Meeting on Parasites and the Invertebrate Vector

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With few exceptions, the power of modern biology has not been effectively harnessed to control vector-borne parasites or the diseases they cause. In Africa, neither chemotherapy nor conventional vector control measures have had a sustained impact on parasitic diseases such as malaria, yet vector control has worked in the past. Clearly, in industrialized countries, vector control was the single most important intervention leading to the eradication of malaria. The problem is that the old tools for vector control have been or are now inadequate for controlling disease in subSaharan Africa and much of the tropics. The task at hand is to exploit the power of modern biology to control the spread of parasites. Rather than trying to eradicate the vector, genetic engineering may make feasible novel approaches for controlling vector-borne diseases, such as control strategies in which susceptible invertebrate vectors are replaced by those that cannot transmit disease. Such strategies may require a detailed understanding of parasite-vector interactions.

Insect defense mechanisms

Insect immunity

In large part due to the foresight of the MacArthur Foundation, who sponsored this meeting on "Parasites and the Invertebrate Host," the research questions being asked by vector biologists have shifted from the vector itself to parasite-vector interactions. For example, what changes occur in the vector and the parasite during the parasite's development in the vector; what makes some vectors susceptible to parasite infections and others resistant; and are there vector-derived factors such as receptors and enzymes that the parasite requires for normal development? Although little is known yet about the natural defense response(s) in vectors to parasites, a fascinating picture of the processes involved in acute phase reactions to bacterial challenge or bodily injury in arthropods is developing. Studying induced antibacterial reactions in insects, namely in *Drosophila*, Dr Jules Hoffmann and his colleagues in Strasbourg are identifying which molecules are involved, how the genes encoding these molecules are regulated, and how the vector recognizes microbial infection. The response to bacterial challenge is both cellular and humoral. The former is mediated by circulating blood cells, the latter by an array of induced peptides and polypeptides.

Several classes of peptides/polypeptides are now recognized that mediate the strong antibacterial activity induced following bacterial challenge or tissue injury of insects. The first to have been described (1980), called cecropins, are peptides of approximately 40 amino acids. They are C-terminally amidated and have helix bend helix structure. The N-terminal helix is amphipathic; the C-terminal is hydrophobic. Cecropins are membrane active and are believed to form voltage dependent channels in both gram negative and gram positive bacteria. Similar peptides have been identified in secretions of various tissues of vertebrates. A second class of inducible antibacterial peptides is the insect defensins, which show some sequence similarities with mammalian defensins that participate in bacterial killing within phagocytes. Insect defensins are small-sized (approximately 40 amino acids), strongly cationic peptides containing six cysteine residues engaged in three intramolecular disulfide bridges. Their three-dimensional structure consists of a flexible N-terminal loop, a central amphipathic α−helix, and a C-terminal anti-parallel ß-sheet. The loop is linked via a disulfide bridge, and the a-helix through two disulfide bridges to the ß-sheet. Insect defensins that are widespread among insects kill gram positive bacteria by forming voltage-gated channels.

In addition to these two well-defined families of antibacterial peptides, close to 15 distinct antibacterial peptides/polypeptides have been isolated and are tentatively classified into proline-rich and glycine-rich molecules. The proline-rich antibacterial peptides are usually small-sized (15 to 30 residues), strongly cationic molecules that are active against gram negative bacteria. Some of these molecules are modified by O-linked glycosylation (N-acetylgalactosamine/galactose), the removal of which decreases their antibacterial activity. In contrast to the proline-rich short pep-

tides, the glycine-rich polypeptides are mostly large molecules in the range of 15 to 30 kDa. They are active against gram negative bacteria. The three dimensional structure and the mode of action of the proline-and glycine-rich peptides/polypeptides await investigation.

The various antibacterial molecules are produced predominately by the insect fat body (a functional homologue of the mammalian liver) and by some blood cell types. The synthesis follows acute phase kinetics: it is a rapid (within 1-2 hours) and transient phenomenon. As a result of this reaction, the hemolymph of challenged or injured insects contains a significant concentration (up to 0.02 mM) of a mixture of peptides/polypeptides with a generally wide spectrum of activity against bacteria.

How is the expression of the genes encoding the antibacterial peptides controlled? Recent analysis of the promoters of some selected genes has shown the presence of cis-acting regulatory elements (CREs) to which several distinct transactivating factors bind. Significantly, all these CRE nucleotide motifs have strong homology with regulatory motifs present in the promoters of genes encoding acute phase reactants in mammals. In particular, this is the case for motifs serving as cytokine-response elements (e.g., IL-6 and IFN-γ) or sequences binding the inducible transactivator NF-kB, which is involved in the control of expression of many genes encoding acute phase reactants in mammals. Experiments based on establishment of transgenic *Drosophila* demonstrate that the replacement of the NF-kB-like motifs in the promoter of immune response genes by random sequences suppresses the inducibility of these genes. Interestingly, the NF-kB-related morphogen *dorsal* is induced in the fat body and in blood cells during the immune response of *Drosophila* and the dorsal protein translocated into the nucleus shortly after bacterial challenge. Although the exact role of the *dorsal* protein in the immune response remains to be established, it is exciting to note that a maternally expressed gene involved in the control of early embryonic development is reused in the host defense response during larval development and in adults.

Insect immunity does not appear to involve somatic rearrangements of gene products, memory, or an innate repertoire as there is in mammalian immune response. Rather pattern-recognition receptors, such as receptors for lipopolysaccharides, peptidoglycans, and complex carbohydrates present in bacteria and fungi, may recognize non-self entities. Because of the similarities between the defense response in insects and the acute phase reaction response in mammals, which is also non-specific and has no memory, it is likely that the means of recognizing non-self in these two systems may be similar. The ease of transfection in the *Drosophila* model should allow the similarities and differences between mammalian acute phase reactions and insect defense responses to be further defined (Cociancich S et al. 1994 *Parasitol Today 10:* 132-139).

Life cycle transitions

The transitions from vector to vertebrate host and back again present formidable challenges to the parasite; thus, the life cycle stages (transition stages) involved in these transitions may be vulnerable to intervention. The immune response in the vertebrate and the immune response in the vector differ substantially, as do the cell surface proteins and the extracellular matrices. As discussed below, significant changes occur on the surface of the parasite to adapt to the different environments of the vector and vertebrate host. Less obvious are significant changes that occur intracellularly, such as the ribosomal RNA content of transition stages in *Plasmodium.*

Ribosomal RNA changes during host transition stages

Studying the biology of these transition stages has been particularly useful in understanding more basic mechanisms such as ribosomal RNA (rRNA) function. One of the original theories proposed for the role of rRNA in development species was that different catalytic rRNA species were involved in translational regulation in different stages of an organism's development. This theory was dismissed almost outright when it was discovered that the same rRNAs were present during multiple stages of differentiation in most eukaryotes. Recent studies of the expression of the 4-7 rRNA genes in the transitional stages of the malaria parasite life cycle have, however, revived this rRNA theory of translational regulation. Previously, Dr McCutchan and his colleagues showed that when the malaria sporozoite left the mosquito salivary gland to invade a mammalian hepatocyte, expression of the rRNA C gene was down-regulated and the A gene up-regulated. The A gene continued to be the predominately expressed rRNA gene throughout the rest of the developmental stages in the vertebrate including sexual differentiation; however, once the sexual forms were ingested by the vector, a switch back to expression of the C gene occurred. Unfortunately, comparison of the structure of these different rRNAs did not reveal an obvious explanation for this switch. Even in *P. berghei*, a murine malaria parasite in which the A and C genes are closely related, the cluster of differences between A and C genes was not in core regions responsible for function or catalytic activity. A closer examination of the expression of

rRNA during the transition of *P. vivax* from vertebrate host to vector revealed a further paradox. Despite rapid growth from day 2 to day 6 in the mosquito midgut, expression of both the A and C genes was extremely low. This gap in rRNAs in the developing oocyst appears to be partially filled by expression of other rRNAs. A series of rRNA B transcripts has been identified in oocysts, in which portions of the rRNA A are present in rRNA B gene products. The role of these novel rRNAs is not presently known, but they do not appear to be present in the developing sporozoites within the oocyst and may be limited to the cytoplasm surrounding the sporozoites. The existence of species-specific and stage-specific rRNAs may be exploited to quantitatively study development of parasites within the vector. For instance, PCR primers that amplify unique A and C gene fragments can be used to determine quantitatively the relative ratio of parasites that have successfully developed from early midgut forms to sporozoite filled oocysts (Li J et al. 1994 *Mol Biochem Parasitol*, in press).

Changes in surface molecules during host transition stages

GPI anchors

Membrane-bound proteins can be stably anchored to the cell surface by transmembrane α-helices or by glycosylphosphatidylinositol (GPI). GPI anchors are ubiquitous in eukaryotes and appear to be the favored means of anchoring surface molecules in many parasites. Dr Michael Ferguson and co-workers have elucidated the carbohydrate-lipid structure of a series of parasite GPI-anchored surface molecules. In *Leishmania* promastigotes (a vector stage of the parasite), three types of GPI-anchored molecules constitute a large portion of the integral surface molecules: a GPI-anchored lipophosphoglycan (LPG) is present in 5 million copies per cell, a GPI-anchored glycoprotein (GP63) is present in 0.5 million copies, and GPI without other moieties attached (referred to as glycoinositol phospholipids or GIPLs) is the most abundant of the three at 20 million copies per cell.

Biophysically similar GPI-anchored molecules are present on the surface of trypanosomes; however, rather than having LPG with its negatively charged, helical disaccharide repeat motif with radiating side chains, procyclic trypanosomes have a glycoprotein, procyclin, which in *Trypanosoma brucei* has a polyanionic stalk structure of Glu-Pro repeats. The procyclin of *T. brucei* has N-linked glycosylation near the N-terminal end of the protein component and does not contain sialic acid. The GPI anchor of the *T. brucei* procyclin has an extremely complex carbohydrate side chain of galactose, N-acetylglucosamine, and five sialic acid residues linked to GPI by an unusual and unknown sugar linkage. How this side chain is sialylated and the role of this complex side chain in the insect stage of the African trypanosomes are presently not known. When the parasites return to the vertebrate host, GPI-anchored surface molecules predominate: in *Leishmania*, amastigotes have abundant GPIs (GIPLs) but almost none have protein moieties attached, whereas the trypomastigotes of *T. brucei* have predominately dimers of variant surface glycoproteins (VSG) anchored by GPI and modified with N-linked glycosylation (Ferguson MAJ 1994 *Parasitol Today 10:* 48-52).

Changes in glycoproteins of trypanosomes during the transition from vertebrate host to vector

In addition to changes in the translational machinery, changes in the surface of parasites occur during the vertebrate host-vector transitional stages of the life cycle. As the *T. brucei* bloodstream forms (trypomastigotes) leave the vertebrate host to transform into procyclic forms in the tsetse fly midgut, the variant surface glycoprotein (VSG) is shed and rapidly replaced by another GPI-anchored glycoprotein, procyclin or procyclic acidic repetitive protein (PARP). PARP is the predominant and immunologically dominant antigen of the 15 or more surface proteins that can be identified by surface labeling of the procyclic stage. A long dipeptide repeat of Glu-Pro in PARP appears to provide a highly negatively charged stalk to position the N-terminal end, with its N-linked glycosylation, on the outermost surface of the parasite. A similar but non-homologous glycoprotein, GARP, is present on the surface of parasites of the subgenus *Nannomonas*, such as *T. congolense*. Instead of an abundance of Glu and Pro residues, the procyclin GARP is Glu- and Ala-rich, and rather than N-linked glycosylation, GARP is probably O-glycosylated. There is no amino acid sequence similarity between the two proteins; however, GARP has a negatively charged stalk region and forms an immunological barrier to the other surface proteins present on the procyclic stage. Both procyclins, PARP and GARP, are hypothesized to mediate protection from antibodies against other surface proteins, complement and other serum factors, and midgut enzymes and trypanocidal factors. The differences in PARP and GARP may be responsible for the tropism observed in the tsetse fly, that is, although the proteins may share similar functions in evading vertebrate and vector defense mechanisms, the differences in their structures may explain why *T. brucei* procyclic stages end up in salivary glands and those of *T. congolense* end up in the mouthparts (Richardson JP et al. 1986 *J Immunol 36:* 2259-2264, Richardson JP et al. 1988 *Mol*

Biochem Parasitol 31: 203-216, Roditi I et al. 1989 *J Cell Biol 108:* 737-746, Roditi I & Pearson TW 1990 *Parasitol Today 6:* 79-81, Beecroft RP et al. 1993 *Mol Biochem Parasitol 61:* 285-294, Bayne RAL et al. 1993 *Mol Biochem Parasitol 61:* 295-296).

Lipophosphoglycans of Leishmania *during transition stages*

In *Leishmania*, as in trypanosomes, the major surface molecules that appear during the transition from vertebrate host to vector are GPI-anchored; however, rather than being glycoproteins, the major surface molecules are lipophosphoglycans (LPG). The salient features of LPG are a cap of neutral oligosaccharides atop a helical stalk of phosphorylated disaccharide repeats that are attached to a conserved core glycan moiety which is anchored to the surface by GPI. The variations observed between LPGs are essentially limited to the disaccharide repeats, although variants in the oligosaccharide cap have also been described. As discussed below, the variations are both species and stage specific. The function of LPG appears to be at least fourfold: 1) to provide a means of attachment to the midgut epithelium; 2) to evade complement-mediated lysis when entering the vertebrate host; 3) to assist in binding to and invading host macrophages; and 4) to mediate initial survival in the hostile environment of the phagolysome by inhibiting protein kinase C (thus inhibiting a mediator of activation of phagolysomes).

The dividing forms of *Leishmania*, called procyclic promastigotes, found early in the midgut development bind to the posterior midgut epithelium by LPG. Procyclic promastigotes are essentially avirulent to the vertebrate host and are sensitive to complement-mediated lysis and macrophage killing. The LPG present on the surface of procyclic promastigotes has a shorter disaccharide repeat region than the LPG found in the later developmental stage, the metacyclic promastigotes. The latter are the nondividing forms in the anterior midgut and mouthparts and are highly virulent to vertebrate hosts and are resistant to complement mediated lysis and macrophage killing. The changes that occur in LPG during parasite development within the vector appear to mediate release of the parasite from the midgut and to facilitate infectivity of the parasite to the vertebrate host. In addition to elongating the repeat region, the side chain substitutions in the metacyclic promastigotes are modified. For instance, in *L. major*, the disaccharide side chains of the metacyclic forms are capped with arabinose, while in *L. amazanensis* the side chains are removed. These changes in terminally exposed sugars control the stage-specific adhesion of procyclic promastigotes to sandfly midgut. The elongation of LPG allow complement factor C3b to bind efficiently to the surface of metacyclic promastigotes but cause the parasites to be resistant to C5b-9 lysis. The surface-bound C3b is used by the parasite to bind to macrophages for invasion. The species specific polymorphisms in LPG structure may also account for the vector specificity of *Leishmania* species. Although almost all species of *Leishmania* can survive within sandflies for two days, only compatible parasites remain at day 5 after a blood meal. The compatibility appears to be due primarily to specificity of midgut binding, although differential effects on the secretion of digestive enzymes might also play a role in reducing the number of incompatible parasites present early in the sandfly infection (Pimenta P F et al. 1992 *Science 256:* 1812-1815).

The metacyclic form of LPG may also be involved in the comparatively quiet invasion of macrophages. Preliminary evidence from work by Dr Albert Descoteaux suggests that inhibition of protein kinase C (PKC) may mediate survival in the phagolysome. PKC is an important regulator of many inducible cellular functions within the macrophage including within the phagolysome and, thus, is a likely target for the parasite to enhance its survival in the macrophage. LPG is a potent inhibitor of PKC activity, and the 1-O-alkylglycerol domain of LPG appears to be the major source of inhibition *in vitro*. In the macrophage, PKC inhibition by LPG does not appear to occur during the translocation of PKC to the membrane, but rather during the activation of PKC once it associates with the inner leaflet of the membrane. How LPG, which presumably is in contact with the outer leaflet of the membrane, inhibits PKC on the inner leaflet is unknown. Because the degree of inhibition by 1-O-alkylglycerol correlates with the length of the alkyl chain, one of many proposed mechanisms of inhibition is that the alkyl chain may reach through the lipid bilayer and influence the fluidity of the inner leaflet or bind directly to PKC. Alternatively, a breakdown product of LPG rather than LPG itself may flip in the membrane to inhibit PKC (Descoteaux A 1993 *Parasitol Today*, in press).

Surface proteins of new world trypanosomes during the vector to vertebrate transition

Several surface proteins present on the insect stage (metacyclic) of *T. cruzi* mediate the initiation of an infection in the vertebrate host. In addition, binding to the extracellular matrix proteins may promote invasion. Dr Yoshida has found that surface iodination of metacyclic trypomastigotes label three predominate proteins of 90 kDa, 82 kDa, and 75 kDa that are metacyclic

stage-specific. Tritium labeling experiments identify a doublet of 50/35 kDa that is not detectable in blood trypomastigotes or intracellular amastigotes. Although the 90 kDa and the 50/35 kDa proteins have strain-specific variation, all of these proteins are present in a wide range of geographical isolates. *In vitro* studies of metacyclic trypomastigote invasion of Vero cells indicate that antibodies to the 90 kDa, the 82 kDa, and the 50/35 kDa proteins can inhibit invasion. Likewise, purified 90 kDa, 82 kDa, and 50/35 kDa antigens directly bind Vero cells, and the purified antigens can inhibit invasion. Because the inhibition is incomplete, the data suggest that the parasite uses one or more of these proteins to invade mammalian cells. Studies comparing strains expressing different amounts of 50/35 kDa protein, or expressing a variant form of the molecule, indicate that less-invasive strains have lower levels of the 50/35 kDa protein, whereas highly infective strains have a variant protein. Preliminary evidence suggests that invasion using the 82 kDa protein is more efficient than that dependent on the 50/35 kDa protein.

Dr Yoshida has characterized three of the surface antigens. The 50/35 kDa is a mucin-like glycoprotein rich in threonine and galactose. It is the major sialic acid acceptor for trans-sialidase catalyzed reactions in the metacyclic stage. The 82 kDa protein has N-linked glycosylation which, when removed, causes the protein backbone to migrate as a 70 kDa protein. Although DNA sequence analysis revealed a consensus sequence for trans-sialidase, enzymatic activity of the 82 kDa protein has not been demonstrable. Comparison of the sequence from the 90 kDa gene shows 60% homology to the 82 kDa protein in the carboxy-terminal end, which may indicate that the two proteins recognize the same mammalian cell surface receptor (Yoshida N et al. 1990 *Mol Biochem Parasitol 39:* 39-46, Yoshida N et al. 1989 *Infect Immun 57:* 1663-1667, Mortara RA et al. 1992 *Infect Immun 60:* 4673-4678, Ruiz RC et al. 1993 *Parasite Immunol 15:* 121-125, Ramirez MI et al. 1993 *Infect Immun 61:* 3636-3641).

Trans-sialidases

Trypanosomes do not synthesize sialic acids *de novo*; therefore, these parasites must rely on the host for sialic acids. Sialidases such as neuraminidase mediate the hydrolysis of sialic acid from glycoconjugates, while trans-sialidases (TS) catalyze the reversible removal of sialic acid from glycoconjugates and transfer it to acceptors containing ß-galactosyl residues. Recently, novel TSs have been identified in trypanosomes that transfer sialic acid from host glycoproteins and glycolipids to parasite surface molecules. In *T. cruzi*, TSs are expressed in both the vector (epimastigote) and vertebrate (trypomastigote) stages; in *T. brucei*, only the procyclic stage expresses TS. Dr Eichinger has cloned and sequenced the genes encoding a number of these TSs. In *T. cruzi*, he identified a multi-copy gene family encoding TSs. The genes encode proteins with four aspartate boxes (SxDxGxTW) in the N-terminal end, the first three of which align with similar boxes in sequences of bacterial sialidases. The fourth aspartate box is degenerate. The C-terminal end of these proteins contain 12-amino acid tandem repeats followed by a GPI-anchor attachment signal sequence. The numbers of repeats vary between the gene family members and are present only in the TS expressed in trypomastigotes, not in the epimastigote stage. The function of the repeats may be in formation of polymeric TS, as suggested by Dr Schenkman, who recently found that papain digestion at the N-terminal end of the repeat region of affinity-purified TS converted the high MW aggregate (400 kDa) to a full-activity monomer of 70 kDa.

Dr Eichinger found that transformation of bacteria with one of three trypomastigote expressed genes confers both TS and NA activity. Using recombinant bacterial expression, the TS activity has been mapped to the middle of the protein. By comparing the sequence of active TS gene to gene family members that do not express TS activity, a Tyr residue was identified and shown to be critical for TS activity.

Insect stages of *T. rangeli*, another new world trypanosomatid, express a sialidase that does not transfer sialic acid. A comparison of the hydropathy profiles of the *T. rangeli* sialidase and the *T. cruzi* TS indicates that they are strikingly similar. By swapping defined regions between the genes encoding these proteins, the specific regions conferring TS activity may be elucidated. Interestingly, comparison of the *T. cruzi* TS and *Salmonella typhimurium* sialidase, whose crystal structure has been determined, revealed conserved residues within the proposed catalytic and sugar-binding domains of the bacterial and protozoan enzymes. One of the predicted contact residues is the Tyr that was shown to be critical for TS activity in the parasite enzyme.

When *T. cruzi* epimastigotes reach stationary phase in the insect, the TS activity of the parasites increases. The 90 kDa monomeric protein having TS activity is not released as the metacyclic stage forms; however, when epimastigotes (noninfective to the vertebrate host) transform into metacyclic forms (infective to the vertebrate host), a TS with high MW, similar to the trypomastigote TS, is released by the parasite. Although 90% homologous to the trypomastigote TS in the N terminal part of the polypeptide, the epimastigote TS has a putative transmembrane

domain with a hydrophilic cytoplasmic tail rather than a repeat region and a GPI anchor sequence. The functional significance for the differences in monomeric associations and .nembrane attachment between the vector and the mammalian forms of the trypanosome TS is as yet unknown. In addition, because most of this work has been done with cultured parasites, further work in the vector is necessary to confirm these observations (Uemura H et al. 1992 *EMBO J 11:* 3837-3844, Crennel SJ et al. 1993 *Proc Natl Acad Sci USA 90:* 9852-9856, Roggentin P et al. 1989 *Glycoconjugate J 6:* 349-353, Schenkman S et al. 1994 *Ann Rev Microbiol, in press).*

Sialic acid receptor molecules

The target molecules, referred to as sialic acid acceptors (SAA) of the trypanosome trans-sialidases, may play a significant role in the interaction of the parasite with its host. Dr Schenkman has found that, in both the insect and the mammalian forms, sialic acid is incorporated into O-linked oligosaccharides of mucin-like, GPI-anchored proteins. The size of the mucin differs in the two stages, in that the trypomastigote mucin migrates as a broad band from 40 to 200 kDa in SDS-PAGE, whereas the metacyclic mucin has a MW of 35 to 50 kDa. When trypomastigotes are cultured in the presence of sialic acid donor molecules, sialic acid is transferred to the parasite mucins. Fab fragments against the SAA block attachment of parasites to mammalian cells. Mammalian cells having low levels of sialic acid are invaded poorly. A possible role for sialic acid residues is in the initial attachment of the parasite to lectins on the surface of host cells. Alternatively, TS itself may bind to a host molecule that acts as a TS receptor mediating attachment. Following attachment, TS or sialidases may break this initial attachment and allow the parasite to invade mammalian cells. Whether sialic acid plays a role in the parasite's interaction with the vector remains to be established (Schenkman S et al. 1992 *J Exp Med 175:* 567-575, Schenkman S et al. 1992 *J Exp Med 175:* 1635-1641, Frevert U et al. 1992 *Infect Immun* 60: 2349-2360, Uemura H et al. 1992 *EMBO J 11:* 3837-3844, Vandekerckhove F et al. 1992 *Glycobiology* 2: 541-548, Schenkman RPF et al. 1993 *Infect Immun 61:* 898-902, Chaves L et al. 1993 *Mol Biochem Parasitol 61:* 97-106, Schenkman S et al. 1994 *J Biol Chem,* in press).

Chitinases

Chitin is a ubiquitous, fibrous polymer of N-acetylglucosamine that is completely hydrogen-bonded (i.e., unhydrated). Insects form chitinous structures in many locations and during many stages of development. Parasites transmitted by blood-sucking insects encounter chitinous structures in the alimentary tract of their vector hosts. Malaria and *Leishmania* parasites encounter the chitinous, sac like structure, referred to as a peritrophic membrane or matrix (PM), that forms around the blood meal. During their egress from the blood meal, these parasites must traverse through this matrix. *Leishmania* parasites also encounter chitin at the cardiac valve. In a fascinating and intricate interaction between the tsetse fly, a rickettsia-like organism (RLO), and the trypanosome parasite, RLO-derived chitinase mediates the susceptibility of the parasite to growth in the fly. Dr Phil Robbins and colleagues have cloned the genes for several yeast chitinases. The filarial chitinase and a number of plant and other fungal chitinases have now been cloned by other investigators. A least some of the yeast chitinases have putative-chitin binding domains. Genes encoding other chitinases appear to have fibronectin-binding domains instead of chitin-binding domains. It may soon be possible to construct a series of PCR primers to clone a wide variety of genes encoding proteins with chitinase activity (Robbins PW et al. 1992 *Gene 111:* 69-76).

The ookinete (midgut) stage of *P. falciparum* must cross the PM before it can invade the midgut epithelium. Ultrastructural studies have shown that the laminated structure of the PM is focally disrupted near the apical end of the parasite, suggesting that penetration of the PM is an enzymatic process mediated by a parasite-produced chitinase. Indeed, *in vitro*-cultured *Plasmodium* ookinetes synthesize and then secrete chitinase at about the time that they would have had to cross the PM. Dr Shahabuddin has found that allosamidin, a potent inhibitor of many non-fungal chitinases, almost completely inhibited malaria parasite chitinase *in vitro* and, when added to an infectious blood meal, completely blocked the parasite from invading the midgut epithelium. Mosquitoes fed fungal chitinase or polyoxin D (an inhibitor of chitin synthase) do not form PMs. To determine whether the blocking effect of allosamidin requires the presence of an intact PM, mosquitoes were fed allosamidin along with these compounds that disrupt PM formation. The transmission-blocking effect was completely reversed, suggesting that the parasite requires chitinase to cross the PM and that inhibition of this chitinase blocks transmission solely by interfering with the parasite's ability to cross the PM.

The chitinase secreted by the parasite is a zymogen. The prochitinase is activated by mosquito midgut trypsin-like proteases, probably by cleavage C-terminal to one or more lysine residue. Inhibition of the protease(s) by adding the serine protease inhibitor leupeptin to an infec-

tious blood meal completely blocked parasite transmission. The blocking effect is reversed by the addition of fungal chitinase to the blood meal, again suggesting that the block occurs as the parasite crosses the PM. Antibodies to midgut-derived black fly trypsin block parasite transmission as well. This work suggests that immunization of the mammalian host with either parasite-produced chitinase or vector-produced protease could elicit transmission-blocking immunity (Huber M et al. 1991 *Proc Natl Acad Sci USA 88:* 2807-2810, Shahabuddin M et al. 1993 *Proc Natl Acad Sci USA 90:* 4266-4270, Shahabuddin M & Kaslow DC *Parasitol Today 9:* 252-255).

Dr Schlein has found that other protozoans of several genera (*Leishmania, Trypanosoma, Leptomonas, Crithidia*, and *Herpetomonas*) secrete chitinase and N acetylglucosaminidase, with the enzymatic activities in *L. major* residing in proteins of approximately 110 kDa and 250 kDa, respectively. As in *Plasmodium*, *L. major* parasites are within a PM in the midgut. The parasites migrate anteriorly within the blood meal. The PM in this region focally disintegrates, apparently by parasite-secreted chitinase, to allow the parasites to exit the blood meal and move to the cardiac valve, the main sphincter of the sandfly food pump. In *L. major*-infected sandflies, the chitinous cuticle that covers the valve detaches and exposes the underlying tissue, thus apparently causing degeneration of the epithelial cells and muscle fibers of the valve. During blood feeding, the damaged cardiac valve apparatus allows the parasites to mix with the blood and to be regurgitated from the gut into the vertebrate host tissue, thus facilitating transmission. Curiously, repeated blood meals appear to inhibit the parasite induced damage to the cardiac valve, perhaps because hemoglobin accumulates in the midgut. Indeed, hemoglobin has been shown to inhibit parasite chitinase activity *in vitro*. These observations led Dr Schlein to study the effect of hemoglobin on parasite infectivity *in vivo*. Hemoglobin was found to inhibit infectivity; thus, vertebrate host-derived hemoglobin may interfere with chitinase-mediated development of infectious parasites in the vector. Whether the disruption of the chitinous cuticle is directly responsible for the damage observed to the cardiac valve has not yet been determined (Schlein Y 1993 *Parasitol Today 9:* 255-258).

The effect of chitinase on the establishment and maturation of African trypanosomes in tsetse flies is altogether different. The source of the chitinase is not parasite derived but rather comes from an opportunistic rickettsia-like organism (RLO). The establishment and maturation of trypanosomes in tsetse flies is highly carohydrate-dependent. Midgut lectins prevent the establishment of a trypanosome infection, and the oligosaccharide products of the RLO-derived chitinase reaction bind to these tsetse-produced lectins, converting an otherwise refractory tsetse into one susceptible to infection. Dr Ian Maudlin has shown that a similar effect can be achieved by feeding tsetse N-acetylglucosamine, indicating that lectins binding N-acetylglucosamine stimulate trypanosome killing. The maturation of trypanosomes in tsetse is even more complex, involving interactions between lectin-mediated signaling, fly sex-limited genes, and the trypanosome genotype. Once established, the trypanosome depends on midgut lectins for maturation signaling. Continuously feeding lectin-binding inhibitors or competitive lectins, i.e., Con A, to infected tsetse prevents metacyclogenesis. These experiments have demonstrated that midgut lectins that bind glucosyl sites promote maturation, presumably by causing surface changes in the parasite that induce cell cell signaling. Efficiency of parasite maturation is also sex-dependent. By producing less exogenous sugars that inhibit the maturation lectin from binding to the parasite, male tsetse allow more efficient maturation of the trypanosomes than female tsetse. The most obvious target receptor of these lectins is procyclin, described above. Subspecies differences in maturation may be due to differences in procyclin binding of lectins, perhaps through differences in the carbohydrate composition of procyclin.

The origin of replication of a plasmid carried by RLOs has now been cloned. That establishment of trypanosome infection in tsetse is regulated by symbionts offers the possibility of creating "pseudotransgenic" tsetse with recombinant RLO that would be refractory to trypanosomes. For instance, by increasing the activity of the N acetylglucosamine permease that transports this sugar into RLOs, midgut concentrations of the sugar would decrease, increasing midgut lectin-mediated killing. Alternatively, by increasing the activity of RLO-derived chitinase, the concentrations of exogenous sugars would increase in the midgut, resulting in inhibition of maturation by mimicking what occurs naturally in female tsetse (Welburn SC et al. 1993 *Parasitology 107:* 141-145, Welburn SC et al. 1994 *Med Vet Entomol 8:* 81-87).

Interactions of parasites with host extracellular matrix proteins

Insect basement membrane

During a number of stages in the life cycle, particularly during host transition stages, parasites have to circumnavigate and interact

with thin deposits of extracellular matrix (ECM) proteins that comprise basement membrane (BM, because the BM is not a true membrane, is now often referred to as the basal lamina). As an example, malaria parasites encounter basal lamina after the ookinete stage traverses the mosquito midgut epithelium, before sporozoites can invade mosquito salivary gland cells and, finally, when the sporozoites pass through the endothelial cells in the liver and in the Space of Disse to invade hepatocytes. Although the collagen type IV, laminins, and peptidoglycans that comprise the basic network of BM are evolutionarily conserved, particularly in their junctional domains, the specific composition of ECM proteins in each of the BM that the parasite encounters may differ depending on the function of the BM. For instances, the BM of capillaries, which functions to contain endothelial cells and provide structural support, differs from the ECM proteins present in the Space of Disse, which undoubtedly differs from the BM that surrounds the insect hemocoel. Even the individual ECM proteins are structurally different: collagen type IV of vertebrates aggregate in groups of four molecules by single binding domains at their amino-terminal ends, whereas *Drosophila* collagen molecules aggregate in other groups by means of multiple binding domains at their amino terminal ends. The attachment site on laminin for the nidogen connectors that associate a sheet of collagen with a network of laminin is also conserved, but the connecting chains between functional domains appear to be evolutionarily divergent.

In insects, the composition of BM and the structure of ECM proteins has been studied in the most detail in *Drosophila*. Dr John Fessler and his colleagues have now characterized a series of ECM proteins expressed during embryogenesis. The production of ECM proteins occurs in a stepwise fashion, starting with proteoglycans and peroxidasin (a peroxidase-type protein), followed by laminin and a protein referred to as Protein Z, then tiggrin (a ligand for integrins), and finally collagen IV. The major source of these ECM proteins appears to be the hemocytes that are widely distributed throughout the developing fly. In addition to producing ECM proteins, hemocytes engulf cells, encapsulate cells, and appear to play a role in programmed cell death and remodelling that occurs during embryogenesis. Peroxidasin, a 170 kDa protein that forms a homotrimer, is a peroxidase homologue that appears early in embryogenesis and may play an important role in removal of apoptotic cells. In the embryo, peroxidasin co-localizes with laminin and collagen as well as hemocytes and may be involved in crosslinking basal lamina monomers as well as in phagocytosis of dead cells during programmed apotosis. A unique ECM protein, tiggrin, localizes to the muscle junction sites of the embryo. This 257 kDa protein has the canonical RGD integrin interaction site and binds $\alpha_{PS2}\beta_{PS}$ integrin of *Drosophila*. Transfection of *in vitro*- cultured insect cells with the genes for the aPS2 and ß integrin chains confers a "spreading" phenotype to the cells. This phenotype can be inhibited by the addition of peptides containing the RGD sequence. As discussed below, the presence of the RGD sequence in the TRAP protein of malaria sporozoites suggests a possible function for this protein in binding to the basal lamina either in the mosquito salivary gland or in the vertebrate liver (Hortsch M & Goodman CS 1991 *Ann Rev Cell Biol 7:* 505-557, Brown NH 1993 *Bioessays* 15: 383-390, Bunch TA & Brower DL 1993 *Curr Top Develop Biol 28:* 81-123, Fessler LI et al. 1994 *Meth Enzymol, in press).*

Role of insect extracellular matrix proteins in parasite development in the midgut

After the *Plasmodium* ookinete traverses a midgut epithelial cell, it encounters the basement membrane that lines the hemocel. Here the motile ookinete rounds up and forms an oocyst inside which sporozoites develop and accumulate. Several lines of evidence suggest a significant role of the basement membrane in the development of an ookinete. Dr Alon Warburg found that *in vitro*-cultured ookinetes injected directly into the hemolymph form clusters of oocysts adherent to the basement membrane throughout the hemocel. Likewise, when ookinetes are added to dissected midguts, the majority bind to the external, basement membrane-covered side. Furthermore, binding of ookinetes to artificial surfaces, such as plastic, is enhanced at least 10-fold by addition of various components of basement membrane (matrigel, collagen IV, and laminin). Parasite-derived basement membrane may also play a significant role in the maturation of the oocysts, as the thick capsule that surrounds the oocyst appears to contain both laminin and collagen IV. The apparent requirement of basement membrane components for the further development of an ookinete into an oocyst has been exploited to promote transformation to occur *in vitro*. *In vitro*-cultured ookinetes aggregate, and an occasional oocyst develops. Addition of matrigel and *Drosophila* L2 cells to the culture markedly enhances this transformation process, even to the point where structurally recognizable sporozoites form. In the presence of matrigel, the L2 cells appear to be required. An attractive hypothesis consistent with these observations is that the ookinete attaches to any basement membrane, but that only insect basement membranes signal the induction of oocyst development. The addition of L2 cells may be

necessary to modify the mammalian-derived matrigel to the insect-type basement membrane required to signal transformation induction or may be providing necessary growth factors for oocyst development (Warburg A & Miller LH 1992 *Science 255:* 448-450).

The transition of oocyst sporozoites to sporozoites residing in the salivary gland is, in many ways, functionally analogous to the transition that occurs in *Leishmania* procyclic parasites during metacyclogenesis. As discussed above, the poorly infective procyclic stage differentiates into an infective metacyclic form during development in the insect. Likewise, poorly infective sporozoites in oocysts become highly infectious after invading the mosquito salivary gland. The structural differences between the two forms of sporozoites are not as marked as those in *Leishmania* metacyclogenesis; however, the oocyst forms do have a thinner surface coat as compared to the thicker coat present on salivary gland forms. The difference in the surface coats may reflect the need of the oocyst form to escape recognition by the mosquito, whereas the salivary gland form needs to escape recognition by the vertebrate host. One of the differences between these two forms of sporozoites is their sensitivity to complement: oocyst sporozoites are sensitive to lysis by the alternative pathway of complement, whereas salivary gland sporozoites are not. Differences between the two forms are also manifested by the inability of the salivary gland sporozoite to re invade the salivary gland when injected into the mosquito hemolymph. It is now clear that multiple vector receptor parasite ligand interactions occur during the movement of the sporozoites from the oocyst into the salivary gland duct. The specificity of oocysts sporozoites for salivary glands suggest that the binding of salivary glands is mediated by receptors.

Ultrastructurally, studies have shown that the surface of sporozoite in the hemolymph closely interact with the basement membrane covering the salivary gland. Once through the basement membrane, the sporozoite invades the salivary gland cells but, unlike the intracellular stages of the vertebrate host, the salivary gland sporozoite quickly escapes from the parasitophorous vacuole. The sporozoites aggregate within the secreting vacuoles and form intercellular junctions with one another that exclude saliva.

Some of the important questions that remain are: what are the receptors and ligands involved in the parasite-vector interactions and which are the most amenable to genetically controlled modification to create a refractory mosquito? To address these questions, Drs Musa Touray and Louis Miller have developed an *in vivo* salivary gland invasion assay and have found that anti-salivary gland antibodies, sulfated glycosaminoglycans, and some lectinsparticularly Suc-WGA and, to a lesser extent, underivatized WGA-block invasion of sporozoites. Although the mechanism of blocking is not yet known, those lectins that block invasion bind to salivary glands but do not bind to sporozoites. The blocking effect of lectins can be reversed by appropriate sugars. Whether the presently known surface proteins, CSP and TRAP, are involved is not yet known (Touray MG et al. 1992 *J Exp Med 175:* 1607-1612, Touray MG et al. 1994 *Exp Parasitol, in press).*

Role of hepatocyte heparan sulfate proteoglycans in the vertebrate host

Malaria parasites complete the transition from vector salivary gland duct to invasion of the vertebrate hepatocyte within minutes after transmission by an infected mosquito. The parasite must find the liver, leave the bloodstream in the hepatic sinusoid, and cross the Space of Disse before invading a hepatocyte. The selectivity and speed of this process strongly suggest that it is mediated by specific receptor-ligand interactions. Because the circumsporozoite protein (CSP) is the predominant surface protein of sporozoites and contains a highly conserved region (region II+) homologous to a cell adhesive motif in thrombospondin, properdin, terminal complement components C6-9, and a number of other vertebrate host proteins, much of the work of Drs Ute Frevert, Photini Sinnis, and Victor Nussenzweig on hepatocyte invasion has focused on CSP-host cell interactions. Previously it was shown that CSP, and more specifically peptides representing the region II+ sequence, bind receptors associated with the hepatocyte microvilli in the Space of Disse. Radiolabeled CSP injected retro-orbitally into mice clears from the bloodstream and accumulates in the liver. By electron microscopy, the CSP accumulates almost exclusively in the Space of Disse, apparently on hepatocyte microvilli. In tissue sections, CSP also binds to heparin-containing granules of connective tissue mast cells and to selected basement membranes in the kidney in a distribution typical for heparan sulfate. Kidneys, however, do not accumulate labeled CSP injected intravenously in mice, suggesting that the CSP binding sites in the kidneys are not accessible to circulating CSP or to sporozoites.

Only aggregated CSP is cleared by the mouse liver; monomeric CSP remains in the circulation. Consistent with the notion that the aggregated form of CSP is responsible for binding and that formation of aggregates is disulfide-dependent, Dr Sinnis found that the aggregated fraction in a crude mixture of region II+ peptides accounts for most of the binding activity to HepG2 cells.

Blocking the cysteine sulfhydryl groups in region II+ peptides results in a loss of binding activity. Binding activity also requires the basic amino acids, arginine and lysine, at the C-terminus of region II+ and hydrophobic amino acids. Preinjection of mice with multivalent region II+ peptides significantly decreases the accumulation of radiolabeled CSP in the liver. Thus, multimerization of region II+ is required for binding. Analysis by SDS-PAGE reveals that a portion of CSP extracted from sporozoites exists as a disulfide-linked multimer on the parasite surface.

The requirement for positively charged residues in region II+ may be explained by the negative charge of the receptor. Treatment of HepG2 cells with chondroitinase ABC has no effect on CSP-binding to HepG2 cells or to liver sections, but removal of heparan sulfate with heparitinase abolishes it, suggesting that CSP-binds specifically to heparan sulfate proteoglycans (HSPG). The CSP-binding HSPGs appear to be of the syndecan type of cell surface proteoglycans, because mild trypsin treatment but not phosphatidylinositol-specific phospholipase C or heparin incubation releases the HSPGs from HepG2 cells. The CSP-binding hepatocyte HSPG has an apparent MW of 150-300 kDa and migrates as a high molecular weight smear on SDS-PAGE. The free HSPG has both heparitinase- and heparinase-sensitive regions. If first complexed with CSP, however, the HSPG is still sensitive to heparitinase but resistant to heparinase, suggesting that CSP binds preferentially to highly sulfated, heparin-like oligosaccharides, rather than to the regions with a lower degree of sulfation. Consistent with this notion, HepG2 cells that have been treated with chlorate, a competitive inhibitor of sulfation, bind poorly CSP and are less susceptible to invasion by sporozoites. Interestingly, syndecans and the CSP-binding HepG2 cell HSPG turn over by internalization, and preliminary evidence suggests that binding of CSP enhances this turnover. Whether the parasite uses this mechanism to invade the hepatocytes is not yet known (Cerami C et al. 1992 *Cell 70:* 1021-1033, Frevert U et al. 1993 *J Exp Med 177:* 1287-1298, Cerami C et al. 1994 *J Exp Med 179:* 693-701).

Insect factors in parasite refractoriness

Encapsulation of parasites

Products of the conversion of tyrosine to melanin via DOPA are used by insects in cuticle tanning, egg shell tanning, and wound healing. A number of enzymes in the pathway, including phenol oxidase (PO), dopa decarboxylase (DDC), and an as-yet-unclassified enzyme (that functions in the conversion of dopachrome to 5,6-dihydroxyindole) play key roles in the formation of the end product, melanin, which is also used as a defense mechanism to encapsulate parasites such as *Plasmodium* and filaria. In adult mosquitoes, the blood meal provides a source of tyrosine. Because melanotic encapsulation uses the pool of tyrosine also necessary for egg shell tanning, a melanization response to parasites can significantly delay the deposition of eggs (oviposition). Indeed, Dr Bruce Christensen has found that a response of *Armigeres subalbatus* mosquitoes against *Brugia malayi* impairs egg and ovary development and delays oviposition. The delay in oviposition can be explained by the observation that >80% of *B. malayi* microfilariae are killed in the mosquito by melanotic encapsulation within 36 hours of ingestion. Dr Frank Collins has been studying encapsulation in *Anopheles gambiae*, the primary vector of malaria in subSaharan Africa. Encapsulated malaria parasites were first observed when a Ceylon strain of the monkey malaria parasite *P. cynomolgi* was fed to the African-derived *An. gambiae* vector. Although many of the malaria oocysts developed normally on the mosquito midgut, some of the parasites were covered by a thick black capsule at a stage in parasite development when the ookinete was completing its passage across the midgut epithelium. Those ookinetes that escaped complete encapsulation could develop into oocysts. Because the capsule appears to be melanin and encapsulation only occurs when the parasite nears the basement membrane, the factor(s) responsible for encapsulation are presumed to be products of tyrosine metabolism and to derive from hemolymph rather than the epithelial cells.

Dr Collins has taken both genetic and biochemical approaches to identify factors involved in encapsulation. Two mosquito lines were genetically selected: one that was fully susceptible to parasite infection by the monkey malaria parasite, *P. cynomolgi*, and one that was completely refractory. The susceptible line was susceptible to a wide range of other strains and species of malaria parasites, while the refractory line was refractory to most but not all strains and the species. One notable exception to refractoriness were strains of *P. falciparum* that originated from Africa, where transmission of malaria is primarily by the *An. gambiae* complex. Early in the genetic analysis of the two lines, an esterase phenotypic marker was found that segregated with susceptibility. Because the esterase marker maps to a specific inversion in the susceptible line (the refractory line is wild-type), the genetic linkage of the esterase phenotype and susceptibility may only be fortuitous rather than causative. The analysis of these lines for the refractoriness gene(s) is further complicated by differences in refractoriness between parasite strains: refractoriness to the Ceylon strain of *P. cynomolgi* is simply inherited recessively, whereas inheritance

of refractoriness to other strains is multigenic and not recessive. Another refractory line has been selected, and at least one of the loci involved maps to the same region as the esterase gene. Other loci appear to be important as well. Thus, preliminary genetic studies suggest that the products of several genetic loci may contribute to malaria parasite encapsulation, and one of the important loci is linked with the esterase locus on the left arm of chromosome 2. Consistent with the notion that the encapsulation process was mediated by enzymes of the dopamine metabolic pathway in hemolymph, hemolymph of the refractory strain shows a higher level of phenol oxidase activity than the *Plasmodium*-susceptible strain of *An. gambiae*. Compared to the susceptible strain, the refractory strain also has elevated levels of serine protease activity after a blood meal. Interestingly, serine proteases have been implicated in phenol oxidase activation in the hemolymph of mosquitoes. One of several *An. gambiae* serine protease genes is elevated in response to both wound healing, and this gene maps to the same inversion as the esterase gene on the left arm of chromosome 2 (Crews Oyen AE et al. 1993 *Am J rop Med Hyg 49:* 341-347, Paskewitz SM et al. 1989 *J Parasitol 75:* 594-600, Vernick KD et al. 1989 *Am J Trop Med Hyg 40:* 585-592, Vernick KD & Collins FH 1989 *Am J Trop Med Hyg 40:* 593-597, Paskewitz SM et al. 1988 *J Parasitol 74:* 432-439, Vernick KD et al. 1988 *Biochem Genet 26:* 367-379, Collins FJ et al. 1986 *Science 234:* 607-610).

Lysis of parasites in the vector midgut

To elucidate mechanisms involved in vector parasite incompatibility, Dr Ken Vernick is studying ookinete development of the avian malaria parasite *P. gallinaceum* in a refractory and a susceptible line of a nonavian malaria parasite vector, *An. gambiae*. In both lines, ookinetes invade midgut epithelial cells in similar numbers. In the susceptible line, ookinetes invade and rapidly cross midgut epithelial cells to form oocysts beneath the basement membrane, whereas in the refractory line, the ookinete migrate through approximately a third of the epithelium and stop. The first ultrastructural evidence of damage to the ookinetes is degradation of internal structures; only later does the surface of the parasite disintegrate, dispersing parasite remnants throughout the epithelial cell. To look for a soluble, inducible, hemolymph-derived killing factor, *in vitro*-cultured ookinetes were injected into the hemocel and individual mosquitoes were monitored for parasite development using the specific rRNA probes described above. Neither the susceptible nor the refractory line maintained ookinetes in the hemolymph. Indirectly, these data suggest that the specific lytic factor(s) in the refractory line are intracellular. Identification of these factors may be exploited in a broader context to kill parasites that invade midgut epithelium. Dr Vernick is taking several approaches to identify these factors: 1) differential binding of radiolabeled midgut extracts from the two lines to ookinetes; 2) subtractive hybridization of cDNA from the epithelial cells of the two lines after feeding ookinetes; 3) genetic crosses; and 4) further ultrastructural studies to determine the mechanism of killing and lysis.

Insect factors in mammalian pathogenesis

Blood-sucking arthropods have developed a wide array of molecules to prevent hemostasis during the blood meal. The vertebrate host responds in three ways to focal blood loss: platelet aggregation, activation of the clotting cascade, and vasoconstriction. For most blood-sucking insects, except those insects that feed from a pool of unclotted blood or for a prolonged period of time, anti-coagulation does not appear to be essential for successful engorgement. Apyrases, which hydrolyze ATP and ADP to AMP and prevent platelet aggregation, are present in a wide variety of insects and have evolved independently by convergence. A diverse array of vasodilatory substances, including nitric oxide (NO), prostaglandins, and novel peptides and enzymes, are present in the saliva of insects. Because the targets of many of these substances are mediators and receptors associated with inflammatory and immune cells, immunomodulation at the site in the skin where the insect saliva and the parasite are deposited may act to potentiate the infectivity of the parasite.

Vasodilators present in insect saliva

Chagas disease is transmitted by triatomine bugs including *Rhodnius prolixus*. The saliva and salivary glands of this insect are particularly amenable to study because the glands can be surgically removed without difficulty and, once removed, the insect will still attempt to take a blood meal. Insects in which the glands have been removed abnormally probe repeatedly and do not successfully engorge. Examination of the skin at the probe site reveals platelet aggregation in the blood vessels. The saliva of these insects has vasodilatory properties similar to NO. Chromogenic substrates for NO synthase specifically label the salivary gland, and the chromogen localizes to the vacuoles of salivary gland cells. The NO, bound to heme-containing proteins stored in the glands, can be readily displaced from the Fe3+ form of heme by histamine or by argon. Histamine and serotonin, released when platelets aggregate, mediate vasoconstriction in

response to local blood loss. Thus, as the mammalian host responds to blood loss during a blood meal, the NO carried by heme proteins in the saliva is released and causes vasodilation. Dr José Ribeiro has found, in studies using pig ileal tissue to measure vasoconstriction, that the amount of NO-loaded heme protein present in a salivary gland is sufficient to have a physiologic effect in inhibiting vasoconstriction mediated by histamine.

Other insects have a variety of vasodilating substances, many of which may also modulate the host immune response to the benefit of the pathogens that the vector transmits. Ticks have prostaglandins in their saliva, including $PGF_2\alpha$ and PGE_2, the latter of which is known to inhibit macrophage and T cell activation. Tick saliva profoundly inhibits T cell proliferation stimulated by Con A or PHA and inhibits IL-2 secretion; however, these effects are not due to PGE_2 but rather to a protease sensitive molecule retained by a low MW dialysis membrane. *Aedes aegypti* mosquitoes have two bioactive peptides, sialokinin I and II, that are similar to tachykinins implicated in immunomodulation. Anopheline mosquitoes have salivary peroxidases that oxidize catecholamines and serotonin.

Dr Richard Titus has examined the saliva of the sandfly for vasodilators and immunomodulators. Addition of sandfly saliva to *L. major* promastigotes dramatically enhances parasite infectivity by inhibiting the ability of macrophages to present antigens and to produce two important defense molecules, hydrogen peroxide and NO. The immune modulating effect of saliva on infectivity is very potent and lasts up to four days in the host. A potent vasodilator (termed erythema-inducing factor, or EIF) present in sandfly saliva causes an intense area of local erythema that lasts for hours after the bite. Although EIF was initially thought to be the immunosuppressive substance in saliva, it may not have immunosuppressive activity. Nevertheless, immunization of mice with EIF neutralized the immunosuppressive effect of saliva and was protective for the host (Ribeiro JMC et al. 1990 *Br J Pharmacol 101:* 932-936, Ribeiro JMC et al. 1993 *Science 260:* 539-541, Ribeiro JMC & Nussensweig RH 1993 *J Exp Biol 179*: 273-287, Ribeiro JMC & Nussensweig RH 1993 *FEBS Lett 330:* 165-168, Champagne D & Ribeiro JMC 1994 *Proc Natl Acad Sci USA 91:* 138-142, Titus RG & Ribeiro JMC 1988 *Science* 239: 1306-1308, Ribeiro JMC et al. 1989 *Science 243:* 212-214, Nong YH et al. 1989 *J Immunol* 143: 45-49, Titus RG & Ribeiro JMC 1990 *Parasitol Today 6:* 157-160, Samuelson J et al. 1991 *J Exp Med* 173: 49-54).

Molecular approaches to understanding the parasite in the vector

In addition to classical genetic techniques, new molecular biological tools have been and are being developed to identified and analyze genetically controlled resistance factors present in vectors and parasites. DNA transfection now allows for genetic analysis of parasites in the absence of genetic crosses, e.g., in protozoan parasites that do not mate. Powerful new marker methods, such as restriction fragment length polymorphism (RFLP) analysis, single-stranded conformation polymorphisms (SSCP), random amplified polymorphic DNA (RAPD), and other PCR-based markers, now allow genetic analysis of vector and parasite crosses in the absence of phenotypic markers. In systems where genetic crosses and/or transfection are technically difficult, new methods of physical mapping such as yeast artificial chromosomes (YACs) and sequence-tagged sites (STS) can be used to fine-map loci of interest.

Genetic analysis of trypanosome surface glycoprotein function

GP72, a surface glycoprotein, is the immunodominant antigen of the insect midgut (epimastigote) form of *T. cruzi*. The protein is also expressed on the infective (metacyclic) form, and immunization with GP72 elicits stage-specific protective immunity. It is present in all strains that have been examined and has been proposed as the membrane acceptor site for C3-binding. GP72 is modified by an immunogenic, novel O-linked phosphoglycan, probably in the Thr- and Pro-rich region of the polypeptide. Antibodies to recombinant GP72 recognize the protein by immunoblot but do not recognize the surface of the parasite by indirect immunofluorescence, most likely due to the carbohydrate modification. Unlike many other surface proteins of trypanosomes, GP72 is encoded by a single copy gene. Using a 4 kb genomic DNA fragment in which the open reading frame was replaced by a G418 resistance marker or hygromycin marker, a doubly resistant null (double knockout) mutant was generated. Morphologically, the flagellum of the null mutant does not adhere normally to the parasite body and the anterior end of the mutant is truncated. Motility of the mutant is impaired. Despite the persistence of the abnormal flagellum, the null mutant developed into a metacyclic form and acquired complement resistance and sialidase expression and was infective to mammalian cells. The mammalian stages showed major morphological abnormalities; however, despite the apparent lack of the trypomastigote forms, the null mutant propagated in culture. When fed to *Triatoma infestans*, the null mutant survived very

poorly. The infectivity of the null mutant is more than four orders of magnitude less than wild type parasites. Whether this is due to the altered motility of the parasite or directly attributable to GP72 itself (e.g., through adhesion to the midgut) is not yet clear (Cooper R et al. 1992 *Mol Biochem Parasitol 39:* 45-60, Cooper R et al. 1993 *J Cell Biol 122:* 149-156, Ribeiro de Jesus A et al. 1993 *J Cell Sci 105:* 1023-1033).

Genetic maps of vectors

In mosquitoes, a number of genetic factors control refractoriness phenotypes to parasitic infection. In *An. gambiae*, very few phenotypic markers are available to rapidly map the refractoriness loci; therefore, other modern methodologies have been employed to map genetic loci in mosquitoes. Dr Liangbiao Zheng and coworkers have developed a dense genetic map using PCR primers that flank microsatelite sequences in *An. gambiae*. About 10,000 copies of these microsatelites (simple sequence repeats) are highly dispersed throughout the genome and are highly polymorphic. Over 100 such markers have been identified and genetically mapped: approximately 40 map to the sex-linked chromosome, about 40 to chromosome 2, and 24 to chromosome 3. Using 248 offspring from five families of backcrosses, the white-eye locus was mapped between two markers each about 1 centi-Morgan away on the sex-linked chromosome. Because of heterozygosity, mapping autosomal markers is more difficult and computer-assisted mapping is necessary. Using 165 offspring from two families of backcrosses, the red-eye locus was mapped on chromosome 3, the lunate locus on the right arm of chromosome 2, and the dieldrin locus on the left arm of chromosome 2. Some of the microsatelite markers have been physically mapped by *in situ* hybridization to polytene chromosomes. The genetic map correlates well with the cytogenetic map (Litt M & Luty JA 1989 *Am J Hum Genet 44:* 397-401, Tautz D 1989 *Nucl Acids Res 17:* 6463-6471, Weber JL & May PE 1989 *Am J Hum Genet* 44: 388-396, Rafalski JA & Tingey SV 1993 *Trends Genet 9:* 275-280, Weissenbach J et al. 1992 *Nature 359:* 794-801, Zheng L et al. 1993 *Science 261:* 605-608).

Drs Bruce Christensen and David Severson have taken a slightly different genetic approach to map loci that confer susceptibility of *Ae. egypti* to filaria and *Plasmodium*. Over 80 RFLP markers, mostly from random cDNA clones, were used to create a saturated genetic linkage map that covers 134 map units across the genome. Using RFLP analysis of F2 populations from crosses between susceptible female and refractory male mosquitoes, quantitative trait loci (QTL) mapping has revealed that two important loci govern susceptibility to filarial infection.

These mapping studies also show a multigene control of susceptibility to *P. gallinaceum* infection. Moreover, this mapping technique allows one to calculate the percent of the phenotype arising from genetic factors in the loci mapped. Mapping of susceptibility loci is also being performed by isolating highly susceptible and refractory lines from a common strain by pairwise matings. Initial studies indicate that one of the genes associated with susceptibility affects the penetration efficiency of microfilaria to cross the midgut wall. A large series of DNA probes has been developed for RFLP analysis of genetic crosses of *Ae. aegypti*; these probes can be used to quickly generate RFLP maps in other mosquitoes, because many of these *Ae. aegypti* probes cross-hybridize to anopheline and culicine mosquitoes. Likewise, insect systems for which a large panel of probes are available, such as *Drosophila*, may be useful in generating RFLP probes for vectors that transmit parasites (Severson DW et al. 1993 *J Hered 84*: 241-247, Severson DW et al. 1994 *Am J Trop Med Hyg 50:* in press, Severson DW et al. 1994 *Insect Mol Biol* in press).

Introducing genes into vector populations

As shown above, genetic factors clearly influence the susceptibility or refractoriness of vectors to parasite infection. Many natural populations have naturally occurring genes for refractoriness to parasite infection, but these genes are not fixed, despite the fact that for some natural parasite-vector combinations, such as filaria in mosquitoes and trypanosomes in tsetse, parasite infection substantially reduces fitness. The apparent enigma of extremely low vector infection rates during epidemic spread of disease (e.g., African sleeping sickness) may be explained, in part, by the very high efficiency of parasite transmission from individual infected vectors. This high efficiency allows transmission to occur even though few individual vectors are infected. By keeping the number of individual infected vectors to a minimum in the population, the parasite may be preventing fixation of genes for refractoriness from occurring in natural populations.

In addition to enhancing natural refractoriness or endogenous resistance mechanisms to parasite infections, importing mechanisms of killing that do not normally occur in the vector but do exist in other insects or organisms may prove to be an effective means of creating resistance. A number of approaches to introduce parasite resistance genes in natural vector populations are being explored, including transposable elements (TE) to introduce and fix genes in the vector genome, arbovirus (such as Sindbus virus) to express resis-

tance genes or anti sense RNA that bind to transcripts encoding susceptibility factors, and recombinantly altered symbionts (such as RLOs of tsetse described above).

Feasibility of using transposable elements to drive genes into vector populations

If a single gene conferring a desired refractoriness phenotype and an efficient means of introducing transposable elements to carry that gene into a vector population were available, would transposable elements drive the desired gene to fixation in a natural population? Dr Kidwell is taking two different, but complementary, approaches to address the question of the feasibility of gene fixation by transposable elements: empirical studies using *Drosophila* population cages and the well-studied P element, and computer simulation studies to examine the critical parameter values to fix a gene in a population. P elements are class II elements that transpose within the germline and without the RNA intermediates used by class I elements. Complete members of this multigene family are encoded in approximately 3 kb of DNA and transpose autonomously. Other, smaller, defective members that lack transposase activity can only move if an autonomous element is present in the same genome. Depending on a number of factors, P elements can cause hybrid dysgenesis and significantly decrease the fitness of the insect host. Nevertheless, over the last 50 years, P elements have completely invaded the cosmopolitan natural population of *D. melanogaster*. Also, in cage studies, they have been shown to spread rapidly through the population, despite significantly decreasing reproductive fitness of the fly. Although P elements may have been horizontally transferred to *D. melanogaster* from another *Drosophila* species, for the most part these elements do not have the potential to be useful in spreading sequences in populations of most other genera. In addition, it appears that the preexistence of P elements may suppress the spread of newly introduced P elements (probably by host derived regulatory factors rather than a lack of insertion sites in the genome); therefore, a new class of transposable elements may be necessary to drive a gene to fixation in populations that have preexisting transposable elements.

To study the spread of loaded P elements in population cages, a series of recombinant P elements has been constructed that appears to confer a slight fitness advantage. The notion is that if, under the optimum conditions of this model system, the marker gene did not spread, then the general approach of using transposable elements to drive genes into natural populations would need to be reconsidered. The cage population model is being used to compare: 1) the efficiency of a single-element system that mimics autonomous elements (containing the marker gene and transposase genes) and a two-element system that distributes the marker gene and the transposase onto separate elements; 2) the effect of varying the initial frequency of transposon-bearing flies on the rate of spread and efficiency of fixation of the marker; 3) the effect of varying the population size; 4) the effect of maintaining populations on discrete versus continuous generation regimens (i.e., the one-versus two-element systems); and 5) the effect that the marker gene (size of the sequence, promotor efficiency, fitness advantage or disadvantage, and the magnitude of the fitness) has on spread and fixation efficiency. Preliminary results indicate that loaded transposons spread rapidly through the cage populations, but the transposon frequency plateaus and, so far, the marker gene has not reached fixation. The reason for this plateau effect is not known. Overall, the early spread of the marker is more rapid than might be expected due to any advantage the marker gene provides. The two-element system appears to be somewhat more efficient in spreading the marker gene than the one-element system, although for both systems varying the frequency (5% vs. 10%) of element-bearing flies at the start of the study made little difference in the rate of spread. Using Southern blot analysis and *in situ* hybridization, up to three copies of the marker genes were observed in individual marked flies. Further experiments are in progress to study the long-term effects of these elements on the population along with their long term stability and molecular integrity.

Drs Ribeiro and Kidwell used a three-parameter density-dependent growth equation in a computer simulation model to determine and examine the critical parameter values required to fix a gene in a given population. The results of the simulations indicate that genes can be driven into and fixed in a population even when introduced at very low frequencies. The rate of fixation depends on the reproductive rate of element-bearing individuals, the transposition efficiency of the element, and the initial population size. Density dependence and the wild-type reproductive rate are not critical parameters in this model. The model predicts that fixation will occur even if the gene confers a substantial disadvantage in fitness to individual carriers.

Novel transposable elements

To date, P elements have not been used successfully to introduce genes through insects other than *Drosophila* genera; therefore, other TEs are being explored. One of them, the Minos element, has been described by Dr Babis Savakis. Minos TEs were isolated from *Drosophila hydei* and are

low copy number (5-15 copies/genome), 1.7 kb DNA sequences with inverted terminal repeats and two non-overlapping open reading frames. They are highly homogeneous both in DNA sequence and size and are related to the Tc1 element family first described in the nematode *Caenorhabditis elegans*. Like Tc1, Minos elements insert into TA target sequences. Minos elements encode a putative transposase of approximately 40 kDa which shows significant similarity to that of Tc1. The Tc1 family of mobile elements is also related to the *mariner* family of elements, members of which are present in a large number of insect species from many orders; both Minos and *mariner* appear to use the same 2 bp insertion site, and their transposases are distantly related. The molecular basis of a Minos-mediated transformation system is now being studied in *D. melanogaster*. Modified Minos elements have been constructed and used to show transposition in the *D. melanogaster* germline by P element-mediated transformation and shown to direct the synthesis of spliced Minos transposase mRNA. The transposase catalyzes the integration of genetically marked, non-autonomous Minos transposons into germline chromosomes after injecting plasmids carrying these transposons into pre-blastoderm embryos of flies carrying the transposase-producing insertion. Similar results were obtained when the transposons were co-injected with a plasmid carrying the gene encoding Minos transposase under the control of a heat shock promotor. Analysis of the individual insertions showed that they contain the complete ends of the element inserted into a TA dinucleotide at the *D. melanogaster* target sequence. In the presence of transposase, the insertions become unstable in both somatic and germline cells, resulting in excision and transposition events. In transformants with a Minos transposon containing a wild-type version of *white*, a dominant marker gene with a cell-autonomous eye color phenotype, somatic instability gives rise to mottled eye phenotypes. When the Minos element excises, it leaves behind a characteristic footprint sequence.

Because transposable elements are autocatalytic, they must be regulatable to survive. Therefore, to introduce foreign genes at different times into a natural population, transformation systems based on several families of transposable elements may need to be pursued (Franz G & Savakis C 1991 *Nucl Acids Res 19:* 6646).

Identifying opportunities for the future

Vector defense mechanisms

How the vector differentiates between self and non-self is the most poorly understood aspect of vector defense mechanisms. The vector probably relies on both cell-cell interactions and humoral factors to distinguish parasites from self. Likely candidates for the cellular component are the hemacyte and the fat body, and likely candidate receptors present on these cells are homologs to those present in vertebrates. In fact, CD36 and CD14 receptor homologs have already been identified in insects. As isolation of hemacytes from mosquitoes or other important vectors is currently a daunting task, an effort to establish immortalized hemocyte cell lines may lead to an entré into the receptors involved, the signaling mechanisms used when ligands bind these receptors, and to how these cells engulf, encapsulate, or dispose of non-self entities. The fat body should also be a focus of intensive investigation, particularly in regard to signaling mechanisms. The cytokine-like modulators described in the *Drosophila* model suggest that cytokine like molecules may be involved in activating fat bodies to release humoral factors that mediate defense mechanisms. Now that some of the mediators of an antibacterial response have been identified, studies of the overlap between these mediators and the antiparasite response are feasible. Establishing whether the vector defense responses are global or pathogen-specific may help to determine what factors mediate parasite resistance/refractoriness. In this regard, the surrogate systems or models may be particular useful, especially *Drosophila*. As new defense and wound-healing mechanisms are identified in these other insect systems, attempts to immediately study their effects on parasites should be undertaken.

Another area that remains poorly understood is the mechanisms used by vectors to neutralize active host components ingested during the blood meal. Are there vector-specific inactivators of these host components that the parasites rely upon? Although it is unclear why many parasites quickly shed their resistance to host complement once inside the vector, modulating the mechanisms the vector uses to neutralize active host components may make it possible to identify a means of creating parasite resistance in susceptible vectors.

Although recognition patterns, such as to LPS in bacteria, present on the surface of vector pathogens may trigger activation of vector defense mechanisms, the vector may also use the absence of highly specific vector-derived molecules as a means of identifying non-self. Leading candidates for such a specific vector molecule are the basement membrane components, particularly the glutactins that line the hemocel. Conceivably, parasites may incorporate vector basement membrane components directly on their surface as they penetrate into the hemocel. Alternatively, parasites may synthesize molecules that either bind extracellular matrix

proteins or mimic their structure. The need for such changes in the surface of parasites as they develop in the vector may explain the loss of resistance to host factors, such as to a complement, and the parasite-vector incompatibility observed when parasites are injected directly into the hemolymph (e.g., the failure of *P. gallinaceum* ookinetes to develop in the hemocel of *An. gambiae* despite normal development in the hemocel of the compatible vector, *Ae. aegypti*).

Vector basement membranes

In addition to playing a potential role in the vector defense mechanism, extracellular matrix proteins may play an important role as signals for the parasite. Regional differences in the thickness and composition of the basement membrane have been shown to provide important signals in embryogenesis of vertebrates and invertebrates. Regional differences of the extracellular matrix proteins in direct contact with the hemolymph need to be further studied, especially in relation to the basement membrane that covers the antilumenal side of midgut epithelial cells and the salivary glands. Yet another function of extracellular matrix proteins that may be worthy of further thought is their effect on receptor ligand interactions that occur in the proximity of basement membranes. Many of the constituents of basement membranes can accelerate ligand binding by a volume exclusion effect (similar to the function of polyethylene glycol or dextran sulfate to stimulate DNA-DNA hybridization during Southern blotting).

A cautionary note when reading the basement membrane literature and doing studies on extracellular matrix proteins: many of the enzymes that are available and have been used to characterize basement membranes are crude and contain other nonspecific enzymatic activities. Care should be given in interpreting results from studies in which these crude preparations have been used.

Glycobiology

Recruitment of additional highly-trained glycobiologists, particularly carbohydrate chemists, to study vector and parasite glycoconjugates is desperately needed. The evidence is quite clear as described multiple times throughout the meeting that many forms of carbohydrates are present on the parasite surface, perhaps because carbohydrates are good building blocks for making ligands with highly specific binding characteristics. Carbohydrates can be charged (by phosphates or sulfates to create ionic interactions), can form hydrogen bonds and, when folded, can create hydrophobic patches; therefore, it is not surprising that carbohydrates are important for conferring specificity to surface molecules. A well-studied example of this type of carbohydrate-mediated specificity has been described for human blood groups. When studying receptor-ligand interactions, it is important to keep in mind that low affinity interactions and the solution in which these interactions are studied (e.g., lectin binding to procyclin in the milieu of a blood meal rather than simple salt solution) may be important, and neither should be overlooked. An example of the importance of searching for low-affinity interactions is the research done on selectins. In solution, selectins form very low-affinity interactions with soluble receptors, yet they bind very well to the surface of cells. Parasites may need to form low-affinity interactions so that they can release themselves as necessary for further development or for invading vertebrate host or vector cells after initial attachment.

A new and expanding armamentarium of tools for studying carbohydrates is available. Carbohydrate analogues to interfere with specific receptor-ligand interactions have been developed, particularly by the pharmaceutical industry, as potential treatment or prophylaxis agents for influenza virus. Biosynthetic inhibitors of glycosidases are now available in the form of donor analogues and acceptor analogues. Because some parasites use unusual sugars such as arabinose or galactosefuranols, specific inhibition of parasite enzymes may be feasible. Transformation-mediated knockouts of parasite or vector glycosidases, sialidases, etc., may also lead to important insights into the function of these carbohydrates. In this regard, peptides that mimic carbohydrate structures may be used to block carbohydrate interactions.

A number of specific questions need to be addressed, including whether trypanosome procyclins bind vector lectins, what changes in lectin binding occur during metacyclogenesis, the function(s) of GIPLs, whether the unusual parasite galactosylfuranols mimic some vertebrate molecule, what the interaction is of GAG with intact sporozoites as compared to CSP, and whether parasites have selectins. More general questions that need to be answered include determining the function of vector-produced lectins, both in the midgut and the hemolymph and perhaps more important, before undertaking the laborious process of interferring with a target; and identifying the best carbohydrate targets on the parasite or in the vector. A meeting designed to prioritize known targets may be useful.

Molecular and genetic approaches

Four major disciplines can be employed to analyze parasite-vector interactions: biochemistry, cell biology, molecular biology, and genetics. Of the four, genetics is the most

powerful and most conclusive in establishing causality *in vivo*. To efficiently attack research problems by either a direct or reverse genetic approach, a series of tools is necessary. To start, strains with various phenotypes and genotypes need to be established. Once established, they need to be stably maintained and stored. For vector genetics, this requires feasible techniques for long-term storage of embryos and stable stock center(s) from which strains can be easily obtained. Neither of these tools is currently available for any of the important vectors that transmit human pathogens. Balancers that reduce genetic rearrangements and chromosomal change should be introduced into these strains to maintain their fidelity. To facilitate screening and mapping, genetic markers must be available, be they visible phenotypic markers, selectable markers, or molecular markers. Many of the marker assays presently employed are labor-intensive, tedious, and simply a "turn-off" to newcomers to the field. Thus, improvement in the assays for markers is necessary (e.g., the development of an elegant rRNA marker assay for monitoring developmental stages of parasites in the mosquito described above). Once these markers are available, detailed genetic maps that are integrated with physical maps need to be generated. Genetic mapping at the current level of 1 centiMorgan is not adequate; in fact, to be most useful, the genetic map must be linked to a physical map. Development and access to automated analysis of DNA markers, especially automated sequencing of large stretches of DNA, would significantly hasten progress in physical mapping of desired genes. Integrated with genetic mapping of vectors must be mapping of parasite genomes. Old barriers, such as the traditional separation of vector biologists and parasitologists into different academic departments or divisions, must be proactively broken down. A multidisciplinary approach should be sought, as should open exchange to create a highly collaborative research community. The latter has been established and maintained for decades in *Drosophila* research and should be fostered in vector/parasite research. In addition, a more formal network for sharing information should be created, such as an easily accessible electronic data base. As these tools and maps become available, it is imperative that they be applied to natural populations as well as to laboratory-reared strains.

Modern biotechnology has markedly advanced our ability to analyze genes and the molecules they encode in parasites and vectors; however, for a number of reasons, some-state-of- the-art methodologies have not yet been applied or have been difficult to apply to highly relevant questions in vectors and parasites. Manual sequencing of large regions of the genome, such as the chloroquine resistance locus in malaria, should be replaced by automated, high-through-put robotics sequencing technology. An even more intensive effort needs to be directed toward establishing routine transformation methodology for both parasites (e.g., malaria) and vectors (e.g., mosquitoes). Transformation is now the preferred choice to directly determine causality *in vivo*, especially when used in rescue analysis in which deficiency phenotypes are complemented by the introduction (or re-introduction) of the candidate gene. Identification of genes that control vector behavior, such as host range specificity and feeding and post-feeding behavior, need to be identified and studied as potential targets for transgenic mosquitoes. In all of these laboratory studies, an attempt needs to be made to confirm that the phenomenon being studied actual occurs in natural populations in a natural setting. Factors such as concomitant infections with other parasites or other organisms (e.g., RLO infection of tsetse), may significantly influence parasite-vector interactions. Perhaps the biggest gap in our understanding of the molecular interactions between parasites and their vectors and vertebrate hosts is in signal and signal transduction. Unlike development in many multicellular organisms, in which temporal blocks in development often lead to death, parasites can be sustained in transition states for variable time periods without loss of viability. Signals received by parasites from vectors and vertebrate hosts undoubtedly trigger the further development to proceed, yet in almost none of the parasite-vector systems currently being studied have any signals or signal transduction pathways been identified or characterized. This is despite almost daily breakthroughs in the identification and characterization of signals and signal transduction mechanisms in other systems. Along these lines, it is important to remember that the interaction is dynamic, that "cross-talk" develops between the parasite and its host. As the parasite evades one defense mechanism, another defense mechanism may be recruited which the parasite then evades, and so on. Genetic variability of the parasite and, likewise, of the host may ultimately evolve as a result of this cross-talk. Lifecycle transition stages are particularly opportune times to look and listen for such cross-talk.