

The Biology of Malarial Parasite in the Mosquito - A Review

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The purpose of this review is to summarize the biology of Plasmodium in the mosquito including recent data to contribute to better understanding of the developmental interaction between mosquito and malarial parasite. The entire sporogonic cycle is discussed taking into consideration different parasite/vector interactions and factors affecting parasite development to the mosquito.

Key words: malaria - mosquito - sporogonic cycle - cell biology

The control and prevention of malaria has been pursued for a long time. Although campaigns against malaria were initially successful in some areas, the emergence of resistance of the parasite to drugs and of the mosquito vector to insecticides, combined with the difficulties in implementing and maintaining effective control schemes have led to a resurgence of the disease in many parts of the world (Wernsdorfer 1991, Schapira et al. 1993, Roush 1993). Much of the current work on malaria focuses on immunological aspects of the disease and there has been remarkable progress in the identification of a variety of parasite antigens in different stages of the parasite development, some of which may be included in a future vaccine (Nussenzweig & Nussenzweig 1989, Mitchel 1989, Targett 1989, Nussenzweig 1990, Hommel 1991, Good 1991a). However, experimental and field studies have shown the complexity of the immune response to parasites, indicating that an efficient malaria vaccine is difficult to achieve (Cattani 1989, Good 1991b, 1992, Philips 1992). Therefore, for control of the disease greater understanding of the biological mechanisms involved in host/parasite/vector interactions is essential.

In recent years an increasing number of studies have concentrated on the mosquito stages of the parasite development. Comprehensive reviews on biological, ultrastructural, biochemical, molecular and immunological aspects of the parasite and the vector can be obtained in the literature (Sinden 1978, 1983, 1984, Carter & Graves 1988, Carter et al. 1988, Alano & Carter 1990, Billingsley 1990, Crampton et al. 1990, 1994, Alano 1991, Brey 1991, Coluzzi 1992, Kaslow et al. 1994a, Sinden

et al. 1996). This review focusses on the cell biology of malarial parasites at different stages of its development in the mosquito and also includes factors that may affect the parasite infectivity.

The sporogonic cycle

Some erythrocytic parasites differentiate into sexual forms called gametocytes. Mature and functional gametocytes ingested by an appropriate species of mosquito in a bloodmeal are stimulated to transform into the stages which establish the parasites in their vector (Garnham 1966). Under the influence of changes in the mosquito midgut environment the gametocytes become extracellular within 8-15 min of ingestion. After emergence from the red blood cell (exflagellation) the male gametes fertilize the female gametes within 60 min of ingestion of blood. The fertilized macrogamete (zygote) differentiates into a single motile ookinete over the next 10-25 hr, which migrates from the bloodmeal through the midgut wall to form an oocyst underneath the basal lamina of the midgut. Each oocyst produces many thousands of invasive sporozoites over a period of 7-12 days. The sporozoites escape from the oocyst and then invade the salivary glands, here they stay for possibly very long periods until injected into another vertebrate host when the next bloodmeal is taken (Sinden 1984, Carter & Graves 1988). A diagram of the sporogonic cycle in the mosquito is shown in Figure.

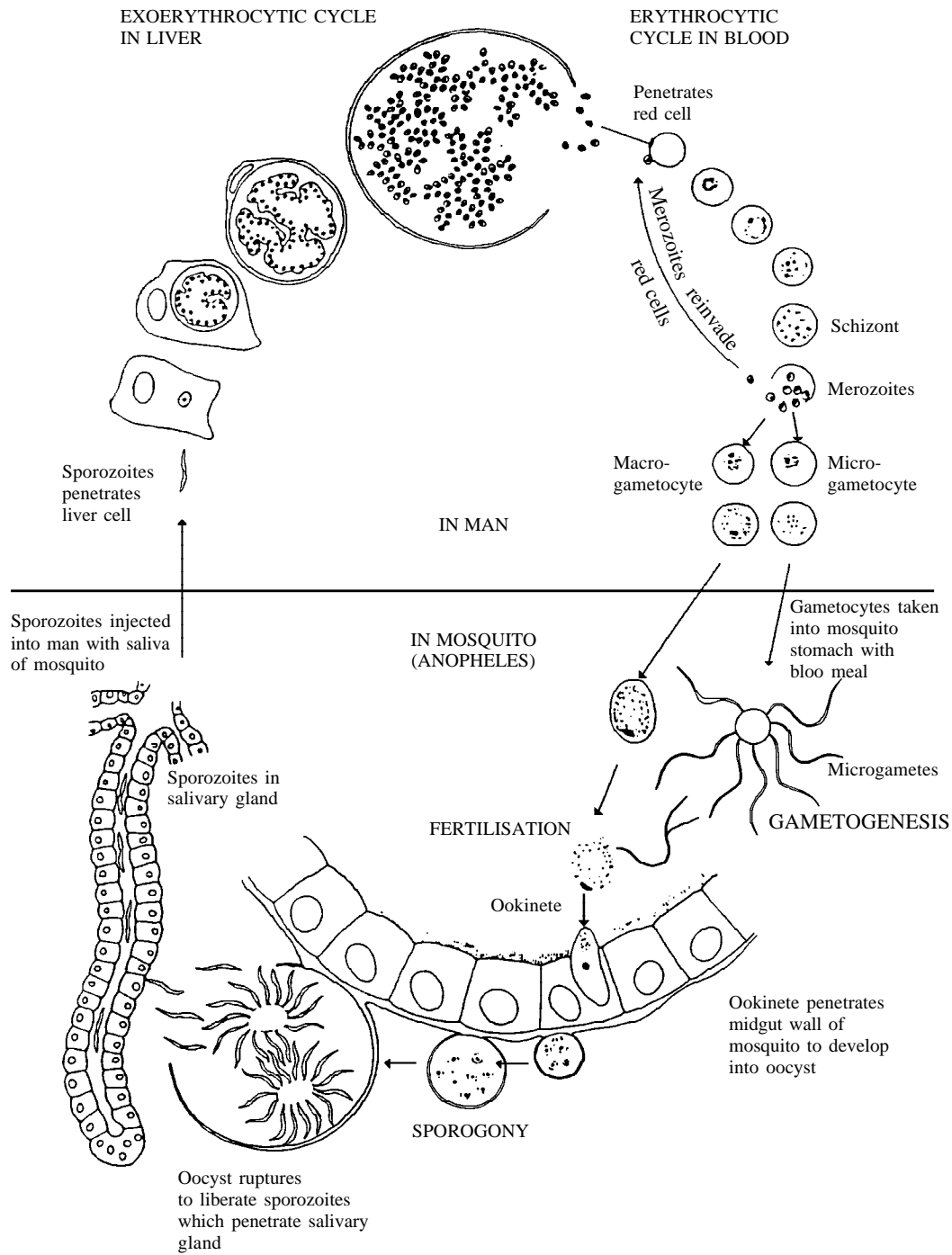
Development of gametocytes (gametocytogenesis)

As with other members of the order Haemosporina, gametocytes of *Plasmodium* can arise from merozoites from pre-erythrocytic parasites. Nevertheless, during natural infections, most gametocytes arise from merozoites of blood-stage origin (Alano & Carter 1990). Two alternative possibilities by which malaria parasites could become committed to either sexual or asexual development have been proposed (Inselburg 1983, Mons 1985, 1986, Bruce et al. 1990): first, the merozoite

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Life cycle of the malaria parasite in mammals (from Vickerman & Cox 1972).

is not committed at the time of invasion of a red blood cell. During early growth (as a ring form), the parasite is susceptible to factors that will commit it to either sexual or asexual development. Second, during growth of an asexual parasite, envi-

ronmental factors influence it so that at maturity the schizont produces merozoites that are precommitted to form asexual parasites or committed to form gametocytes upon subsequent invasion of a red blood cell.

A number of studies have attempted to identify factors that may influence the differentiation and production of sexual stage parasites including cAMP, presence of antimalarial drugs and other environmental factors associated to the host such as the presence of serum undetermined components (reviewed by Sinden 1983, Mons 1986, Carter & Graves 1988, Dearsly et al. 1990). In a recent report (Schneweis et al. 1991) it was claimed that the production of infective gametocytes *in vitro* can be enhanced by products of haemolysis but the nature of the erythrocytic factor was not identified.

Gametocyte development takes longer than asexual schizogony, e.g. 26 hr as opposed to 22.5 hr in *P. berghei* (Mons et al. 1985) or in the more extreme case of *P. falciparum* 8-12 days as opposed to 48 hr (Sinden 1983). The proportion of parasites that develop into gametocytes varies greatly during the course of natural infections even at its peak but is very low in relation to the total parasitaemia (Smalley et al. 1981). The growth and differentiation of gametocytes of *P. falciparum* has been divided into five stages (I to V) covering about eight days from merozoite invasion to mature gametocyte, each stage being distinguished by successive changes in the organization of the cell (Hawking et al. 1971).

Little is known about commitment of gametocytes to be male or female. The fact that both female and male can be produced by haploid blood-stage parasite, the sex of a gametocyte does not result from chromosomal differences between both types of cell but their development must be due to selective gene expression. In general, the number of female gametocytes predominates over the number of male, but this predominance may vary at different times between cloned infections (Cornelissen 1988, Schall 1989).

Gametogenesis

Mature and functional gametocytes ingested by the mosquito in a bloodmeal are stimulated by the midgut environment to transform into gametes. Studies indicate that various triggers induce gametocytes to undergo differentiation. Microgametogenesis *in vitro* is, optimally, dependent upon a rise in pH (Nijhout & Carter 1978), a rise in pCO₂ and bicarbonate levels (Carter & Nijhout 1977, Nijhout & Carter 1978), a fall in temperature of a few degrees below that of the vertebrate host (Sinden & Croll, 1975) or a very potent factor termed mosquito exflagellation factor (MEF). The latter is a small heat stable molecule from the mosquito head and gut which stimulates gametogenesis via a bicarbonate- and pH-independent mechanism (Nijhout 1979). Recently, Kawamoto et al. (1991)

showed *in vitro* that induction of exflagellation of *P. berghei* is triggered by a rise in the intracellular pH (pH_i) which is mediated by Ca⁺⁺ and cGMP regulation. pH_i can be modulated by alkaline media and is controlled by a complex series of interdependent ion pumps and channels controlling Na⁺, K⁺, Cl⁻ and HCO₃⁻ transport between the parasite and the environment. Other influential factors described include cAMP analogues and inhibitors of phosphodiesterase (Martin et al. 1978). The duration of microgametogenesis is both temperature and species dependent, e.g. at 20-22°C it may take 7-15 min for *P. falciparum in vitro*, although exflagellation may be detected after shorter periods in the fluid excreted by feeding *Anopheles* (Sinden 1983). There is no evidence that exflagellation is influenced by factors released by digestion of the blood meal since digestion normally begins several hours later (Graf et al. 1986). Microgamete formation involves three mitotic divisions with a rapid assembly of eight axonemes on the single microtubule organizing centre that divides and passes to the spindle poles. This division simultaneously segregates the genome and the axoneme so that each of the eight emergent gametes receives a single axoneme and haploid genome, both being connected to a common microtubule organizing centre. After exflagellation the microgametes, normally bearing a single axoneme, a single condensed nucleus and a single kinetosome with its sphere and granule at the distal end, are torn from the microgametocyte surface and rapidly move away into the blood meal (Sinden & Croll 1975).

Macrogametogenesis at the morphological level involves little more than escape from the host cell (Sinden 1984). At the cellular level there is *de novo* synthesis of the proteins which are expressed on the surface of macrogamete (Kumar & Carter 1984). It was recently identified a gametocyte specific protein of *P. falciparum* called Pf11-1 and there is some evidence that this protein might be involved in the emergence of gametes from the infected erythrocyte (Scherf et al. 1992, 1993).

Interactions between the vertebrate host and the parasite do not cease when the blood meal is taken. Following induction of gametogenesis the parasite is liberated into the blood meal. It has been shown that the gametes are susceptible to the phagocytes in the host's blood (Rutledge et al. 1969, Sinden & Smalley 1976) and that they are susceptible to activation of the components of the complement pathway (Grotendorst et al. 1986, 1987).

Zygote formation (fertilization) and ookinete development

Fertilization is rapid usually occurring within

1 hr of gamete formation, the plasmalemma of the two gametes fuse and the microgamete axoneme and nucleus enter the macrogamete cytoplasm (Sinden 1983). During zygote development, structural changes occur and its transformation to ookinete is in part determined by the gradual assembly of the apical complex including the collar and collar rings, rhoptries and micronemes (Canning & Sinden 1973, Davies 1974, Sinden 1978). Intensive protein synthesis also begins in the fertilized macrogamete and continues in the zygote and developing ookinete as reported for different *Plasmodium* species (Kaushal et al. 1983a, Kaushal & Carter 1984, Kumar & Carter 1985, Vermeulen et al. 1985a, 1986, Sinden et al. 1987). Included in this repertoire of proteins are many of the targets of proposed transmission blocking immunity. It has been reported that *An. gambiae* can concentrate the bloodmeal by a factor of 1.2 to 1.8 reducing the production of ookinetes when compared to *An. freeborni* which can not concentrate (Sinden et al. 1996). The mature ookinete is a motile cell that varies from 7 to 18 μm in length and 2 to 4 μm in diameter (Sinden, 1978). Locomotion of the ookinete has been described in different *Plasmodium* species as a linear or snake-like gliding motion (Freyvogel 1966). Studying *P. berghei* ookinetes in primary mosquito cell cultures, Speer et al. (1974) described spiral waves on the surface of some ookinetes, especially in the anterior half of the body, which might be involved in ookinete locomotion.

Some factors could influence the development, survival and infectivity of the parasite during its residence in the midgut lumen. Eyles (1952) has showed that the parasite development ceases at the ookinete stage unless a macromolecular (non-dialyzable) component is present in the blood meal. Studying the influence of red blood cells on the ability of *P. gallinaceum* zygotes fertilized *in vitro* to infect *Aedes aegypti* Rosenberg et al. (1984) found a linear relationship between erythrocyte density and the number of oocysts up to a 50% hematocrit. Furthermore, they deduced that there are one or more nondialyzable substances (erythrocytic factors) contained in normal erythrocytes, and released by mosquito digestion, that are essential for ookinete invasion of the gut epithelium. When they added trypsin inhibitor to the bloodmeal there was an inhibition of midgut penetration by ookinetes. Recent studies have shown that when mosquitoes are fed with cultured *P. falciparum* (Ponnudurai et al. 1989) and *P. berghei* (Sinden 1989) gametocytes, upon dilution with fresh red cells, more oocysts result at initial (low) dilutions whereas further dilution reduces oocyst counts. The involvement of blood factors and/or its digestive

products in infectivity has been studied in different parasite-vector models. Using a selected line of *An. stephensi*, Feldmann and Ponnudurai (1989) found mature *P. falciparum* ookinetes in the midgut lumen of refractory mosquitoes but no further penetration of the gut epithelium was observed. The reasons for this limited development in non-compatible mosquitoes could be related to digestive function since early initiation of hemoglobin degradation and higher aminopeptidase activity have been described in refractory strains of *An. stephensi* (Feldmann et al. 1990). It has also been shown that *P. gallinaceum* develops up to the ookinete stage in the non-compatible mosquito *An. stephensi*, this development occurring over the same time period and with the same success as in the compatible vector *Ae. aegypti*. However, *P. gallinaceum* ookinetes did not escape from the midgut lumen in *An. stephensi* mosquitoes (Rudin et al. 1991).

Possible mechanism inhibiting parasite development involves damage of the parasite by digestive enzymes present in the vector. Trypsin and aminopeptidases are the major proteolytic enzymes involved in blood digestion by female mosquitoes, and are produced by the midgut cells in direct response to blood feeding (Briegel & Lea 1975, Graf & Briegel 1982, Billingsley 1990, Billingsley & Hecker 1991). *P. gallinaceum* ookinetes 0-10 hr old (i.e. zygote to ookinete transition) were shown to be susceptible to mosquito enzymes in double feeding experiments (Gass 1977) and *in vitro* damage was observed to cultured ookinetes by proteinases from *Ae. aegypti* (Gass & Yeates, 1979). However, results found by Shahabuddin et al. (1993) using the same parasite/vector system suggest that the parasite secretes an inactive or partially active chitinase that is activated by a mosquito-produced serine protease. In a recent study Chege et al. (1996) examined the effect of digestive enzymes on the kinetics of *P. falciparum* ookinete development and oocyst infection rates in *An. albimanus*, *An. freeborni* and *An. gambiae*. Their data indicated that proteolytic enzymes alone do not limit the early stages of sporogonic development in these vector species of *Anopheles*.

Peritrophic layer

The peritrophic layer (PL) of the insect midgut forms a cylindrical sheet separating the midgut contents from the single cell-layered midgut endothelium. It is secreted within hours of the blood meal at different rates depending on the mosquito species (Billingsley 1990). Apical secretion granules are present in the midgut cells of *Anopheles* species, which are released into the periphery of the posterior midgut lumen during the feeding process in response to stretching of the gut wall. The

vesicle contents coalesce and then condense to form a compacted PL between 8 and 24 hr (Hecker 1977, Berner et al. 1983). In culicine mosquitoes the PL is formed *de novo* by the posterior midgut cells in the proventriculus. In *A. aegypti*, formation starts immediately after the blood meal, but about 12 hr later it is a mature structure (Perrone & Spielman, 1988).

The role of the mosquito PL as a barrier to ookinete invasion of the gut wall and pathogenic effects of *Plasmodium* species upon the vector are controversial. In ultrastructural studies on the interaction of *P. falciparum* ookinetes with the midgut epithelium of *An. stephensi* Meis & Ponnudurai (1987) frequently found parasites trapped in the membrane. They also observed that if the PL was dissected out 36 hr after the blood meal many ookinetes were attached to its external surface. In the same study it was mentioned that the ookinete was capable of penetrating the newly formed, but not the thickened and hardened PL 36 hr after the feeding. A failure to cross this barrier or retarded penetration might increase the length of exposure of ookinetes to mosquito trypsin to which it is known to be sensitive. In contrast, using *P. berghei*-infected *An. atroparvus* mosquitoes, Sluiters et al. (1986) concluded that the PL would not function as physical barrier against migrating ookinetes which can pass through fenestrations. Billingsley and Rudin (1992) observed that infectivity of *An. stephensi* by *P. berghei*, measured by oocyst counts, was unaffected by the presence or absence of the PL. However, in *A. aegypti* infected with *P. gallinaceum* its presence serves to reduce rather than prevent infection (Ponnudurai et al. 1988, Billingsley & Rudin 1992). These observations suggested to the authors that in compatible vector-parasite combinations, the PL acts as a limiting, rather than absolute barrier to the penetrating ookinete while in incompatible combinations the PL appears to be an absolute barrier to ookinete penetration.

To reach the midgut epithelium the ookinetes must first cross the partially or fully formed PL. The PL may act as the recognition site for the penetrating ookinete via lectin-mediated mechanisms. The occurrence of sugar residues which could be involved in vector recognition by the parasite has been demonstrated in the PL of *An. stephensi* and *Ae. aegypti* (Berner et al. 1983). Rudin and Hecker (1989) showed the presence of binding sites for different lectins in midguts of *P. berghei*-infected *An. stephensi*, with high specificity for N-acetyl-D-galactosamine (GalNAc), and *P. gallinaceum*-infected *Ae. aegypti*, with high specificity for N-acetyl-D-glucosamine (GlcNAc). The authors concluded that it seems likely that lectin-binding phe-

nomena play a role in the orientation of the parasites on their way out of the midgut lumen and that the PM and/or glycocalyx may be crucial structures for the penetration of the gut epithelium by the ookinete. This does not exclude the possibility that ookinetes penetrate the PL by an enzymatic process (see below). Ultrastructural observations on *Ae. aegypti* infected with *P. gallinaceum* showing an electron dense amorphous material in front of the parasite were consistent with a blockade of parasites within the PL (Sieber et al. 1991). The ookinetes appeared to disrupt the layers of the PL, suggesting an enzymatic mechanism for penetration. These observations were further investigated by Huber et al. (1991) who, using the same vector/parasite system, also suggested a possible role for GlcNAc in the binding of the ookinete to the PL, and demonstrated the presence of chitin in the PL. They observed that mature ookinetes transiently secrete a soluble chitinase, thought to be responsible for the digestion of the PL. Recently, Shahabuddin et al. (1993) reported an inhibition of *P. gallinaceum* chitinase and a transmission blocking activity of a chitinase inhibitor, allosamidin, on the sporogonic development of *P. gallinaceum* and *P. falciparum* in the respective vectors *Ae. aegypti* and *An. freeborni*. However, enzymatic mechanisms of penetration may differ in other vector/parasite systems since the PL of *An. stephensi* appears not to contain chitin (Berner et al. 1983).

Penetration of midgut epithelium

Ookinetes found in the epithelial cell layer between 24 and 48 hr post-infection have successfully evaded the obstacles presented by the midgut lumen; however, they still might fail to develop at the normal site of development (Sinden 1984, Ponnudurai et al. 1988). It has been shown that the midgut wall is negatively charged (Houk et al. 1986), but there is no evidence of electrical charge interactions between ookinetes and epithelial cells. It has been a matter for discussion as to whether the ookinete follows an intra- or intercellular route to reach the ultimate site of development and encystment in the outer wall of the midgut epithelium.

Intercellular movement of *P. gallinaceum* ookinetes was first described by Stohler (1957), but Mehlhorn et al. (1980) have found the same parasite in an intracellular position. Recently, Torii et al. (1992a) observed *P. gallinaceum* ookinetes in both intracellular and intercellular positions in the midgut epithelium of the mosquito *Ae. aegypti*, which they interpret as that they first enter into the epithelium, then exit into the intercellular space and move to the basal lamina. Garnham et al.

(1962) showed that the ookinete of *P. cynomolgi bastianelli* enters the epithelial cell by liquifying the cell membrane. Davies (1974) again postulated intercellular movement by *P. berghei nigeriensis* ookinetes. Although describing the ookinete of *P. berghei* in an intracellular position, as did Garnham et al. (1969), Canning and Sinden (1973) stated that the parasite might also migrate by an intercellular route. More recently, *P. yoelii nigeriensis* ookinetes have been described to take an intracellular route to the external wall of the midgut (Maier et al. 1987). However, when the same parasite was used to infect *An. omorii*, an intercellular route was mostly undertaken, although the intracellular occurrence was also observed (Syaffruddin et al. 1991). Meis and Ponnudurai (1987) presented evidence that *P. falciparum* ookinetes migrate between the epithelial midgut cells. Using a specific monoclonal antibody they also observed a track in the PL, which is related to the shedding of a 25 kD surface protein (Pfs25) during movement. The authors suggested that this protein may bind to receptors on the epithelial cells prior to an intercellular invasion, since it is reported to have epidermal growth factor (EGF)-like domains (Kaslow et al. 1988). It was recently shown that Pfs25 persists in the oocyst wall during parasite development in the mosquito (Lensen et al. 1993). The same group studied the migration of *P. falciparum* and *P. berghei* ookinetes through the midgut epithelium in *An. stephensi* by using ruthenium red staining (Meis et al. 1989). The results of previous studies were confirmed: *P. falciparum* ookinetes penetrated by intercellular route, but the rodent parasite *P. berghei* appeared to take an intracellular position, confirming that both mechanisms occur and are species-dependent. In the case of *P. berghei* a protein of 21 kD (Pbs21) present on the surface of the ookinete (Sinden et al. 1987) could play a role during the intracellular invasion of the midgut epithelium of *An. stephensi* mosquitoes. It was demonstrated in the midgut of *P. berghei* infected mosquitoes that expression of Pbs21 was predominantly localized on the ookinete surface one day after the infectious blood meal and thereafter expression declined to a minimum on days 2 and 3, the time of onset of oocyst development (Simonetti et al. 1993).

The mode of penetration by ookinetes can perhaps be related to damage of the epithelial lining of the midgut. Intercellular migration may not damage cell membranes, and increased mortality does not occur in *P. falciparum*-infected *An. stephensi* and *An. gambiae* during this period, even with very heavy parasite loads (Meis & Ponnudurai, 1987). Similar observations have been reported in *P. gallinaceum*-infected *Ae. aegypti* (Freier &

Friedman, 1987). They observed similar mortality rates in infected and uninfected batches of mosquitoes. However, when ookinetes use an intracellular route, as described previously, increased damage to midgut cells might occur, resulting in higher mosquito mortality. This is probably mediated by invasion of the hemolymph by opportunistic gram-negative bacteria and/or microsporidia (Maier et al. 1987, Seitz et al. 1987).

Ultimately the ookinete penetrates the basement membrane, but fails to pass through the basal lamina of the midgut adjacent to hemocoel (Sinden 1984). Whether this is due to the inability of the ookinete to penetrate the basal lamina, or to the specific recognition of the lamina and consequent shut-down of the incisive process is not known. Interactions of parasites with vector extracellular matrix proteins (ECM) cannot be discounted (Kaslow et al. 1994b).

Oocyst development

Oocyst development is predominantly extracellular (Duncan et al. 1960, Garnham et al. 1969, Howells & Davies 1971, Sinden 1975), but occasionally occurs within the midgut epithelial cell (Vanderberg et al. 1967, Bafort 1971, Beaudoin et al. 1974). The ookinete usually comes to rest beneath the basal lamina 18-24 hr after the infective blood meal (Sinden 1978). It rapidly rounds up between 18 and 72 hr after feeding and the apical complex is resorbed into the oocyst cytoplasm (Garnham et al. 1969). There is some evidence suggesting a significant role of the basal lamina in the development of the ookinete (Kaslow et al. 1994b). It was found that *in vitro*-cultured ookinetes injected directly into the hemolymph form clusters of oocysts adherent to the basal lamina throughout the hemocoel. Furthermore, binding of ookinetes to artificial surfaces, such as plastic, is enhanced at least 10-fold by addition of various components of basal lamina such as matrigel, collagen IV, and laminin (Warburg & Miller, 1992). The young oocyst is enveloped by a thick plasmalemma that is covered on the hemocoelomic surface by a fibrous basal lamina. Oocysts from the second day onwards are also covered by an amorphous capsule which becomes reduced in thickness at maturity (Vanderberg et al. 1967, Aikawa 1971, Strome & Beaudoin 1974, Sinden 1975).

Despite the usual growth of the oocyst under the basal lamina of the midgut, oocyst development is not site specific. Weathersby (1952, 1954, 1960) has shown by injection of gametocytes directly into the hemocoel of susceptible mosquitoes that oocysts would develop to maturity if attached to other parts of the body than the stomach

or even if they were floating freely in the hemocoel fluid. In his experiments he used different parasite-host combinations and concluded that the site of oocyst development is probably not a critical factor in the maturation process. Furthermore, the factors that are responsible for the death of parasite in refractory lines are not confined to the stomach wall. These results were supported by those reported by Ball and Chao (1957, 1960, 1961, 1976) who showed that oocysts of *P. relictum* may develop *in vitro* away from the intact stomach of the mosquito. The overall results of this series of studies by Ball and Chao demonstrated *in vitro* development of all stages from ookinetes to fully infective sporozoites without attachment of oocysts to the midgut. However, it was not possible to obtain complete sporogonic development in a single preparation. Rosenberg and Koontz (1984) injected cultured *P. gallinaceum* zygotes into the hemocoel of *Ae. aegypti* mosquitoes and observed development of ectopic oocysts in approximately 50% of the mosquitoes, with sporozoites being found in the salivary glands. These observations suggest that oocyst metabolism is not dependent upon direct transfer of nutrients from the midgut epithelium. Occasional intracellular oocyst development has been reported for *P. berghei* in *An. stephensi* and *An. quadrimaculatus* (Vanderberg et al. 1967). The same localization was described in *P. vinckei* by Bafort (1971) who concluded that both mechanical pressure and physiological mechanisms play a role in the movement of oocysts to the hemocoelomic surface. Studying the sporogonic development of *P. berghei* in *An. stephensi*, Beaudoin et al. (1974) found oocysts developing ectopically within the midgut epithelium following normal infection, eventually emptying their sporozoite content into the tissue itself or the midgut lumen. In addition, they observed no morphological and structural abnormalities in the luminal parasites which displayed good viability. In contrast to *P. berghei*, no ectopic development was seen in *P. falciparum*-infected *An. stephensi* mosquitoes (Meis et al. 1992b), confirming previous results observed with *P. falciparum* in naturally infected *An. gambiae* (Sinden & Strong 1978). Recent reports described an enhancement of oocyst development *in vitro* for *P. berghei* (Syaffruddin et al. 1992), *P. gallinaceum* (Warburg & Miller, 1992) and *P. falciparum* (Warburg & Schneider, 1993), when insect cell lines were added into the culture medium. From these observations it appears that nutritional or other regulatory requirements of the developing parasite can be met without a direct contact with midgut epithelium or haemolymph. The question of how the oocyst is supplied with nutritive material is an intriguing one.

Little information is available on the uptake and source of nutrients for oocyst development. It is assumed that *in vivo* the source of nutrients is the hemolymph. Mack and Vanderberg (1978) analyzed hemolymph of *An. stephensi* collected from uninfected and *P. berghei*-infected mosquitoes at different stages of the parasite development. It was found that four days after the blood meal the osmotic pressure and the specific gravity were lower in infected mosquitoes compared with uninfected ones. The difference, however, was attributed to indirect effects of the quality of the ingested blood meal. These studies were complemented by analysis of the concentration of free amino acids in the hemolymph collected in similar conditions with results showing significantly lower concentrations in infected mosquitoes with decreases in valine and histidine, and a total loss of detectable methionine suggesting it is incorporated (Mack et al. 1979). This difference could be due to the utilization of some of these amino acids by the developing oocyst as suggested by Ball and Chao (1976) who analyzed the uptake of amino acids by *P. relictum* oocysts *in vitro*, comparing growth of uninfected and infected guts of *Culex tarsalis* in Grace's insect culture medium. They found significant decreases in the concentration of certain amino acids including arginine, asparagine, proline and histidine, and less marked decrease in concentrations of others like methionine, valine, leucine and isoleucine. From these studies it appears that the reduced amount of free amino acids in the hemolymph is due to oocyst metabolism. Autoradiographic studies with *P. gallinaceum* in *Ae. aegypti* mosquitoes indicated that ³H-leucine is uniformly incorporated through the oocyst within 15 min of injection into hemocoel (Vanderberg et al. 1967).

Sporogony

With increasing maturation the oocyst undergoes considerable cytoplasmic subdivision. Initially the plasmalemma forms invaginations and clefts that penetrate even deeper into the cytoplasm, thus subdividing the cell (Vanderberg et al. 1967, Terzakis 1971, Posthuma et al. 1988). In a transmission electron microscopy study of *P. falciparum* oocysts it was suggested that cleft formation was due to dilation of endoplasmic reticulum (Sinden & Strong 1978). Using immunogold labelling technique during sporogonic stage of the same parasite, Posthuma et al. (1988) considered the latter explanation unlikely. With increasing activity the cytoplasmic clefts become extended and the expanding vacuolar space more pronounced leading to the sporoblast formation. Along the clefts sporozoites are formed by a budding process at the

face of the limiting membrane (Vanderberg et al. 1967, Howells & Davies 1971, Canning & Sinden 1973, Sinden & Strong 1978).

As the sporozoite continues to bud off, a nucleus and various cytoplasmic components are passed into it from the sporoblast. The membranes of the developing sporozoite pellicle are formed and other organelles like microtubules and rhoptries become discernible (Vanderberg et al. 1967, Sinden & Garnham 1973). When sporogony is completed (about 10-12 days after the infective feed), the oocyst is filled with sporozoites and one or more residual bodies (Sinden 1984). Estimations of number of sporozoites per oocyst have ranged widely. Garnham (1966) reported that the number in a single *P. vivax* oocyst varies from 1,000 to 10,000. Pringle (1965) estimated that a single oocyst of *P. falciparum* contains nearly 10,000 sporozoites. Studies carried out with mosquitoes fed on infected volunteers from Thailand showed a mean count of approximately 3,700 sporozoites per oocyst for *P. vivax* and 3,400 for *P. falciparum*, whereas for *P. cynomolgi*-infected mosquitoes a single oocyst contained about 7,500 (Rosenberg & Rungsiwongse 1991). Dependent upon species, the mature sporozoite varies from 9 to 16.5 μm in length and from 0.4 to 2.7 μm in diameter; aberrant forms have been described up to 40 μm long (Sinden 1978).

So far, studies on synthesis and expression of proteins during sporogonic development have focussed on a polypeptide called the circumsporozoite protein (CSP) found on the mature sporozoite. Observations on the origin of CSP and its distribution through the mosquito stage were reported by several authors. It is now well established that these proteins are synthesized in maturing oocysts of different *Plasmodium* species from 6-7 days after the infective blood meal, before sporozoites are visible (Nagasawa et al. 1987, 1988, Posthuma et al. 1988, Hamilton et al. 1988, Boulanger et al. 1988, Torii et al. 1992b, Meis et al. 1992a). At this stage, CSP is present on the plasmalemma and at various sites within the cytoplasm and endoplasmic reticulum of the sporoblast. When the sporozoites bud from the sporoblast they are already covered with CSP which is also found in salivary gland sporozoites (Yoshida et al. 1981, Santoro et al. 1983, Tsuji et al. 1992).

Humoral encapsulation of oocysts, which in malaria infected mosquitoes is known as Ross' black spores, has been described in *P. berghei nigeriensis* and *P. vivax* and seems to occur mainly in older oocysts which have begun to produce sporozoites (Sinden & Garnham, 1973). This phenomenon was studied in a selected line of *An. gambiae* that encapsulates different *Plasmodium*

species (Collins et al. 1986). The authors demonstrated that refractoriness is manifested by melanization of the ookinete after its passage through the midgut epithelium. Paskewitz et al. (1989) localized phenoloxidase activity in the basal lamina of the epithelial cells of both encapsulating and susceptible mosquitoes prior to blood feeding. However, after an infective blood meal, this activity was still observed close to invading ookinetes in refractory mosquitoes but it was reduced or absent in susceptible mosquitoes. When the non-compatible vector *An. gambiae* was fed with ookinetes of *P. gallinaceum*, invasion of the midgut epithelium by the ookinetes occurred but oocysts were infrequently formed. Using the same system Vernick et al. (1989) and Vernick and Collins (1989) tried to elucidate mechanisms involved in vector-parasite incompatibility by injecting *in vitro*-cultured ookinetes into the hemocoel of mosquitoes and monitoring parasite development using specific rRNA probes. As no differences were found between susceptible and refractory mosquitoes the authors suggested that the specific lytic factor(s) in the refractory line are intracellular.

The sporozoite and salivary gland invasion

The motile sporozoites emerge into the hemocoel through holes from an area of weakness in the oocyst wall. Holes are possibly produced by a combination of the muscular action of the gut wall and the activity of the sporozoites (Sinden 1978, Meis et al. 1992b). Within the hemocoel the sporozoites are distributed throughout the mosquito and can initially be found in many parts of the insect, even in the maxillary palps; within a day or two of their release from oocysts the sporozoites invade the salivary glands where they accumulate and remain until delivery (Vaughan et al. 1992). Thus, sporozoites do not adhere to most tissues, except for the salivary glands and rarely the midgut wall, hemocytes or thoracic muscles (Vanderberg 1974, Sinden 1975, 1978, Golenda et al. 1990, Vaughan et al. 1992). The latter observation, especially in heavily infected mosquitoes, could be associated with an impairment of flight activity in malaria-infected *An. stephensi* vectors demonstrated by some authors (Rowland & Boersma 1988).

It has been estimated that in mosquitoes fed on individuals with naturally acquired *P. vivax* about 850 sporozoites per oocyst reached the glands (Sattabongkot et al. 1991) which follows, by calculation, that approximately 23 % of all *P. vivax* sporozoites released into the hemocoel subsequently reach the salivary glands (Rosenberg & Rungsiwongse 1991). Vaughan et al. (1992), using regression analysis, calculated the approxi-

mately 650 salivary gland sporozoites were produced per oocyst and reported that virtually all oocyst infections produced salivary gland infections in *An. gambiae* infected with *P. falciparum* by membrane feeding. The same group has found a similar number by studying the sporogonic development of cultured *P. falciparum* in six species of laboratory-reared *Anopheles* mosquitoes (Vaughan et al. 1994). This is in contrast to observations on wild-caught *An. gambiae* from Burkina-Faso (Lombardi et al. 1987) and western Kenya (Beier et al. 1990) where sporozoites failed to enter the salivary glands in 43% and 10% of infected mosquitoes, respectively. However, when sporozoites from *P. gallinaceum* oocysts were injected into *Ae. aegypti* female mosquitoes only 6.5 to 10.4% of inoculated sporozoites invaded the salivary glands. Interestingly, injected salivary gland sporozoites did not reinvade the glands (Touray et al. 1992). Recently, a laboratory study on *An. tessellatus* mosquitoes infected with different isolates of *P. vivax* and *P. falciparum* from patients living in Sri Lanka showed that approximately 15% of mosquito batches in which oocysts developed failed to produce salivary gland sporozoites (Gamage-Mendis et al. 1993). This discrepancy between naturally and laboratory-infected mosquitoes could be attributed to different environmental conditions or mixed mosquito populations.

It could be expected sporozoites would elicit a humoral response in the mosquito by activation of the prophenoloxidase cascade and as a result be killed by melanization. However, sporozoites in the hemocoel are rarely seen to be melanized (Brey 1991). Sporozoites might be protected from mosquito defense reactions against 'non-self' by antigen sharing. This has been demonstrated as a potential mechanism for avoidance of mosquito defence reactions by microfilariae of *Brugia pahangi* (Maier et al. 1987). Immunoreactivity to CSP was observed on the midgut wall of mosquitoes infected with *P. falciparum* (Boulanger et al. 1988) and *P. yoelii* (Beaudoin et al. 1990). The latter authors also detected reactivity on uninfected midguts and suggest the presence of a common determinant between the parasite and the mosquito.

The migration of sporozoites from oocysts to salivary glands could be active (in contact with basal lamina), passive (in suspension) or both. If active, the parasite would use a gliding motility to move across the basal lamina and reach the gland. Vanderberg (1974) described circular gliding and attached waving locomotion and movement in sporozoites of different species when bovine serum albumin was added to Medium 199. Sporozoites that move over a substratum *in vitro* leave behind trails of CS proteins (Stewart & Vanderberg

1988, 1991, 1992). Soluble CSP was shown to be present throughout the hemocoel (Robert et al. 1988). Beier et al. (1992a) concluded that the release of CSP by sporozoites is a normal but complex mechanism that they interpreted to be associated with sporozoite survival in the host, is not site-specific and it would be regulated in response to background levels of soluble CSP in the environment (negative feedback mechanism). This idea is supported by previous results showing that the parasites cease to synthesize CSP during their journey through the hemolymph but shedding still occurs (Posthuma et al. 1988, 1989). There is no evidence yet for chemotaxis but the congregation of sporozoites in the vicinity of the glands before invasion may support, according to some workers, a tactile mechanism (Golenda et al. 1990, Meis et al. 1992b). Several polysaccharides have been identified that may orient protozoa towards or away from a stimulus (Van Houten 1988).

The selective invasion of mosquito salivary glands by malarial parasites is not well understood. Specific recognition of the glands was shown in *P. knowlesi*. Oocysts developed normally on the gut of *An. freeborni* but sporozoites were never found in salivary glands. When glands from the susceptible vector *An. dirus* were implanted into the abdomen, they did become infected (Rosenberg 1985). These experiments demonstrated that the salivary glands themselves determined specificity. Although invasion of salivary glands seems to require specific recognition, the mechanism by which sporozoites recognize, attach to, and penetrate the glands remains to be determined.

Sporozoites preferentially invade the medial lobe and the distal portions of the lateral lobes of the salivary glands where the salivary duct is not chitinous in *Anopheles* species (Sterling et al. 1973, James & Rossignol 1991, Ponnudurai et al. 1991). Hence, the occurrence of a specific receptor-mediated invasion by sporozoites is plausible. Perrone et al. (1986) used lectins to characterize carbohydrate moieties on the basal lamina of *Ae. aegypti* salivary glands. As the median and distal lateral lobes bound a common lectin, RCA 120, whose substrate is β -D-galactose, the authors suggested that sugars that bind this lectin serve as candidate residues to which sporozoites may attach. In contrast, the sporozoite coat binds some lectins with very low efficiency (Schulman et al. 1980, Turner & Gregson 1982). However, in a recent scanning electron microscopic study Meis et al. (1992b) localized *P. falciparum* sporozoites in proximal and distal parts but were unable to identify any specific regions on the glands where sporozoites penetrate. These authors also showed sites where the sporozoites have pierced the basal lamina, which

probably explains the presence of CSP on the basal lamina induced by shedding during penetration (Posthuma et al. 1989) and the presence of immunoreactive spots of 1-2 μm on the surface of infected glands (Golenda et al. 1990). Recently, Touray et al. (1994) developed an *in vivo* salivary gland invasion assay and have found that anti-salivary gland antibodies, sulfated glycosaminoglycans and some lectins, particularly Suc-WGA, block invasion of sporozoites. Although the mechanism of blocking is not yet known, those lectins that blocked invasion bound to salivary glands but did not bind to sporozoites. Beier et al. (1992a) and Beier (1993) proposed that as sporozoites invade the salivary glands, the build-up of CSP is the signal for sporozoites to halt their active motility, and thus their release of CSP (down-regulation). The involvement of CSP and other proteins distinct from CSP such as PySSP2 (Charoenvit et al. 1987) and PfSSP2 (Robson et al. 1988, Rogers et al. 1992a,b) in binding of sporozoites to the basal lamina of mosquito salivary glands is speculative. As they share similarities in their structure they might be related to this process since it has been suggested region II of CSP is involved in hepatocyte invasion (Cerami et al. 1992a,b, Pancake et al. 1992).

Rosenberg (1985) and King (1988) have suggested that sporozoites invade the salivary gland cells using a mechanism similar to invasion of erythrocytes by merozoites although there is no evidence of parasitophorous vacuole membrane formation observed in other stages of the parasite cycle (Sinden & Strong 1978, Meis & Verhave 1988). Membrane-limited vacuoles beneath the plasma membrane in *An. stephensi* distal salivary gland cells invaded by *P. berghei* have been described (Sterling et al. 1973). Penetration could also involve an unusual intercellular route as suggested by Golenda et al. (1990) who detected CSP in the region between cells of the median lobe of the gland. Posthuma et al. (1989) observed many sporozoites on the basal side of the cells, but also found trail-like CSP immunoreactivity at the lateral space between the cells.

After penetration, sporozoites are present in bundles in the accini of gland cells in both proximal and distal areas. Most probably, the sporozoites which are present in proximal areas of the glands are unable to reach the draining duct because of the chitinized layer in that area whereas distally localized sporozoites reach the draining duct via the large unchitinized collecting tube (Meis et al. 1992b). This explanation would not be valid for *Aedes* species since the salivary ducts are lined with chitin and extend the full length of the glands (James & Rossignol 1991). Penetration by sporo-

zoites could cause pathological vesiculation and cytoskeletal changes in salivary gland tissues as the infected cells are often deformed and swollen (Sterling et al. 1973, Maier et al. 1987). The efficiency of salivary gland invasion is poorly understood. It has been estimated that the median sporozoite load in the glands is less than 10,000 in colonized or wild *Anopheles* species (Shute 1945, Pringle 1966, Wirtz et al. 1987, Beier et al. 1991b, Sattabongkot et al. 1991) or slightly higher (Ponnudurai et al. 1991).

The development of infectivity by the sporozoites appears to be asynchronous, in some cases taking place in the hemocoel, while in others not occurring until after they have invaded the salivary glands. Thus, it seems to be time-dependent rather than site-dependent (Vanderberg 1975, Daher & Kretzli 1980). It was demonstrated in *P. berghei*-infected mice that populations of salivary gland sporozoites were more than 10,000 times as infective to the vertebrate host as populations of oocyst sporozoites from the same mosquitoes (Vanderberg 1975). Touray et al. (1992) found that as few as 10-50 salivary gland *P. gallinaceum* sporozoites were required to induce infection in chickens compared to 5,000 oocyst sporozoites. Sporozoite infectivity increases with time during the first week after the invasion of the salivary gland (Vanderberg 1975). Degeneration of sporozoites is not frequent in nature as observed by Barber (1936) who studied anophelines collected in Mediterranean areas. When degeneration occurred in a salivary gland or a lobe of a gland, in another gland or lobe the sporozoites were normal or in a different stage of degeneration.

The number of sporozoites injected into the tissue or capillary of the vertebrate is very small compared to that found in the salivary glands. It has been estimated, by employing different methods, that each bite delivers fewer than 50 sporozoites with a tendency for most sporozoites to be ejected in the first droplets of saliva (Vanderberg 1977, Rosenberg et al. 1990, Ponnudurai et al. 1991, Beier et al. 1991a,b, Beier et al. 1992b, Li et al. 1992). Although a correlation between salivary gland sporozoite load and sporozoite inoculum has been reported (Rosenberg et al. 1990) ejection of sporozoites is probably a random process, more related to the architecture of the salivary gland duct system than to the number of sporozoites in this organ (Ponnudurai et al. 1991).

Some studies reported that *P. falciparum*-infected mosquitoes deliver sporozoites in an unpredictable fashion, sometimes not at all (Ponnudurai et al. 1991), and others transmit inconsistently (Rickman et al. 1990). Clumping of sporozoites has been reported in infected salivary glands (Ster-

ling et al. 1973) and clusters of sporozoites were detected after delivery when *An. stephensi* mosquitoes infected with *P. falciparum* were allowed to feed through fresh mouse skin (Ponnudurai et al. 1991). It has been observed that salivary glands are not depleted of sporozoites even in vectors that feed up to 15 times (Shute 1945), which allows infected mosquitoes to remain potentially infectious for life.

The low sporozoite inoculum and the low entomological inoculation rates in natural conditions (Mendis et al. 1990a, Gordon et al. 1991) demonstrates the high efficiency with which injected sporozoites will develop malaria.

Factors affecting parasite development to the mosquito

The process of infection of mosquitoes is exceedingly complex and regulated by a range of factors originating from the parasite, the vertebrate host and the mosquito vector, and from the interactions between all three (Sinden 1991). Many of these and other factors are known to affect fertilization and subsequent stages of parasite development, thus having great influence on transmission of the disease. To have an idea, when sporogonic development of cultured *P. falciparum* was evaluated in six species of *Anopheles* mosquitoes there was a total loss of approximately 31,600-fold in the parasite population from macrogametocyte to oocyst stage (Vaughan et al. 1994).

a) Host location and feeding behaviour - Of primary importance in malaria transmission is the proportion of mosquito feeds taken from humans and the proportion of these feeds taken from infected individuals. In nature a vector needs to survive longer than the sporogonic period after taking an infective blood meal; during this period the mosquito probably takes blood meals every 2-3 days, depending on its gonotrophic cycle and the availability of breeding sites (Ponnudurai et al. 1989). Parasitemic hosts tend to be sick and often less irritated by mosquito feeding. Additionally, the thrombocytopenia which is commonly associated with blood-borne parasitic diseases leads to facilitation of vessel location, resulting in increased feeding success by mosquitoes on parasitemic hosts (Rossignol et al. 1985). Salivary glands and saliva contain a whole range of components with pharmacological activities important for blood feeding success and subsequent bloodmeal processing, including anticoagulants, anti-inflammatory, vasodilatory and immunosuppressive compounds (Ribeiro et al. 1984, 1989, Ribeiro 1987, Titus & Ribeiro 1990, James & Rossignol 1991). Rossignol et al. (1984) concluded that the lesions caused by *P. gallinaceum* sporozoites in the salivary glands

of *Ae. aegypti* result in reduced levels of salivary apyrase. Mosquitoes deficient in salivary apyrase experience difficulty in locating host blood and engorging; they therefore probe more often and may attempt to feed on several hosts in succession (Rossignol et al. 1984, 1986, Ribeiro et al. 1985). Li et al. (1992) demonstrated that probing time of *P. berghei*-infected *An. stephensi* is not affected by sporozoite invasion of salivary glands.

Blood meal size may influence infectivity since it determines the number of gametocytes ingested and therefore subsequent infection. It has been shown that larger *An. dirus* females took larger bloodmeals by artificial feeding with cultured *P. falciparum* and developed significantly more oocysts (Kitthawee et al. 1990). In a field study, Lyimo and Koella (1992) reported that the proportion of *An. gambiae* mosquitoes infected with *P. falciparum* during a blood meal was independent of size but the number of oocysts harboured by infected mosquitoes increased with size of the mosquito.

b) Gametocyte carriers/transmission blocking immunity - Malarial infections induce host responses to both asexual and sexual stage malaria parasites that may modulate gametocyte infectivity. Great heterogeneity in infectiousness of different carriers has been noted, with apparently poor correlation between infectiousness and gametocyte density (Graves et al. 1988). However, it remains unclear whether symptomatic and asymptomatic asexual infections differ in their ability to influence gametocyte infectivity. It has been reported that asymptomatic *P. falciparum* patients were more infectious than symptomatic patients (Boyd & Kitchen 1937, Jeffery & Eyles 1955, Muirhead-Thomson 1957, Carter & Graves 1988) while, in contrast, another report suggested that asymptomatic and symptomatic *P. malariae* patients were equally infective (Young & Burgess 1961).

Specific and non-specific responses are believed to modulate parasite transmission. The induction of specific immunological responses to *Plasmodium* shows marked heterogeneity within human populations (Mendis et al. 1987, Graves et al. 1988, Targett 1990, Snow et al. 1993). The sexual stages can induce transmission blocking immunity (TBI) and effective targets include antigens identified on the surface of the macro- and/or microgametes ('pre-fertilization' antigens) as well as antigens present on the surface of the gamete, zygote and ookinete ('post-fertilization' antigens). Antibodies to 'pre-fertilization' antigens of *P. falciparum* such as Pfs230, Pfs48/50 and Pfs16 have been detected in humans (Graves et al. 1988,

Premawansa et al. 1994, Hogh et al. 1994). A significant association between lacking of infectivity of *P. falciparum* gametocyte carriers and recognition of epitope IIa on Pfs48/50 by antibodies in their sera has been observed (Graves et al. 1992). Naturally acquired TBI to *P. vivax* sexual stage antigens has also been demonstrated (Mendis et al. 1987, 1990b, Mendis & Carter 1991, Ranawaka et al. 1988, Goonewardene et al. 1990, Gamage-Mendis et al. 1992) and appears to play a role on transmission of the disease (de Zoysa et al. 1988). In *P. vivax* malaria antibodies, at low concentrations, can also have a transmission-enhancing effect on infectivity of malarial parasite to mosquitoes (Mendis et al. 1987, Peiris et al. 1988, Naotunne et al. 1990, Gamage-Mendis et al. 1992). A recent report described TBI in *P. vivax* malaria when antibodies raised against a peptide blocked parasite development in the mosquito *An. tessellatus* (Snewin et al. 1995). As shown in a variety of *Plasmodium* species, within the mosquito vector antibody to the 'pre-fertilization' antigens may prevent fertilization by any of four mechanisms: (1) the agglutination of macro/microgametes limiting their mobility; (2) antibody coating of macro/microgametes inhibiting cell-cell recognition; (3) opsonization in the bloodmeal or (4) complement dependent/independent lysis (Gwadz 1976, Carter & Chen 1976, Carter et al. 1979, 1985, 1990, Kaushal et al. 1983a,b, Renner et al. 1983, Harte et al. 1985a, Vermeulen et al. 1985b, Grotendorst et al. 1986, Grotendorst & Carter 1987, Quakyi et al. 1987, Peiris et al. 1988, Premawansa et al. 1990). Cellular responses are also involved in TBI and may have some influence on parasite infectivity (Harte et al. 1985b, Mendis et al. 1990b, Riley & Greenwood 1990, Goonewardene et al. 1990).

Immunity to 'post-fertilization' antigens such as Pfs25, Pfs21, Pgs25 and Prs25 also plays an important role on transmission of the disease by suppressing parasite infectivity at different stages of its development in the mosquito as demonstrated by many workers in different *Plasmodium* species (Grotendorst et al. 1984, Vermeulen et al. 1985a, Sinden et al. 1987, Winger et al. 1988, Fries et al. 1989, Carter & Kaushal 1984, Carter et al. 1989a,b, Kaslow et al. 1991, 1992, 1994b, Foo et al. 1991, Sieber et al. 1991, Tirawanchai et al. 1991, Duffy et al. 1993, Paton et al. 1993, Ranawaka et al. 1993, 1994). The mechanisms of blockade could be the same four described above and/or the antibody may act by damaging the parasite surface coat (Ponnudurai et al. 1987).

Non-specific responses to the asexual stages are believed to modulate parasite transmission (Naotunne et al. 1991, Kwiatkowski 1992). Numerous non-specific factors may correlate with

changes in gametocyte infectivity. Acute phase reactants like C-reactive protein (CRP), which are non-specific indicators of inflammatory activity, are elevated in patients with *P. falciparum* malaria (Ree 1971, Naik & Voller 1984, Chagnon et al. 1992). Some cytokines such as interferon (IFN- γ), tumor necrosis factor (TNF- α) and interleukin 6 (IL-6) are elevated in sera from patients with *P. falciparum* and *P. vivax* malaria (Grau et al. 1989, Kern et al. 1989, Kwiatkowski et al. 1990, Mendis et al. 1990c, Karunaweera et al. 1992). Both IFN- γ and TNF- α appear to cause a transient but marked drop in the infectivity of gametocytes to mosquitoes due to the intraerythrocytic killing of parasites (Naotunne et al. 1991, Karunaweera et al. 1992). However, a recent study has shown no elevation in blood levels of cytokines IL-2, IL-6, TNF- α and IFN- γ nor reactive nitrogen intermediates (Hogh et al. 1994). The authors suggested that this could be explained by the inability of asymptomatic gametocyte carriers, unlikely to harbour high asexual parasitaemias, to promote the responses.

The concept that anti-sexual stage immunity may regulate infection of the mosquito vector by gametocyte-infected malarial blood has gained considerable support and must be considered for the development of malaria transmission blocking vaccines (Kaslow et al. 1992).

c) Antibodies against sporozoites - There is evidence that naturally acquired or experimentally elicited anti-sporozoite antibodies ingested by mosquitoes may affect the dynamics of the sporogonic development in the vector. Several studies with *P. falciparum*-infected *Anopheles* species (Vaughan et al. 1988, Beier et al. 1989, Hollingdale & Rosario 1989) showed that (1) ingested human CSP antibodies were detected in the blood meal of field collected mosquitoes up to 36 hr after feeding, (2) antibodies crossing the midgut into hemocoel persist from 4 to 36 hr post-infection in hemolymph, (3) ingested CSP antibodies on day 5 after infection bound to developing oocysts, (4) enhancement of the sporozoite production, (5) ingestion of CSP antibodies on day 10 after feeding had no effect on oocyst maturation or sporozoite production, (6) contact between CSP antibodies and sporozoites in the hemocoel did not block sporozoite invasion of salivary glands, (7) exposure to CSP antibody increased sporozoite infectivity and (8) human IgG antibodies were present on salivary gland sporozoites from field-collected mosquitoes. It has been recently demonstrated that antibodies to *P. gallinaceum* CSP prevent sporozoites from invading salivary glands of *Ae. aegypti* (Warburg et al. 1992). Ponnudurai et al. (1989) did not find any influence of anti-sporo-

zoite antibodies on the number of salivary gland sporozoites but concluded that a second blood meal, with or without antibody, simply functions as a nutritional stimulus for faster oocyst maturation. However, when transmission blocking antibodies anti-Pbs21 (a surface antigen present on the surface of *P. berghei* zygote/ookinete) were added to second bloodfeeds at different stages of parasite development in the mosquito, a significant reduction in oocyst intensity but no detectable change in prevalence occurred. Furthermore, at all times anti-Pbs21 reduced sporozoite number in the thorax but highest gland intensities were obtained when the second bloodfeed was given on day 4 (Ranawaka et al. 1993). These results were interpreted as two opposing roles of second bloodfeeds containing transmission blocking antibody: (1) inhibition of parasite development and (2) the supply of nutrients which permit more sporozoites to be produced by each oocyst. Despite some controversy these results potentially have significant implications for natural malaria transmission and for a possible vaccine development.

d) Anti-mosquito antibodies - In addition to anti-parasite antibodies, it has been tested experimentally the effect on malaria transmission of antibodies raised against parts of the mosquito which could be included in a malaria vaccine. Ramasamy and Ramasamy (1990), studying the *P. berghei/An. farauti* model, found that mosquitoes feeding on mice immunised with midgut antigens exhibited a reduction in mosquito infection rates. Similar results were reported by Billingsley et al. (1990) using monoclonal antibodies produced against mosquito midgut tissue in *P. berghei/An. stephensi* system.

e) Genetic manipulation of the vector - Experiments with refractory lines of *An. gambiae* (Collins et al. 1986) and studies on the effects of broad antimicrobial and antiparasitic components, e.g. magainins and cecropins (Gwadz et al. 1989), showed that it may be feasible to induce effective disruption in the normal development of *Plasmodium* species in the vector by the introduction and expression of appropriate genes into the mosquito genome. Two types of useful target genes can be used in transgenic mosquitoes. First, those that render populations vulnerable to subsequent control measures, such as insecticide susceptibility or temperature sensitivity, and second, those that interrupt disease transmission by replacing vector with non-vector forms (Crampton et al. 1990, Kidwell & Ribeiro 1992, Crampton 1994). Although many technical, and perhaps ethical, problems associated with the wild-release of transgenic insects have yet to be overcome, the potential of this technology has received greater attention recently (Brey 1991,

Coluzzi 1992, Collins 1994, Curtis 1994). Introduction and expression of genes coding for antibodies against target antigens present on the ookinete surface into the mosquito embryos is one of the possibilities to examine the potential of this technology (Crampton et al. 1993).

f) Anti-malarial drugs - Sub-therapeutic doses of antimalarial drugs have been reported to enhance infectivity of *Plasmodium* species to their vectors (Shute & Maryon 1954). Additionally, numerous compounds including chloroquine (Wilkinson et al. 1976), sulphamethoxazole-trimethoprim (Wilkinson et al. 1973), pyrimethamine (Shute & Maryon 1951), Fansidar (Carter & Graves 1988) and Berenil (Ono et al. 1993) have been suggested to induce gametocyte formation but no influence of chloroquine (Jeffery et al. 1956, Smalley 1977, Chutmongkonkul et al. 1992, Hogg et al. 1994) and Fansidar (Hogg et al. 1994) on gametocyte infectivity was observed by some investigators. It was demonstrated that pyrimethamine- and halofantrine-treated gametocytes of *P. falciparum* are more infective to *An. stephensi* mosquitoes than untreated controls (Chutmongkonkul et al. 1992). Other studies examined the effects of some schizontocidal agents on the sporogonic cycle of *P. falciparum* and *P. berghei* in anopheline mosquitoes (Coleman et al. 1988, do Rosario et al. 1988). It was found that chloroquine, when fed during late sporogony (10-12 days post-infection), may increase the vectorial capacity of some mosquito species. The effects of chloroquine on the infectivity of chloroquine-sensitive and -resistant populations of *P. yoelii nigeriensis* to *An. stephensi* mosquitoes were studied by Ichimori et al. (1990). The results showed an enhancement of infectivity in sensitive strains but no effect was detected in resistant clones and sublines. Chloroquine use and the subsequent development of resistance over the past years is associated with an increasing human malaria infectiousness (Lines et al. 1991) which may be indirect effects of parasitaemia on the host. The sporontocidal activity of chloroquine, halofantrine and pyrimethamine was evaluated by administration to *An. stephensi* mosquitoes, either in the first bloodmeal containing *P. falciparum* gametocytes from *in vitro* cultures, or in the second, parasite-free bloodmeal, given four days after infection. A sporontocidal effect was observed only when pyrimethamine was administered with the infective bloodmeal (Chutmongkonkul et al. 1992). It has been demonstrated recently an inhibitory action of the anti-malarial Atovaquone (566C80) against ookinete, oocysts and sporozoites of *Plasmodium berghei* in *An. stephensi* (Fowler et al. 1994, 1995).

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