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Effects of Magainins and Cecropins on the Sporogonic Development of Malaria Parasites in Mosquitoes

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Magainins and cecropins are families of peptides with broad antimicrobial and antiparasitic activities derived respectively from the skin of frogs or from giant silk moths. In insects, cecropins function as part of an inducible immune system against a number of bacterial infections. When injected into anopheline mosquitoes previously infected with a variety of *Plasmodium* species, both magainins and cecropins disrupt sporogonic development by aborting the normal development of oocysts; sporozoites are not formed and the vector cannot transmit the parasite to another host. It may be possible to induce effective transmission-blocking immunity in the mosquito vector by the introduction and expression of genes coding for magainins, cecropins, or similarly acting parasiticidal peptides into the mosquito genome.

The capacity of a mosquito to support the sporogonic development of a malaria parasite depends on a series of highly complex physiological interactions facilitating a continuum of events from gametogenesis to sporozoite invasion of the salivary glands. Most mosquito species are refractory to malarial infection; they fail to promote development at any of a number of points in the life cycle of the parasite. In susceptible species, individuals or whole populations may show various forms of refractoriness. At the same time, mosquito species can be susceptible to one or more species of malaria parasites while being totally refractory to others. When studied, susceptibility or refractoriness has been found to be an inherited trait and amenable to selection.

The concept of malaria control through alteration of the capacity of mosquito populations to transmit the parasite provides an attractive alternative to traditional insecticide-dependent programs. In these schemes involving population replacement, naturally occurring refractory mechanisms would be identified and genetically selected; refractory mosquitoes would then be mass produced and released into the field at a time when they could establish themselves at the expense of the native population of the same species. The introduced population would continue to feed on the human host, but malaria would not be transmitted.

Concurrent with our search for mosquito-derived refractory mechanisms (4) and techniques for manipulating genes (10), we have been examining compounds from other organisms which might have parasiticidal effects in mosquitoes. In this report, we describe the effects of magainins and cecropins on the sporogonic development of various malaria species. Magainins (16) are a class of potent antimicrobial compounds from vertebrates, while cecropins and their relatives (2) may be ubiquitous in insects. The magainins are active against several bacterial species and protozoans such as *Paramecium caudatum*, *Amoeba proteus*, and *Euglena gracilis*. These peptides caused perturbation of membrane functions responsible for osmotic balance. After being exposed, protozoa began to swell until they eventually burst. These compounds are nonhemolytic and have little effect on mammalian cells. In vitro studies with the erythrocytic and sexual stages of *Plasmodium falciparum*, *P. gallinaceum*, and *P. knowlesi* showed that the magainin peptide could disrupt extracellular stages of the parasites but that it had no effect on intracellular development (D. Kaslow, unpublished data). The cecropins are also capable of causing bacterial cell lysis in vivo and in vitro. For protozoa, Jaynes et al. (7) have reported in vitro results with a synthetic derivative of cecropin on the growth of *P. falciparum* and *Trypanosoma cruzi*.

MATERIALS AND METHODS

Magainins. Magainins were originally isolated from the skin of the African clawed frog *Xenopus laevis* (16). Two closely related forms, each consisting of 23 amino acids, have been described. The carboxy amide of magainin 2 used in this study was synthesized by Jean Rivier of the Salk Institute using techniques previously described (1, 9, 17). An analog (Z-12) of magainin 2 in which all Lys and Phe residues have been replaced with D enantiomers (12) was also tested.

Cecropins. Cecropins are a group of insect-derived inducible antibiotic peptides from the giant silk moth *Hyalophora cecropia* (13, 15), best described by Boman and co-workers (2, 13). The cecropin peptide is 35 amino acids long. Cecropin B was synthesized by solid-phase methodology (8) with an automated peptide synthesizer. Standard *tert*-butyloxycarbonyl (t-Boc) chemistries were used, and the peptides were cleaved from the solid support by liquid HF. The cleaved peptides were purified by gel filtration on a P-2 column (Bio-Rad Laboratories, Richmond, Calif.), and the purified peptide was analyzed by amino acid composition and reversed-phase high-pressure liquid chromatography. The carboxy amide of cecropin B was tested along with a

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TABLE 1. Toxic effects of various concentrations of magainin 2, cecropin B, and two analogs on *A. gambiae* females injected 5 days after a noninfectious blood meal[a]

Concn (μg/μl)	Mortality (% dead at 24 h) with:				
	Magainin 2	Z-12	Phosphate-buffered saline	Cecropin B	Truncated (1–21)
20	100	100	0	ND[b]	ND
5	100	40	0	100	100
1	14[c]	0	0	63	10
0.5	5	0	0	40	0
0.05	0	0	0	0	0

[a] Each treatment represents a minimum of 20 injections.
[b] ND, Not determined.
[c] Majority of survivors were moribund at 48 h postinjection.

truncated peptide consisting of amino acids 1 to 21 from the N terminus.

Mosquito species. Most of these studies used the G-3 strain of *Anopheles gambiae*. However, the effects of the various treatments were also examined in *A. freeborni* and *A. dirus*. Mosquitoes were reared and maintained as described previously (14).

Parasite strains. The Ceylon strain of *Plasmodium cynomolgi* and the H strain of *P. knowlesi* were used in most of these studies. These parasites were maintained in previously splenectomized rhesus monkeys (*Macaca mulatta*); mosquitoes were fed directly on anesthetized monkeys between 2100 and 2400 h when the rising parasitemia was between 1 and 10% (ring-stage parasites counted on a thin blood film taken at 1400 h). Mosquitoes were fed at night to maximize the infectivity of these synchronous parasites (5).

In some studies, *A. freeborni* and *A. gambiae* were infected with *P. falciparum* (3D7 clone of the NF54 strain or the NF54 parent strain). These parasites were reared in vitro (6) and fed to mosquitoes through a membrane (11).

Injection procedures. Magainins and cecropins in phosphate-buffered saline were injected into the thoraces of mosquitoes with a glass needle through a membranous area between pleural sclerites either before or at various times after an infectious blood meal. Each mosquito received approximately 0.5 μl of solution.

Mosquitoes were dissected at intervals after injection; their midguts were removed and stained with 2% mercurochrome, and effects on oocyst growth were examined with a compound microscope. Oocysts affected by the various peptides showed a number of characteristic pathologic indicators. Growth ceased, the central body of the parasite became dense and irregular, and mercurochrome was taken up to the point that damaged parasites appeared dark red. In some cases, mosquitoes were held for 14 days, their salivary glands were removed, and the presence or absence of sporozoites was determined by phase-contrast optics. Sporozoite numbers were estimated and reflected the number of viable oocysts from which they originated.

RESULTS

Peptide concentrations. To determine levels at which magainins or cecropins might be toxic to the mosquito host, we injected various concentrations of these peptides and their analogs into noninfected *A. gambiae* mosquitoes. The 50% lethal dose for magainin 2 was between 0.5 and 1 μg/μl and that for cecropin B was approximately 0.5 μg/μl (Table 1).

Parasite sensitivity. At an insectary temperature of 27°C, the time from the infecting blood meal with *P. cynomolgi* to the first appearance of sporozoites in the salivary glands of *A. gambiae* was approximately 12 days. Developing oocysts were first visible by light microscopy at 4 to 5 days after a blood meal. Developing oocysts were not uniformly sensitive to treatment. Oocyst development was not affected in most mosquitoes injected before or up to 2.5 days after an infectious blood meal (Fig. 1). In the group of mosquitoes injected 36 h postinfection, the number of normally developing oocysts was significantly reduced; aborted oocysts were not visible, although one would not expect to see affected oocysts in which growth was disrupted at 36 h. Oocysts were fully susceptible only when treated 5 or more

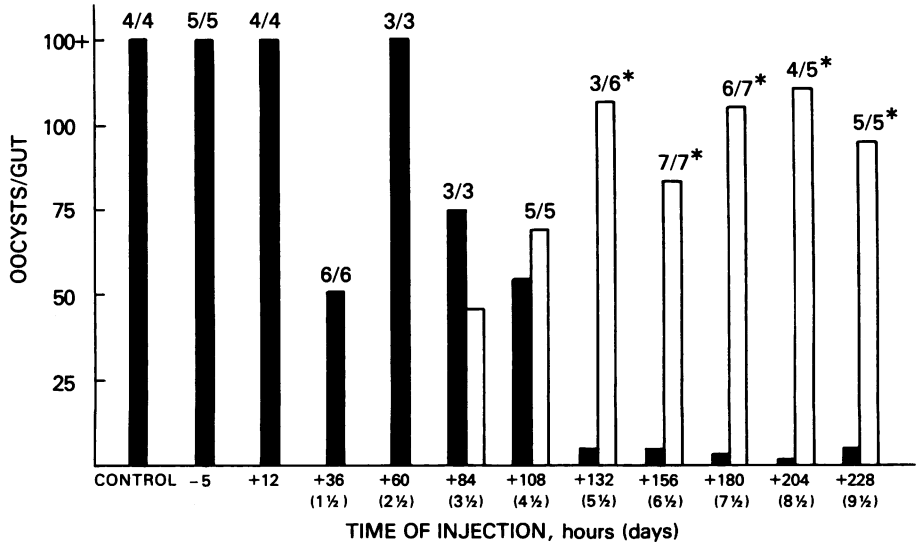

FIG. 1. Effects in *A. gambiae* of a single injection (0.4 μg/μl) of magainin 2 at various times before or after an infecting blood meal with *P. cynomolgi*. Normal oocysts are represented by the black bars and affected oocysts are shown by the clear bars, with a minimum of 20 females per treatment. Oocysts were examined 48 h after injection or at 5 days postinfection for the earlier treatments. The numbers above the bars represent mosquitoes held 14 days after infection with sporozoites in their salivary glands; *, very low sporozoite levels in the glands.

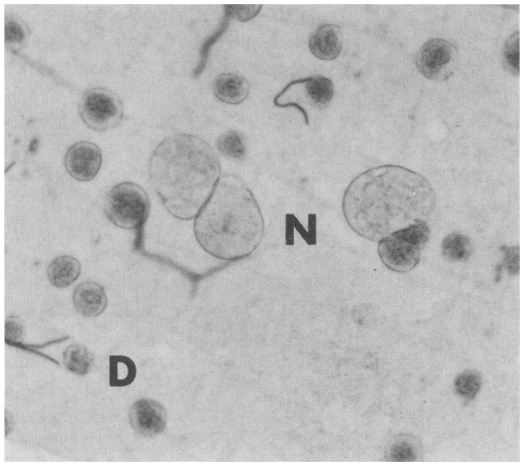

FIG. 2. Midgut of *A. gambiae* infected with *P. cynomolgi* at 8 days postinfection. Magainin 2 (0.3 μg/μl) was injected at 5.5 days postinfection. Note dark-staining degenerated oocysts (D) and normal 8-day oocysts (N).

days after infection. Sporozoites, once in the salivary glands, did not appear to be affected. Sporozoites treated with either magainin or cecropin appeared normal by light microscopy 24 and 48 h after treatment; infectivity was not assayed. The effects of magainins and cecropins on developing oocysts in mosquitoes injected 5 or more days postinfection were dramatic. Oocyst development was arrested and characterized by degeneration and dark staining with mercurochrome (Fig. 2). More mature oocysts (7 to 10 days postinfection) showed shrinkage of the central mass of the parasite from its outer membrane (Fig. 3).

Dose response. A dose response was evident in *A. gambiae* mosquitoes injected 5.5 days after infection, with a greater proportion of oocysts affected at higher concentrations of magainin 2 (Fig. 4A) or cecropin B (Fig. 4B). However, at even higher concentrations, some oocysts appeared unaffected after a single injection; sporozoites were present in the salivary glands of most treated females, although sporozoite numbers were less than in control mosquitoes. The analog of magainin 2 with the Lys and Phe residues replaced (Z-12) and also the truncated cecropin had no effect on oocyst development at concentrations comparable to those of the parent compounds.

Repeated injections of either magainin or cecropin produced a marked effect on both oocyst development and sporozoite production. Mosquitoes injected with magainin 2 at 5.5 and 9.5 days postinfection matured few oocysts and no salivary gland sporozoites (Fig. 4A). Most of the oocysts were affected by the first injection; the majority of the remaining oocysts showed the effects of the second injection. The few normal-appearing oocysts failed to produce viable sporozoites capable of invading the salivary glands.

Table 2 presents the effects of magainin and cecropin injected 5.5 days postinfection on various combinations of mosquito vectors and malaria-causing species. The damage to developing oocysts following treatment with either of the two peptides appeared similar in all combinations.

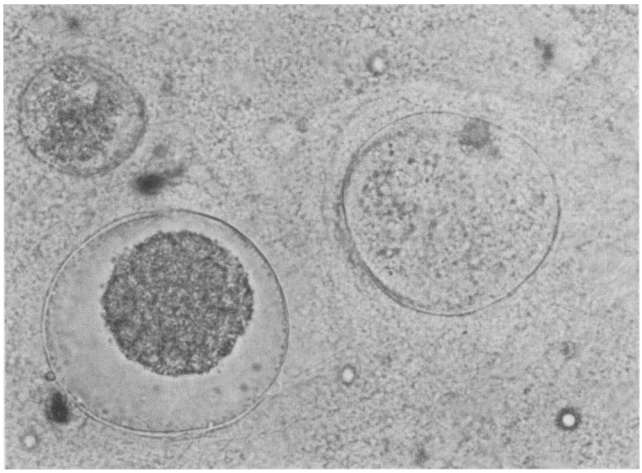

FIG. 3. Midgut of *A. gambiae* infected with *P. cynomolgi* at 11 days postinfection. Magainin 2 (0.3 μg/μl) was injected at 9 days postinfection. Note degenerated oocyst with clear area inside the parasite membrane (left) and normal 11-day oocyst on right.

DISCUSSION

Both magainin 2 and cecropin B were able to arrest development of oocysts of several species of malaria-causing parasites in a variety of mosquito hosts. Examination of infected mosquito midguts by light microscopy showed deformation of the developing oocysts and an increased permeability to mercurochrome stain consistent with damage to parasite membranes. Christensen et al. (3) reported that the antibacterial activity of cecropins was due to the formation of large pores in the cell membranes. Similarly, Zasloff (16) has suggested that magainins work through perturbation of membrane functions.

The concentrations of magainin 2 and cecropin B needed to produce parasiticidal effects in vivo in this study were significantly higher than those used to demonstrate bactericidal or fungicidal effects in vitro, e.g., the MIC of magainin 2 for *Escherichia coli* was 10 to 50 μg/ml, and for *Candida albicans* it was 200 to 500 μg/ml (12). The requirement for pharmacological doses to affect oocysts in vivo might reflect metabolism of the synthetic peptides by the mosquito host, the structure of the synthesized peptides, or the greater resistance of oocysts to these peptides; however, not all stages of malaria parasites were equally resistant. Zygotes, ookinetes, and merozoites were affected in vitro at concentrations of magainin 2 between 30 and 100 μg/ml (D. Kaslow, unpublished data).

TABLE 2. Effect of a single injection of magainin 2 (0.5 μg/μl) or cecropin B (0.5 μg/μl) on oocyst development in a variety of malaria parasite-mosquito vector combinations[a]

Mosquito species	Malaria species (strain)	% Aborted oocysts with:	
		Magainin 2	Cecropin B
A. gambiae	*P. cynomolgi*	94	92
	P. knowlesi	95	94
	P. falciparum (NF54)	82	85
	P. falciparum (3D7)	86	81
A. dirus	*P. cynomolgi*	91	ND[b]
A. freeborni	*P. falciparum* (NF54)	85	ND
	P. falciparum (3D7)	83	ND

[a] The peptide was injected 5.5 to 6 days after the infecting blood meal, and oocysts were examined 48 h later. Each treatment represents a minimum of 20 mosquitoes.

[b] ND, Not determined.

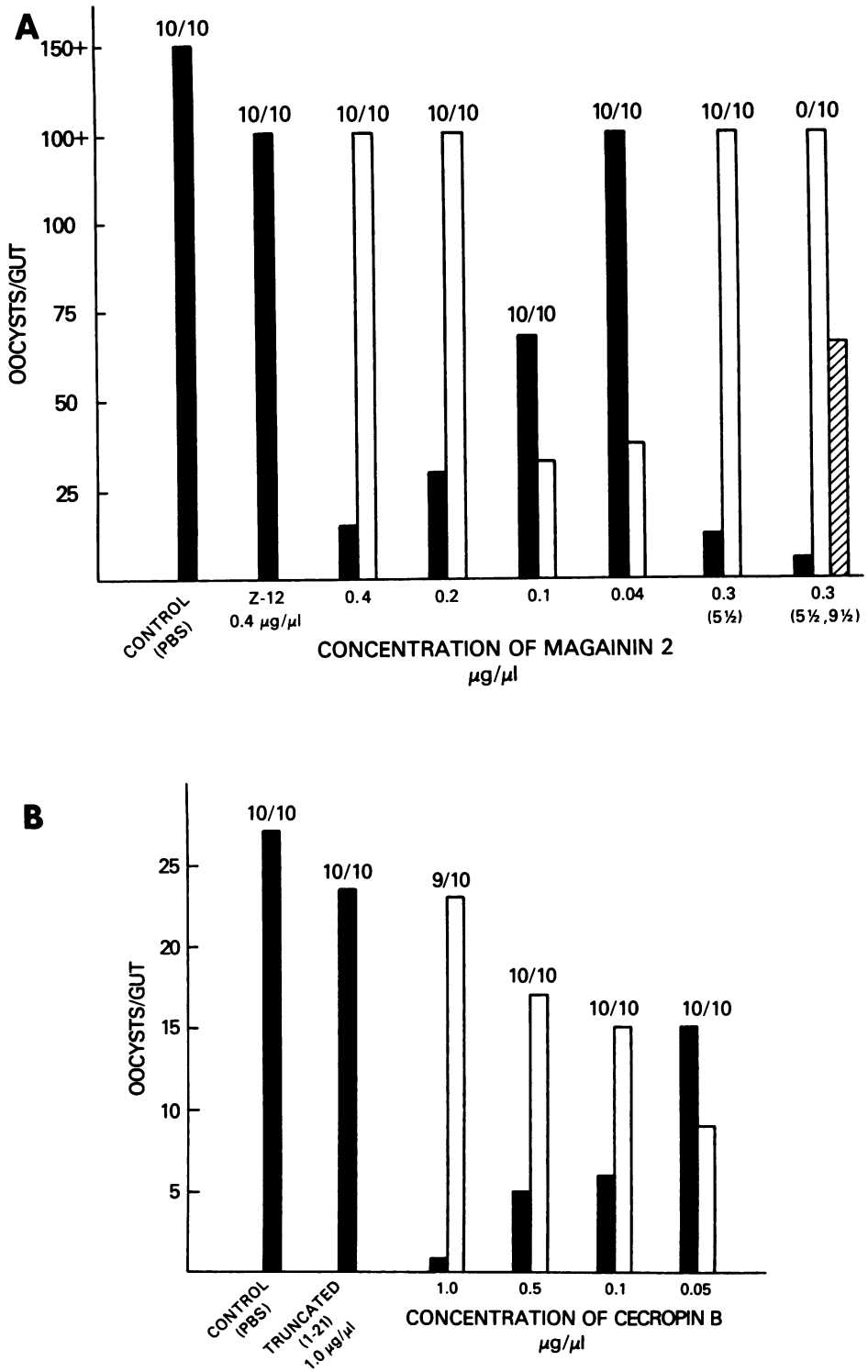

FIG. 4. Effects in *A. gambiae* of injections of various concentrations of magainin 2 (A) or cecropin B (B) 5.5 days after an infecting blood meal with *P. cynomolgi*. One group of mosquitoes received a second injection of magainin 2 9.5 days after infection. The black bars represent normal oocysts, the clear bars represent affected oocysts, and the hatched bar represents oocysts affected by the second injection. Each treatment included a minimum of 20 females. Numbers above the bars are the proportion of mosquitoes with sporozoites in their salivary glands at 14 days postinfection. Mosquitoes treated with magainin 2 at 0.2 to 0.4 μg/μl showed reductions in total sporozoite numbers in the salivary glands. PBS, Phosphate-buffered saline.

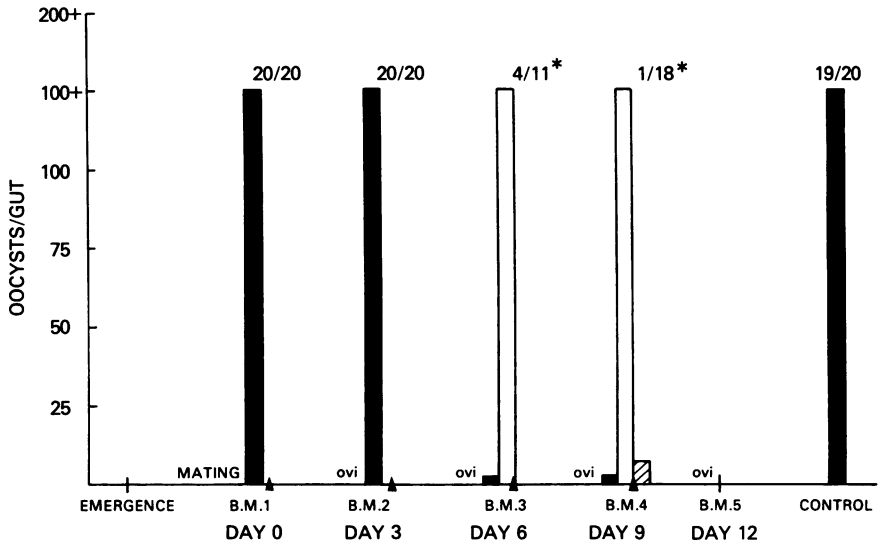

FIG. 5. Effects in *A. gambiae* of sequential injections of magainin 2 (0.3 μg/μl) given 12 h after each of four blood meals (B.M.). The first blood meal was infectious with *P. cynomolgi*. The mosquitoes were allowed to lay their eggs 2.5 days after each blood meal. The black bars represent normal oocysts, the clear bars represent oocysts affected by the third injection, and the hatched bar represents oocysts affected by the fourth injection. Each group represents at least 20 injected females. The numbers above the bars reflect the proportion of each group with salivary gland sporozoites; *, fourfold decrease in sporozoite numbers in a single female. ovi, Oviposition.

The Z-12 analog of magainin 2 showed little activity against oocysts; against bacteria, it displayed markedly reduced activity, requiring concentrations greater than 1,000 μg/ml to produce an effect (16). The truncated cecropin B containing residues 1 to 21 includes the amphipathic helix indicative of a strong membrane association potential (3), but this peptide failed to significantly affect oocyst development in mosquitoes.

One scheme for the mobilization of magainins or cecropins within the mosquito envisions introducing the genes coding for these parasiticidal peptides linked to a blood meal-activated promoter such as that which controls vitellogenin synthesis. Figure 5 presents such a scheme in which magainin would be produced in the mosquito following each blood meal. In this model, magainin was injected after each blood feeding; the first feeding included infectious gametocytes. The first two releases of magainin (on days 0 and 3) had no effect on oocyst development or sporozoite production; oocysts were normal and salivary glands were heavily infected. However, as a result of the third and fourth magainin releases, oocyst development was significantly affected. More importantly, only 1 of 18 mosquitoes had salivary gland sporozoites and that one had them at a level fourfold less than control mosquitoes did. In nature, one would expect oocyst levels far below that seen in the laboratory. Indeed, most infected mosquitoes usually carry only one or two oocysts. Moreover, under natural conditions, one could expect four to six blood meals between the infecting blood meal and the appearance of sporozoites in the salivary glands. If endogenous peptide levels with each blood meal were sufficient, then complete destruction of oocysts could be expected.

It is clear that the parasiticidal levels of magainin and cecropin used in this study were perilously closer to the 50% lethal dose levels for these compounds in mosquitoes. Efforts are under way to develop peptide configurations with maximum effects on the parasite and minimum deleterious effects on the mosquito. At the same time, it is unclear what levels of toxicity to the parasite or the mosquito host will occur when the genes coding for these peptides are expressed in the mosquito.

The feasibility of introducing parasiticidal genes into the mosquito genome with their expression at a time and level sufficient to destroy malarial parasites remains to be demonstrated. The difficulties involved in producing a heritable gene introduction with no deleterious side effects are numerous. Moreover, the ethical and practical problems associated with the release of a genetically engineered insect vector will be considerable. The problem of malaria is so enormous, the need for alternative control strategies so pressing, that these research efforts are clearly justified.

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