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Review

Blocking malaria parasite invasion of mosquito salivary glands

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Summary

Release of genetically engineered mosquitoes resistant to parasite infections has been proposed as a novel way to control malaria transmission, and several important advances have been made in anticipation of testing this approach. In particular, the development of synthetic effector genes that block parasite development in mosquito hosts has exploited a number of different mechanisms that result in parasite-resistant phenotypes, and those that target specifically the sporozoites are reviewed here. The use of a number of synthetic genes based on different mechanisms in transgenic mosquitoes will make the selection of resistant parasites unlikely.

Key words: sporozoite, salivary gland, transgenic, mosquito, genetic control, lectin, blocking antibody.

Introduction

The development of malaria parasites in mosquitoes involves significant interactions of the pathogens with host tissues. As detailed by other authors in this volume, ookinetes must successfully negotiate the midgut environment by avoiding digestive enzymes and the developing peritrophic matrix to penetrate and lodge at the basal surface of the midgut epithelium. The resulting oocysts must avoid the host immune responses as the sporozoites develop within. Finally, the sporozoites must navigate the open circulatory system, avoiding humoral and cellular immune responses, to reach and invade their final destination in the mosquito host, the salivary glands. The specificity of these parasite–vector interactions makes them attractive targets for those working to develop synthetic refractory mechanisms in mosquitoes. Some years ago, we proposed to block malaria transmission by interfering with sporozoites in salivary glands (James et al., 1989). This could be achieved by disabling parasites once they invaded the salivary glands, or by blocking the initial invasion. As the tools for testing this strategy have evolved over the last few years, the focus has shifted from the former to the latter approach.

Mosquito salivary glands

The salivary glands of adult mosquitoes are sexually dimorphic and it is clear that the structural and functional differences between the male and female organs reflect the ability of the latter to engage successfully in hematophagy (James, 1994; Stark and James, 1996). The glands are paired structures and are much larger in females than in males (Fig. 1). Each gland consists of three lobes that are attached to a common salivary duct. The duct in culicine mosquitoes extends the length of each lobe, whereas in anophelines, it extends only part-way along the lobe. Each lobe comprises a secretory epithelium surrounding a duct into which saliva is released. The cells in each lobe are organized into a single-layer epithelium with characteristic basal and apical surfaces. The basal ends of the epithelial cells form the outside surface of the glands and are in contact with a basement membrane that provides the cohesiveness of the glands.

In general, the three lobes of male salivary glands appear similar to one another and likely all have the same secretory capabilities (James, 1994). Female glands are differentiated into two lateral and one medial lobes. The proximal regions of the lateral lobes in females express and secrete salivary gland products such as amylases and α1-4 glucosidase that are involved in sugar feeding, and these lobes appear to overlap functionally the male salivary glands (James, 1994; Arcá et al., 1999). In contrast, the medial lobe and distal-lateral lobes express genes whose products such as apyrases, anticoagulants and vasodilatory agents are involved in hematophagy (Champagne et al., 1995; Smartt et al., 1995; Beerntsen et al., 1999; Stark and James, 1998; Arcá et al., 1999).

In addition to their gene expression characteristics, the surface properties of the different salivary gland lobes are also variable. A number of studies with lectins and monoclonal antibodies raised to whole salivary glands show differential binding of these agents to the lobes of the female glands (Perrone et al., 1986; Barreau et al., 1995, 1999). Some of these reagents recognize specifically the distal-lateral and/or medial

lobes, indicating a differentiation among the regions of the glands. These differences are particularly important because the results of a number of studies have been interpreted to indicate that sporozoites preferentially invade the distal-lateral and medial lobes of the female glands (Sterling et al., 1973; Rossignol et al., 1984; Golenda et al., 1990; Pimenta et al., 1994). In a striking demonstration of this specificity, a peptide, SM1, binds to the distal-lateral and medial lobes of female glands of *An. gambiae* and *An. stephensi*, and blocks slightly more than 90% of *P. berghei* sporozoite invasion in the latter species (Ghosh et al., 2001). The authors conclude that the peptide competes with the sporozoites for a salivary ligand.

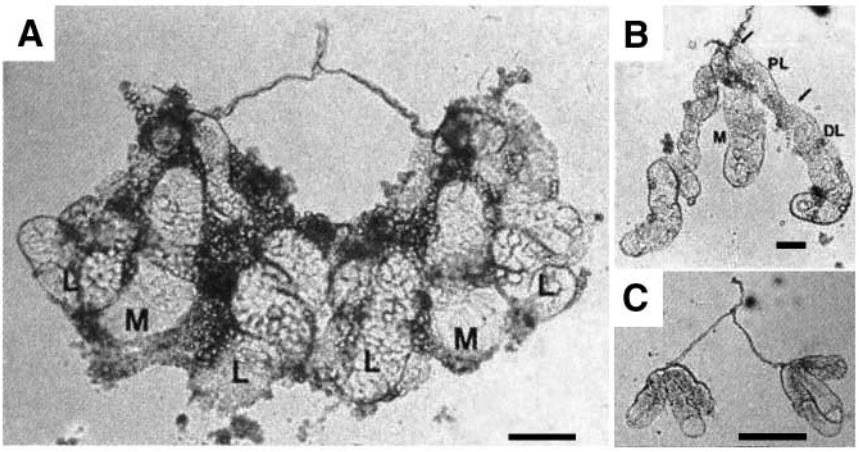

Fig. 1. Adult salivary glands of *Aedes aegypti*. (A) Paired salivary glands of an adult female. The lateral (L) and medial (M) lobes are associated with the fat body tissue. (B) Single female salivary gland showing medial (M), proximal-lateral (PL) and distal-lateral (DL) lobes of the glands. (C) Paired salivary glands of an adult male. Scale bars: 100 μm.

Some of the most exciting work being done in vector physiology is the discovery and characterization of a large number of proteins and their corresponding genes that are involved in facilitating hematophagy. Classes of proteins that appear common to all bloodfeeding arthropods include polyphyletic groups of enzymes that prevent coagulation, cause vasodilation and prevent platelet aggregation (Stark and James, 1996). Furthermore, proteomics approaches have provided comprehensive lists of the individual gene products in the 'sialomes' of various mosquito vectors including *Aedes aegypti*, *Anopheles gambiae* and *An. stephensi* (Valenzuela et al., 2002, 2003; Francischetti et al., 2002). These studies have revealed an amazing diversity in the recruitment of members of gene families to roles in hematophagy as well as a remarkable amount of apparent redundancy in each recognized functional class. For example, proteins that function as vasodilatory agents include small peptides, the sialokinins, from *Ae. aegypti* (Champagne et al., 1995), and larger enzymes, the catechol oxidases/peroxidases, in *An. albimanus* (Ribeiro and Nussenzveig, 1993). Furthermore, the products of a number of different genes function as anti-coagulants in individual mosquito species (Stark and James, 1998; Valenzuela et al., 2002, 2003; Francischetti et al., 2002).

Parasite–salivary gland interactions

While the role of mosquito salivary gland proteins in preventing vertebrate host hemostasis is established, whether or not these proteins contribute to the enhancement of malaria parasite infections in the vertebrate host is unresolved. Clear evidence of the involvement of mosquito saliva in enhancement of viral pathogenesis exists (Edwards et al., 1998; Osorio et al, 1996; Limesand et al., 2003), but no unequivocal studies have yet shown a specific enhancement of malaria parasite infections. Malaria parasites do appear to damage the glands and impair normal secretory processes. Parasitized mosquitoes were observed to secrete less apyrase and probed for longer periods of time before obtaining a bloodmeal (Rossignol et al., 1984). This prolonged feeding creates a greater opportunity for the inoculation of parasites and presumably increases transmission rates.

The invasion of salivary glands by sporozoites is thought to be mediated by receptor–ligand-like interactions resulting from the binding of parasite surface ligands to specific receptors on the salivary glands (Beerntsen et al., 2000). The receptor–ligand hypothesis derives support from observations that the majority of sporozoites released from the oocysts are found in the salivary glands (Golenda et al., 1990). This is interpreted to indicate that sporozoites have some mechanism for differentiating among the multiple mosquito organs suspended in the hemocoel. Electron microscope studies of sporozoite interactions with salivary glands also lend support for a receptor–ligand model. Analyses of *P. gallinaceum* invasion of *Ae. aegypti* glands show filamentous attachments of the sporozoites to the basal lamina of the glands, suggesting the presence of a receptor–ligand complex (Pimenta et al., 1994). Interestingly, initial contact of the sporozoite with the gland was followed by a reorientation of the sporozoite so that the 'anterior tip' (apical end) is in close association with the plasma membrane of the salivary gland cells. This behavior strongly supports the hypothesis that additional parasite surface molecules are needed for invasion of the gland following the initial binding.

Rosenberg (1985) showed that there were species-specific recognition properties of sporozoites for salivary glands. *Plasmodium knowlesi* sporozoites could recognize and invade salivary glands from *An. dirus* even when the glands were transplanted to a non-permissive host, *An. freeborni*. Conversely, these sporozoites could not infect *An. freeborni* salivary glands under any circumstances. These experiments have been interpreted by many to indicate that the presence

(*An. dirus*) or absence (*An. freeborni*) of species-specific receptor molecules is an important component of vector competence for sporozoites to invade the salivary glands.

Potential parasite ligands for salivary gland recognition include the circumsporozoite protein (CSP). The CSP is the major protein on the surface of sporozoites, and may account for as much as 10% of the protein located there (Nussenzweig and Nussenzweig, 1989). Sidjanski et al. (1997) bound recombinant *P. falciparum* CSP to the medial (strongly) and distal-lateral (weakly) lobes of *An. stephensi* salivary glands. This group was able to show that a specific domain (region 1) was the likely salivary binding domain of CSP. Others have shown that CSP shed during invasion of glands by sporozoites remains on the surface of the glands (Posthuma et al., 1988; Golenda et al., 1990). Furthermore, some of the monoclonal antibodies made to *P. gallinaceum* CSP blocked sporozoite invasion of *Ae. aegypti* salivary glands (Warburg et al., 1992). These results could occur as a consequence of blocking a receptor or steric hindrance. Interestingly, the SM1 peptide is not similar in amino acid sequence to the CSP (Ghosh et al., 2001), indicating that other molecules also could function as ligands.

Other molecules on the surface of sporozoites such as the thrombospondin-related anonymous protein (TRAP) and the apical membrane antigen/erythrocyte binding-like protein (MAEBL) may have more complex interactions with the salivary glands (reviewed in Kappe et al., 2003). These proteins may be involved with the invasion phase of sporozoite infection that occurs after the initial attachment.

Surprisingly, the putative salivary gland receptor molecules have yet to be identified. Despite the long-time availability of reagents (lectins, monoclonal antibodies and peptides) that compete or block sporozoite invasion of the glands, the molecules with which these reagents interact remain elusive. The molecular complexity of the basement membrane surrounding the salivary gland epithelium may make difficult the identification of a single molecule that functions as the receptor. Furthermore, there may not be a single molecule that fulfills this role, but multiple interchangeable molecules or a molecular complex may be the sporozoite target.

Antisporozoite refractory phenotypes

Three different sites within the mosquito are available for targeting the sporozoites. First, their development in oocysts can be impaired. Discovery of parasite genes necessary for sporozoite development maybe provide a number of specific targets for interfering with this stage. A recent study by Matuschewki and colleagues (2002) identified as many as 30 proteins that are expressed specifically in developing sporozoites and these could be potential targets. The methods for inactivating any one of these targets remain to be worked out, but specific antibodies, small peptide agonists, drugs and perhaps even RNAi may interfere with these developmentally regulated genes.

Sporozoites are potentially vulnerable when they are in the salivary glands. Promoters from genes expressed specifically in the glands can be used to drive the expression of an effector molecule that would disable the parasites and prevent them from being secreted or abrogate their infectivity to the vertebrate host.

The most attractive target is provided by the sporozoites as they make their way through the hemolymph from the oocysts to the salivary glands. This approach provides the opportunity to bathe the sporozoite in a solution (hemolymph) containing an effector molecule. Furthermore, the mosquito has a number of genes that express proteins such as vitellogeninins and lipophorins that are made in the fat body and then transported into the hemolymph (Raikhel et al., 2002; van Heusden et al., 1998). The promoters and other control sequences of these genes direct the expression of large amounts of the corresponding proteins and therefore these sequences are good candidates for donating the control portions of synthetic effector genes.

Various effector strategies and molecules were reviewed recently by Nirmala and James (2003). They described five categories of approaches, based on the target of action. Effector molecules can interact with ligands on the parasite surface, or their corresponding receptors on mosquito tissues. They can block the activity of parasite-expressed proteins important for invasion of tissues, or they can be toxins that destroy parasites. Finally, immune-system components could be regulated and expressed in the vector to incapacitate the parasite. A list of approaches that have been applied to salivary glands is presented in Table 1.

Effector molecules targeting salivary gland receptors were some of the first to be tested for transmission blocking. Carbohydrate moieties were implicated in parasite receptor recognition when it was shown that *P. gallinaceum* sporozoites could not invade salivary glands of *Aedes aegypti* treated with

Table 1. Potential antisporozoite effector mechanisms*

Effector strategy (target)	Molecule	Target parasite	References
Parasite ligands	N2 scFv	*Plasmodium gallinaceum*	Capurro et al., 2000
Tissue recognition (receptors)	Lectins, mAbs	*Plasmodium gallinaceum*	Barreau et al., 1995
	SM1 peptide	*Plasmodium berghei*	Ito et al., 2002
Immune response effectors	Defensins	*Plasmodium gallinaceum*	Shahabuddin et al., 1998

*Adapted from Nirmala and James (2003).

lectins (Barreau et al., 1995). *Plasmodium berghei* numbers are reduced in mosquitoes treated with a peptide, SM1, which binds to *An. stephensi* midguts and salivary glands (Ito et al., 2002). Transgenic mosquitoes expressing SM1 have fewer oocysts, and concomitantly fewer sporozoites, when compared with controls. It is intriguing that SM1 binds both the apical surface of the midgut and the basal surface of the distal lobes of the salivary glands, suggesting the presence of similar receptors on these organs.

Single-chain antibody fragments (scFv) composed of fused heavy- and light-chain variable regions can preserve the specificity of an antibody and be expressed as the product of a single gene. An scFv that binds CSP, N2scFv, reduced by 99% the number of *P. gallinaceum* sporozoites in salivary glands (Capurro et al., 2000). These studies demonstrate that parasite ligands are good targets for effector molecules. Furthermore, it is anticipated that targeting the parasite will impose less of a genetic load on the transgenic mosquito than will interfering with surface molecules on mosquito tissues.

Immune system interactions with sporozoites could provide the basis of an effector strategy. Both exogenously and endogenously derived immune peptides have been evaluated for their effects on malaria parasites. Defensins isolated from *Aeschna cyanea* and *Phormia terranovae* reduced the number of viable *P. gallinaceum* oocysts and sporozoites by 50% when injected into *Ae. aegypti* (Shahabuddin et al., 1998). Natural immune responses of mosquitoes to infection are also being studied as possible effector mechanisms for transmission blocking. Microarray analyses coupled with the sequence of the *An. gambiae* genome have provided the first comprehensive look at immune responses in parasite-infected mosquitoes (Dimopoulos et al., 2003; Holt et al., 2002), and there are possibilities of modulating responses that will affect sporozoite development.

The development of antisporozoite effector genes is ongoing work. Combining these genes with others that target ookinetes and prevent oocyst formation should permit producing a multigenic phenotype of 'no sporozoites' in the salivary glands. Furthermore, the use of multiple effector genes maybe necessary to prevent the selection of resistance to any one mechanism. This could prevent the breakdown of a control strategy based on a genetics approach. In addition, a balance among fitness effects, effectiveness of the molecule, ease of engineering of the phenotypes and mechanism for spreading the phenotypes through a population will dictate the practicality of any one strategy. Ultimately, saving lives will be the most important measure of these approaches.

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