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What are relevant assays for refractoriness?

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Abstract

The engineering and testing of genes that result in refractory phenotypes are important components of the continuing effort towards the use of population replacement strategies for vector control. Both 'endogenous' and 'synthetic' refractory phenotypes are being considered. Additional research is required to determine the prevalence of such phenotypes in the various vector–pathogen combinations, and the threshold levels of activity of genes conferring transmission blocking, as well as to develop efficient methods for the evaluation of their entire spectrum of biological effects.
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Current state of the art

Refractoriness phenotypes could be divided into 'endogenous' and 'synthetic'. To date, a number of endogenous refractory phenotypes have been recognized, including melanotic encapsulation and lytic destruction of malaria parasites in the mosquito midgut (Christophides, Vlachou and Kafatos 2004) and RNA-interference (RNAi)-mediated suppression of viruses (Sanchez-Vargas et al. 2004). A small number of additional refractory phenotypes have been recorded; however, their specifics are, to a large extent, unknown and could be attributed to differences in vector physiology (e.g. lack of vector factors).

Melanization is an insect immune reaction that has been reported for many mosquito–parasite combinations. In a genetically selected *Plasmodium*-refractory strain of *Anopheles gambiae*, parasite melanization occurs immediately after the ookinete has traversed the midgut epithelium, between the epithelial cells and the basal lamina (Collins et al. 1986; Paskewitz et al. 1988). Although these refractory mosquitoes block development of the primate malaria parasite, *P. cynomolgi*, the rodent parasite, *P. berghei*, and allopatric strains of the human parasite *P. falciparum*,

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they fail to melanize sympatric *P. falciparum* populations (Collins et al. 1986), suggesting that the reaction is based on specific recognition and is most probably regulated by parasite-induced immune evasion. Recent data showing that silencing of specific immunity genes unrelated to the final steps of the melanization reaction suppress the melanization phenotype allowing parasite development and support a hypothesis that specific parasite recognition and killing may precede melanization (Blandin et al. 2004; Osta et al. 2004). Three quantitative-trait loci (*Pen1*, *Pen2* and *Pen3*) have been found to be responsible for the melanization phenotype, with one (*Pen1*) having the most important contribution (Zheng et al. 1997). However, the specific genes responsible for the quantitative traits have yet to be identified. Furthermore, a multidisciplinary morphological, biochemical and genomic approach has demonstrated broad physiological differences between the refractory and susceptible mosquitoes, implicating the level of reactive oxygen species as a factor contributing to the observed phenotype (Kumar et al. 2003).

A similar refractoriness phenotype has been described in a genetically selected line of *An. dirus* that is fully refractory to the rodent parasite, *P. yoelii* (Somboon, Prapanthadara and Suwonkerd 1999). Melanization starts early after midgut invasion and ranges from small melanized parasites to fully encapsulated oocysts. However, these mosquitoes are susceptible to natural infections with the human parasites *P. falciparum* and *P. vivax*, again emphasizing the specificity of the mosquito–parasite interaction.

The second refractoriness mechanism, lytic destruction of parasites, was reported first in a genetically selected strain of *An. gambiae* that kills *P. gallinaceum* ookinetes inside the midgut epithelial cells (Vernick et al. 1995). The ookinetes appear to be initially vacuolated and subsequently lysed while still in the cytoplasm of the midgut cells. A single dominant gene is responsible for this phenotype. Two recent studies have shown that mosquito innate immunity plays a central role in lysis of *P. berghei* ookinetes in the midgut epithelium (Blandin et al. 2004; Osta et al. 2004). RNAi-mediated gene silencing of two immunity genes, *TEP1*, a complement-like protein, and *LRIM1*, a leucine-rich repeat immune protein, results in an average fourfold increase in parasite numbers. However, two other mosquito genes encoding C-type lectins, CTL4 and CTLMA2, protect the parasites from immune reaction, since their absence leads to parasite killing and melanization (Osta et al. 2004). Specifically, the two parasite agonists protect the ookinetes from the immune action of LRIM1. Whether the protective function of the C-type lectins is the result of evolutionary adaptation remains to be examined (Osta, Christophides and Kafatos 2004).

A proteomic approach in a strain of *An. stephensi* showing reduced susceptibility to infection with *P. falciparum* (Feldmann and Ponnudurai 1989) revealed many differences in the midgut expression profile following a blood meal, when compared to a susceptible strain (Prevot et al. 1998). A factor responsible for the death of *Plasmodium* in the midgut of the mosquitoes is the production of nitric oxide (NO) and nitrite/nitrate radicals, all products of NO synthase (NOS) activity. NOS is induced in the *Anopheles* midgut upon parasite infection, resulting in elevated levels of these highly reactive species, and inhibition of this enzyme promotes parasite development (Luckhart et al. 1998).

A field-based study (Southern Tanzania) has revealed that *Plasmodium* melanization also is detected in natural mosquito populations (Schwartz and Koella 2002). However, the same study provided evidence that melanization of Sephadex beads, which is commonly used to measure the mosquito melanization capacity, does not accurately model *An. gambiae* susceptibility to *P. falciparum* in field conditions.

Strong evidence for genetic variability of *An. gambiae* in terms of susceptibility to natural malaria populations, manifested as reduced *P. falciparum* oocyst numbers detected in the mosquito midgut, has also been revealed (Niare et al. 2002). This phenotype is attributed to segregating alleles of two chromosomal loci (*Pfin1* and *Pfin2*). The apparently high natural frequency of resistance alleles suggests that natural mosquito populations exhibit significant variation in permissiveness for parasite development, and supports the hypothesis that malaria parasites exert a significant selective pressure on vector populations.

Synthetic refractoriness phenotypes are the result of genetic engineering of the vectors through the introduction of genes that determine anti-pathogen activities. These effector molecules could be exogenous or endogenous and act as pathogen anti-ligands (e.g. single-chain antibodies against parasite surface proteins), host mosquito anti-receptors (e.g. peptides blocking mosquito midgut invasion by the parasite), immune factors (e.g. antimicrobial peptides; AMPs) and toxins, or molecules acting through RNAi interference specific for pathogen or vector genes (Nirmala and James 2003).

Along these lines, a short-chain peptide, SM1 that binds to the *An. gambiae* midgut and salivary glands has been transformed into *An. stephensi* generating mosquitoes that express the peptide in the midgut lumen (Ito et al. 2002). These mosquitoes exhibit strongly reduced capacity to support *P. berghei* transmission. Similarly, a *P. berghei*-refractory *An. stephensi* has been generated by midgut-specific expression of a bee-venom phospholipase (Moreira et al. 2002). The mode of action of the two effectors remains unknown. Transgenic overexpresion of a gene encoding the AMP Cecropin A (*CEC1*) in the midgut of *A. gambiae* reduced the number of developing *P. berghei* oocysts by 60% (Kim et al. 2004). Similarly, in the yellow-fever mosquito *Aedes aegypti*, transgenic overexpression of the endogenous AMPs Defensin A and Cecropin A under the control of the *vitellogenin* gene promoter leads to robust inhibition of *P. gallinaceum* development (Shin, Kokoza and Raikhel 2003). Furthermore, *Ae. aegypti* resistant to *P. gallinaceum* have been developed via a different approach (De Lara Capurro et al. 2000): single-chain antibodies, engineered from a cDNA encoding an anti-*Plasmodium* monoclonal antibody, bind to *P. gallinaceum* sporozoites and largely prevent infection of the salivary glands when expressed by Sindbis virus.

Issues and challenges

Much work remains to be done on the identification and full characterization of effector traits as well as the construction and analysis of penetration and expression of transgenes targeting human malaria parasites. In addition, issues such as phenotypic variation depending on environmental conditions, development of resistance or even increased virulence of the pathogen, must also be addressed. Assuming that a strategy is chosen that does not rely on a complete replacement of populations, the relative frequency (prevalence) of the transgenes will be important and the threshold levels of refractoriness needed to interrupt transmission will need to be determined. Field tests of specific genes and outcome evaluation parameters also are necessary, as are assays, possibly based on membrane-feeding of gametocytemic blood, to evaluate refractoriness to endogenous malaria pathogens.

Research and control opportunities

Vector-borne diseases are fast increasing in most parts of the developing world, and there is an immediate need to develop novel strategies for disease control. Recent advances toward development of methods for vector transgenesis and identification and engineering of anti-pathogen effectors make the concept of vector population replacement with innocuous vector populations particularly attractive. However, many technical issues remain to be resolved, including the development of ideal effector traits that confer full resistance against human pathogens in natural transmission conditions. To date, efforts to characterize endogenous or synthetic effector genes have focused on model vector–pathogen combinations. However, a limited number of studies have suggested considerable differences among the various vector–pathogen combinations, and therefore, experimental approaches focusing on the main mosquito vectors and human pathogens should be undertaken in the future.

Furthermore, experiments will have to be performed to determine the genetic characteristics (dominance relations, penetration and expression) of mosquito genes and effects of both endogenous and synthetic refractoriness genes on the selection of resistance or increased virulence of the target pathogen. In addition, research on combinations of effector mechanisms to thwart the emergence of new pathogen phenotypes is needed. Additional studies of natural refractoriness and its potential to adapt as a 'death-on-infection' effector mechanism also are needed. Field-based work is necessary to assay the potential for phenotypic variation in refractory gene function in response to different environmental conditions, and protocols for field tests and outcome evaluation are required. Work with both laboratory- and field-based components includes modelling and experiments to establish the needed frequency of genes with a refractoriness phenotype in mosquito populations, in order to achieve the threshold levels needed for interrupting transmission.

Finally, research is needed to identify key qualitative and quantitative endpoints for the efficacy of effector genes. This includes routine and reliable methods for transmission-blocking assays that do not pose threats to humans. For this, protocols for human subjects must be standardized.

Future directions for research and capacity/partnership building

The future direction of research in this area that will bridge the gap between laboratory and field work will likely include the further analysis of endogenous, field-derived refractoriness mechanisms, the development of cage-trial protocols that allow the evaluation of engineered genes under (semi)natural conditions, the refinement of socially acceptable assays for transmission blocking, and outcome evaluation protocols that measure accurately the impact of a refractoriness gene on transmission dynamics.

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