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Interrupting malaria transmission by genetic manipulation of anopheline mosquitoes

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Malaria ranks among the deadliest infectious diseases that kills more than one million persons every year. The mosquito is an obligatory vector for malaria transmission. In the mosquito, *Plasmodium* undergoes a complex series of developmental events that includes transformation into several distinct morphological forms and the crossing of two different epithelia—midgut and salivary gland. Circumstantial evidence suggests that crossing of the epithelia requires specific interactions between *Plasmodium* and epithelial surface molecules. By use of a phage display library we have identified a small peptide-SM1—that binds to the surfaces of the mosquito midgut and salivary glands. Transgenic *Anopheles stephensi* mosquitoes expressing a SM1 tetramer from a blood-inducible and gut-specific promoter are substantially impaired in their ability to sustain parasite development and transmission. A second effector gene, phospholipase A2, also impairs parasite transmission in transgenic mosquitoes. These findings have important implications for the development of new strategies for malaria control.

Key words Germ line transformation – malaria vector – mosquito – phospholipase A2 – SM1 tetramer – transgenic

Malaria is among the deadliest infectious diseases in the world and kills an estimated 1 to 3 million people (mostly African children) per year. More worrisome, however, is the fact that, as the parasite becomes resistant to drugs and the mosquito resistant to insecticides, malaria is becoming increasingly difficult to control. If no new measures to fight the disease are introduced, it is predicted that the number of malaria cases will double in the next 20 years[1]. Currently, only two types of weapons are available to fight malaria—drugs that kill the parasite in humans and insecticides that kill the mosquito vector. Both approaches suffer from problems of development of resistance by the target organisms and from expense and complicated logistics for implementation of control measures. Despite intensive efforts in the last few decades, an effective vaccine has not yet been developed. Clearly, new approaches to combat malaria are urgently needed. This article reviews progress made to date towards the development of a novel approach–genetic modification of mosquito vector competence to control malaria.

A basic tenet of the new approach is that malaria transmission has an absolute requirement for passage of the parasite through the mosquito vector. The possibility of transmission by blood transfusion is not considered here because it is negligible in malaria epidemiology. Thus, if it were possible to introduce into the mosquito a gene that interferes with parasite development, transmission by this modified mosquito would be cut correspondingly. There are three basic requirements to achieve genetic modification of mosquitoes: (i) a method to introduce foreign genes into the germ line (transformation); (ii) the availability of a suitable

promoter to drive the expression of foreign genes in the appropriate tissue and at the appropriate time; and (iii) the identification of appropriate gene products (*effector genes*) capable of interfering with the development of the parasite. Each of these requirements are reviewed below.

Germ line transformation : *Drosophila* is the first multicellular organism ever to be transformed, and this was accomplished by use of the *P* transposable element[2]. Transformation of the first mosquito came only 16 years later[3]. This delay was caused in part by the fact that scientists initially did not realise that the *P* element does not transpose in non-drosophilid organisms, presumably because it requires some host-specific factor(s). The delay was also caused by the lack of an effective marker to detect transformed individuals. Presently, a variety of transposable element vectors for germ line transformation are available for the transformation of insects[4] and effective transformation markers have been developed. Early transformation experiments relied on eye colour markers to detect transformed individuals. This had the disadvantage that both, an eye colour mutant of the target organism and the corresponding cloned wild type gene to correct the mutation, had to be available. A major breakthrough was the discovery that the enhanced green fluorescent protein (eGFP) could be used as a dominant marker gene, thus obviating the need to generate insect eye colour mutants and cloning of their genes. Transformation vectors that use ubiquitous promoters (e.g. actin)[5]; or tissue-specific (e.g. eye)[6] are available. The latter are preferable because strong expression of a foreign protein in many tissues might confer a fitness load on the insect carrying the gene.

Promoters to drive transgene expression : As mentioned above targeting transgene expression to specific tissues is desirable because it minimises possible fitness load. Other important considerations in choosing a promoter are their spatial and temporal profile and their strength of expression. In principle, stronger promoters are better because effectiveness of the transgene is expected to increase as the abundance of the corresponding protein product increases. Moreover, tissue specificity and time of expression of the promoter related to the development of the parasite in the mosquito should be carefully considered. *Plasmodium* develops in two mosquito compartments (midgut lumen and haemocoel) and is stored in a third compartment (salivary gland lumen). The parasite is acquired when the mosquito ingests an infected blood meal. Initial development to mobile ookinetes (about one day) occurs in the midgut lumen. The ookinetes invade and traverse the midgut epithelium, transforming into oocysts that face the mosquito body cavity or hemocoel (the second compartment). After ~10 days each oocyst releases several thousand sporozoites, and these invade the salivary gland epithelium. Sporozoites are stored in the salivary gland lumen (the third compartment) until they are released with saliva when the mosquito feeds on another host. Carboxypeptidase is an effective promoter to target the midgut lumen[7,8]. In addition to being robust, the temporal pattern of expression of the carboxypeptidase promoter is favourable in that it is activated in synchrony with the arrival of the malaria parasite in the midgut lumen. Targeting of the hemocoel has been accomplished with the vitellogenin promoter[9]. Its expression is also robust but the temporal profile of expression (peak at 24 h) is favourable only to target the initial stages of oocyst development. Targeting of sporozoites is more problematic because expression from the vitellogenin promoter drops to basal levels within two days of blood intake and sporozoites are released on about Day 10. Thus, effectiveness of this promoter has to rely on high stability of the transgenic protein in the hemolymph and/or re-activation of the promoter by a subsequent blood meal. While a promoter active in salivary glands has been identified, its use to drive transgene expression is diminished by its relative weakness[10]. The identification of a strong salivary gland promoter should receive a high priority.

When devising a strategy for interfering with the *Plasmodium* life cycle in the mosquito, it is important to consider parasite numbers. A severe bottleneck occurs during the ookinete-to-oocyst transition, when parasite

numbers reach their lowest point[11]. As mentioned above, parasite numbers increase by a factor of several thousand during the oocyst-to-sporozoite transition. On this basis, ookinetes are a considerably more effective target than sporozoites. The advantage of targeting salivary gland sporozoites (if a strong promoter can be identified; see above) is that the parasites remain in contact with the salivary gland secretions for relatively long times (many days). The disadvantage is of course, the large numbers of sporozoites (thousands) that are normally found in the salivary gland, meaning that inactivation of sporozoites needs to be very efficient. Note that one, or a few sporozoites is (are) sufficient to start an infection in the vertebrate host.

Effector genes : An ideal effector gene blocks development of the parasite or kills it with 100% efficiency and does not impose a fitness load to the mosquito. While effector genes that fulfill all these requisites do not yet exist, several have been identified. Examples are monoclonal antibodies that recognise antigens on the parasite surface[12], proteins that selectively kill the parasite[13] or proteins that interfere with parasite invasion of the midgut or salivary gland. Our laboratory has examined two genes belonging to the latter class.

A phage display peptide library is a large collection of recombinant phages, each displaying on its surface a different peptide. We used a phage display library displaying 12-amino acid peptides of the structure XCX_8CX, where X is any amino acid and C a cysteine. The two cysteines presumably form a disulphide bridge exposing an 8-amino acid loop. Screening this library for phages that bind to the midgut and salivary gland epithelia identified a peptide, termed SM1 (for salivary gland and midgut binding peptide 1) that bound specifically to the epithelial surfaces that are invaded by *Plasmodium*—salivary gland and midgut. Importantly, binding of the peptide to these epithelia interfered with parasite invasion[14]. Presumably, SM1 and the parasite both bind to a common receptor required for midgut invasion. Encouraged by these results we constructed transgenic *An*. *stephensi* that express a SM1 tetramer in the midgut from a carboxypeptidase promoter. *P. berghei* oocyst formation in these mosquitoes was inhibited by ~ 80% and vectorial capacity of the mosquitoes was dramatically reduced (100% blockage of transmission from one mouse to another in two out of three experiments[15]. The effectiveness of SM1 in interfering with development of human parasites in the mosquito remains to be tested.

Previously, bee venom phospholipase A2 (PLA2) had been shown to strongly interfere with *Plasmodium* invasion of the midgut[16]. We have constructed *An*. *stephensi* transgenic strains that express PLA2 from a carboxypeptidase promoter. As for SM1, oocyst formation and vectorial capacity were significantly reduced[17]. The mechanism of PLA2 action is not well understood. One possibility is that PLA2 modifies the properties of the midgut epithelial membrane by inserting itself into the lipid by (layer PLA2) hydrophobicity seems to be important for inhibition of parasite invasion[16].

Transgenic mosquito fitness : The ultimate goal of the transgenic mosquito approach is to introduce the genes that confer *Plasmodium* refractoriness into wild populations. For this, it is desirable that the transgene has the minimum possible fitness load on the mosquito. We have measured by a variety of approaches the fitness of mosquitoes carrying both the transgenes (SM1 and PLA2). The experiments included measurements of longevity, fecundity (number of eggs per female) and fertility (proportion of eggs that hatch into larvae). In all experiments SM1 transgenics were as fit as non-transgenic siblings, while fecundity of PLA2 transgenics was significantly lower. The absence of SM1 fitness load was confirmed in cage experiments whereby a mixture of transgenic and non-transgenic mosquitoes were maintained blindly through five generations [18]. A recent report claimed that transgenic mosquitoes expressing a GFP gene from an actin promoter are less fit than their non-transgenic counterparts[19]. However, in this case reduced fitness was likely to be caused by inbreeding and ubiquitous expression of the transgene

in many tissues. As the experiments with SM1 transgenic showed, loss of fitness is not an inherent property of transgenic mosquitoes.

Summary and future prospects

Considerable progress has been made in the last few years in our ability to genetically manipulate mosquito vectorial capacity. We can now introduce genes at will both in culicine and anopheline mosquitoes. A number of promoters have been identified that can drive robust gene expression and secretion of transgenic proteins in the two main mosquito compartments where the parasite develops and candidate effector genes were identified. Importantly, we now know that it is possible to genetically manipulate mosquito vectorial capacity and that at least one effector gene does not impose a significant fitness load on the mosquito. While all these developments are encouraging, much work remains to be done before this strategy can be tested with field experiments. For use in the field, mosquitoes carrying multiple effector genes will have to be produced to minimise the risk of parasites overcoming the barriers imposed by the transgenes. The biggest unanswered question is how to introduce transgenes into wild populations. While examples exist in *Drosophila* that transposable elements can be used for this purpose, no experiments have been done with mosquitoes. The use of other approaches — symbiotic Wolbachia bacteria or meiotic drive genes have been proposed but their feasibility has not been tested in mosquitoes. Introduction of transgenes into wild populations also requires a better understanding of population structure, since there is evidence that different reproductively isolated vector *An. gambiae* populations can co-exist in the same area (sympatric). Introduction of a transgene into 100% of the mosquitoes in any given area is obviously important. Moreover, ethical, social and political questions need to be addressed such as educating affected populations, government officials and local scientists about the risks and benefits of a possible release of recombinant mosquitoes. Development of new weapons to fight malaria is extremely important and there is reason to be optimistic that this goal is attainable.

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