Mini review

Mosquito immune responses and malaria transmission: lessons from insect model systems and implications for vertebrate innate immunity and vaccine development

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Abstract

The introduction of novel biochemical, genetic, molecular and cell biology tools to the study of insect immunity has generated an information explosion in recent years. Due to the biodiversity of insects, complementary model systems have been developed. The conceptual framework built based on these systems is used to discuss our current understanding of mosquito immune responses and their implications for malaria transmission. The areas of insect and vertebrate innate immunity are merging as new information confirms the remarkable extent of the evolutionary conservation, at a molecular level, in the signaling pathways mediating these responses in such distant species. Our current understanding of the molecular language that allows the vertebrate innate immune system to identify parasites, such as malaria, and direct the acquired immune system to mount a protective immune response is very limited. Insect vectors of parasitic diseases, such as mosquitoes, could represent excellent models to understand the molecular responses of epithelial cells to parasite invasion. This information could broaden our understanding of vertebrate responses to parasitic infection and could have extensive implications for anti-malarial vaccine development. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Mosquito; Malaria; Vaccine; Insect; Innate immunity

1. Introduction

After a century of control efforts, vector-borne diseases continue to pose a serious threat to public health worldwide. Malaria, a mosquito-transmitted disease that was predicted nearly five decades ago to be an affliction amenable to eradication, still affects more than 300 million people each year and causes 1.5–2.7 million deaths, mostly in children under 5 years of age. The recent surge in malaria cases, the spread of multi-drug resistant parasites, and the emergence of insecticide-resistant anopheline mosquitoes has emphasized the need for effective malaria control strategies and bolstered research efforts towards the development of vaccines, drugs and mosquito control methods. This has rekindled interest in research aimed at improving our understanding of the genetic basis of vector–parasite compatibility and the biology of cellular interactions between the mosquito and the various parasite developmental stages.

In recent years, enormous progress has been made in our understanding of the molecular mechanisms mediating the physiological responses of insects to pathogenic organisms. The biodiversity of insects has provided excellent model systems that have generated complementary information. The biochemical purification of individual components from large insects such as Manduca sexta, in conjunction with the use of powerful genetic tools from Drosophila melanogaster and molecular biology analysis in many different insect species have provided new insight into the organization and regulation of the insect immune system. It is now clear that insects have evolved a pathogen recognition system that enables them to activate sophisticated networks of signaling pathways and coordinate the expression of
multiple effector genes. Insects lack the acquired immune responses of vertebrates, such as genomic rearrangement of antigen-recognition receptor molecules, clonal expansion of specific cell populations and the induction of long-lasting pathogen-specific “memory” cells. However, the regulatory pathways mediating the innate immune responses in insects have been conserved throughout evolution all the way to vertebrates, including humans. Immunological studies in Drosophila have provided new insight into the receptor molecules mediating the initial responses of the vertebrate innate immune system to pathogens (reviewed by Hoffmann et al., 1999). This is particularly important in light of the recent evidence suggesting that the interaction of microorganisms with the innate immune system generates signals which set the stage for the generation of pathogen-specific effector cells of the adaptive immune system (reviewed by Fearon and Locksley, 1996).

Our current understanding of the insect immune system is based mostly on the responses of model organisms to bacterial or fungal infections. We will use this information as a frame of reference to discuss in detail the responses of mosquitoes to bacterial and malarial infections. Future directions and the potential of insects as model systems to study the anti-parasitic responses of the vertebrate immune system will be addressed. Elucidating the molecular nature of Plasmodium recognition and the activation of specific regulatory pathways could be the key to the rational development of new adjuvants for anti-malarial vaccines. Adjuvants consist of a carrier containing a mixture of molecules of microbial origin which are necessary to elicit an effective immune response to foreign antigens. The nature of the Plasmodium molecules capable of inducing the innate immune system to generate the signals required by the adaptive immune system to confer long-lasting protection to malaria infection is not known.

### 2. Pathogen recognition and activation of the immune system

In order for an organism to mount an immune response to a pathogen it has to detect its presence and be able to distinguish it from self-tissues. The pattern recognition theory establishes that immune recognition is mediated by products encoded by the genomes of two organisms (that of the host and the pathogen) with conflicting selective pressures (Medzhitov and Janeway, 1997b). The development of a recognition system imposes a selective advantage to the host, whereas mechanisms to avoid recognition will be beneficial to the pathogen. As a result, the innate immune system of the host has evolved pattern recognition receptors (PRRs) that interact with conserved molecular structures, pathogen-associated molecular patterns (PAMPs), that are essential for survival of the pathogen. Examples of such molecules are lipopolysaccharide (LPS) and teichoic acids of Gram-negative and Gram-positive bacteria, respectively; double-stranded RNA of RNA viruses and mannans in yeast cell walls (Medzhitov and Janeway, 1997b).

This theory would then predict that PRRs are ancient molecules that arose early on and have been conserved throughout evolution. In fact, several recognition molecules that bind to β-1,3-glucan, mannan and LPS have been reported in both vertebrates (reviewed by Fearon and Locksley, 1996) and invertebrates (reviewed by Söderhäll and Cerenius, 1998). For example, a Gram-negative binding protein (GNBP) from the silk moth B. mori has sequence similarity to a glucanase polysaccharide-binding domain and is inducible by bacterial challenge (Lee et al., 1996). A GNBP homolog, isolated from the mosquito An. gambiae (AgGNBP), has been shown to be induced in response to bacterial and malarial infection (Dimopoulos et al., 1997). Recently, the cDNA of a multi-domain protein, Sp22D, has been cloned from An. gambiae. Analysis of the predicted amino acid sequence indicates that this protein contains multiple potential pattern recognition domains linked to a C-terminal proteolytic domain. Sp22D has a signal peptide followed by a histidine–proline–glutamine-rich region, two chitin-binding domains, one mucin-like region, two low density lipoprotein receptor class A-like domains, two scavenger receptor cysteine-rich domains and a serine protease catalytic domain. Based on quantitative Northern blot analysis, Sp22D is slightly up-regulated after injection of bacteria but not by sterile wounding (Maureen Gorman and Susan Paskewitz, personal communication). Sp22D has the features one would expect to find in those molecules involved in pathogen recognition and activation of signaling pathways by proteolytic processing.

Binding of a receptor to a pathogen surface can trigger different types of events. Some soluble receptors in the hemolymph agglutinate pathogens directly, while others could trigger the activation of proteolytic cascades. Interaction with membrane-bound receptors could also facilitate phagocytosis by hemocytes or activate intracellular signaling pathways. Proteolytic processing is necessary for the activation of some immune responses, such as the prophenol oxidase (PPO) cascade and signaling by cytokine-like molecules (Fig. 1).

One of the best understood regulatory cascades in arthropods, mediated by a series of proteolytic processing steps, is the activation of the coagulation response in the horseshoe crab (Tachypleus). Two recognition molecules which activate the hemolymph clotting cascade have been characterized. Factor C is a biosensor whose active form is autocatalytically generated by binding of LPS (or synthetic lipid A analogs). This clotting cascade can also be activated by β-1,3-glucan binding to factor G zymogen. Factor G is a heterodimer
Fig. 1. Schematic representation of immune responses in insects. Binding of a recognition receptor to a pathogen surface can trigger different types of events such as agglutination, phagocytosis by hemocytes or activation of proteolytic cascades. Proteolytic processing is necessary for the activation of some immune responses, such as the prophenol oxidase (PPO) cascade and signaling by cytokine-like peptides, such as *Drosophila* Spätzle.

composed of two subunits derived from separate genes. Subunit α contains the β-1,3-glucan binding site and subunit β a serine protease domain (reviewed by Muta and Iwanaga, 1996).

In *An. gambiae*, three clip-domain serine proteases cDNAs, Sp14D1, Sp14D2, and Sp14A, have been cloned from hemolymph. They have sequence similarity to the three prophenol oxidase (PPO) activating enzymes in GenBank and to *Drosophila* Easter. The mRNAs of Sp14D1 and Sp14D2 are induced by bacterial challenge, while Sp14A is repressed (Paskewitz et al., 1999; Gorman et al., 2000). These proteases could be involved in the activation of PPO or cytokine-like molecules.

### 3. The prophenol oxidase cascade

Phenoloxidase (PO) catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine and the oxidation of dihydroxyphenylalanine and dopamine to their respective quinones. The reactive quinones serve as a defense response by mediating protein cross-linking and polymerizing to form a melanin layer that immobilizes the pathogen, and they are also thought to be toxic to the invading organisms (reviewed by Söderhäll and Cerenius, 1998). Phenoloxidase is present in insect hemolymph as an inactive pro-phenoloxidase (PPO) zymogen. PPO cDNA clones have been isolated from several insect species, including *M. sexta*, *B. mori*, *An. gambiae* and *D. melanogaster* (Hall et al., 1995; Jiang et al., 1997b,a; Kawabata et al., 1995; Lee et al., 1998b; Müller et al., 1999; Fujimoto et al., 1995).

A C-type lectin from *M. sexta*, Immulectin, has two carbohydrate recognition domains and agglutinates Gram-positive and Gram-negative bacteria and yeast. Addition of recombinant Immulectin to *M. sexta* plasma stimulated PPO activation in vitro, and this effect was enhanced by the addition of *E. coli* LPS (Yu et al., 1999). A β-1-3-glucan-binding-protein (GBP), recently characterized from *M. sexta*, has sequence similarity to *B. mori* GNBP and aggregates yeast, *E. coli* and *S. aureus* in vitro at physiological concentrations. A recombinant form of GBP also activates PPO in the presence of laminarin, whereas laminarin alone does not. It has been proposed that Immulectin and GBP may undergo a conformational change following LPS or β-1-3-glucan binding, respectively, which leads to activation of PPO. However, it remains to be determined if Immulectin and GBP mediate pathogen recognition in vivo (Yu et al., 1999; Ma and Kanost, 2000). In addition to Immulectin and GBP-mediated PPO activation, PPO-activating proteinase (PAPs) have been characterized from *M. sexta*, *B. mori* and *Holotrichia diomphalia*. PAPs have sequence homology to *Drosophila* Easter and belong to a family of arthropod serine proteases containing a carboxyl-terminal proteinase domain and an amino-terminal clip domain (Jiang et al., 1998; Satoh et al., 1999; Lee et al., 1998a,b).

The quinones generated by the activated PO enzyme are toxic not only to the pathogens, but also to the insect and could lead to a potentially lethal generalized melanization of the host. Thus, PPO activation has to be a tightly regulated process. Arthropod hemolymph contains proteins that inhibit serine protease activity (reviewed by Kanost, 1999). A member of the serpin family, serpin-J from *M. sexta*, inhibits PPO activation in vitro, while other serpins have no effect (Michael Kanost, personal communication). A family of serpins has also been characterized and cloned in *An. gambiae* (Alberto Danielli and Fotis Kafatos, personal communication).

### 4. Melanotic encapsulation and refractoriness to malaria infection

An *An. gambiae* strain, refractory to *Plasmodium* infection (L35) and a susceptible one (44rr), have been the subject of extensive study. The L35 strain blocks parasite development by melanotic encapsulation of
early oocysts (Collins et al., 1986). We will only discuss this system briefly, as a comprehensive review on this subject has been recently published (Paskewitz and Gorman, 1999). The gene responsible for the refractory phenotype is inherited in an autosomal dominant fashion but its identity is still unknown (Zheng et al., 1997). Two alternative approaches are currently underway to identify this gene, a genetic mapping approach and the cloning of candidate genes.

A high resolution genetic map of An. gambiae, based on microsatellite markers, has been generated (Zheng et al., 1996); and the degree of linkage between the refractory trait and the different markers was determined by using quantitative trait locus analysis (QTL) (Zheng et al., 1997). Two major Plasmodium encapsulation (Pen) intervals were defined, Pen1 and Pen2 which explain 60 and 19% of the phenotype, respectively. When Pen1 and Pen2 were fixed, this revealed a third interval, Pen3. The combined actions of Pen1, Pen2 and Pen3 control 76% of the trait (Zheng et al., 1997). Besides parasite encapsulation, the two strains also differ in their ability to melanize CM–Sephadex beads. These beads elicit a strong encapsulation response in the L35 strain, but not in the 4arr strain (Gorman and Paskewitz, 1997). Bead encapsulation mapped to the same region as Pen1 (Gorman et al., 1997). The Pen intervals cover large genomic DNA regions, so that further refinement of the map, by increasing the density of the markers in those regions, together with the elaboration of bacterial artificial chromosome (BAC) library contigs and large scale sequencing, would be required to identify the genes mediating refractoriness by a genetic mapping approach (Collins et al., 1997).

From the physiological perspective, it is clear that the two strains differ in their encapsulation responses. One could envision that differences at the level of non-self recognition, activation of the proteolytic cascade, regulation of proteolytic activity by protease inhibitors, or in the PPO gene itself could all lead to differential PPO activation. Alternatively, PPOs could be activated with the same efficiency, but differ in their catalytic activity. All the genes involved in the encapsulation process are good candidates, and those responsible for the refractory phenotype should map to the Pen1, Pen2 or Pen3 regions.

Six different ProPO cDNAs have been characterized in the mosquito Anopheles gambiae (Lee et al., 1998b; Jiang et al., 1997a; Müller et al., 1999). They differ in their temporal expression pattern throughout development. PPO5 and PPO6 are not transcribed in embryos and are mainly present in the pupal and adult stages. The mRNA levels of PPO1 through PPO4 increase 24 h after blood feeding, PPO6 expression does not change and PPO5 levels actually decrease. PPO protein has been detected in hemocytes but not in fat body cells of adult females by immunofluorescence. However, non-cross reacting antibodies would be required to determine the specific tissue distribution of the different PPOs (Müller et al., 1999). The chromosomal location of PPO1 (Lee et al., 1998a,b), PPO2 (Maureen Gorman, personal communication) and PPO4 through PPO6 (Müller et al., 1999) has been established, but none of these genes maps to the Pen regions. It is still important to determine which of the PPOs mediates the Plasmodium encapsulation response, and to study its expression and activation. These studies could reveal the nature of the refractoriness gene if it is acting upstream of PPO. For example, one could observe an increased rate of PPO activation or higher expression levels in the L35 strain.

5. Expression of effector genes in fat body cells

In response to infection, the fat body synthesizes a variety of proteins and peptides with antibiotic activity. Anti-bacterial peptides were first characterized from the moth Hyalophora cecropia (Steiner et al., 1981), and since then they have been isolated from multiple species, including mammals (reviewed by: Lehrer and Ganz, 1999; Ganz and Lehrer, 1998; Zanetti et al., 1995). In Drosophila six anti-bacterial peptides (Defensin, Cecropins, Diptericin, Drosocin, Attacin and Metchnikowin) and one anti-fungal peptide (Drosomycin) have been characterized (reviewed by Hoffmann et al., 1999 and Hoffmann and Reichhart, 1997). The genes encoding insect immune peptides contain regulatory DNA sequences, such as the kB-like elements first described in Hyalophora cecropia (Sun and Faye, 1992), which are known to be involved in the regulation of immunoglobulin (Sen and Baltimore, 1986) and acute phase response genes in mammals (Böhnlein et al., 1988; Hoyos et al., 1989; Lenardo and Baltimore, 1989). Mutagenesis analysis in Drosophila has shown that these elements are necessary for induction of the Diptericin and Cecropin genes (Engström et al., 1993; Kappler et al., 1993).

6. Transcription factors activating gene expression in response to infection

Three Drosophila transcription factors of the rel-family, Dorsal, Dif and Relish, translocate to the nucleus of fat body cells in response to bacterial challenge (Lemaitre et al., 1995b; Ip et al., 1993; Hedengren et al., 1999). Loss-of-function mutants of Dorsal have normal induction of all known immune peptides (Lemaitre et al., 1996), while Dif mutants are impaired in the induction of Drosomycin and Defensin (Meng et al., 1999). In Relish mutants there is a complete loss of Cecropin and Diptericin inducibility and the induction of Attacin, Drosomycin and Metchnikowin is 10–20% of the levels achieved
in wild type flies (Hedengren et al., 1999). Clones of fat body cells homozygous for a deficiency covering both the dorsal and dif genes were generated using a genomic recombination system. Cells lacking both genes failed to induce Drosomycin in response to infection, while Diptericin induction was not affected. Drosomycin inducibility could be rescued by overexpression of either dorsal or dif under the control of a heat-shock promoter, suggesting a functional redundancy between these Rel proteins (Manfruelli et al., 1999).

7. Signaling pathways activating transcription

Dorsal is activated by the Toll pathway and plays the role of morphogen in establishing the dorso-ventral polarity in the embryo (Steward 1987, 1989). The systematic analysis of flies mutant for genes in this pathway revealed that spätzle, toll, tube, pelle and cactus, are also essential for the activation of Drosomycin in response to infection in adults (Lemaitre et al., 1996). The Spätzle protein is the ligand that activates the Toll receptor in embryos and it has a cysteine knot motif also found in extracellular ligands such as nerve growth factor (NGF), transforming growth factor β2 (TGF-β2) and platelet-derived growth factor BB (PDGF-BB) (McDonald and Hendrickson, 1993). Furthermore, in recent experiments, cleavage of Spätzle to an active form has been observed 1 h after bacterial challenge (Levashina et al., 1999). A serine protease inhibitor of the serpin family, Spn43Ac, has a central role in regulating the Spätzle activation cascade. The necrotic Drosophila mutants have a loss of function of three serpin genes and this results in constitutive activation of Spätzle, leading to activation of Drosomycin in the absence of immune challenge, while expression of Diptericin and Cecropin A1 is unaffected. This mutant phenotype is rescued by a single copy of Spn43Ac (Levashina et al., 1999). In double loss-of-function mutants, lacking either both spätzle and Spn43Ac, or toll and Spn43Ac, the constitutive expression of Drosomycin is not observed, indicating that spätzle and toll are acting downstream of Spn43Ac in the activation cascade (Levashina et al., 1999).

The activation of the anti-bacterial peptides is under the control of multiple regulatory pathways. Besides the well-characterized toll pathway, mutagenesis analysis has revealed the participation of at least two other pathways, immune deficiency (imd) and immune response deficient (ird) (Lemaitre et al., 1995a; Wu and Anderson, 1998). The identity of these genes remains to be determined. Several genes coding for members of the Toll family of receptors are present in Drosophila (Jean-Luc Imler and Jules Hoffmann, personal communication). The 18-Wheeler receptor belongs to this family and mediates nuclear translocation of Dif and expression of Attacin (Williams et al., 1997). There is increasing evidence indicating that more than one pathway can influence the expression of a given anti-bacterial peptide (Lemaitre et al., 1996; Levashina et al., 1998). It may be more appropriate to think not in terms of regulatory pathways but of networks with extensive cross-talk between pathways.

8. Anti-bacterial responses in mosquitoes

The first mosquito Defensins were characterized and cloned in Aedes aegypti (Chalk et al., 1994; Lowenberger et al., 1995; Cho et al., 1996, 1997). Since then, several different molecules with anti-bacterial activity such as, defensin, cecropin, lysozyme and nitric oxide synthase (NOS), which generates nitric oxide (NO) that is toxic to bacteria, as well as several genes induced by bacterial challenge, such as transferrin, a putative serine protease (ISPL5), a putative Gram-negative binding protein (Ag-GNBP), a lectin-like protein (IGALE20) and a chitinase (ICHIT), have been characterized from different mosquito species (Table 1). Further characterization and cell biology studies will be required to establish the precise role of several of these induced markers and to determine how their expression is regulated during the immune response. Two transcription factors, Gambif1 (gambiae immune factor 1) which belongs to the rel-family (Barillas-Mury et al., 1996) and Ag-STAT, a member of the STAT-family of receptors, are known to be activated in response to bacterial infection in An. gambiae (Barillas-Mury et al., 1999).

Gambif1 is most similar to Drosophila Dorsal. Gambif1 protein is translocated to the nucleus in fat body cells in response to bacterial challenge, although the mRNA is present at low levels in all developmental stages and is not induced by infection. DNA binding activity to the κB-like sites in the An. gambiae Defensin and the Drosophila Diptericin and Cecropin promoters is induced in larval nuclear extracts following bacterial infection. Recombinant Gambif1 has the ability to bind to κB-like sites in vitro, and co-transfection assays in Drosophila mbn-2 cells show that Gambif1 can activate transcription by interacting with the Drosophila Diptericin regulatory elements. Gambif1 protein translocation to the nucleus and appearance of κB-like DNA binding activity serve as molecular markers of activation of the immune system (Barillas-Mury et al., 1996). Gambif1 is the only insect rel-family protein whose crystal structure has been solved (Cramer et al., 1999).

In vertebrates, cytokines such as interleukins and interferons play a central role in regulating and coordinating the immune response (reviewed by Leaman et al., 1996). Several of these cytokines interact with specific membrane receptors and result in activation of members of the JAK (Janus kinase) family, which in turn activate members of the STAT (signal transducers and activators
Molecular markers induced by infection

### A. Anti-bacterial responses in mosquitoes

<table>
<thead>
<tr>
<th>Species</th>
<th>Molecular markers</th>
<th>Developmental stage</th>
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<tr>
<td><em>An. gambiae</em></td>
<td>Defensin</td>
<td>L, A1, A2;</td>
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<td></td>
<td>Lysozyme</td>
<td>CL5</td>
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<td></td>
<td>Ag-GNBP</td>
<td>A*</td>
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<td>ISPL5</td>
<td>L, A1, [tx, mg, ov].CL5</td>
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<td></td>
<td>IGALE20</td>
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<td>ICHIT</td>
<td>L, A6</td>
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<td></td>
<td>Ag-NOS</td>
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### B. Anti-malarial responses in mosquitoes

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<td>mg, ab</td>
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<td>3 d</td>
<td>mg, car17</td>
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* Gram-negative bacteria-binding protein (GNBP). Immune-related serine protease-like sequence 5 (ISPL5). Immune-related serine protease-like sequence 13 (ISP13). Putative infection-responsive galactose-binding lectin (IGALE20). Putative chitin-binding protein (ICHIT). Nitric oxide synthase (NOS). Putative clip-domain serine protease (Ester-like SP) and putative non-receptor tyrosine kinase cDNA fragment (CHED). Expression of all molecular markers is induced by infection unless otherwise indicated (constitutive expression is indicated by * and decreased levels by #). A. Anti-bacterial responses — developmental stages are indicated by L=larvae; A=adult; CL=cell line and tissue specificity by [ ]; tx=thorax; mg=midgut; ab=abdomen and ov=ovary. B. Anti-malarial responses — the induction at different times post-infection is indicated, ND=not done and car=carcass (whole mosquito without midgut tissue). References are indicated by numbers as subscripts. (1) Richman et al., 1996; (2) Lowenberger et al., 1999b; (3) Müller et al., 1999; (4) Kang et al., 1996; (5) Dimopoulos et al., 1997; (6) Dimopoulos et al., 1998; (7) Paszewitz et al., 1999; (8) Moreira-Ferro et al., 1998; (9) Chiou et al., 1998; (10) Lowenberger et al., 1999a; (11) Yoshiga et al., 1997; (12) Cho et al., 1996; (13) Lowenberger et al., 1999c; (14) Sun et al., 1998; (15) Sun et al., 1999; (16) Richman et al., 1997 and (17) Luckhart et al., 1998. For complete bibliographical information see Reference section.

of transcription) family of transcription factors (reviewed by Ihle, 1996; O’Shea, 1997 and Darnell, 1997). Gene disruption experiments in mice have shown that three of seven STAT family members have non-redundant functions, regulating distinct aspects of the immune response: STAT1 participates in the innate immune response to viral and bacterial infections (Meraz et al., 1996), while STAT4 (Kaplan et al., 1996) and STAT6 (Takeda et al., 1996) are involved in the regulation of acquired immune responses that favor cellular immunity (Th1) or antibody production (Th2), respectively.

*An. gambiae* STAT (Ag-STAT) is most similar to *Drosophila* D-STAT and to vertebrate STATs 5 and 6 (Barillas-Mury et al., 1999). Ag-STAT mRNA is expressed at all developmental stages, and the protein is present in hemocytes, pericardial cells, midgut, skeletal muscle and fat body cells. There is no evidence of transcriptional activation following bacterial challenge. However, bacterial challenge results in nuclear translocation of Ag-STAT protein in fat body cells, and induction of DNA binding activity that recognizes a STAT domain. The formation of melanotic tumors (Hanratty and Perrimon, 1987) is associated with hypertrophy of the larval lymph glands (the hematopoietic organs), a leukemia-like phenotype and the formation of melanotic tumors (Hanratty and Dearolf, 1993; Harrison et al., 1995). Interestingly, a newly identified family of complement C3a2-macroglobulin-like molecules induced in response to infection in *Drosophila*, are under the control of the Toll and JAK pathways (Marie Lagueux and Jules Hoffmann, personal communication). Members of the complement C3a2-macroglobulin-like molecules have been also identified in *An. gambiae* and shown to be induced in response to bacterial infection (Elena Levashina and Fotis Kafatos, personal communication).
9. Epithelial immune responses to Plasmodium invasion

In insects, the fat body is the main site of immune peptide biosynthesis (reviewed by Hoffmann and Reichhart, 1997), but other organs may be the first ones to come into contact with the invading organism: examples are the midgut in the case of malarial infection of mosquitoes, or the epidermis in the case of cuticle wounding in the presence of fungi or bacteria. Infection of An. gambiae with P. berghei results in transcriptional activation of several immune markers (Table 1). AgGNBP, Defensin, ICHIT, ISPL5, IGAL20 and NOS mRNA levels increase in the midgut and abdominal wall tissues 24 h post-malarial infection (Dimopoulos et al. 1997, 1998; Richman et al., 1997). Interestingly, Defensin C is also expressed in the midgut of Aedes aegypti, but the mRNA levels remain constant following P. gallinaceum infection (Lowenberger et al., 1999b). The An. gambiae immune markers are also induced in the salivary glands and abdominal wall at 20 and 25 days post-infection (a time when sporozoites have invaded the salivary glands); with the exception of NOS, whose mRNA levels increase in the abdominal wall but decrease in the salivary gland at this time (Dimopoulos et al., 1998).

Studies in An. stephensi have also shown transcriptional activation of AsNOS (nitric oxide synthase) in the midgut and systemically in response to P. berghei infection. Furthermore, dietary provision of the NOS substrate L-arginine significantly increased the number of oocysts that proliferate in P. berghei-infected mosquitoes, while a NOS inhibitor significantly increased the number of oocysts that developed (Luckhart et al., 1998). STAT-like and NF-κB-like DNA target sequences have been recently reported in the regulatory regions of the AsNOS gene (Luckhart and Rosenberg, 1999). NOS has also been cloned from An. gambiae, and the midgut has been established as the tissue expressing the highest levels of AgNOS mRNA, which increase at 24 h following malarial infection (Dimopoulos et al., 1998).

In vertebrates, the STAT pathway has been shown to regulate inducible NOS (iNOS) expression. The promoter of the mouse iNOS gene confers inducibility by interferon gamma (Xie et al., 1993), and STAT1 is known to have a central role in signaling responses to this cytokine (Meraz et al., 1996). It is very likely that AgNOS is regulated by Ag-STAT, as we have observed inducible DNA binding activity to the STAT target sequences in response to malaria infection in both midgut and body wall tissues; the midgut being the main organ involved this response (Yeon Soo Han and Carolina Barillas-Mury, unpublished).

There is extensive experimental evidence now establishing the midgut as an immune-responsive organ. It is clear that the mosquito is capable of detecting the presence of the parasite and mounting a response, suggesting that the mosquito could be actively controlling the level of Plasmodium infection. If the activation of specific pathways mediating these responses was blocked, would that result in an increase in the number of parasites that successfully invade the midgut? Would constitutive activation result in a mosquito refractory to malaria infection?

It has been reported that the ookinetes of P. gallinaceum selectively invade a cell type in the midgut of the mosquito Aedes aegypti expressing high levels of vesicular ATPase (v-ATPase) (Shahabuddin and Pimenta, 1998). They have been named “Ross cells”; and differ from other epithelial cells in that they do not stain with toluidine blue (a basophilic dye), are less osmiophilic, they contain minimal endoplasmic reticulum, lack secretory granules and have few microvilli (Shahabuddin and Pimenta, 1998). The vATPase-positive cells are also present in the midgut on An. gambiae mosquitoes and P. gallinaceum appears to also be invading this cell type (Cociancich et al., 1999). The identity of the specific midgut cells mounting the immune response to malaria, as well as the immune competence of the Ross cells remain to be determined.

10. Evolutionary conservation of the signaling pathways mediating activation of innate immune responses

There are many organisms that can cause human disease, and due to their different biology, the body needs to use different defense strategies to fight them. For the human immune system to elicit the appropriate response, the innate immune system must detect the presence of a non-self antigen together with molecules recognized as originating from a specific pathogen. Following this initial recognition, the innate immune system not only activates early resistance mechanisms to contain invading pathogens, but also dictates the direction that the ensuing adaptive immune response should take (Janeway, 1998; Fearon and Locksley, 1996; Medzhitov and Janeway, 1997a). Toll-like receptors have been characterized in mammals, and it has become apparent that they are involved in transducing the initial signals resulting in the transcriptional activation of specific cytokines and co-stimulatory molecules. These signals may direct the acquired immune system either towards a cellular response (effective against intracellular pathogens) or to the production of specific antibodies (which interact with extracellular pathogens). The balance between these two types of responses is critical to mount an efficient response, especially in the case of pathogens such as Plasmodium, which have complex life cycles and may require different responses depending on the developmental stage of the parasite.

The initial observations that drew attention to the
resemblance between the *Drosophila* Toll/Dorsal signaling pathway and the mammalian IL-1R/NF-κB signaling cascade (Wasserman, 1993), were followed by the breakthrough discovery of the first human Toll homolog (Medzhitov et al., 1997). Since then, an explosion of information has confirmed and expanded the structural and functional similarities between the various components of the signaling systems that mediate innate defense responses in insects and mammals (reviewed by Hoffmann et al., 1999). These findings have led to an in-depth search for additional parallels between the effector mechanisms that execute the innate immune responses in multicellular organisms.

To date, six human homologs of the *Drosophila* Toll receptor, termed Toll-like receptors (TLRs) have been cloned (Medzhitov et al., 1997; Yang et al., 1998; Rock et al., 1998; Takeuchi et al., 1999b). An active form of TLR4 constitutively expressed in the human monocytic cell line THP-1 demonstrated for the first time that this vertebrate homolog functions like *Drosophila* Toll, that is, as a receptor capable of inducing NF-κB-dependent transcription of immune response genes (Medzhitov et al., 1997). While the Toll pathway induces the production of the anti-fungal peptide drosomycin in the adult fly (Lemaitre et al., 1996), the TLR4/NF-κB signaling system controls the expression of the pro-inflammatory cytokines and chemokines IL-1, IL-6 and IL-8 and that of the co-stimulatory molecule B7.1 in humans (Medzhitov et al., 1997).

Since lipopolysaccharide (LPS) represents a conserved molecular pattern shared by Gram-negative bacteria (Medzhitov and Janeway, 1997b) and has been known to trigger several intracellular signaling cascades, it was reasonable to hypothesize that a TLR could be the receptor responsible for transducing the LPS signal across the plasma membrane. LPS was known to activate NF-κB in human monocytes and macrophages (reviewed by Ulevitch and Tobias, 1999), with the subsequent production and release of various pro-inflammatory mediators, such as IL-1β, IL-6, IL-8 and TNF-α (reviewed by Schletter et al., 1995). Indeed, TLR2 and TLR4 were found to be highly expressed in LPS-responsive cells, such as peripheral blood leukocytes, monocytes and macrophages (Medzhitov et al., 1997; Yang et al., 1998), and capable of inducing LPS-mediated cellular signaling in a NF-κB-dependent fashion (Yang et al., 1998, Kirschning et al., 1998; Hoshino et al., 1999; Chow et al., 1999).

CD14 and LPS-binding protein participate in the LPS-sensing mechanisms of both TLR2 and TLR4 (reviewed by Ulevitch, 1999; Yang et al., 1998; Hoffmann et al., 1999). TLR4 is an essential component of the LPS signaling receptor complex inasmuch as mice homozygous for a missense or a null mutation of TLR4 display an LPS-unresponsive phenotype. Because of their inability to transduce the LPS signal, these mice are not only highly resistant to endotoxin, but also seriously predisposed to Gram-negative infections (Poltorak et al., 1998).

Recent studies demonstrated that TLR2 is a signal transducer of bacterial lipoproteins (BLPs) (Brightbill et al., 1999; Aliprantis et al., 1999) and soluble peptidoglycans from Gram-positive bacteria (Yoshimura et al., 1999). As shown for LPS, BLP signaling through TLR2 can mediate the activation of NF-κB and the subsequent production of the cytokine IL-12 (Brightbill et al., 1999). Interestingly, BLPs induce through TLR2 not only the transcriptional activation of iNOS in macrophages (Brightbill et al., 1999) and the activation of the respiratory burst in peripheral blood leukocytes, but they also induce apoptosis in monocytic cells. The induction of apoptosis suggests that TLR signaling may also participate in a feedback pathway that regulates the extent of an immune response (Aliprantis et al., 1999).

Further analyses aimed at the elucidation of the contributing roles that TLR2 and TLR4 signaling pathways play in the defense against microbial stimuli have conclusively revealed that these two receptors are capable of specifically discriminating the cell wall components from different pathogens (Underhill et al., 1999; Takeuchi et al., 1999a). Through a novel in vitro system using macrophages expressing dominant-negative TLR mutants, it was found that TLR2 signals TNF-α production during the phagocytosis of yeast, Gram-positive bacteria or their cell wall components but not of Gram-negative bacteria or LPS. TLR4, in contrast, couples cytokine production with the phagocytosis of Gram-negative but not of Gram-positive bacteria (Underhill et al., 1999). Similar conclusions were obtained using macrophages from TLR2- and TLR4-deficient mice, except for the finding that macrophages from TLR4-deficient mice also lacked the response to Gram-positive lipoteichoic acids (Takeuchi et al., 1999a).

11. Innate immunity to malaria sporozoites and implications for vaccine development

Sporozoites represent the stage of *Plasmodium* spp. malaria parasites that are delivered by an infected anopheles mosquito to a susceptible vertebrate host. In minutes, infective sporozoites reach the liver and selectively invade hepatocytes where they eventually develop into the erythrocyte-infective stages that are responsible for the clinical symptoms associated with malaria. It has been known for decades that a sterile and long-lasting protective immunity against challenge with sporozoites can be obtained by the immunization of rodents, non-human primates and humans with irradiated sporozoites of their respective host-specific *Plasmodium* species (reviewed by Hoffman et al., 1996). Because the use of radiation-attenuated sporozoites would be impractical for
large-scale immunization, enormous research efforts have been aimed at designing malaria vaccines that would be capable of emulating the protective immune mechanisms elicited by the immunization with irradiated sporozoites. Evidence gathered thus far implicates various immune components as playing critical roles in sporozoite-induced protection. However, a comprehensive view of all the effector mechanisms triggered by the irradiated sporozoite vaccine has not yet been fully attained.

Because irradiated sporozoites retain their ability to invade hepatocytes, and only irradiated but not killed sporozoites induce protection against sporozoite-induced malaria, it is evident that the intracellular location of the parasite is required for generating at least some of the signals that will mount a protective immune response. Thus far, most studies on sporozoite-induced protective immunity have focused on the signals and events that take place at the onset and during an ongoing adaptive immune response. In contrast, aside from a few studies indicating that the cytokines IL-1, IL-6, interferon-γ and TNF participate early in a signaling cascade, that ultimately interferes with the development of liver stage malaria parasites (Melloul et al., 1987; Pied et al., 1992; Nussler et al., 1991b), very little is known about the early recognition events by the innate immune system.

Extensive evidence indicates that CD8+ T lymphocytes, a key adaptive effector cell subset in sporozoite-induced protection, recognize parasite-derived peptides bound to MHC class I molecules on the surface of infected hepatocytes, and this recognition results in the destruction or inactivation of the intracellular parasite. The elimination of malaria liver stage parasites by activated CD8+ T cells may operate via the release of IFN-γ, which in turn induces parasiticidal levels of NO within the infected hepatocyte, and via a cytotoxic mechanism which directly destroys the malaria-infected cell (reviewed by Hoffman et al., 1996). IL-12, a cytokine that links innate and adaptive immunity, has been shown to play a crucial role in sporozoite-elicited protection by mediating IFN-γ-dependent induction of NO (Sedegah et al., 1994). IL-12 has also been implicated, along with natural killer (NK) cells, in a novel positive feedback loop of adaptive immunity aimed at augmenting the levels of IFN-γ released by CD8+ T cells to the levels required for iNOS activation and NO-mediated destruction of intrahepatic malaria parasites (Doolan and Hoffman, 1999). Thus, only the events that succeed and not those that precede IL-12 production have been analyzed. With the recent appreciation of the importance that innate immunity has on the conduct of adaptive immune responses, it is clear that in order to develop a vaccine that would reproduce the signals elicited by irradiated sporozoites, it is crucial to elucidate the molecular and cellular components that generate such signals.

Given that innate defense mechanisms have remained conserved throughout evolution, it is likely that studying the response of the mosquito midgut epithelium to ookinetes invasion could shed important information about the response of vertebrate hepatocytes to sporozoite invasion. For instance, in both cases the parasites invade the epithelial cells and shed their surface molecules within their cytoplasm (Fig. 2). Sporozoites release circumsporozoite (CS) protein within the hepatocytes (Frevert et al., 1998), while P. berghei ookinetes release the surface protein Pbs21 within midgut epithelial cells (Yeon Soo Han and Carolina Barillas-Mury, unpublished). Furthermore, both epithelia display inducible synthesis of NO, which limits parasite development in the midgut and liver (Luckhart et al., 1998; Nussler et al., 1991a). A modest amount of NO was produced by naïve rats following a sporozoite challenge (less than 5% of the infected hepatocytes expressed iNOS), as compared to the robust response observed in animals that had been previously immunized with irradiated sporozoites (81% of infected hepatocytes expressed iNOS) (Klotz et al., 1995). When immunized rats were treated with an iNOS inhibitor (aminoguanidine) and challenged with sporozoites, the number of protected animals was reduced to 25%, as compared to the 100% protection observed in untreated controls. Besides the role of NO as an effector molecule with parasiticidal activity, it has been proposed that it could also serve as a mediator of inflammation promoting recruitment of other cells to the infected hepatocyte (Klotz et al., 1995). That an early NO production is essential during an anti-parasitic response, and that it is a prerequisite for the function of IL-12, was recently demonstrated by genetically deleting iNOS in mice that...
are normally resistant to infection by the Leishmania major parasite. In mice with iNOS deficiency, inhibition of early parasite spreading, the up-regulation of IFN-γ, and the induction of NK cell cytotoxicity at day 1 of infection were abolished (Diefenbach et al., 1999). In Anopheline mosquitoes experimentally fed with a blood meal containing infectious malaria parasites, the inhibition of the iNOS homolog significantly increased the load of midgut oocysts, indicating that NO is a defense reaction that limits Plasmodium development (Luckhart et al., 1998). This example illustrates the likelihood that some of the regulatory pathways that activate NOS in the insect, such as Ag-STAT, may be similar in vertebrate hepatocytes.

Based on the pattern recognition theory, malaria susceptible hosts are likely to recognize defined molecular arrays expressed by sporozoites through pattern recognition receptors. This initial interaction with sporozoite molecular patterns would trigger a series of signaling cascades that transfer the information to other cells in order to mount a coordinated and effective response. Such a response would include the generation of immunological memory, one of the hallmarks of adaptive immunity. However, adequate protection can only be achieved if the correct decision is made regarding the nature of sporozoites.

Given that there is little or no information about the identity and the order in which the various molecules and cells of the innate response participate in the response to viable sporozoites, it seems reasonable to formulate a series of questions which might provide some direction to ongoing and future research projects. First, in relation to pathogen recognition: Are there specific sporozoite-associated molecular patterns? What is the chemical nature of these molecules? Based on what we know about other pathogens, one would predict that they consist of conserved molecules on the parasite’s surface. Does recognition take place extra- or intracellularly? Do they have adjuvant activity? Second, in regards to pattern-recognition receptors recognizing sporozoites: Are they intracellular or membrane-bound receptors? Are there parasite-specific TLRs transducing their signals? Can insect model systems provide a clue to their identity? Besides hepatocytes, what is the role of hepatic and extra-hepatic phagocytic cells such as monocytes, macrophages, Kupffer cells and dendritic cells? In sum, the answers to these and other related questions might hold the key to developing successful malaria vaccines.

12. Future perspectives

The number of genes participating in immune responses in mosquitoes that have been characterized has increased rapidly over the last few years, and this process will be further accelerated by the ongoing projects aimed at generating expressed sequence tags (ESTs) from Ae. aegypti (Michael Wells, personal communication) and An. gambiae (Fotis Kafatos, personal communication) by randomly sequencing large numbers of cDNAs from different tissues. As a result, one of the major challenges over the following years will be to develop strategies to assess gene function.

The extent to which specific immuno-regulatory pathways and immune effector genes influence the development of the malaria parasite in the mosquito remains to be defined. Disruption of individual genes in a given organism has been extremely powerful in elucidating gene function in other systems such as mice, yeast and Drosophila. Classic methods, such as mass mutagenesis are not applicable to mosquitoes due to technical and practical limitations. For example, in An. gambiae, single pair matings do not occur spontaneously so they have to be “forced” individually; and keeping hundreds of individual stocks is unpractical due to the requirement for blood-feeding. An alternative approach is to perform “functional knockouts” by expressing an exogenous gene product that interferes with the function or expression of a specific endogenous gene. Recently, stable transformation of the mosquito Ae. aegypti, resulting from legitimate recombination, has been successful using the Hermes (Jasinskiene et al., 1998) and Mariner transposable elements (Coates et al., 1998). The Sindbis (SIN) virus transducing systems, which allow constitutive cytoplasmic expression of exogenous mRNAs (Reviewed by Olson et al., 1998), represent another useful alternative. Viral systems to express exogenous gene products in mosquitoes are only available for Ae. aegypti at the moment but are currently being developed for anopheline mosquitoes (Kenneth Olson and Barry Beaty, personal communication).

If one could block the activation of specific signaling pathways normally activated in response to malaria infection, would that result in a significant increase in the number of developing oocysts? How would this affect the expression of different effector genes? Furthermore, if one could manipulate the activation of these pathways, would it be possible to block Plasmodium development? In order to design such experiments, one would need to know where and when to express the exogenous genes and to characterize candidate molecules to manipulate the immune responses. One also needs to consider the temporal and sub-cellular localization of specific gene products.

Due to the ancient nature and conservation of the innate immune system throughout evolution, the information generated from the Drosophila model system has already opened a new area of intense study in vertebrate immunity. The finding that resistance to endotoxin and susceptibility to Gram-negative bacterial infections in mice is due to a mutation in TLR4, was the first conclusive evidence that TLRs could transduce the key signals...
that direct the immune system in the direction appropriate for a given pathogen (Poltorak et al., 1998). Furthermore, recent experiments have revealed that the TLR2 and TLR4 receptors are capable of specifically discriminating the cell wall components from Gram-positive and Gram-negative bacteria, respectively (Underhill et al., 1999; Takeuchi et al., 1999a). Does each of the six known members of the TLR family transduce the signals for a specific type of pathogen? Gene disruption of the other TLRs in mice, as well as the elucidation of their specific ligands will be required to confirm this hypothesis. These findings have broad implications, particularly for the area of vaccine development. Despite the efforts over the last decades to develop effective vaccines against parasitic infections, none are available to date. Our current understanding of the molecular language that the innate immune system uses to identify and direct the responses to parasites is very limited. Insect vectors of vertebrate parasitic disease, such as malaria, could represent valuable model systems to study the initial responses of epithelial cells to infection. Such information may provide new insight into the responses of vertebrate epithelial cells and open the possibility to develop a new class of anti-malarial vaccine adjuvants. The key to developing a successful vaccine against malaria may be to let the parasite itself tell us what we need to do.

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