New Weapons in an Ancient War: Fighting Malaria with Recent Research on the *Anopheles* Vector and the *Plasmodium* Parasite

by

STARTING THE WAR: THE CHARACTERS

Malaria

Malaria is a potentially fatal parasitic disease caused by unicellular protozoa of the genus *Plasmodium*, transmitted between hosts by mosquitos of the genus *Anopheles*. The name derives from "mal aria" or "bad air," as it was once thought that the disease was caused by fetid marshes. It is endemic in 91 predominantly tropical countries (many of which are among the world's poorest), putting 40% of the world's population at risk for malaria infection. There are 300 million cases of acute malarial illness each year and at least 100 million deaths. One quarter of the yearly dead are children under the age of five; every 12 seconds a child dies of malaria (Spielman & D'Antonio, 2001). In Africa, malaria accounts for one third of all hospital admissions, and 90% of the world's malarial deaths occur in sub-Saharan Africa, where the prevalence of the most virulent type of malaria in relation to the prevalence of other strains is highest.

Human symptoms of malaria infection typically appear within 9-30 days of infection. Common complaints associated with the disease are irregular high fevers, headaches, aches and pains all over the body, nausea, diarrhea and abdominal pain. Without treatment, deterioration into anemia, jaundice, organ failure, convulsions, shock and/or unconsciousness can occur, followed by persistent coma and then death (Spielman & D'Antonio, 2001).

A summarized history of malaria illuminates the undeniable fact that the proportions of the current epidemic have been at least partly caused by serious regressions in progress towards understanding and controlling the epidemic. Fossils from 30 million years ago show the presence of *Plasmodium* inside *Anopheles*. Hippocrates is responsible for the earliest written descriptions of the illness, the ancient Romans implemented a relatively effective measure against it by draining stagnant water, and in the 1600s Jesuits used a tree bark containing quinine, an anti-malarial drug, to treat it. Not until 1889 was the protozoan cause of malaria established; a few years later, the *Anopheles* vector was identified. The push for the development of

malaria cures began in the 1920s and produced chloroquine in 1943; the year before that, the insecticide dichlorodiphenyltrichloroethane (DDT) came into existence in its current form. The remainder of the 1940s was subjected to the dream of global malaria eradication, in the forms of widespread use of cheap and then effective treatment drugs, insecticide-soaked mosquito nets, DDT, paraffin to coat marshes and stagnant water drainage efforts. Subsequent decades warned of the deterioration of the world malaria situation into its current state when DDT resistance in *Anopheles*, chloroquine resistance in *Plasmodium* and the harmful effects of DDT on human and environmental health were observed (Spielman & D'Antonio, 2001).

Today, malaria eradication is not viewed as a realistic goal for the foreseeable future. Trends in vector and parasite resistance to insecticides and treatment drugs are worse than ever before. Overpopulation, poor health care, limited resources and poverty in malaria endemic areas are further complicated by the increasing emergence of high risk populations such as minorities, foreigners without immunity, marginalized populations and refugee communities. And inevitably, social, economic and political factors convolute whatever attempts at malaria control there are.

Though however bleak this situation may look, advances in scientific technology are providing new possibilities for potentially effective methods of malaria control. Within the past year, the complete genomes of an *Anopheles* species, a *Plasmodium* species and the human species have been sequenced, and many new molecular research and manipulation techniques have become available. This is incredibly exciting for everyone involved in malaria research, as progress away from the old failed attempts at disease eradication can continue, and new possibilities for malaria control can be fostered.

Anopheles

The word "mosquito" is Spanish for "little fly." Mosquitoes, being true flies (they possess one pair of frontal true wings and one pair of hind wings modified into halteres for balance), belong to the taxonomic order Diptera, family Culicidae, and are divided into several genuses and more than 3500 different species. They inhabit virtually every ecosystem found on Earth, from the equator to the Arctic to high in the Himalayas, though some environments are clearly more favorable to their survival and success than others (Morel et al., 2002).

One of the common characteristics to all of these populations is the necessity for water to breed on (but lending to their environmental adaptability is their ability to utilize a wide variety of aquatic environments; the source of water can be permanent or transient, fresh or brackish, stagnant or even slightly circulating). A gravid female mosquito alights on the water surface, and deposits approximately 300 elliptical eggs between

her hind legs, fertilizes them with stored sperm, and arranges them standing upright in a floating canoe-shaped raft (Spielman & D'Antonio, 2001).

After two days, the enclosed embryos burst out into the water head first as segmented larvae, with whiskered heads for feeding hanging into the water and tails equipped with air-breathing apparatuses extending up to the surface. Metamorphosis into the adult form is through stages of voracious, carnivorous larval feeding and growth, cuticle shedding to expose progressively larger larval cuticles and finally the pupae (non-feeding, air-breathing, comma-shaped and extremely motile), and then cuticle shedding again to release the adult. This whole cycle takes one or two weeks, varying mainly by temperature and species (Spielman and D'Antonio, 2001). For the first few days of their lives, both sexes of adult mosquitoes feed solely on plant sugar sources, and when they are physiologically prepared for mating, the male deposits his sperm with the female and flies off to mate again, while the female stores the sperm as her only male gamete source for the many fertilization events she may undergo.

At this point, the one thing the female mosquito must still acquire to reproduce is a concentrated nutrient source. For most mosquitoes, the blood meal satisfies this criterion. There are some examples of mosquito reproduction without blood meal ingestion, but these subspecies of mosquitoes are restricted to larval development in nutrient-rich waters and/or one reproductive event per lifetime that produces few eggs. The few micrograms of blood she ingests are three times her own body weight of one of the most nutrient-rich sources in nature (Morel et al., 2002). To access the blood meal, the mosquito alights on an organism of her choosing and uses two pairs of parallel carving-knife-like fixtures from inside her proboscis to slice through the skin of the host. She then begins probing for a blood vessel with her proboscis, withdrawing and re-pricking up to 20 times until a vessel is located. Then one hollow proboscis tube withdraws blood as the other injects saliva, the active components of which are anti-hemostatic and anti-inflammatory compounds to reduce blood clotting and pain reception in the host. A period of digestion follows the blood meal, after which reproduction is possible (Spielman & D'Antonio, 2001).

Mosquitoes are parasitized by a variety of pathogens. The female mosquito's need for a blood meal to successfully reproduce has created an ideal vector for protozoa (such as Plasmodium), filarial worms (nematodes which can invade the human lymph system) and arboviruses (inclusive of Dengue fever and encephalitic viruses), all of which use mosquitoes as vectors for their spread among a variety of vertebrate hosts. To map mosquito population distribution worldwide by species is to map the prevalence of vector-borne diseases. The coincidence of geographical patterns of malaria infection and *Anopheles gambiae* population distribution can be used as an example: there is species-specific affinity observed between the four

Plasmodium and over 60 *Anopheles* species; the mosquito species of most interest and concern is *Anopheles gambiae* because of the extent to which it has been studied, its resistance to insecticides, its affinity for the human host, and most importantly its ability to carry all four species of *Plasmodium* and its widespread distribution (in many areas through especially vulnerable human populations)(Morel et al., 2002).

Because of its life history, the mosquito has secured a reputation as a double-edged sword. While it is a possible ecological necessity, it is also one of the most dangerous and reviled organisms on earth. In terms of the malaria epidemic, which would not exist as we know it if not for *Anopheles*, the mosquito is clearly a major factor to be considered. Many of the molecular mechanisms underlying the life cycle and environmental interactions of the mosquito (especially with *Plasmodium*) have already been detailed, and a great deal of mosquito-focused research is currently being conducted. Managing the epidemic by mosquito control is viewed as one of the more hopeful possibilities.

Plasmodium

Malaria in humans can be caused by any of four species of *Plasmodium* (*falciparum, vivax, ovale and malariae*), which are all carried by *Anopheles* mosquitoes; these four species differ at least in their geographical distribution and morphology (Zheng, 1997). In its complete life cycle from one vertebrate host to another via mosquito vector, *Plasmodium* passes through a series of developmental stages that differ from one another in their morphology, protein expression and cell division status. *Plasmodium* predictably and precisely regulates the transitions to and from each developmental stage by maintaining tight control over gene expression, taking cues from both external and internal signals (Doerig, 1997).

In the vertebrate host's bloodstream, haploid *Plasmodium* gametocytes circulate within red blood cells. Minutes after mosquito ingestion and midgut entry, emergence from vertebrate red blood cells and differentiation into male and female gametes occurs (Sinden, 2002). The female gametocyte becomes a single macrogamete, to be fertilized by one of the eight flagellated male microgametes from each male gametocyte. The resulting diploid zygote differentiates within 15 hours of the bloodmeal into a motile ookinete and crosses two physical barriers in the midgut of the mosquito, the peritrophic matrix and the midgut epithelium, to reach the hemocoel (mosquito body cavity) and settle into the basal lamina (a layer of the gut wall) as a stationary oocyst. The oocyst rapidly divides over the next 7-30 days (depending on species and temperature) as it grows and secretes a cyst wall through which nutrient exchange occurs (Sinden, 2002). The products of these cell divisions, motile sporozoites, emerge from the oocyst and contact many mosquito cell and tissue types in their journey to the salivary glands. Upon the mosquito's next bloodmeal, they are injected into their new

vertebrate host's bloodstream.

While one single sporozoite may be capable of initiating a malaria infection in the host, the number actually injected can be in the thousands. Within a few minutes, hepatocyte (liver tissue cell) invasion occurs, and the asexual phase of the *Plasmodium* life cycle begins (Doolan & Hoffman, 1997). The mononucleated sporozoite multiplies asexually in hepatocyte cytoplasm, and each sporozoite undergoes nuclear division to produce a multinucleated schizont. Each schizont ruptures and releases mononucleated merozoites which invade erythrocytes. (It is interesting to note that the length of time spent in the liver stage of the life cycle and the total number of merozoites released is determined by the specific *Plasmodium* species, involving less time (5 days where 15 is possible) and producing more merozoites (30,000 where 2,000 is possible) in *Plasmodium falciparum*, the species responsible for the most virulent form of malaria (Ghosh, 2002). Within host erythrocytes, merozoites develop into multinucleated schizonts, and eventually the schizont merozoites are released by erythrocyte rupture (Sinden, 2002). These erythrocytic rupture cycles will recur until immune response, chemotherapy or host death occurs, though by the third generation of merozoites, some alternative differentiation into gametocytes instead of schizonts also occurs within the erythrocytes. Erythrocyte rupture and release of these gametocytes into the host bloodstream enables the mosquito vector to spread the malaria infection to another host (Stefan et al., 2001; Zheng, 1997).

FIGHTING THE WAR: CHARACTER CONFLICT

The success of a vector borne disease depends on the pathogen establishing a niche in a vector life cycle. In the case of malaria, a variety of molecular interactions between *Plasmodium* and *Anopheles* are necessary for successful malaria transmission, as *Plasmodium* must overcome a variety of physical and chemical barriers within the mosquito in passage between vertebrate hosts (Doerig, 1997). Recent developments in molecular technology, such as the ability to culture *P. falciparum in vitro* and the now complete sequences of the *P. falciparum* and *A. gambiae* genomes, along with a better understanding of the chemical signaling pathways which regulate passage to and from each *Plasmodium* developmental stage and the ways in which *Anopheles* responds to infection, can help create complete understanding of the molecular processes involved in vector-parasite interactions which may make controlling the malaria epidemic possible (Warburg & Schneider, 1993).

Plasmodium artillery

Understanding the ways in which the *Plasmodium* parasite navigates the inhospitable body of the

Anopheles vector to get from host to host in order to propagate may lead to the development of new strategies to control the malaria epidemic. Combating *Plasmodium* during its phase of vector inhabitation is a new and exciting prospect, in some ways more feasible than targeting it during its phase of human inhabitation.

Crossing physical barriers

The first physical barrier *Plasmodium* must cross in its vector-borne phase is the peritrophic matrix (PM). *Plasmodium* enters *Anopheles* when the mosquito takes a bloodmeal which is digested in the mosquito midgut; within 12 hours of bloodmeal ingestion, epithelial cells of the midgut lining have secreted the molecular components of the PM, an epithelial lining (Warburg & Schneider, 1993). The PM has been shown to contain the protein chitin, which must be destroyed by the ookinete for successful passage through the epithelial cells to the hemocoel (Ghosh et al., 2002). Ookinetes punch holes in the PM by producing the protein chitinase, which degrades chitin.

Surprisingly, the chitinase produced by *Plasmodium in vitro* is inactive until exposure to the protease (enzyme for protein breakdown) trypsin, which is produced by mosquito midgut cells for use in blood meal digestion (Doerig, 1997). Several experiments with trypsin and chitinase have demonstrated the effects of these molecules on *Plasmodium*. Although trypsin has been implicated in both apoptosis (death) of ookinetes and earlier reduction of initially invading female gametocyte numbers (of which only ten percent typically successfully form macrogametes), elevated trypsin levels also enhance oocyst formation in the hemocoel. Protease inhibitors can block oocyst formation completely (possibly indicating the effectiveness of the PM as a physical barrier to *Plasmodium*), though blocking chitin synthesis does not necessarily lead to higher oocyst numbers in the midgut epithelium (demonstrating that the PM is not the principle barrier to *Plasmodium*)(Sinden, 2002; Zheng, 1997). Increasing ability to understand and regulate elements of mosquito-parasite interactions, such as chitin, chitinase and trypsin, could lead to *Plasmodium* blocking methods involving manipulation of those chemicals within the mosquito.

The second physical barrier *Plasmodium* encounters after crossing the PM is the midgut epithelium. Ookinetes must cross this barrier to settle in the hemocoel as oocysts, but it is not known whether the cell barrier is crossed primarily inter- or intracellularly (as both methods have been observed), or which of the three cell types present in the midgut epithelium (digestive, endocrine or regenerative) is preferred as a crossing target (Bonnet et al., 2001). It has been shown, however, that epithelial invasion comprises two distinct steps: recognition and adhesion to the midgut surface (inhibition of this step by flooding with

competitors for cell adhesion has been demonstrated) followed by penetration. Though prevention of midgut epithelial crossing by *Plasmodium* has been experimentally achieved, it is still unclear precisely which cell receptors and adhesion molecules are essential to epithelial invasion by ookinetes (Shahabuddin et al., 1998). One possible mechanism, discussed below, is implicated in epithelial crossing by sporozoites as well.

Sporozoite development and invasion

Plasmodium sporozoites invade both salivary glands in mosquitos and hepatocyte cells in vertebrates. They are produced from stationary oocysts, and are motile by the use of actin/myosin (protein) motors. Little is known about the molecular mechanisms of the morphological reconfigurations (such as membrane reformation) involved in *Plasmodium* sporozoite formation, but many genes and gene products and signaling pathways have been identified in the two aforementioned sporozoite invasions, which appear to be quite similar to each other.

The major surface protein and antigen of sporozoites has been shown to be circumsporozoite protein (CSP), and the disruption of CSP formation by gene manipulation blocks sporozoite formation (and manipulation of a second gene has been shown to completely prevented sporozoite formation as well (Stefan et al., 2001).

Once differentiated, sporozoites either actively migrate from the hemocoel past several tissue types and to the salivary glands or else are passively transported and then bind selectively to the glands (Sinden, 2002). Arguing the case for passive hemolymph (fluid of the mosquito's open circulatory system) transport is the demonstration that CSP is an effective adhesive molecule to salivary glands but not to other mosquito organs, along with motility/adhesivity experiments with other sporozoite surface proteins such as TRAP (Stefan et al., 2001).

The role of the sporozoite-specific transmembrane protein thrombospondin-related anonymous protein (TRAP) in sporozoite motility and cell invasion has been assessed by Matuschewski et al. (2002). In its extracellular domain TRAP has two types of adhesivity modules (known as A-domains and thrombospondin type I repeats (TSR), both also found in CSP) which are known to participate in mammalian protein adhesion systems. The discovery and location of *Plasmodium* gene sequences for both adhesion modules allowed creation of phenotypically identical but molecularly altered A-domain and TSR modules with no adhesive abilities. Insertion plasmids (small DNA molecules sometimes used in genetic manipulation experiments by insertion into an organism's cells and incorporation into the cells' genomes) with mutated adhesion module sequences were successfully incorporated into *Plasmodium* genomes, and wild type, A-

domain mutated, TSR mutated and dual-module mutated *Plasmodium* oocysts were monitored in their progress through hemolymph to salivary glands. Results were that the parasites with mutations in both adhesion modules were 95% reduced in salivary glands. Lesser reductions occurred in those subjects with mutations in only one of the two modules. Motility was shown to be unaffected by the mutations, as wild type and mutant strains displayed reasonably identical gliding motions on albumin (protein)-coated slides. One hypothesis based on these results is that the two adhesion modules play their role in gland invasion by acting as ligands (molecules which bind selectively to other molecules) for host receptors on target cells (Stefan et al., 2001). Similar experiments with sporozoite invasion of the rodent liver produced similar conclusions about the role of TRAP in sporozoite success.

Other experiments with sporozoites in vertebrate hosts with other cell types for sporozoites to cross such as the cells of capillary walls (*Plasmodium* has been found in the blood streams of mice that have only undergone skin probing by an infectious vector) have clearly demonstrated the role of CSP in motility by using anti-CSP antibodies to block cell invasion. Additionally, cytoplasmic CSP trails left behind by sporozoites after intracellular membrane transversal have been observed (Stefan et al., 2001; Zheng, 1997).

It may be interesting to also note that a protein known as CSP and TRAP related protein (CTRP) expressed by the ookinete has been associated with midgut epithelium invasion, though since CTRP gene disruption also blocks motility, it is still undetermined whether blocking production of this protein inhibits epithelial invasion by blocking the motile, invasive or midgut ligand properties of CTRP (Sinden, 2002).

Understanding the significance of sporozoite-characteristic molecules may prove to be an excellent tool for controlling malaria. For example, CSP's antigenic properties have been used to induce antibody resistance to sporozoites in mosquitoes. Mosquitoes genetically altered to produce CSP antibodies have nearly completely blocked sporozoite invasion of their salivary glands, with *Plasmodium* inhibition rates approaching 100% (Ghosh et al., 2002). This is one demonstration of the importance of transgenic mosquito research. More evidence supporting the assertion that molecular sporozoite analysis may lead to novel methods of malaria control is a recently quantified protein number: all told, 1049 proteins have been found (through proteomic analysis) to be expressed by *Plasmodium* sporozoites. Of these, 513 are expressed uniquely by sporozoites and in no other phases of the *Plasmodium* life cycle (Stefan et al., 2001). This is of considerable interest because the gene products which prove to be the most useful targets in malaria control efforts will likely be the ones that are the most organism- (and ideally, developmental phase-) specific.

Signal transduction

Examination of the transitions between life stages of *Plasmodium* begins to reveal the complex molecular processes that must be precisely regulated in order for successful development to occur in the right places and at the right times, all within potentially inhospitable environments. For *Plasmodium*, as for other organisms, developmental events can be viewed as little more than a series of signal transduction pathways (processes by which cells and/or their components communicate, often with the use of messenger molecules such as hormones and proteins) that activate and repress each other to regulate genes that manufacture proteins and/or regulate other genes.

Doerig (1997) detailed some recent research on signal transduction pathways. There are two main approaches to studying signal transduction in *Plasmodium*: to search for signaling pathways known to exist in other organisms or to search for signaling pathways in *Plasmodium*-specific processes, such as sporozoite invasion of mosquito salivary glands. (At this point it is important to realize how logical the first approach is, due largely to extensive research on the fruit fly *Drosophila melanogaster*. There is a wealth of information on the genome, developmental stages and life cycle of *D. melanogaster* useful for comparative research on a huge number of molecular processes observed in many other organisms.) A goal of using these two approaches in tandem is to find the similarities and differences in signal transduction pathways between parasite, vector and host (now more feasible with the genome sequencing of *P. falciparum*, *A. gambiae* and *H. sapiens* completed). Understanding these similarities and differences will make interference in signaling pathways for the purpose of *P. falciparum* control without effect on *A. gambiae* or human signaling pathways, for example, possible. Here are some notable signal transduction pathways observed in *Plasmodium*:

1) The cAMP-dependent pathway. Cyclic adenosine monophosphate (cAMP) activity is controlled by cell membrane-bound surface receptors, as these receptors respond to the binding of extracellular proteins known as G-proteins to adenylyl cyclase (an enzyme that catalyzes the formation of cAMP). cAMP binds to the catalytic though currently inactive protein kinase A (PKA) subunit, activating the subunit and therefore the kinase function of PKA so that a wide variety of substrates such as gene transcription factors can be phosphorylated (activated by the addition of phosphate groups), and gene expression can be altered. The cAMP-dependent pathway is one common to many eukaryotes, and has been associated with a variety of gene expression events. However, in *Plasmodium*, the pathway has been shown to be different in two ways from homologous pathways in mammals: the substrate of *Plasmodium* adenylyl cyclase (manganese) differs from that of mammalian adenylyl cyclase (magnesium), and in *Plasmodium*, unlike in mammals, regulation of the G-protein has been shown to have no effect on the rest of the signaling pathway. In *Plasmodium*, the cAMP-dependent pathway is involved in the sexual differentiation of gametes, and PKA activity has been observed at

lower levels in *Plasmodium* clones incapable of sexual differentiation. A gene encoding PKA has been identified in some *Plasmodium* species.

2) The PI pathway. Surface receptor activation causes the enzyme phospholipase C (PLC) to hydrolyze a phosphatidylinositol (PI) molecule into two molecules, one of which releases calcium stored in the cell's endoplasmic reticulum, and the other of which can phosphorylate substrates. Again, this is a common and important pathway in cellular processes in many other organisms. In *Plasmodium*, this pathway has been linked to the exflagellation of gametes (the extrusion of 8 microgametes from what was the male gametocyte, after division of the gametocyte into 8 daughter nuclei each with a flagellum for motility). The PI pathway is also necessary for erythrocyte invasion by merozoites, as a protease bound in the merozoite by a PI anchor must be released and solubilized for merozoite entry.

3) Calcium signaling. Sometimes associated with the PI pathway (which involves calcium release), the role of calcium in cell signaling is once again common to a multitude of signaling pathways in many organisms. An alternative (to the endoplasmic reticulum) calcium source is the extracellular environment, from which calcium can enter the cell through transmembrane channels, often mediated by the calcium-binding protein calmodulin. Calcium has been shown to play roles both in *Plasmodium* gametogenesis and in the invasion of erythrocytes by merozoites (in an unsurprising parallel to the necessity of calcium for eukaryotic fertilization, which also involves membrane lysis and content exocytosis), and a *Plasmodium* calmodulin homologue has been isolated, along with two genes for calcium-dependent protein kinases, one of which may be a feature of the merozoitic cell surface.

Anopheles artillery

Research on the immunity *Anopheles* displays to *Plasmodium* (now heavily reliant on the completed genome sequences of *A. gambiae* and *P. falciparum*) has brought to light and will further elucidate specific aspects of the mosquito response to parasitization. This will allow artificial induction of even more effective immune responses by cloning, enhancing and/or creating genes that encode specific immunologically advantageous proteins.

Upon *Plasmodium* invasion, a number of immunological genes are activated in the mosquito. Monitoring of this genetic upregulation has shown both spatial coincidence with *Plasmodium* transversal of epithelial barriers and temporal coincidence with peaks in parasite death trends (Dimopoulos, 2001). The majority of the mosquito response to pathogens is mediated by the hemocytes (blood cells) of the hemolymph, though other cells, such as those of the midgut, also display immunological properties (Richman

& Kafatos, 1995). The immunity of the mosquito comprises a number of signal transduction pathways and other cellular mechanisms, on which further research is crucial if the enhancement of mosquito defense against vector-borne disease is to be possible.

Pathogen recognition

Recent research efforts based largely on the now complete *Anopheles* genome sequence have identified 242 genes involved in mosquito immunity. There is now an extensive effort to create categories of these genes' products based on specific protein roles in the immune system (such as pathogen recognition, signal transduction and gene regulation) and to demonstrate their similarities and differences to their 185 more thoroughly researched *Drosophila* homologues (Morel et al., 2002).

While some molecular mechanisms of bacterial recognition and response in the mosquito have been defined, mechanisms of parasitic recognition and response are still largely unknown. It is possible that the processes of antibacterial peptide production in response to bacterial infection have homologous counterparts in antiparasitic gene regulation; it is also possible that antibacterial peptides play antiparasitic roles as well (some antibacterial proteins appear to also have deleterious effects on parasites)(Dimopoulos, 2001).

In *Anopheles*, at least four antibacterial peptide genes have been isolated and mapped. One of these peptides (*gambicin*) has been demonstrated to be an effective toxin against *Plasmodium* oocysts and sporozoites, while another (*cecropin*) inhibits sporozoite development and another (*defensin*) kills sporozoites (Richman & Kafatos, 1995). The possibility of antibacterial peptide action on mosquito parasites is further reinforced by the observation that antibacterial peptide gene production is upregulated after feeding to combat bacterial growth in the bloodmeal, and in conjunction, severe gametocyte population reduction (upon initial mosquito gut entry) occurs. Additional supporting evidence comes from studies suggesting that genes for antibacterial proteins such as *defensin* can be regulated by gametocyte ingestion (Tahar et al., 2002).

Parasite death

In *Drosophila*, defense mechanisms to pathogen invasion have been shown to be the results of a variety of signaling pathways usually involving serine proteases (proteins which break down other proteins), the production of which has been mapped to many genes. In *Anopheles*, several genes encoding serine proteases have been identified, the majority of which are expressed in hemocytes. A number of these genes also carry sequences for production of the enzyme phenoloxidase (PO), which is responsible for inducing one of the two known signaling pathways by which mosquitos kill internal parasites (Dimpopoulos, 2001).

In this first pathway, known as the encapsulation reaction, PO activity initiates melanin synthesis in any midgut epithelial cells in contact with *Plasmodium* oocysts, and the melanin encapsulates and infiltrates the oocysts, causing them to disintegrate (Zheng, 1997). In the second pathway, the enzyme nitric oxide synthase (NOS) is activated, also in the midgut, and toxic nitric oxide that kills parasites is produced (Dimopoulos, 2001).

An additional mosquito defense against *Plasmodium* is the destruction of zygotes by proteases; however, proteases have also been shown to hinder other defenses to parasites both by destroying ingested host blood cells that could otherwise have beneficial immunological functions within the mosquito and by activating the *Plasmodium* enzyme chitinase that degrades PM chitin. Phagocytosis (recognition and destruction of foreign objects by blood cells) by the host leukocytes (white blood cells) ingested in the bloodmeal that escape protease destruction is thought to occur in the midgut, as the number of oocysts in the epithelium has been shown to be inversely related to the number of leukocytes present in the bloodmeal (Shahabuddin et al., 1998).

Signal transduction

Besides the aforementioned serine protease signal transduction pathway responsible for the melanin encapsulation of oocytes, there are other important signaling pathways associated with the recognition of and response to pathogens. The best-studied is the Toll pathway, which has been extensively researched in *Drosophila* for its involvement in developmental and immune response events (Dimopoulos, 2001). Four of the 11 known *Anopheles* Toll genes are *Drosophila* homologues, indicative of both high levels of gene conservation and species-specific genome expansion by divergent evolution. The Toll pathway can be briefly summarized (like many other signal transduction pathways) as such: it is initiated by cleavage of a peptide ligand, entails a series of phosphorylation events, and concludes with the activation of numerous genes, some of which have been demonstrated to encode antimicrobial peptides (Morel et al., 2002).

Transgenic mosquitoes

Genetic modification of mosquito vectors to render them refractory to (able to resist) parasitization by *Plasmodium* has now become an attractive concept in malaria control. Some of the many indications that mosquito genome manipulation is one of the shining lights in the often bleak world of progress towards amelioration of the malaria epidemic are the completed *Anopheles* and *Plasmodium* genome sequences, the new technologies for genetic manipulation, our exponentially increasing understanding of the molecular interactions between malaria vectors and parasites, our awareness of limitations to malaria control within the human host, the increasing unacceptability of using insecticides and the wide range of laboratory procedures permitted for use on non-human subjects.

Recombinant DNA technology

To create a transgenic organism, a gene appropriate for insertion into the wild type genome must first be identified and located. To this end, experiments such as the ones resulting in the previously discussed observations on molecular interactions between vector and parasite can demonstrate gene desirability where refractoriness is the goal. One powerful experimental tool for screening gene activity is the phage display library. Each library consists of many bacteriophages (viruses which infect bacteria, bacteriophages are easily genetically manipulated due to their simple DNA structure), each of which displays one specific surface protein. The different phage types are created by DNA insertion of the genes for the desired proteins. They can be used experimentally to determine the effects of their surface proteins and therefore the genes that manufacture those proteins (Ghosh et al., 2002).

To effectively evaluate most genetic experiments, quantity of information alone dictates the necessity of a precise organization system for the data. Quantitative trait loci (QTL) mapping using a high-resolution image of the genome establishes a road map of the genome onto which specific gene sequences can be mapped (Atkinson & Michel, 2002). With the *Anopheles* genome now entirely sequenced, it is now theoretically possible to establish the location of every known gene in the genome and deem every bit of the genome part of a gene or "junk DNA" (non-coding DNA sequences). If a foreign gene is identified as a desirable component of a genome, it can be incorporated into that genome. One method of DNA delivery into insect embryos is direct microinjection (now by a patented gene delivery system known as the Gene Gun). In this procedure, a large amount of DNA is physically injected into a cell. Results are usually disappointing, with DNA degradation and/or limited DNA uptake often occurring. Better methods of recombining DNA involve vectors (DNA molecules into which desired foreign DNA can be inserted that are capable of replication

in appropriated cells). Vector possibilities include a number of transposable elements and viruses.

Transposable elements (or "transposons" or "jumping genes") are genetic elements that can copy and insert themselves around the genome. The best example is the famous P-element of *Drosophila*, the first vector identified as having introduced foreign genes to a genome. The P-element is thought to have infiltrated cells of the *Drosophila* germ line within the past 100 years, and its potential for transposon use is demonstrated by the fact that it is now found in all wild *Drosophila* populations worldwide. Unfortunately, the P-element is probably an effective transposon only in *Drosophila*, but four transposons effective for mosquito transformation have been found (nicknamed *Hermes, piggyBac, Minos and Mos1*). Of these four transposons, *piggyBac* has been found to be most effective; *Hermes* often integrates into the mosquito genome incorrectly, even though its integration rate is considerably higher than *Minos* and *Mos1*, which are often not incorporated into the genome at all (Atkinson & Michel, 2002).

Viral vectors are another vehicle of DNA delivery. They are natural infectious agents, so the problem of mediating DNA delivery is solved, but other problems present themselves within this vector system. Viral tropisms (affinities for specific cell types) can make the selection of a good vector difficult, though recent methods for altering viral envelope proteins and therefore viral tropisms can ameliorate this limitation. Viruses impose size limitations on DNA inserts due to the short lengths of their own DNA, whereas transposons do not impose absolute outer limits (though genome incorporation of smaller sequences occurs with a higher success rate)(O'Brochta & Atkinson, 1997).

Once a transgene has been inserted into a genome, it requires a specific promoter to activate it at the appropriate time. A good example of this promoter specificity requirement would be a transgenic mosquito that expresses large quantities of the protein salivary gland and midgut peptide 1 (SM1), which is known to bind to specific regions of mosquito salivary glands. Failure of sporozoites to invade salivary glands when competing with phages displaying SM1 surface peptides has been demonstrated; it is hypothesized that this is due to competitive sporozoite-versus-SM1 binding to salivary gland receptor molecules (Ghosh et al, 2002). So a mosquito transgenic for heavy SM1 expression would ideally have a promoter to activate the transgene at the time when sporozoites attempt salivary gland invasion. Promoter timing considerations (as opposed to temporally indiscriminate promoter activation) are important because of the possibility of a fitness load on an organism with constant transgene transcription (Moreira et al., 2002). This occurs if the transgene product is actually deleterious to the organism, such as would be the case for a transgene to enhance melanin encapsulation of *Plasmodium* ookinetes in the *Anopheles* midgut. Melanin is actually toxic to the mosquito, and in wild type encapsulation reactions it is present only in small quantities at specific times. Ongoing

research to identify transgene promoters that are products of genes expressed during time windows of desired transgene expression has revealed several candidates. For transcribing transgenes against *Plasmodium* in the hemolymph of the mosquito following a bloodmeal, the *defensin* protein is a good promoter candidate. Its expression in the hemolymph is induced by blood ingestion, and it remains there as a stable protein for up to three weeks after a bloodmeal. Another candidate is the promoter utilized by a gene for a PM protein, also expressed in response to a bloodmeal (Moreira et al., 2002).

A good gene-delivery system cannot be developed and cannot be effective without a method of quickly identifying successfully genetically manipulated individuals. So a last important tool of transgenic technology is that of the genetic marker, or a noticeable trait whose appearance is induced upon activation of a transgene if the transgene effect itself is not immediately noticeable enough. Candidates for genetic markers include recessive gene alleles (alternative gene forms) for phenotypic mutations, such as the white-eye color mutation in some *Anopheles* species; however, problems with inducing recessive alleles as genetic markers, such as limited availability of such alleles or the mistake of altering gene expression beyond just the phenotypic variation, are hard to avoid. For unknown reasons, inducing the white-eye color mutation in an *Anopheles* mosquito also causes the mosquito to poorly support *Plasmodium* development, rendering the mutation virtually useless as a genetic marker (Moreira et al., 2002). One genetic marker currently being used in transgene experiments is the jellyfish gene for the protein green fluorescent protein (GFP), which causes the transgenic organism to produce a green pigment, although its effectiveness in transgenic mosquito experiments is limited due to the opaqueness of the mosquito cuticle. More appropriate as markers for mosquitos are insecticide resistance genes, which confer resistance to specific chemicals on those organisms successfully engineered. Where resistance genes are used as markers, only the mosquitos that have not been transformed will die upon exposure to insecticides.

Experiments

There have been many attempts to create transgenic mosquitoes that are refractory to malaria. Moreira, et al. (2002) has detailed some of the experiments to identify genes possibly desirable for addition to a wild-type mosquito genome to create a malaria-resistant strain. In these examples, the important principle is that the fitness of the mosquito is not compromised by the action of the gene that either blocks *Plasmodium* development or directly kills the parasite. The genes investigated for transgenic use produced a number of proteins in mosquitos which had a variety of effects on *Plasmodium*:

1. *Defensin.* The protein *defensin*, normally produced upon bloodmeal ingestion and active against bacteria for the next 22 days, had an indeterminate effect on sporozoites.

2. CSP antibodies. CSP has been identified as the major surface protein of the sporozoite and as a requirement for salivary gland invasion. When a viral vector was used to introduce a transgene for the production of a CSP antibody, sporozoite invasion of the salivary glands was inhibited from 96.8-99.9%.

3. SM1. A synthetic gene for SM1 (a peptide that has been shown to compete with sporozoites for salivary gland binding) was used to create a transgenic mosquito that expressed SM1 in midgut cells following a bloodmeal. Oocyst formation was inhibited 69-95%, and malaria transmission ability of the vector was severely impaired.

4. PLA2. PLA2 is a bee venom protein that inhibits ookinete invasion of the midgut. A mosquito transgenic for the PLA2 gene both reduced oocyst formation by 87% and was displayed inhibited malaria transmission ability.

Out of the lab: feasibility and desirability

Two types of transgenic transformation are possible: that of the germ-line and that of a somatic cell. Somatic engineering targets specific differentiated cell types, and the genetic changes that result are not inheritable. Germ-line engineering targets cells before they have differentiated into adult cell types, and the genetic changes that result are inheritable. Germ-line transformation can be accomplished in two ways. It is possible to manipulate either the germ cells of an adult organism (offspring from the transgenic gametes will inherit the changes and display them in every cell) or a zygotic cell (which will develop into an organism with the changes displayed in every cell).

To create a refractory strain of mosquito, especially if they are to be released with the expectation of their dominance over wild-type mosquitos, germ-line transformation will be necessary. It is important to note that the four transgenic mosquito types previously discussed were all somatically transformed. Germ-line transformation in *Anopheles* was achieved for the first time only two years ago, with the successful microinjection of GFP genes into mosquito eggs, five years after the first non-*Drosophila* (Mediterranean fruit fly) germ-line transformation. Another non-*Drosophila* insect that has undergone successful germ-line transformation is the screw-worm fly. Transgenic screw-worm flies, modified to produce only sterile males, have been released into the wild, and there has been significant progress towards eradicating this pest.

Even though successful germ-line transformation in an *Anopheles* species has now been achieved, our grasp on this technology is still tenuous at best. The problem of identifying desirable transgenes for creation of an *Anopheles* strain refractory to malaria remains. More knowledge of the molecular interactions between *Anopheles* and *Plasmodium* is needed. And even if a good transgenic strain is developed, this may mark a point closer to the beginning than the end of the crusade to eradicate malaria by vector resistance to parasite. There are at least two major foreseeable hurdles: the feasibility of a transgenic mosquito population overtaking a wild-type population and the question of whether attempting this population transition is even a desirable prospect.

In June of 2002, an international meeting of vector ecologists convened in the Netherlands to come up with a list of criteria that they recommended be met before the wild release of transgenic mosquitoes is even considered (Morel et al., 2002). A primary concern was whether a transgenic mosquito population will be able to replace a wild one to the point that malaria control actually occurs. To overcome a wild type gene, a transgene must spread through a population at faster than average Mendelian inheritance rates. To predict the likelihood of this happening between transgenic and wild type *Anopheles* populations, gene flow patterns and their effectors, such as geographical features, seasonal effect and mate selection preference, must be understood (a pattern of random mating is not necessarily a safe assumption). It must then be hypothesized how the introduction of transgenic populations will change those natural patterns.

Transgenic mosquito introduction raises many other questions. Release of transgenic mosquitoes will not meet with success if the genetic manipulation has adversely affected the insects' fitness. Refractoriness to malaria will not spread into wild *Anopheles* populations if the natural environment compromises the refractoriness of the transgenic population. And there are dangers that engineered populations may pose. Transgenic mosquitoes refractory to malaria could prove to be more capable of transmitting alternative diseases. *Plasmodium* could mutate to better overcome new vector resistance, which could be catastrophic if there had been a window of malaria decline prior to *Plasmodium* mutation (because of elimination of accrued human immunity to malaria during that window). Transgenic organisms may be overly susceptible to dangerous genetic mutations by erroneous transposon insertion.

To further complicate these issues is a plethora of social and financial and logistical pressures. However, there are still enough scientific unknowns surrounding the distant possibility of a project to release transgenic mosquitos that research efforts to clear these limitations should continue. A tiny insect embryo injected with some DNA molecules somewhere in a laboratory may prove to be the resolution to centuries of suffering and death caused by malaria.

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