probe (0.15 mm) was dipped into the moist bacterial pellet and inserted into the hemocoel through the neck membrane (Lowenberger *et al.* 1995). Because initial inoculations of *An. gambiae* resulted in mortality of the majority of mosquitoes, this species subsequently was inoculated with a needle dipped into a mixture of the overnight cultures of *E. coli* and *M. luteus* without centrifugation and concentration, a process that produced >90% survival. To control for factors produced in response to wounding, a second group of mosquitoes was inoculated with a stainless steel probe that had been dipped in sterile *Aedes* saline (Hayes 1953). A third group of mosquitoes was not inoculated. All *Ae. aegypti* and *An. gambiae* used in this study were inoculated, or sham inoculated, within 48 and 72 h postemergence, respectively, and then were returned to the environmental chamber. In each mosquito-*Plasmodium* combination, we used groups of mosquitoes inoculated at various times: those inoculated 1 day prior to bloodfeeding (day -1), those inoculated 30-60 min after bloodfeeding (day 0), and those inoculated on days 1, 2, 3, 4, or 5 after bloodfeeding. Control groups from the same cohort of mosquitoes remained noninoculated or were inoculated with a needle dipped in sterile saline. In the three trials a subset of 10 *Ae. aegypti* exposed to *P. gallinaceum* was inoculated with bacteria on day 7 post bloodfeeding and on day 10 post bloodfeeding the salivary glands were dissected to determine if immune activation affected the sporozoites as they moved from the oocysts through the mosquito hemolymph to the salivary glands. Salivary glands were removed from the mosquitoes and transferred to microcentrifuge tubes and the glands were macerated in 100 µl distilled water. The numbers of sporozoites were counted on a hemacytometer under 400× magnification as described by Kelly and Edman (1997).

Parasite exposure. All mosquitoes were exposed to parasites on day 0. *Ae. aegypti* were deprived of sucrose for 8-12 h and then were exposed to a White Leghorn chicken with a parasitemia of ~10% and a gametocytemia of about 1%. *An. gambiae* were supplied constantly with sucrose and were exposed to a mouse with a parasitemia of 5-8%. Mosquitoes were allowed 15-30 min to feed, and fully engorged females were separated, supplied with 10% sucrose solution *ad libitum*, and returned to the environmental chamber. Mosquitoes were dissected 7 days later to determine the prevalence and mean intensity of oocyst infection.

Hemolymph collection and mature protein analysis. Hemolymph from a subset of bacteria-inoculated, bloodfed, and naive mosquitoes was collected 24 h after inoculation or bloodfeeding by tearing the last abdominal segments and perfusing the hemocoel contents by injecting sterile distilled water with 0.05% TFA into the thorax as described by Beerntsen and Christensen (1990). Three drops of hemolymph were collected per mosquito and the hemolymph of five mosquitoes was combined per tube. Hemolymph samples were filtered through 30,000 molecular mass filters (Centricon 30; Amicon, Beverly, MA) for 10

min at 12,000 rpm. The resultant hemolymph was vacuum dried, and the pellet was resuspended in 25 μ l distilled water containing 0.05% TFA. Samples were analyzed using a Gilson HPLC (Gilson) with an Aquapore OD 300, 7- μ m, 220 \times 2.1-mm column (Applied Biosystems). Samples (5 μ l) were injected and the areas under the peak corresponding to defensin were compared with a standard curve derived by injecting known concentrations of purified *Ae. aegypti* defensin (Lowenberger *et al.* 1995). Protein levels were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules CA).

Northern analyses. Mosquito defensin transcriptional activity was assessed in a subset of each group of immune-activated and blood-fed mosquitoes. Five *Ae. aegypti* or 10 *An. gambiae* were cold anesthetized and total RNA was collected from whole bodies, dissected fat bodies, or midguts, using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction isolation method (Chomczynski and Sacchi 1987). RNA from each group of mosquitoes was separated on formaldehyde-agarose gels as described by Sambrook *et al.* (1989). Following transfer, membranes were air-dried and UV cross-linked (UV Stratallinker; Stratagene, La Jolla, Ca). 32 P probes were generated using specific primers to amplify 50 ng of template in a PCR described previously (Severson and Kassner 1995). Templates used were the cloned 120-bp sequence of the *Ae. aegypti* mature defensin isoforms A and C (Lowenberger *et al.* 1995) or a cloned cDNA from *An. gambiae* amplified using primers described by Richman *et al.* (1996). Membranes were hybridized simultaneously or sequentially with appropriate loading controls. Hybridizations and washes were conducted at 60°C in glass bottles in a rotating oven (National Labnet). Membranes were washed initially in 2 \times SSC with 0.1% SDS at room temperature for 15 min, then at 60°C for 15 min, and then twice in 0.2 \times SSC with 0.1% SDS at 60°C for 15 min each. The membranes then were exposed to Kodak XAR film (Eastman-Kodak) at -80°C with an intensifying screen.

RT-PCR. RNA was extracted as described above from the carcasses of 5 *Ae. aegypti* or 40 midguts at various times after inoculation with bacteria or ingestion of an infectious or noninfectious bloodmeal. RNA was treated with RNase-free DNase (Promega, Madison, WI), phenol-chloroform extracted, and quantified by spectrophotometer, and 2 μ g of total RNA was reverse transcribed as described previously (Ausubel *et al.* 1997). Primers used in PCR were isoform A/B, atgaagtcacatcgtcatttg and acagacgcagaccttctgg; isoform C, atgcgtaccctcatcgtcg and acagacgcagaccttttgcg; all isoforms, atgaagtcacatcgtcatttg/atgcgtaccctcatcgtcg and tcaatttcgacagacgcagacct. The PCR program to amplify defensins was 94°C for 2 min and then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. To discriminate between isoforms, the annealing temperature was increased to 63°C. Positive controls used were 100 ng of a purified cDNA encoding either isoform A or C that, under the stringent conditions (63°C annealing temperature), renders only a product with the appropriate primer pairs. The PCR conditions were standardized using S7-specific primers (Richman *et al.* 1996). PCR products were analyzed and separated on a 1% agarose gel, stained with ethidium bromide, and compared using an Eagle Eye II Still Video system (Stratagene).

Statistical analysis. Three independent replicates were done in each mosquito-*Plasmodium* system, involving 25 mosquitoes/group/replicate. A two-way Analysis of variance demonstrated no significant differences between replicates (*Ae. aegypti* $F = 0.0272$, $P = 0.9732$; *An. gambiae* $F = 0.505$, $P = 0.4028$), indicating the consistency of experimental procedures between replicates. However, this analysis identified significant differences among the treatment groups (*Ae. aegypti* $F = 17.13$, $P < 0.0001$; *An. gambiae* $F = 7.65$, $P < 0.0001$). The

same statistical relationship was found between the different treatment groups in each replicate. A χ^2 analysis was used to compare the prevalence of infection among the treatments with the prevalence of the noninoculated control group of mosquitoes serving as the expected prevalence against which treated mosquitoes were compared. Because the distribution of oocysts on the midguts was not normally distributed, nonparametric statistics (Kruskal Wallis analysis of variance [KW]) were used. Because there were no significant differences in the same treatment groups among the three replicates (KW: $P > 0.05$), data from the same treatment groups from the different replicates were pooled for subsequent analysis. After the KW test, Bonferroni's multiple comparison procedure was used to compare mean intensities of infection (mean number of parasites/infected host) between and among groups. Statistical analyses were done using SigmaStat (Jandel, San Rafael, CA) and differences were considered significant at $P < 0.05$.

RESULTS

In immune-activated mosquitoes, the prevalence of infection was significantly lower in mosquitoes immune activated on days -1 and 0 compared with the noninoculated controls (χ^2 analysis: *Ae. aegypti* day -1 $P < 0.001$, day 0 $P < 0.001$; *An. gambiae* day -1 $P = 0.0196$, day 0 $P = 0.007$) (Tables I and II). There were no significant differences in prevalence of infection between mosquitoes inoculated on days 1 and 2 post bloodfeeding and the noninoculated controls (χ^2 analysis: *Ae. aegypti* day 1 $P = 0.65$, day 2+ $P = 0.89$; *An. gambiae* day 1 $P = 1.00$, day 2 $P = 0.323$) (Tables I and II). In the control group inoculated with sterile saline there were no statistically significant differences in prevalence of infection between any of the inoculated groups and noninoculated controls (Table I).

There were significant differences when mean intensities of infection were compared among all treatment groups inoculated with bacteria (KW: *Ae. aegypti* $H = 94.4$, $df = 4$, $P < 0.0001$; *An. gambiae* $H = 38.3$, $df = 4$, $P < 0.001$). In *Ae. aegypti* inoculated with bacteria, three statistically significant groups were determined: The control group and the day 2+ group were not significantly different (KW: $H = 1.33$, $df = 1$, $P = 0.2485$), nor were the groups of mosquitoes inoculated on days -1 and 0 (KW: $H = 0.248$, $df = 1$, $P = 0.6188$). Intermediate between these groups, and significantly different from both, was the group of mosquitoes inoculated on day 1 post bloodfeeding (Table I). Analysis of the mean intensities of infection among saline-inoculated *Ae. aegypti* revealed two significantly distinct groups: The mean intensities of infection of mosquitoes inoculated on days -1, 1, and 2 and of the controls were not significantly different from each other (KW: $H = 3.15$, $df = 3$, $P = 0.3695$), but there was a significant difference when the

TABLE I
Prevalence and Mean Intensity of Infection of *Plasmodium gallinaceum* in *Aedes aegypti* When Mosquitoes Had Been Immune Activated with Bacteria or Sterile Saline at Various Times before or after Bloodfeeding

Bacteria inoculated				Saline inoculated			
Time of treatment	N	Prevalence (%) ¹	Mean intensity (SE) ²	Time of treatment	N	Prevalence (%) ¹	Mean intensity (SE) ²
Control	80	96.3 ^a	24.05 (1.5) ^a	Control	76	91.6 ^a	18.8 (1.4) ^a
-1	37	62.2 ^b	6.52 (2.8) ^b	-1	52	89.7 ^a	22.1 (2.3) ^a
0	75	65.3 ^b	7.55 (1.9) ^b	0	62	91.2 ^a	13.5 (1.6) ^b
1	75	93.3 ^a	17.24 (1.6) ^c	1	45	91.8 ^a	16.4 (1.8) ^a
2+	188	96.8 ^a	20.86 (1.0) ^a	2+	55	91.7 ^a	20.3 (2.2) ^a

Note. Numbers represent pooled samples from three replicates as explained in the text.

¹ Within treatment groups, comparisons of prevalence of infection values followed by the same letter as the control value are not significantly different from the control as determined by χ^2 analysis ($P > 0.05$).

² Within treatment groups, comparisons of mean intensity of infection values followed by the same letter are not significantly different from each other as determined by analysis of variance and Bonferroni's pairwise multiple comparison procedure.

mosquitoes inoculated on day 0 were included in the analysis (KW: $H = 15.5$, $df = 4$, $P = 0.0038$).

In comparing mean intensities of infection with *P. gallinaceum* between *Ae. aegypti* inoculated with bacteria and those inoculated with saline, there were significantly fewer oocysts found in the bacteria-inoculated mosquitoes compared with saline-inoculated mosquitoes on day -1 (KW: $P < 0.0001$) and day 0 (KW: $P = 0.0017$). However, there were no significant differences between mosquitoes inoculated with saline or bacteria on days 1 or 2+ or the noninoculated controls (KW: $P > 0.05$).

TABLE II
Prevalence and Mean Intensity of Infection of *Plasmodium berghei* in *Anopheles gambiae* Immune Activated with Bacteria at Various Times before or after Bloodfeeding

Time of treatment	N	Prevalence (%) ¹	Mean intensity (SE) ²
Control	43	97.8 ^a	10.44 (1.1) ^a
-1	20	80.0 ^b	6.25 (0.7) ^b
0	34	79.5 ^b	5.56 (0.7) ^b
1	40	100 ^a	11.30 (0.9) ^a
2+	33	91.9 ^a	11.24 (1.0) ^a

¹ In comparisons of prevalence of infection, values followed by the same letter as the control value are not significantly different from the control as determined by χ^2 analysis ($P > 0.05$).

² In comparisons of mean intensity of infection, values followed by the same letter are not significantly different from each other as determined by Kruskal Wallis analysis of variance on ranks and Bonferroni's pairwise multiple comparison procedure.

In *An. gambiae* there were only two significantly different groups: those inoculated on days 1 and 2+ and the controls were not significantly different from each other (KW: $H = 2.0$, $df = 2$, $P = 0.3676$), but were significantly different from those mosquitoes inoculated on days -1 and 0 (KW: $P < 0.001$) (Table II). The mean intensities of infection of mosquitoes inoculated with bacteria on days -1 and 0 were not significantly different (KW: $H = 2.64$, $df = 1$, $P = 0.1044$).

The frequency distributions of oocysts demonstrated that inoculation of mosquitoes with bacteria on days -1 and 0 shifted the distribution of parasites, resulting in an overdispersion of oocysts, with most mosquitoes having very few oocysts (Fig. 1). This effect was more pronounced in *Ae. aegypti* than in *An. gambiae* (Fig. 1). The inoculation of mosquitoes 7 days after ingesting a *P. gallinaceum*-infectious bloodmeal did not prevent the invasion of the salivary glands by sporozoites: inoculated and noninoculated mosquitoes all were positive for sporozoites.

Ae. aegypti inoculated with bacteria demonstrated rapid and significant transcription of insect defensin as did saline-inoculated *Ae. aegypti*, albeit at reduced levels (Fig. 2). *An. gambiae* also demonstrated transcriptional activity in bacteria-inoculated mosquitoes, but not in naive females (Fig. 2). Northern analysis could not detect transcriptional activity of insect defensins in RNA extracted from whole bodies nor in RNA isolated solely from midguts of *Ae. aegypti* at any time after bloodfeeding on a *P. gallinaceum*-infected host (Fig. 3).

RT-PCR detected mosquito defensins in the midguts of

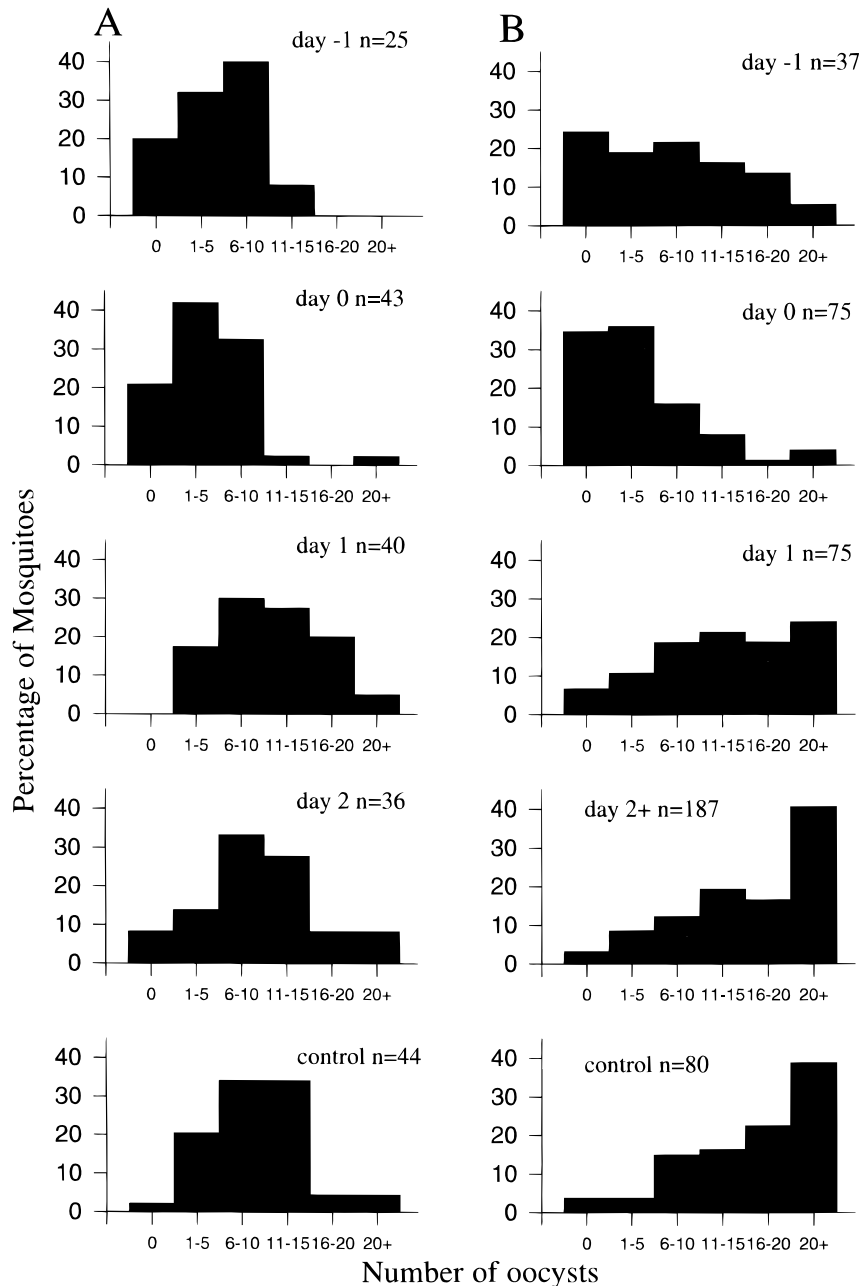


FIG. 1. Frequency distribution of oocysts found on the midguts of *An. gambiae* (A) and *Ae. aegypti* (B) 7 days post-ingestion. Mosquitoes were inoculated with bacteria at various times before or after ingestion of an infective bloodmeal as indicated in the text.

naive mosquitoes as well as in those mosquitoes fed on an infectious or noninfectious bloodmeal (Fig. 4). No significant increase in transcription was associated with the infectious bloodmeal as opposed to a bloodmeal containing no parasites. Similarly, in mosquito carcasses, there were no

differences in the expression of defensin mRNAs between mosquitoes exposed to an infectious or noninfectious bloodmeal (data not shown). The primer sets used in RT-PCR were designed to amplify defensin A/B, defensin C, or all *Ae. aegypti* defensins. Although there were no discernible

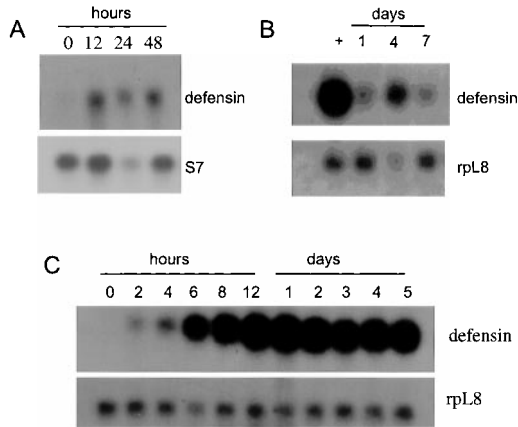


FIG. 2. Northern blot autoradiograph demonstrating transcriptional activity for insect defensins in *Ae. aegypti* and *An. gambiae*. (A) Northern autoradiograph of total RNA isolated from *An. gambiae* at various times (h) postinoculation with bacteria. (B) Northern autoradiograph of total RNA isolated from abdominal fat bodies of *Ae. aegypti* at various times after inoculation with a sterile needle. (C) Northern autoradiograph of total RNA isolated from abdominal fat bodies of *Ae. aegypti* at various times after inoculation with bacteria. Each lane represents 5 μ g of total RNA isolated from abdominal fat bodies of *Ae. aegypti* or 10 μ g of total RNA from *An. gambiae*. The positive control (+) is 5 μ g total RNA isolated from *Ae. aegypti* 8 h postinoculation with bacteria. 32 P probes were generated using specific primers to amplify 50 ng of template: the cloned 120-bp sequence of the *Ae. aegypti* mature defensin isoforms A and C (Lowenberger *et al.* 1995) or a cloned cDNA from *An. gambiae* amplified using primers described by Richman *et al.* (1996). Loading controls were rpL8 in *Ae. aegypti* and S7 in *An. gambiae*.

differences in the products amplified from parasite-exposed and bloodfed mosquitoes, defensin isoform C was expressed more highly in the midguts compared with isoforms A/B (Fig. 4).

HPLC analysis demonstrated that naive mosquitoes inoculated with bacteria had significant levels of mature defensin in the hemolymph (Fig. 5). The levels of defensin in control, noninoculated mosquitoes were not significantly different from those of mosquitoes that fed upon an infectious or noninfectious bloodmeal. Defensin levels in bacteria-inoculated mosquitoes ranged from 120 to 200 ng/mosquito, which corresponds to approximately 6% of the protein contained in the insects' hemolymph.

DISCUSSION

In mosquitoes that support the development of *Plasmodium* spp., the ookinete moves through the cytoplasm of the

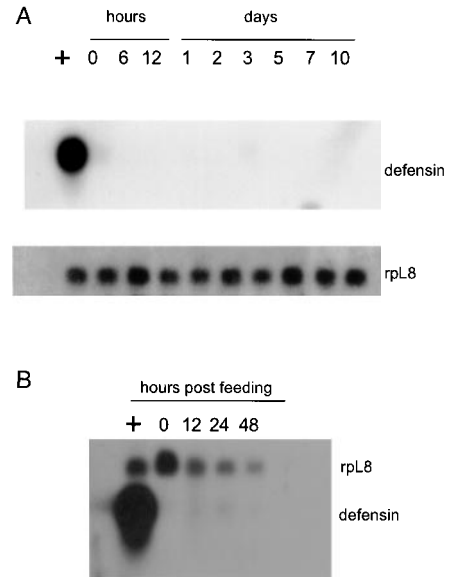


FIG. 3. Northern blot autoradiography demonstrating defensin transcriptional activity for insect defensins in *Ae. aegypti* at various times after ingesting a *P. gallinaceum* infectious bloodmeal. (A) Northern autoradiography of total RNA isolated from whole bodies of *Ae. aegypti* with the midguts removed. (B) Northern autoradiography of total RNA isolated from the midguts of *Ae. aegypti* removed from the mosquito at various times after ingestion of a *P. gallinaceum* infectious bloodmeal. Each lane represents 5 μ g of total RNA (A) or 10 μ g of total RNA from midguts (B). The positive control (+) is 5 μ g of RNA isolated from *Ae. aegypti* 8 h postinoculation with bacteria. 32 P probes were generated using specific primers to amplify 50 ng of the cloned 120-bp sequence of the *Ae. aegypti* mature defensin isoforms A and C (Lowenberger *et al.* 1995). A probe generated from a ribosomal protein-encoding cDNA, rpL8, was used as a loading control.

epithelial cell to the intercellular space or to the space between the epithelial cell and the basal lamina (Torii *et al.* 1992; Shahabuddin and Pimenta 1998) where they form oocysts. The timing of events is important; sedentary round zygotes of *Plasmodium* spp. require about 18–24 h (depending on species) to develop into the motile, elongated ookinetes (Torii *et al.* 1992; Shahabuddin *et al.* 1995) that penetrate the mosquito midgut. This is also the period required for the production of maximum levels of defensins, and undoubtedly other immune compounds, in the hemolymph after immune activation. Our data suggest that susceptible mosquitoes immune activated prior to, or within 1 h of, ingesting a bloodmeal become resistant to ingested parasites. Vernick *et al.* (1995) selected a strain of *An. gambiae* refractory to *P. gallinaceum* in which ookinetes died after invading the midgut epithelial cells. In this selected strain there were clusters of pigment granules on the midguts, but no oocyst formation (Vernick *et al.* 1995). Similarly, we

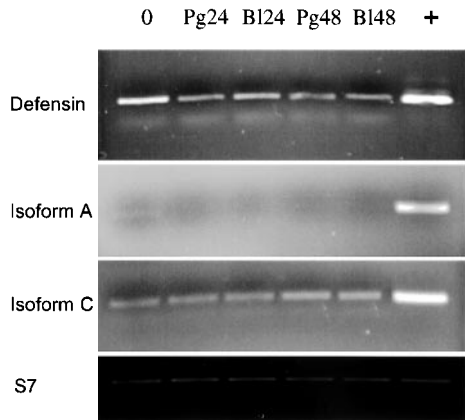


FIG. 4. RT-PCR analysis of gene expression for defensins in *Ae. aegypti* midguts at various times after feeding on a *P. gallinaceum*-infectious bloodmeal (Pg) or a noninfectious bloodmeal (B1). Expression of a gene encoding the ribosomal protein S7 was used as a control. RT-PCR was performed as explained in the text. The template used in the positive controls was 100 ng of a cDNA isolated from the fat bodies of bacteria-inoculated mosquitoes.

have observed pigment clusters on the midguts of some bacteria-inoculated mosquitoes, suggesting that the parasites were killed after penetration of the peritrophic matrix and before oocyst formation. This response was seen only in mosquitoes inoculated prior to, or on the day of, ingesting a bloodmeal.

Insect antibacterial peptides, such as the defensins, reach maximum concentrations in the hemolymph about 24 h after induction (Hoffmann and Hetru 1992; Dimarcq *et al.* 1990). Therefore, in order to have parasite killing of preoocyst stages, induction of resistance mechanisms must take place soon after blood feeding, because induction by inoculation 24 or 48 h after bloodfeeding (and subsequent high levels of immune compounds 48–72 h post bloodfeeding) produces no significant reduction in oocyst numbers. This timing of events suggests that the ookinete, as it moves from the bloodmeal to the midgut wall 18–36 h post bloodfeeding, is the stage susceptible to immune factors. When mosquitoes were immune activated after oocyst formation there was no significant reduction in the number of oocysts on the midguts compared with control mosquitoes. We found some oocysts

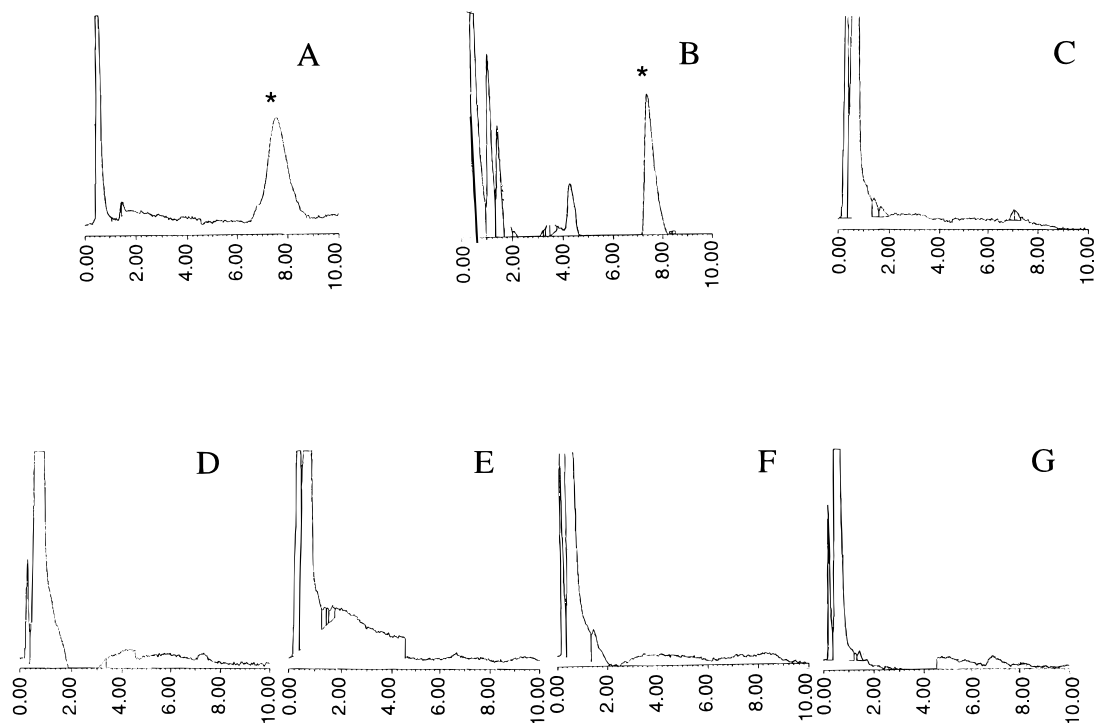


FIG. 5. HPLC profile of mature defensin protein in the hemolymph of *Ae. aegypti* at various times after immune activation or ingestion of a bloodmeal. The samples were prepared as described under Materials and Methods. The asterisk denotes the peak of defensin: (A) Purified defensin standard, (B) bacteria-inoculated mosquitoes, (C) naive mosquitoes, (D) mosquitoes fed on a noninfectious blood meal 24 h post-bloodfeeding, (E) mosquitoes fed on a noninfectious blood meal 48 h post-bloodfeeding, (F) *P. gallinaceum* fed mosquitoes 24 h post bloodfeeding, (G) *P. gallinaceum* fed mosquitoes 48 h post-bloodfeeding.

in immune-activated mosquitoes which may have been due to improper immune activation or possibly to the location of the oocyst. Torii *et al.* (1992) reported that ookinetes may be inter- or intracellular when they round up to form oocysts. Conceivably, parasites that are intracellular may be more protected from immune peptides than those in the intercellular position. Shahabuddin and Pimenta (1998), however, reported that ookinetes of *P. gallinaceum* preferentially invade one type of vesicular ATPase-expressing cells in the midgut wall. Alternatively, some ookinetes may have penetrated the midgut wall and formed oocysts before high concentrations of killing molecules were present.

Although immune activation with bacteria on days -1 and 0 significantly reduced the prevalence and mean intensities of infection in *Ae. aegypti*, this was not the case with mosquitoes inoculated with a needle dipped in sterile saline. In the latter case there was no significant reduction in prevalence of infection in any treatment group, and there was a reduction in mean intensity of infection only in mosquitoes inoculated within 60 min of ingesting an infective bloodmeal. These data suggest that violation of the cuticle alone does not in itself activate the immune system to the same extent as does the introduction of bacteria into the hemocoel. This was demonstrated previously when we showed that levels of insect defensins found in the hemolymph of saline-inoculated mosquitoes were only about one-third of levels in the hemolymph of mosquitoes inoculated with bacteria (Lowenberger *et al.* 1995). In the present study, inoculation with bacteria was more successful in reducing the prevalence and mean intensity of infection than was the inoculation of sterile saline. The production of parasite-killing compounds in saline-inoculated mosquitoes may not have been induced or perhaps not produced in the concentrations required for lethal effects on ingested parasites. We reported similar results in a previous study looking at the development of *B. malayi* in immune-activated mosquitoes, in which saline inoculation did not result in the same level of parasite killing as did inoculation with bacteria (Lowenberger *et al.* 1996). There are several differences in the biology of the two parasites: whereas microfilariae of *Brugia* sp. penetrate the midgut and traverse the hemocoel within 30 min after ingestion (Christensen and Sutherland 1984; Perrone and Spielman 1986) and before the deposition of physical barriers such as the chitinous peritrophic matrix, ookinetes of *Plasmodium* sp. move from the blood bolus, actively penetrate the peritrophic matrix using endogenous chitinase (Shahabuddin *et al.* 1993; Shahabuddin and Kaslow 1994), move through the midgut epithelium, and form oocysts beneath the basal lamina of the midgut (Sinden 1984; Shahabuddin *et al.* 1995;

Torii *et al.* 1992), where they lie physically separated from the hemolymph.

Shahabuddin *et al.* (1998) reported that injecting insect defensins from the dragonfly, *Aeschna cyanea*, and the flesh fly, *Phormia terranova*, did not affect the prevalence of infection, but reduced the mean number of oocysts in *P. gallinaceum*-exposed *Ae. aegypti* and that a significant number of surviving oocysts were morphologically abnormal. This study by Shahabuddin *et al.* (1998) also reported that later stages of oocysts and sporozoites are susceptible to insect defensins at levels of 1–25 μ M, which are within the range found in bacteria-inoculated mosquitoes (Lowenberger *et al.* 1999). Our study could not demonstrate a reduction in the number of oocysts in bacteria-inoculated mosquitoes from day 2 to 5 post bloodfeeding but we did not examine critically oocyst morphology. Whereas Shahabuddin *et al.* (1998) demonstrated a significant killing effect of these defensins on *P. gallinaceum* sporozoites *in vitro*, we did not see a similar effect *in vivo* in mosquitoes inoculated with bacteria 7 days after bloodfeeding. These contrasts may be due to differential effects of endogenous and exogenous compounds or differences among strains of mosquitoes and strains of parasites.

Northern analysis failed to detect defensins in whole bodies of *Ae. aegypti* after ingestion of an infectious bloodmeal. This finding is at odds with the report by Richman *et al.* (1997) that demonstrated a threefold increase in defensin transcripts in *P. berghei*-infected *An. gambiae*. We used significantly less RNA in our Northern blots compared with theirs, which may have affected our result, and there may be differences due to species and strains of mosquito and/or parasite. On another Northern blot containing 10 μ g of RNA isolated solely from *Ae. aegypti* midguts, no significant up-regulation of defensin was seen 12 and 24 h post bloodfeeding (Fig. 3). RT-PCR did detect defensins in the midguts of naive mosquitoes and in those from mosquitoes that had ingested a parasitemic or nonparasitemic bloodmeal. This observation suggests that low levels of transcription occur in the midgut, but we did not detect a significant increase in transcription for defensins as a result of ingestion of an infectious bloodmeal, as reported in *An. gambiae* by Richman *et al.* (1997), or a noninfectious bloodmeal. These differences also may be a result of behavioral or pathological differences between the *Plasmodium* species in their insect hosts. Richman *et al.* (1997) also reported a systemic effect of midgut defensin expression that induced the expression of defensin in other tissues. In our study, RT-PCR also detected defensin in the carcass of mosquitoes, including naive controls, from which the midguts were removed (data not

shown). Whether these low levels of defensin are constitutively expressed, were initiated due to our manipulations, are a consequence of resident midgut bacteria, or are a carryover from the pupal stages in which mosquito defensins are expressed (Richman *et al.* 1996; Lowenberger *et al.* 1999) is not known. Regardless of their origins, the levels of transcription of defensins in bloodfed *Ae. aegypti* are significantly lower than in those immune-activated with bacteria or sterile injury. We also determined by RT-PCR that there was a differential expression of defensin isoforms in the midgut tissues; under stringent conditions only isoform C was amplified. This isoform was originally found in lower levels than were isoforms A and B in the hemolymph of bacteria-inoculated mosquitoes (Lowenberger *et al.* 1995), and the signal peptide region of isoform C is quite different from those of isoforms A and B (Lowenberger *et al.* 1999), which may represent some tissue specificity of defensin isoform expression. Mature defensin levels in the hemolymph are significantly lower in bloodfed than in immune-activated mosquitoes and did not differ significantly from naive controls at any time postingestion. These data suggest that, in *Ae. aegypti*, low levels of constitutive midgut transcription activity for insect defensins do not result in high levels of hemolymph immune compounds required for the killing of ingested parasites. Similarly, in *An. gambiae*, the low levels of defensins induced by the parasite (Richman *et al.* 1997) apparently do not negatively influence its development in the midgut.

Only fully engorged mosquitoes were used in these studies. We have demonstrated previously (Lowenberger *et al.* 1996) that the volume of blood ingested by immune-activated or naive *Ae. aegypti* is not significantly different, indicating that the reduced parasitemias, as demonstrated here, are not due to ingestion of different blood volumes. Rather, some factor induced by the immune activation process itself is implicated in the parasite killing. Insect defensins are about 4 kDa, which places them in the size range that could cross the basal membrane from the hemolymph and affect parasites. Although we cannot identify the compound or mechanism responsible for the parasite killing demonstrated here, it would seem that the susceptible stage of the parasite is the ookinete.

Although both *Ae. aegypti* and *An. gambiae*, inoculated with bacteria on day 0, had reduced mean intensities of infection, there was not as dramatic an increase in the transcriptional response for insect defensins in *An. gambiae* as was seen in *Ae. aegypti*. Defensin production is induced in *An. gambiae* after inoculation with bacteria (Richman *et al.* 1996), albeit at lower levels than those found in *Ae. aegypti* (Lowenberger *et al.* 1995). Our present study suggests that

immune activation of *An. gambiae* with bacteria induces the production of immune compounds that reduce the development of ingested *P. berghei*, but not to the levels seen in *Ae. aegypti* exposed to *P. gallinaceum*. However, if defensin induction is dependent on the number of bacteria injected, we might expect this result because *An. gambiae* were injected with significantly fewer bacteria than were *Ae. aegypti*.

One proposal for the control of vector-borne diseases involves the introduction of genes for parasite resistance or refractoriness into mosquito populations (Miller 1992; James 1992; Collins and Besansky 1994; Vernick *et al.* 1995). The identification of specific compounds responsible for parasite killing, as demonstrated here, will provide the molecules to use in transformation experiments to express parasite-killing compounds to coincide temporally and spatially with susceptible stages of ingested parasites.

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