Rendering Mosquitoes Resistant to Arboviruses through RNA Interference

Genetically enhancing RNA interference in mosquitoes can greatly reduce their ability to act as vectors for dengue virus

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Dengue viruses (DENV) are the most important of the arthropod-borne viruses, or arboviruses. Recognized in the 1870s, dengue fever is considered a reemerging disease due to recent increases in numbers and the widespread distribution of severe cases during the last 30 years. Thus, DENV infect 50 to 100 million humans—more than 2.5 billion are at risk—each year in more than 100 countries in tropical and subtropical regions. Infections with DENV can result in self-limiting dengue fever or more severe hemorrhagic fever.

DENV refers specifically to four serologically related arboviruses that are transmitted to humans by mosquitoes, principally Aedes aegypti. DENV, which are members of the family Flaviviridae, genus Flavivirus, are small, spherical, enveloped particles with a positive-sense RNA genome of about 11,000 nucleotides. The genome encodes a polyprotein that is co- and posttranslationally cleaved to yield three structural and seven nonstructural proteins. As is typical of arboviruses, DENV can infect and replicate in cultured primate cells, causing cytopathic effects and death, and in cultured mosquito cells, establishing persistent, noncytopathic infections. These persistent infections mimic the insect phase of the natural cycle of this virus, where it establishes a lifelong infection but also remains capable of causing serious disease when transmitted to humans and amplified in this vertebrate host.

The immunopathologic nature of dengue disease makes development of a safe, effective, quadrivalent vaccine a difficult challenge, and none is currently licensed. Although insecticides and breeding-site reduction efforts proved effective during a Pan American Health Organization (PAHO) campaign in the 1950s and 1960s, A. aegypti subsequently reinfested formerly mosquito-free areas throughout the Western Hemisphere. Thus, new strategies and a coordinated campaign are needed to combat dengue diseases.

Combating Dengue by Interrupting the Virus Transmission Cycle

In the mid-1990s we formulated a genetic strategy for combating dengue diseases, namely by interrupting the viral transmission cycle in mosquitoes. The central idea is to render A. aegypti resistant to DENV infection. Part of the technical approach to realizing this strategy borrows from findings elsewhere in biology. In particu-
lar, plant virologists learned that using a heterologous virus expression vector to deliver a fragment of viral RNA to plant cells makes them resistant to that (latter) virus. We adapted this approach in 1996, using it to engineer RNA-based resistance to DENV-2 initially in cultured mosquito cells and then in adult female mosquitoes. Specifically, we inserted a fragment of RNA that was complementary to the prM gene of DENV-2 into a Sindbis virus (SINV) vector. When that inserted RNA fragment is expressed, it renders the recipient cells resistant to subsequent challenges with DENV-2.

Although this resistance lasted only for the life of the mosquito, it marked an important early step toward our goal. Like DENV, SINV is an arbovirus (Togaviridae, Alphavirus). We engineered its positive-sense RNA genome to contain an extra promoter, from which inserted foreign genes or gene fragments can be expressed. Because SINV readily infects most mosquito cells, we used it as an expression vector in a series of experiments intended to define the parameters of RNA-mediated virus resistance in mosquitoes.

However, some of our control experiments left us puzzled because we did not expect an insert with the same polarity as the DENV genome itself also to render mosquitoes resistant to this virus. In 1998, several other research groups described a similar phenomenon—that is, interference with expression of endogenous genes induced by RNA. However, those findings involved very different organisms, namely the nematode Caenorhabditis elegans and the fruitfly Drosophila melanogaster. In both these animal species, interference followed injection of double-stranded (ds) RNA cognates to the mRNA corresponding to the suppressed genes.

This phenomenon, which came to be known as RNA interference (RNAi), proved to have a mechanism similar to the posttranscriptional gene silencing (PTGS) observed earlier in plants and has since been observed in a variety of eukaryotic organisms. Studies of this phenomenon in Drosophila, involving an insect that is similar in many ways to mosquitoes, have been particularly valuable in guiding our studies. Some of those findings helped to explain what triggers virus interference in mosquitoes. When SINV replicates, it forms a dsRNA intermediate. Thus, in our perplexing control experiments, a dsRNA version of the DENV prM insert formed and interfered with the DENV replication within recipient mosquito cells.

**Harnessing RNA Interference to Break Viral Transmission by Insects**

RNAi is part of an invertebrate mechanism for protecting cells against dsRNA or aberrant
mRNA. In vertebrate cells, dsRNA molecules that contain more than 30 bp activate interferon-γ and related antiviral proteins. However, this innate immune response to virus infection apparently does not occur in invertebrates. Instead, a dsRNase known as Dicer recognizes and cleaves long dsRNA into small interfering (si)RNA molecules that are about 21 bp. These smaller molecules are denatured and incorporated into a multicomponent RNA-induced silencing complex (RISC) to act as guides for sequence-specific endonucleolytic cleavage of complementary mRNA (Fig. 1). An important component of the Drosophila RISC is an endonuclease called Argonaute 2. Human and other vertebrate genomes encode Dicer homologues, but they are thought to be involved in producing microRNA (miRNA) from endogenous transcripts. These miRNA molecules function during development and also regulate translation. Human silencing complexes can incorporate endogenous miRNA or exogenously formed siRNA.

We infected mosquito cells with a series of engineered versions of SINV—to define the polarity, size, optimum time of expression, and location along the DENV genome of the RNA fragments that trigger viral interference in those recipient cells. In addition, we transformed a mosquito cell culture line (C6/36, A. albopictus) with a plasmid that produces an inverted-repeat transcript derived from the prM gene of DENV; it forms a 290-bp dsRNA. The transformed cells are permanently resistant to DENV-2 challenge, and they contain both sense and antisense siRNA with prM sequences. After these cells are infected with DENV-2, neither viral RNA nor viral antigens accumulate. These findings in cultivated cells indicate that intact mosquitoes might also be genetically manipulated to induce stable resistance to arboviruses. Recognizing that mosquito cells apparently contain all the machinery in the RNAi response pathway, we tested whether naturally occurring RNAi is a broadly acting, antiviral defense mechanism. As part of this test, we infected mosquito cell lines with arboviruses from the families Flaviviridae, Togaviridae, and Bunyaviridae and then used Northern blot hybridizations at intervals to analyze virus RNA-derived siRNA molecules. Although all these arbovirus infections elicited virus-specific siRNA molecules, the level and timing of their appearance varied widely. In particular, we did not detect siRNA in SIN-TE3/2J-infected cells until about 48 hours postinfection (PI). As the level of siRNA increases to a maximum about 120 hours PI, the amount of virus mRNA decreases dramatically, suggesting that RNAi modulates arbovirus infections in mosquitoes. However, the burst of virus RNA synthesis in mosquito cells immediately after an alphavirus infects them apparently overwhelms that RNAi machinery, thus allowing the virus to become established.

The DEN virus, in contrast, elicits very little siRNA production, suggesting a different mode of RNAi evasion, possibly by sequestering the
dsRNA replication intermediate. Moreover, some arboviruses might encode a suppressor of RNAi, as occurs with some plant viruses.

**Harnessing RNAi Requires Rebalancing Innate Immune Responses**

Thus, although RNAi certainly is part of the mosquito innate immune response to arbovirus infections, we must bypass the viral counterdefenses if we are to tip the balance in favor of interference and use this phenomenon to control dengue disease. Specifically, using RNAi to develop resistant mosquitoes likely will require enhancing the production of siRNA early during the viral replication cycle and in the right host tissues.

To identify the molecular components of the mosquito RNAi pathway, we took advantage of published information about the genome sequence of *Anopheles gambiae*, the most important vector of malaria. Using the nucleotide sequences of the *Drosophila dicer* and *argonaute* gene families as BLAST queries against this database, we identified two *Anopheles dicer* (*AgDcr*) and five *Anopheles argonaute* (*AgAgo*) genes.

To verify their roles in RNAi, we infected adult female mosquitoes with the only arbovirus known to be transmitted by *Anopheles gambiae*, the alphavirus O’nyong nyong virus (ONNV). After ONNV infects *A. gambiae* mosquitoes, it replicates and disseminates through their tissues over a nine-day course. However, even this moderate infection is dramatically reduced when we coinject dsRNA derived from one of the ONNV nonstructural genes along with intact ONNV.

In contrast, when the expression of AgAgo2 is knocked down by coinjecting AgAgo2 dsRNA simultaneously with virus, ONNV spreads to virtually all tissues of the mosquito within three days (Fig. 2), indicating that this gene product ordinarily interferes with arbovirus replication. Viral replication is also enhanced after knockdown by cognate dsRNA of AgDcr1, AgDcr2, and AgAgo3, suggesting that these three proteins also participate in the mosquito RNAi pathway. We later used the more recently available *A. aegypti* genome database to identify homologous genes from the *dicer* and *argonaute* families in the DENV vector.

**Enhancing RNAi Can Render Mosquitoes Resistant to Dengue Virus**

With this knowledge of how to enhance RNAi in *A. aegypti*, we renewed our efforts to render
them resistant to DENV. Thus, we transformed A. aegypti embryos by injecting a nonautonomous transposable element that expresses an inverted repeat RNA that derives from the DENV2 prM gene and is under control of the midgut-specific carboxypeptidase A (AeCPA) promoter. With that promoter in place, mosquitoes express the 578-bp dsRNA product after they imbibe a bloodmeal. When one transgenic mosquito family, designated Carb 77, was challenged with DENV2 contained in an artificial bloodmeal, dsRNA was expressed, interfering with viral replication and thereby keeping the virus from disseminating to the host salivary glands (Fig. 3). Small interfering RNA derived from the prM gene is present in midguts of Carb 77 mosquitoes after they receive a bloodmeal, and the resistance phenotype is lost when the RNAi pathway is interrupted by injection of dsRNA from AaAgo2. In other words, the RNAi response is responsible for DENV2 resistance. Thus, we can genetically manipulate A. aegypti to enhance RNAi and thereby greatly reduce the competence of such mosquitoes to serve as a vector for DENV2.

We are further optimizing our genetic construct to express dsRNA from the highly conserved DENV NS5 gene, and plan to use it to target all four DENV serotypes. We are also evaluating promoters that will act specifically in additional critical tissues such as the fat body and salivary glands. Further, we are studying the mechanisms of viral counterdefenses to the mosquito innate immune response, and we are developing mosquito population replacement strategies to follow when introducing virus-resistant mosquitoes in DENV-endemic regions as a way of reducing the global dengue disease burden.

ACKNOWLEDGMENTS

This article expands on a talk given during the 2006 ASM Biodefense Research Meeting in the Symposium “Vector, Food, and Waterborne Transmission.” We are grateful to Dr. Kimberly M Keene for photomicrographs of ONNV-infected A. gambiae. Our research is funded by NIH grants AI34014 and AI48740 and from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health Initiative.

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