

A snake venom phospholipase A₂ blocks malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface

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Summary

Oocyst formation is a critical stage in the development of the malaria parasite in the mosquito. We have discovered that the phospholipase A₂ (PLA₂) from the venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*) inhibits oocyst formation when added to infected chicken blood and fed to mosquitoes. A similar transmission-blocking activity was demonstrated for PLA₂s from the venom of other snakes and from the honeybee. This effect is seen both with the avian malaria parasite *Plasmodium gallinaceum* and with the human parasite *Plasmodium falciparum* developing in their respective mosquito hosts. The inhibition occurs even in the presence of an irreversible inhibitor of the active site of PLA₂, indicating that the hydrolytic activity of the enzyme is not required for the antiparasitic effect. Inhibition is also seen when the enzyme is fed to

mosquitoes together with ookinetes, suggesting that the inhibition occurs after ookinete maturation. PLA₂ has no direct effect on the parasite. However, pretreatment of midguts with PLA₂ (catalytically active or inactive) dramatically lowers the level of ookinete/midgut association *in vitro*. It appears, therefore, that PLA₂ is acting by associating with the midgut surface and preventing ookinete attachment to this surface. Thus, PLA₂ is an excellent candidate for expression in transgenic mosquitoes as a means of inhibiting the transmission of malaria.

Key words: malaria, ookinete, transmission blocking, phospholipase, *p*-bromophenacyl bromide, eastern diamondback rattlesnake, *Crotalus adamanteus*, *Plasmodium gallinaceum*, *Plasmodium falciparum*, *Aedes aegypti*.

Introduction

Despite a century of research and effort, malaria is still a major cause of morbidity and infant mortality in tropical regions. New, promising approaches to malaria control seek to disrupt the transmission cycle in vector mosquitoes *via* transmission-blocking vaccines or controlled release of parasite-refractory strains of mosquitoes (Crampton et al., 1994; Gwadz, 1994). Before these interventions are possible, however, we need to have a full molecular understanding of the sporogonic development of the parasite in the mosquito.

The malaria parasite begins its development shortly after the mosquito ingests an infected blood meal. Male and female gametes emerge from infected red blood cells and mate to form a zygote. Over the next 16–24 h, the zygote transforms into a motile ookinete, which then leaves the blood meal, penetrates through the chitinous peritrophic membrane, makes contact with the midgut epithelium and invades a midgut cell. It crosses the epithelium until it reaches the basement membrane on the other side of the midgut wall and rounds up against this structure to form an oocyst. The oocyst enlarges and produces numerous sporozoites. These are released and migrate to, and invade, the salivary glands, from where they

are injected into a new vertebrate during blood-feeding on the host.

Little is known about how the parasite recognizes and invades the mosquito midgut epithelium. Studies using light and electron microscopy have shed some light on the events occurring between ookinete invasion of the midgut epithelium and oocyst formation (Mehlhorn et al., 1980; Meis and Ponnudurai, 1987; Meis et al., 1989; Sieber et al., 1991; Syafruddin et al., 1991; Torii et al., 1992). Recently, the establishment of an *in vitro* system has allowed more detailed studies of the interactions between *Plasmodium gallinaceum* ookinetes and the midgut of *Aedes aegypti* (Shahabuddin et al., 1997; Shahabuddin and Pimenta, 1998; Zieler et al., 1998, 1999). *In vitro*, the ookinetes bind to the microvillated midgut surface and invade midgut cells, reflecting their normal behavior in the mosquito host (Shahabuddin et al., 1997; Shahabuddin and Pimenta, 1998). Using these novel methods, we and others have been able to observe specific adhesion of ookinetes to the mosquito midgut (Shahabuddin et al., 1997; Zieler et al., 1999), invasion of the epithelium (Zieler and Dvorak, 2000), changes in the morphology of the invaded cells

(Shahabuddin and Pimenta, 1998) and the resulting death of the invaded cells (Zieler and Dvorak, 2000; Han et al., 2000).

During the course of our attempts to understand the binding of ookinetes to the midgut surface, we discovered that a phospholipase A₂ (PLA₂) from the eastern diamondback rattlesnake (*Crotalus adamanteus*) inhibits ookinete adhesion and oocyst formation. The *C. adamanteus* PLA₂ belongs to a large and well-studied class of highly conserved secreted PLA₂s that are commonly found in snake and insect venoms and in vertebrate pancreatic excretions (for reviews, see Slotboom et al., 1982; Waite, 1987). These enzymes are small (approximately 15 kDa) and extremely stable because of the presence of seven disulfide bridges in the globular structures. PLA₂s hydrolyze stereospecifically the acyl ester in position 2 of 3-*sn*-phosphoglycerides to form lysophospholipids and fatty acids, causing the degradation of phospholipids in membrane bilayers.

Although they are highly water-soluble, PLA₂s are much more active towards aggregated phospholipid present in micelles, monolayers or natural or artificial bilayers than towards dispersed monomeric substrates (Dennis, 1973, 1983; Verger et al., 1973; Hønger et al., 1997). The reason for this preference is the ability of PLA₂s to interact efficiently with membranes and insert into the lipid bilayer to bind to their substrates (interfacial binding). Several amino acid residues found on the enzyme surface participate in interfacial binding in a manner independent of hydrolytic activity (Keith et al., 1981; Brunie et al., 1985; White et al., 1990; Jain et al., 1995; Gelb et al., 1999). Because of their preference for the interfacial membrane environment, PLA₂s are also very sensitive to the physical state of membranes, and their binding is strongly influenced by lateral lipid pressure, lipid composition, surface charge, membrane curvature, temperature and the presence of gel or fluid lipid phases in the membrane (op den Kamp et al., 1974; Kensil and Dennis, 1979; Goormaghtigh et al., 1981; Apiz-Castro et al., 1982; Dawson et al., 1984; Thuren et al., 1987; Burack et al., 1993; Lehtonen and Kinunnen, 1995; Hønger et al., 1997). Most PLA₂s prefer to bind to membranes perturbed by a variety of factors that cause lipid packing defects. Furthermore, the PLA₂s are very diverse in terms of their ability to penetrate lipid bilayers, which has made them into useful tools for studying lateral lipid pressures in biological membranes (Demel et al., 1975; Waite, 1987).

Because of their interfacial binding characteristics, PLA₂s bind efficiently to aggregated lipids even in the absence of enzymatic activity (Tinker et al., 1980; Condrea et al., 1981; Kini and Evans, 1989). Studies of this property have benefited from the use of *p*-bromophenacyl bromide (pBPPB), which reacts irreversibly with an essential histidine residue in the active site of the enzyme (Volwerk et al., 1974). It has been found that pBPPB-inactivated PLA₂s display the same binding characteristics as the catalytically active enzyme. As a result, many of the venom PLA₂s retain some or all of their toxic effects in animals after inactivation with pBPPB (for reviews, see Rosenberg, 1986; Kini and Evans, 1989).

Because of their binding properties, small size, stability and heterogeneity, the PLA₂s represent a useful and convenient tool for probing interactions between cell surfaces. We report here that a snake venom PLA₂ inhibits oocyst formation of malaria parasites by blocking the association between ookinetes and the midgut surface. We propose that the gene coding for this enzyme may be useful as a genetic tool for interfering with malaria parasite development in mosquitoes.

Materials and methods

Enzymes and reagents

All PLA₂ enzymes were bought from Sigma-Aldrich (St Louis, MO, USA). With the exception of the *Crotalus adamanteus* PLA₂, which was purified to homogeneity for all experiments, the PLA₂s were used as supplied from Sigma, but were desalted by dialysis. All protein concentrations throughout this study were determined using a BCA assay kit (Pierce Chemical Company, Rockford, IL, USA).

Chickens, mosquitoes and parasites

The 8A strain of *Plasmodium gallinaceum* was used throughout this study. Parasites were maintained in white Leghorn chickens by serial blood passage. Mosquitoes were raised and fed using standard techniques (Gerberg et al., 1994; Higgs and Beaty, 1996). Zygotes were purified from the blood of infected chickens as described in detail elsewhere (Zieler et al., 1999). After purification of zygotes, the parasites were resuspended in M199 medium supplemented with 2 mmol l⁻¹ L-glutamine, 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (ookinete medium) at a density of 5×10⁶ zygotes ml⁻¹ and incubated for 16–24 h at 25 °C to allow development of ookinetes. Oocyst formation assays and transmission-blocking assays (Sieber et al., 1991; Higgs and Beaty, 1996) were performed as described previously (Zieler et al., 1999). Oocyst numbers in infected mosquitoes were compared amongst samples using Mann–Whitney rank sum analysis.

Purification to homogeneity of commercially available

Crotalus adamanteus PLA₂

The *C. adamanteus* PLA₂ used in all procedures was bought from Sigma-Aldrich and further purified to homogeneity by a single step of reversed-phase high-performance liquid chromatography (HPLC). The enzyme (1–2 mg, approximately 80 % pure) was dissolved in 20 % acetonitrile, 0.1 % trifluoroacetic acid (TFA) and injected into an Alltech Macrosphere C18 column (30 nm, 250 mm×4.6 mm) equilibrated with 20 % acetonitrile, 0.1 % TFA, run at a flow rate of 0.5 ml min⁻¹ with a 90 min gradient from 20 % to 60 % acetonitrile, 0.1 % TFA, with collection of 0.5 ml fractions. The PLA₂ was the major peak, eluting at approximately 60–65 min. The fractions containing PLA₂ activity were pooled and dried under vacuum in a SpeedVac. The protein was redissolved in 100 mmol l⁻¹ Hepes, pH 7.5, and dialyzed extensively against 1 mmol l⁻¹ Hepes, pH 7.5, in a

Slide-a-Lyzer dialysis cassette (3.5 kDa molecular mass cut-off).

Labeling of Crotalus adamanteus PLA2 with Alexa 488

Since PLA2s are generally sensitive to reaction with amine-reactive compounds (Wells, 1973; Hazlett and Dennis, 1985), the PLA2 was fluorescently labeled using a technique that modifies carboxylate groups in the protein (Staros et al., 1986; Grabarek and Gergely, 1990). These carboxylate groups are not thought to be involved in interfacial binding of the enzyme (Fleer et al., 1981; Waite, 1987; Gelb et al., 1999). The active-site aspartate was protected from modification by addition of calcium to the reaction (Fleer et al., 1981). Coupling of Alexa 488 (Molecular Probes, Eugene, OR, USA) to PLA2 was performed at room temperature (approximately 23 °C) in 0.1 mol l⁻¹ MES, 0.5 mol l⁻¹ NaCl, 1 mol l⁻¹ CaCl₂, pH 6.0. An enzyme solution of 1 mg ml⁻¹ was prepared in this buffer; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Sigma) was added to a final concentration of 2 mmol l⁻¹ and *N*-hydroxysulfosuccinimide (sulfo-NHS; Pierce) was added to a final concentration of 5 mmol l⁻¹. After 15 min at 23 °C, the reaction was stopped by the addition of 2-mercaptoethanol to a final concentration of 20 mmol l⁻¹. Alexa 488 hydrazide (Molecular Probes) was then added to a final concentration of 2 mmol l⁻¹, the pH of the mixture was raised to 7.0, and the reaction was allowed to proceed for 2 h at room temperature (Staros et al., 1986; Grabarek and Gergely, 1990). Finally, hydroxylamine (Sigma) was added to a final concentration of 50 mmol l⁻¹ and was allowed to react at room temperature for 3 h.

The modified enzyme was desalted by dialysis in a Slide-A-Lyzer dialysis cassette (3.5 kDa molecular mass cut-off; Pierce), followed by purification over a SepPak Plus C18 cartridge (Waters Corporation, Milford, MA, USA) and re-dialysis. Spectrophotometric and fluorescence determinations showed an approximately 2:1 ratio of enzyme molecule to dye molecule (one dye molecule per enzyme dimer). This was determined by measuring the approximate enzyme concentration by absorbance at 280 nm, establishing a standard fluorescence curve of serial dilutions of a known concentration of the Alexa 488 dye and correlating the observed fluorescence of the labeled enzyme sample with the standard curve.

Staining of midguts and ookinetes with fluorescent PLA2

Midgut epithelia or ookinetes were suspended in ookinete medium +10% heat-inactivated chicken serum (adhesion medium). Fluorescently labeled PLA2 was added to a final concentration of 2.9 µmol l⁻¹, and the samples were incubated for 10 min at room temperature. The samples were then washed twice with 1 ml of adhesion medium, transferred to a glass slide, spread out and fixed with 4% paraformaldehyde, 2.5% glutaraldehyde in phosphate-buffered saline (PBS). The fixed cells or midguts were transferred to a fresh glass slide, covered with a coverslip and examined by widefield fluorescence microscopy. Alternatively, midguts or ookinetes were examined unfixed by fluorescence microscopy, with similar

results being obtained in both cases. The fluorescence of stained, unfixed midguts was also measured in a Fluorolite 1000 fluorimeter (Dynatech Laboratories, Chantilly, VA, USA), which confirmed the observation of PLA2 binding to midguts.

Preparation of isolated midgut epithelia, staining of ookinetes with PKH26 and ookinete/midgut adhesion assay

These procedures were performed as described in previous publications (Shahabuddin et al., 1997; Zieler et al., 1999). Briefly, midguts were removed from female mosquitoes 24 h after a blood meal, cut in half with a scalpel blade, and the two half-midgut epithelia (midgut sheets) were separated from the blood meal. Each pair of midgut sheets was transferred into a separate Eppendorf tube containing adhesion medium. For pre-treatment with PLA2, the midguts were mixed with 20 µl of adhesion medium containing a known quantity of PLA2 and incubated at room temperature for 10 min, while control midguts were incubated with adhesion medium only. After the incubation, 80 µl of adhesion medium was added to each tube to dilute the PLA2, followed by 50 µl of ookinetes stained with PKH26 and suspended in adhesion medium (roughly 3 × 10⁵ to 10⁶ ookinetes added to each pair of midgut sheets). Staining of ookinetes with PKH26 (Sigma) was performed by diluting the PKH26 solution supplied by Sigma 1:40 in 5.4% glucose, using this solution to resuspend a pellet of ookinetes, incubating the parasites for 30 s and then washing them three times with adhesion medium by successive centrifugation and resuspension to remove excess PKH26. Adhesion of ookinetes to the midguts was achieved by centrifuging the tubes containing midgut sheets and parasites three times at 300g in a microfuge and resuspending the midgut sheets and parasites in each tube after each centrifugation by flicking the tube. The midgut sheets were then washed twice with 1 ml of adhesion medium, spread out flat on glass slides with the luminal side facing up, fixed by the addition of 2.5% glutaraldehyde, 4% paraformaldehyde in PBS, and examined by fluorescence microscopy to count the bound ookinetes. All adhesion experiments were performed with quadruplicate samples. Statistical analyses of differences between sample groups were performed using the *t*-test.

Results

Snake venom PLA2 inhibits oocyst formation of Plasmodium gallinaceum and Plasmodium falciparum

We fortuitously discovered an activity, present as a contamination in a commercial preparation of *Crotalus adamanteus* phosphodiesterase (Sigma), that inhibited oocyst formation of malaria parasites when fed to mosquitoes. The active principle in this preparation was purified to homogeneity by gel-filtration chromatography and reversed-phase chromatography and was sequenced using Edman degradation. The N-terminal sequence matched the published sequence of the *Crotalus adamanteus* PLA2. We formally proved that the active principle corresponded to the PLA2 by raising a rabbit

Table 1. *Effects of Crotalus adamanteus PLA2 on oocyst formation when feeding mosquitoes with infected blood or mature ookinetes*

Sample	Prevalence of infection ^a	Oocyst number (% of control) ^b	Oocyst number (range)	Intensity of infection ^c	<i>P</i> (compared with control) ^d
<i>Infected blood feed of Plasmodium gallinaceum</i>					
PLA2 ^e	7/21	0.3 (0.8)	0–2	1.1	<0.0001
PLA2+pBPB ^f	8/21	0.8 (2.1)	0–8	3.4	<0.0001
pBPB only ^f	21/21	16.9 (42.7)	4–176	16.9	0.002
Buffer only	25/25	39.6 (100)	2–148	39.6	–
<i>Ookinete feed of Plasmodium gallinaceum</i>					
PLA2 ^e	20/30	1.8 (16.4)	0–155	3.2	<0.0001
PLA2+pBPB ^f	16/23	2.1 (19.1)	0–121	3.5	<0.0001
pBPB only ^f	21/22	8.4 (76.4)	0–209	9.0	0.28
Buffer only	26/26	11.0 (100)	1–300	11.0	–
<i>Gametocyte feed of Plasmodium falciparum</i>					
<i>Anopheles gambiae</i> +PLA2 ^e	4/34	0.10 (0.5)	0–2	1.19	<0.0001
<i>Anopheles gambiae</i> +buffer	24/24	18.22 (100)	3–60	18.22	–
<i>Anopheles stephensi</i> +PLA2 ^e	2/35	0.05 (0.3)	0–2	1.41	<0.0001
<i>Anopheles stephensi</i> +buffer	31/31	3.54 (100)	1–13	3.54	–

^aNumber of infected mosquitoes/total number of mosquitoes dissected.

^bGeometric mean of the oocyst numbers in gravid female mosquitoes; in parentheses, geometric mean as a percentage of the buffer control.

^cGeometric mean of the oocyst numbers in infected mosquitoes only.

^d*P*-values were computed using Mann–Whitney rank-sum analysis.

^ePLA2 was mixed into the infected blood at a final concentration of 0.5 µmol l⁻¹ in infected blood feeds, 0.6 µmol l⁻¹ in gametocyte feeds and 2.5 µmol l⁻¹ in ookinete feeds.

^f*p*-Bromophenacyl bromide (pBPB) was preincubated with the PLA2 at a final concentration in the blood meal of 100 µmol l⁻¹.

polyclonal antiserum to a synthetic peptide corresponding to amino acid residues 24–53 of the PLA2; this antiserum was compared with the preimmune serum for its capacity to neutralize the ability of the PLA2 to reduce ookinete binding in ookinete/midgut adhesion assays (described in the Materials and methods section and below). The ookinete binding levels were 100±5 % to control midguts, 29±8 % to midguts pretreated with 1 µmol l⁻¹ PLA2, 24±6 % to midguts pretreated with the same amount of PLA2 premixed with preimmune serum and 89±9 % (means ± S.E.M., *N*=6) to midguts pretreated with the same amount of PLA2 premixed with PLA2-immune serum. Thus, the immune serum raised against a portion of the PLA2 sequence was able to neutralize the effects of the purified enzyme on ookinete interaction with mosquito tissues (see below for a detailed description of this effect), proving that the active

principle purified from the commercial phosphodiesterase preparation was indeed the *C. adamanteus* PLA2.

When fed to *Aedes aegypti* mosquitoes as part of an infected blood meal, the PLA2 strongly inhibits oocyst formation (Table 1). This effect does not require the hydrolytic activity of the enzyme; when PLA2 was preincubated with the irreversible inhibitor *p*-bromophenacyl bromide (pBPB), oocyst formation was still strongly reduced. The inhibitor alone had some effect on oocyst numbers (Table 1). However, the enzyme in the presence of the inhibitor was completely inactive in our assays. The effect of the PLA2 is concentration-dependent (Fig. 1) both in the presence and in the absence of pBPB. We determined that the lowest concentration of phospholipase that resulted in a statistically significant reduction in oocyst frequency when feeding infected blood is

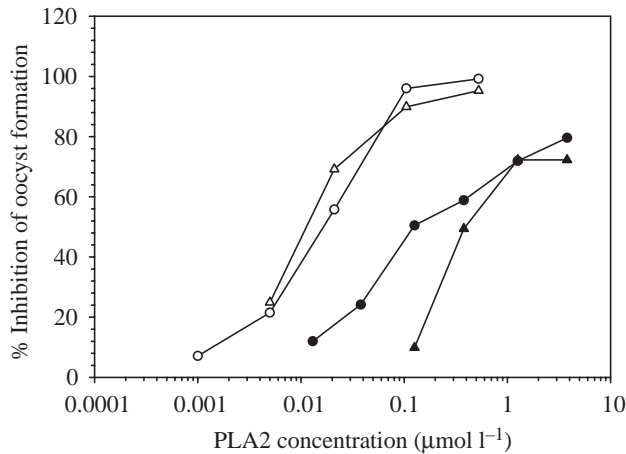


Fig. 1. Oocyst numbers from membrane feeds of purified *Crotalus adamanteus* PLA2. Open circles, the PLA2 was mixed with washed, infected chicken blood (16% parasitemia) and fed to mosquitoes. Open triangles, the PLA2 was pretreated with *p*-bromophenacyl bromide (pBPB) at a final concentration of $100 \mu\text{mol l}^{-1}$ and then mixed with washed, infected chicken blood (16% parasitemia) and fed to mosquitoes. Filled circles, the PLA2 was added to an ookinete suspension in naïve chicken blood and fed to mosquitoes. Filled triangles, the PLA2 was pretreated with pBPB, then added to an ookinete suspension in naïve chicken blood and fed to mosquitoes. Oocyst numbers were scored 8 days after the membrane feed. Each data point represents the geometric mean of oocyst numbers from at least 20 mosquitoes expressed as percentage inhibition relative to control mosquitoes fed either with infected blood plus buffer or with infected blood plus inhibitor alone.

21 nmol l^{-1} (Fig. 1). The PLA2 was also fed to *Anopheles gambiae* and *Anopheles stephensi* together with cultured gametocytes of the human malaria parasite *Plasmodium falciparum*. Similar levels of inhibition were seen in both anopheline species (Table 1), and the concentration-dependence of PLA2 inhibition was similar to that observed in *P. gallinaceum*.

To determine whether the inhibition of oocyst formation occurs before or after the maturation of ookinetes, we fed PLA2 to mosquitoes together with purified, mature ookinetes suspended in naïve chicken blood. The PLA2 significantly inhibited oocyst formation, although the effect was less dramatic than when feeding infected blood (Table 1). The inhibition was also concentration-dependent but, in comparison with feeds with infected blood, higher PLA2 concentrations were necessary to achieve a given level of inhibition (Fig. 1). The lowest PLA2 concentration needed to achieve significantly different oocyst numbers compared with the control was $0.38 \mu\text{mol l}^{-1}$. Once again, hydrolytic activity of the enzyme was not required for inhibition.

PLA2 has no effect on ookinete development or viability

To determine whether any pre-ookinete developmental stage is sensitive to the PLA2, we added the enzyme during the course of exflagellation and purification of macrogametes and zygotes during routine preparation of parasites from

Table 2. *Crotalus adamanteus* PLA2 has no effect on parasite development when added during exflagellation and zygote formation

Parasite stage	Parasite numbers ^a			
	Experiment 1 ^b ($0.28 \mu\text{mol l}^{-1}$)		Experiment 2 ^b ($0.33 \mu\text{mol l}^{-1}$)	
	+PLA2	-PLA2	+PLA2	-PLA2
Microgametes ^c	9.8×10^7	9.4×10^7	2.8×10^8	3.1×10^8
Zygotes/macrogametes ^d	4.0×10^7	4.3×10^7	10^8	9.5×10^7
Ookinetes ^e	6.2×10^6	4.7×10^6	2.5×10^7	2.2×10^7

C. adamanteus PLA2 was added to exflagellating, washed chicken blood at the beginning of a parasite purification procedure. The numbers of microgametes were determined 15 min later by counting exflagellation centers; the total numbers of macrogametes or zygotes were counted at the end of the parasite purification, and the ookinete numbers were determined 1 day later, after ookinete development had been completed.

^aNumbers of parasites obtained from parasite preparations treated with PLA2 or left untreated; PLA2 treatment occurred during exflagellation gradient purification; parasite densities in all samples were counted in quadruplicate and averaged.

^bDifferent PLA2 concentrations were used in the two experiments (as indicated).

^cTotal number of microgametes (exflagellation centers) during exflagellation.

^dTotal number of macrogametes and zygotes obtained at the end of the parasite purification.

^eTotal number of ookinetes obtained after overnight incubation of zygotes and macrogametes.

infected chicken blood. There was no difference in the number of exflagellation events in the presence or absence of PLA2, nor did the PLA2 have a significant effect on the total yield of macrogametes/zygotes obtained at the end of the purification (Table 2). The ookinete yields from the treated parasites were also unaffected. When the PLA2 was added to purified zygotes, which were then incubated in ookinete medium (for 24 h) to allow transformation into ookinetes, there was no effect on ookinete development in terms of either frequency (Table 3) or timing (data not shown). Some of the samples listed in Table 3 had PLA2 present continuously from exflagellation through ookinete formation and showed no significant differences in ookinete numbers compared with the controls. Furthermore, the ookinetes in all the samples displayed normal motility on glass slides. We conclude from these data that the PLA2 does not affect parasite development in the blood meal.

The effect of PLA2 on the viability of ookinetes was also determined. PLA2 was added to ookinete suspensions at various concentrations. At two times after PLA2 addition, a sample of ookinetes was removed and stained with ethidium homodimer, a dye that specifically stains necrotic or dead cells (Kaneshiro et al., 1993; Papadopoulos et al., 1994; Shahabuddin et al., 1998). There was no effect of the PLA2 treatment on the fraction of ookinetes staining with ethidium

Table 3. *Crotalus adamanteus* PLA2 has no effect on the transformation of zygotes to ookinetes

PLA2 concentration in zygote treatment ($\mu\text{mol l}^{-1}$)	Ookinete densities (parasites ml^{-1}) ^a			
	Experiment 1		Experiment 2	
	+PLA2 in exflagellation ^b	–PLA2 in exflagellation ^b	+PLA2 in exflagellation ^b	–PLA2 in exflagellation ^b
0	–	1.3×10^6	2.8×10^6	2.4×10^6
0.08	–	10^6	2.5×10^6	2.3×10^6
0.16	–	10^6	2.6×10^6	2.3×10^6
0.32	–	9.9×10^5	2.2×10^6	2.3×10^6
0.63	–	–	2.4×10^6	2.8×10^6
1.6	–	–	2.4×10^6	2.3×10^6

Zygotes purified from chicken blood were incubated with various concentrations of ookinetes during ookinete development. The columns referring to + or –PLA2 in exflagellation refer to whether or not the zygotes used for ookinete development were derived from parasite purifications that had been treated with PLA2 during the exflagellation (i.e. from treated samples in Table 2); PLA2 treatment of the zygotes occurred continuously for 24 h during maturation of ookinetes from zygotes.

^aOokinete densities in duplicate samples that were either treated with the PLA2 concentration indicated in the left column or treated with buffer only ($0 \mu\text{mol l}^{-1}$); parasite densities in all samples were counted in quadruplicate and averaged.

^bZygotes were taken from parasite preparations that had either received PLA2 treatment ($0.74 \mu\text{mol l}^{-1}$) during exflagellation and gradient purification as described in Table 2 or not, as indicated.

homodimer (Table 4), showing that the enzyme does not affect ookinete viability.

As a final test of whether the PLA2 has an effect on the parasite, ookinetes were treated with $1.2 \mu\text{mol l}^{-1}$ PLA2 for 4 h. The parasites were then washed to remove the enzyme, resuspended in naive chicken blood and fed to mosquitoes. Untreated ookinetes from the same batch were handled in the same manner and used as controls. Midguts were dissected and oocysts counted 8 days after feeding. There was no significant difference in oocyst numbers between treated and untreated ookinetes (Table 5), suggesting that PLA2 treatment does not affect the ability of the ookinetes to continue their development and form oocysts.

Inhibition of ookinete adhesion to mosquito midguts by PLA2

The data presented above argue that the PLA2 acts on the midgut surface, rather than on the ookinete, in blocking a critical step of the migration of the parasite. To test whether the PLA2 affects the ability of ookinetes to adhere to midguts,

we pretreated midgut epithelia with PLA2 *in vitro*, and then used these epithelia in adhesion assays. Ookinetes adhering to midguts were counted, and the numbers were normalized and averaged. As shown in Fig. 2, pretreatment of midguts with PLA2 significantly lowered the number of midgut-bound ookinetes. The effect was the same when pBPB-inactivated enzyme is used, but pBPB by itself had no effect on adhesion. A second inhibitor of the active site of PLA2s, ZnCl_2 , was used to duplicate the results with pBPB (Fig. 2). In the presence of $100 \mu\text{mol l}^{-1}$ pBPB or $100 \mu\text{mol l}^{-1}$ ZnCl_2 , no enzymatic activity (hydrolytic activity) could be detected. We conclude that the PLA2 inhibits the association of ookinetes with the midgut surface and that this effect is dependent on a structural feature of the phospholipase rather than on its catalytic activity. We also tested the inhibition of adhesion at various concentrations of PLA2 and found the lowest concentration at which significant inhibition was observed to be $0.5 \mu\text{mol l}^{-1}$.

In the adhesion protocol described above, the PLA2 was diluted in the assay rather than being washed away after pretreatment of the midguts. To determine whether the inhibition would still occur after removing all excess PLA2, the midguts were thoroughly washed after pretreatment and then used in adhesion assays. Control midguts showed a level of ookinete binding of $100.0 \pm 2.3\%$, ookinete binding of midguts pretreated with PLA2 alone was reduced to $70.3 \pm 5.0\%$ and ookinete binding of midguts pretreated with pBPB-inhibited PLA2 was reduced to $67.8 \pm 8.0\%$ (normalized means \pm S.E.M., $N=6$). The differences between the PLA2-treated midguts and the control midguts are statistically significant in both cases. In the converse experiment, ookinetes were treated with $3.2 \mu\text{mol l}^{-1}$ of PLA2 for 1 h, after which they were washed and compared with mock-treated ookinetes (treated only with buffer) in adhesion assays. The treated ookinetes adhered to midguts at the same levels ($99.9 \pm 4.7\%$)

Table 4. *Crotalus adamanteus* PLA2 has no effect on ookinete viability

PLA2 concentration ($\mu\text{mol l}^{-1}$)	% of ookinetes stained with ethidium ^a	
	30 min treatment	4 h treatment
0	1.0	1.9
0.03	1.3	1.2
0.32	0.7	0.9
3.2	0.4	1.3

^aStaining with ethidium homodimer reflects the percentage of dead or necrotic cells; at least 500 cells were examined in each sample to determine the percentages; the differences in percentages of staining cells are not significant.

Table 5. *Ookinetes pretreated with PLA2 form oocysts at normal frequencies*

Sample	Prevalence of infection ^a	Oocyst number (% of control)	Oocyst number ^b	<i>P</i> (compared with control) ^c
Ookinetes pretreated with PLA2 ^d	24/24	21.8 (80.7)	0–400	0.66
Ookinetes pretreated with buffer	24/24	27.0 (100)	0–300	–

^aNumber of infected mosquitoes/total number of mosquitoes dissected.

^bGeometric mean of the oocyst numbers in gravid female mosquitoes; in parentheses, geometric mean as a percentage of the buffer control.

^c*P*-values were computed using Mann–Whitney rank-sum analysis.

^dOokinetes were pretreated in $1.2\mu\text{mol l}^{-1}$ PLA2 for 4 h, then washed, resuspended in naive chicken blood and fed to mosquitoes.

as the mock-treated ookinets ($100.0\pm 3.0\%$) (normalized means \pm S.E.M., $N=4$). On the basis of these results, we conclude that the enzyme changes the properties of the midgut epithelium, but does not affect the adhesiveness of ookinets.

The adhesion assays performed in the presence and absence of PLA2 allowed us to observe shedding of lipids from the posterior ends of ookinets, which can be detected as the capping of fluorescent stain on the posterior end of the parasite, and which correlates with ookinete motility. In certain ookinete preparations, all parasites bound to the midgut surface

show capping after the adhesion is complete and the fixed midguts are being examined for the number of bound ookinets. In 10 pairs of midgut sheets where capping was observed, there were never any visible differences in the posterior capping of fluorescent stains in the presence or absence of PLA2. It is likely, therefore, that the PLA2 does not interfere with ookinete motility.

Adhesion assays using PLA2s from other organisms were used to determine whether the ability of the *C. adamanteus* enzyme to inhibit ookinete/midgut binding is a general feature of PLA2s. As shown in Fig. 3, PLA2s from the venoms of various snakes, as well as honeybee venom PLA2 and mammalian pancreatic PLA2s, were used to pretreat midguts in adhesion assays. The results (Fig. 3) indicate that the PLA2s derived from snake and insect venoms strongly inhibit ookinete binding, while the mammalian pancreatic enzymes do not. The extent of inhibition of oocyst formation by the different PLA2s in oocyst formation assays mirrors their inhibition of *in vitro* adhesion (data not shown).

Fluorescently labeled PLA2 binds to mosquito midguts but not to ookinets

The results from the adhesion assays suggested that the PLA2 affects the mosquito midgut, but not ookinets. To test whether the enzyme interacts with midguts, we prepared fluorescently labeled *C. adamanteus* PLA2 and measured its ability to bind to midguts and parasites. The labeled enzyme retained some of its hydrolytic activity and was identical to unlabeled enzyme in its ability to block oocyst formation when mixed with infected blood and fed to mosquitoes (data not shown).

The labeled PLA2 was used to stain isolated midguts and ookinets. Staining was performed in the presence of 10 % chicken serum, as a non-specific competitor, and the stained tissues or cells were washed extensively after staining to remove non-specifically bound PLA2. The protein readily bound to midgut epithelia and showed a diffuse staining on both the luminal surface and the basement membrane on the other side of the epithelium (Fig. 4). The staining of the luminal surface was often irregular, with certain areas or individual cells staining more brightly for unknown reasons. Higher magnifications clearly showed surface staining of the

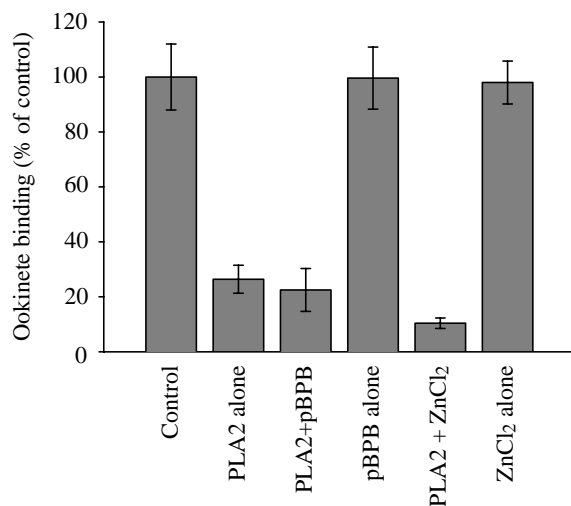


Fig. 2. Midguts were pretreated with $3.2\mu\text{mol l}^{-1}$ PLA2 in M199 medium for 10 min, and the treated guts were then used in adhesion assays. Control, midguts treated with buffer only; PLA2 alone, midguts treated with active PLA2; PLA2+pBPB, midguts treated with PLA2 in the presence of $100\mu\text{mol l}^{-1}$ *p*-bromophenacyl bromide (pBPB), an inhibitor of the enzyme; pBPB alone, midguts pretreated with the inhibitor pBPB only; PLA2+ZnCl₂, midguts pretreated with PLA2 in the presence of $100\mu\text{mol l}^{-1}$ ZnCl₂, an inhibitor of the enzyme; ZnCl₂ alone, midguts treated with $100\mu\text{mol l}^{-1}$ ZnCl₂. Each column is the mean of 1–2 experiments, each performed in quadruplicate. The error bars represent \pm S.E.M. ($N=4$ –8). *P* values for each column are as follows: PLA2 alone, $P=0.0002$; PLA2+pBPB, $P<0.0001$; pBPB alone, $P=0.98$; PLA2+ZnCl₂, $P=0.004$; ZnCl₂ alone, $P=0.91$.

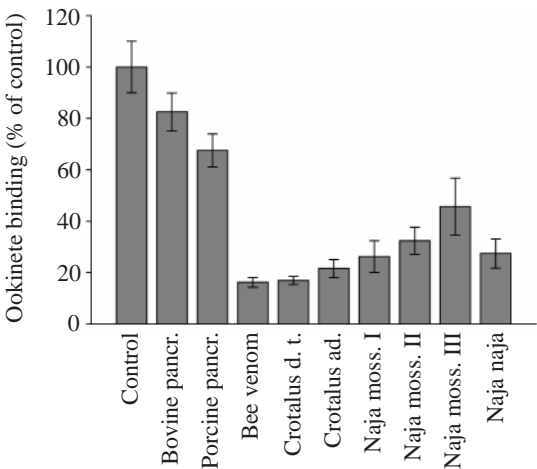


Fig. 3. Midguts were pretreated with different PLA2s (final concentration $10\mu\text{mol l}^{-1}$) in M199 medium for 10 min and then used in adhesion assays. Control, midguts treated with buffer only. The PLA2s used in treatments were as follows: Bovine pancr., bovine pancreatic PLA2; Porcine pancr., porcine pancreatic PLA2; Bee venom, honeybee venom PLA2; *Crotalus d. t.*, *Crotalus durissus terrificus* venom PLA2 (crotoxin); *Crotalus ad.*, *Crotalus adamanteus* venom PLA2 ($7\mu\text{mol l}^{-1}$); *Naja moss. I*, *Naja mossambica mossambica* venom PLA2 isozyme I (pI=6.5); *Naja moss. II*, *Naja mossambica mossambica* venom PLA2 isozyme II (pI=8.8); *Naja moss. III*, *Naja mossambica mossambica* venom PLA2 isozyme III (pI=9.6); *Naja naja*, *Naja naja* venom PLA2. The PLA2s were dialyzed extensively before use in the assays. Each column represents the results of a single experiment, performed in quadruplicate. Error bars represent \pm S.E.M. ($N=4$). *P* values for each column are as follows: Bovine pancr., $P=0.23$; Porcine pancr., $P=0.05$; Bee venom, $P=0.001$; *Crotalus d. t.*, $P=0.001$; *Crotalus ad.*, $P=0.002$; *Naja moss. I*, $P=0.003$; *Naja moss. II*, $P=0.004$; *Naja moss. III*, $P=0.02$; *Naja naja*, $P=0.003$.

midgut cells (Fig. 4D), although the lack of resolution made it difficult to determine whether the PLA2 was associating with the microvilli-associated network observed on the midgut surface in earlier studies (Zieler et al., 1998, 2000). Although the unstained midguts are not shown for comparison, the degree of autofluorescence was low compared with the stained midgut and resulted in a very dim image at the lamp intensity and gain settings used for the digital camera. We also

demonstrated that the binding of labeled PLA2 to the midguts could be inhibited by an excess of unlabeled enzyme. In contrast to the midgut staining by the labeled PLA2, however, labeled PLA2 completely failed to bind to ookinetes. In addition, the PLA2 failed to label isolated peritrophic membranes, suggesting that its ligand is found only on the midgut surface (data not shown).

C. adamanteus and honeybee venom PLA2s are suitable candidates for refractory genes

The *C. adamanteus* and honeybee venom PLA2s were fed to mosquitoes to test the premise that the genes encoding these proteins would be suitable as refractory genes. The percentage of mosquitoes that fed on chicken blood containing the PLA2s or on control blood was determined and was not significantly different on any of the feeds (Table 6). No difference in viability or in egg production was observed between mosquitoes fed the PLA2 compared with the control group (Table 6). We conclude that the PLA2s have no strong effect on the abilities of mosquitoes to engorge or reproduce. These results suggest that it may be feasible to express PLA2 genes in transgenic mosquitoes in a midgut-specific fashion.

Discussion

We have shown that the *Crotalus adamanteus* PLA2 greatly reduces the efficiency of oocyst formation of malaria parasites. This enzyme blocks the development of *Plasmodium falciparum* as efficiently as of the model organism *P. gallinaceum*, enhancing the significance of our finding and implying that a conserved part of the plasmodial life cycle is affected. Furthermore, the reduction in oocyst numbers correlates with an inhibition of ookinete adhesion to the midgut surface *in vitro*. The PLA2 has no effect on any of the mosquito stages of the malaria parasite *in vitro*, nor does it alter the adhesion ability of ookinetes or their ability to form oocysts after pretreatment with the enzyme. The gliding motility of treated ookinetes on glass slides was also normal, and ookinetes shed their surface lipids normally in the presence of the enzyme. These results, together with the observation that fluorescently labeled PLA2 binds to mosquito midguts but not to ookinetes, suggest that the phospholipase binds to or

Table 6. *Crotalus adamanteus* PLA2 has no effect on mosquito viability or fecundity

PLA2 fed to mosquitoes	% Engorgement (number fed/total) ^a	Viability 4 days post blood feed (%) ^b	Egg count per gravid female ^c
+1.2 $\mu\text{mol l}^{-1}$ <i>Crotalus</i> PLA2 ^d	52.3 (45/86)	88.9	55.0 \pm 7.8
+0.2 $\mu\text{mol l}^{-1}$ <i>Crotalus</i> PLA2	51.6 (32/62)	90.6	57.3 \pm 6.0
+1.5 $\mu\text{mol l}^{-1}$ bee venom PLA2	69.1 (47/68)	89.4	55.8 \pm 11.3
Control (+buffer only)	59.4 (37/69)	90.2	56.7 \pm 12.6

^aPercentage of mosquitoes with visible blood meals in their abdomens immediately after membrane feeding on blood with or without PLA2.
^bPercentage of the blood-fed mosquitoes still alive 4 days after membrane feeding.
^cMean number of eggs in six randomly selected gravid females \pm S.D.
^dFinal concentration of the PLA2s in the blood used for membrane feeding.

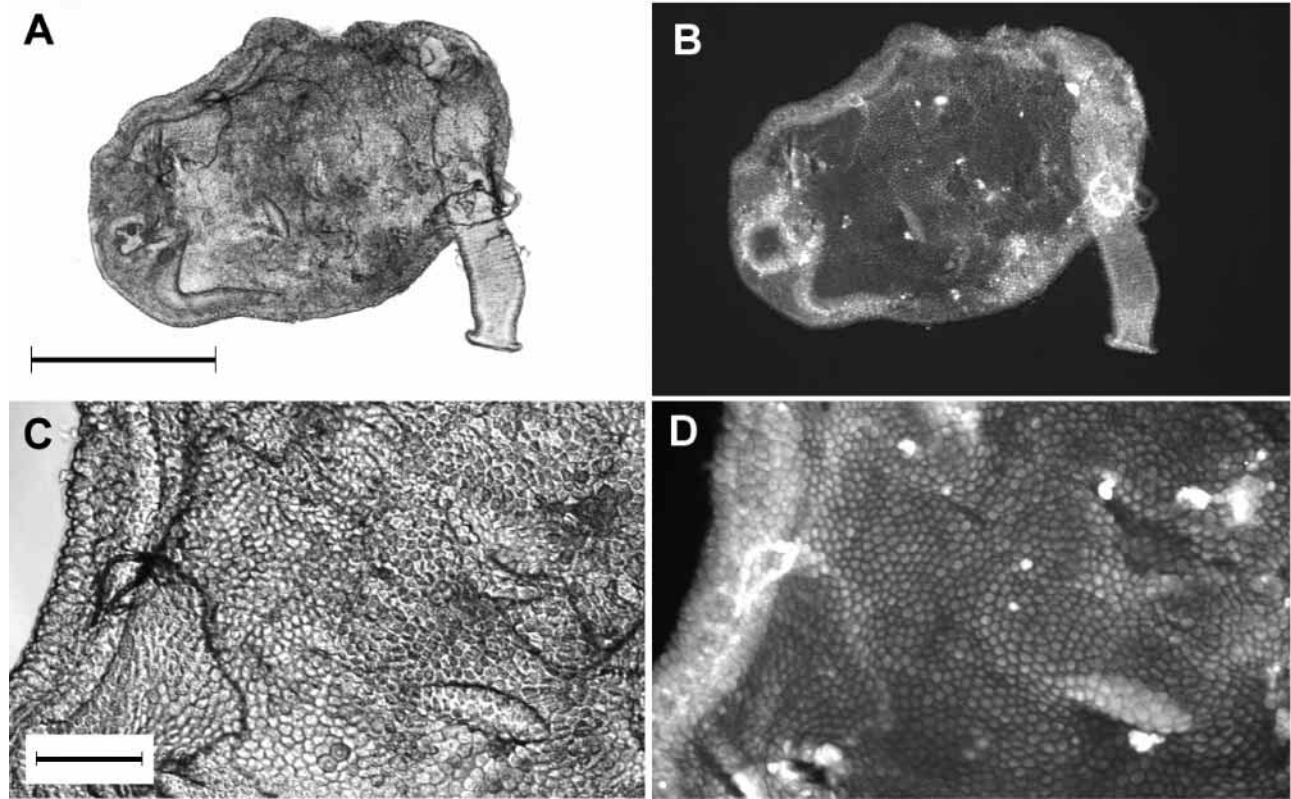


Fig. 4. Bright-field (A,C) and fluorescence (B,D) images of an *Aedes aegypti* blood-fed midgut sheet stained with fluorescently labeled *Crotalus adamanteus* PLA2 and fixed. The images in C and D are a higher magnification of the upper portion of the midgut shown in A and B. Scale bars: A, 500 µm; C, 100 µm.

obscures an essential ligand on the midgut surface that is needed for the ookinete successfully to traverse the midgut and form oocysts. The development of *P. gallinaceum* occurs in mosquitoes of the genus *Aedes* and that of *P. falciparum* in mosquitoes of the genus *Anopheles*. Our results suggest that a similar midgut ligand is used by both parasite species to recognize the midgut of their mosquito hosts and that this molecule is essential for the parasite to identify its target.

PLA2s from the venoms of other snakes and from honeybees appear to have the same property as the PLA2 from *Crotalus adamanteus* (see Fig. 3). In contrast, the mammalian pancreatic PLA2s do not efficiently block adhesion. The data in Fig. 3 suggest a correlation between the membrane-inserting abilities of a PLA2 and its ability to block ookinete adhesion and oocyst formation. The mammalian pancreatic PLA2s tend to have a very low membrane-inserting ability, while the honeybee venom PLA2 has the highest inserting ability measured in any phospholipase (Demel et al., 1975; Waite, 1987). This result is consistent with our finding that the hydrolytic activity of the PLA2 is not an essential part of its antiparasitic activity and suggests that the inhibition of ookinete adhesion by the PLA2 is primarily dependent on the enzyme's property of binding to exposed membrane lipids.

We have previously reported a novel membranous structure on the midgut surface that we have termed the microvilli-associated network (MN) and which seems to be the primary

structure with which ookinetes associate on the midgut surface (Zieler et al., 1998, 2000). It is likely that the PLA2 binds to the MN. Unfortunately, this structure is difficult to see using light microscopy, and in our fluorescence labeling experiments we were unable to determine whether the labeled PLA2 does indeed bind to MN strands.

The inhibition by the PLA2 of ookinete association with the midgut surface could be a result of its direct binding to the same periodate-sensitive carbohydrate ligand (Zieler et al., 1999) recognized by the ookinete. In this case, the ookinete ligand would probably be a glycolipid or a highly periodate-sensitive phospholipid such as phosphatidylglycerol. We were unable to test whether periodate treatment of midguts affects PLA2 binding, since extensive cell damage occurs during periodate treatment and the PLA2 binds more efficiently to the perturbed membranes in damaged cells (Dawson et al., 1984; Thuren et al., 1987).

In contrast, it is unlikely that inhibition of ookinete adhesion by PLA2s is related to their ability to bind to heparin and other anionic glycosaminoglycans. First, this property of PLA2s is best-documented in the pancreatic enzymes (Diccianni et al., 1990, 1991), which are not very active in our assays. Second, we have tried heparin, as well as a variety of other anionic glycosaminoglycans, as competitors in adhesion assays and have been unable to detect any inhibition at concentrations as high as 500 µg ml⁻¹. Third, the *Crotalus adamanteus* PLA2

inhibits ookinete adhesion even in the presence of heparin (data not shown).

In the adhesion assay, we also tested human annexin V, a protein known to bind to acidic phospholipids (such as phosphatidylserine) normally present in the inner leaflet of the plasma membrane bilayer (Swairjo et al., 1995; Reutlingsperger and van Heerde, 1996), and found no inhibition of adhesion. Annexins are Ca^{2+} -dependent phospholipid-binding proteins that compete with PLA2s for binding to membranes (Davidson et al., 1987; Buckland and Wilton, 1998a,b). The phospholipid preference of PLA2s, or the manner in which they disrupt lipid bilayers during binding, is probably an important characteristic that enables these enzymes specifically to disrupt ookinete/midgut interactions.

The PLA2s have a number of unique properties that make them especially suitable for expression in mosquito midguts as refractory genes. First, they are exceptionally stable and resistant to protease cleavage, an important property in the degradative environment of the midgut lumen. Second, they are generally secreted as proenzymes, which are activated by trypsin cleavage (Pieterse et al., 1974; Waite, 1987). Proenzyme activation will occur after secretion into the midgut lumen, as happens with midgut chitinases (Shen and Jacobs-Lorena, 1997). The feasibility of expressing PLA2 enzymes as proenzymes in a variety of heterologous expression systems has already been demonstrated (Verheij and de Haas, 1991). Third, the hydrolytic activity of PLA2s is not required for inhibition of parasite development, implying that the midgut-secreted enzyme can be catalytically inactivated by mutation if necessary. We have shown with membrane feeds that ingestion of the active PLA2 has no measurable effect on the female mosquitoes (Table 6), arguing that a midgut-expressed PLA2 would be well-tolerated by transgenic mosquitoes. PLA2-expressing transgenic *Aedes aegypti* are currently being generated using the recently developed transformation methods for this organism (Coates et al., 1998; Jasinskiene et al., 1998). The PLA2 gene will thus be one of the first genes to be tested in transgenic mosquitoes for its ability to confer refractoriness to malaria parasites.

Our long-term studies are aimed at a complete characterization of the interaction between malaria parasites and mosquito tissues. In the process of this work, we will further our understanding of the biology of this parasite, and we hope that this knowledge will be a starting point for developing new methods of malaria control.

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