Malaria, sexual development and transmission: retrospect and prospect

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SUMMARY

It is difficult to recapture the excitement of recent research into the malaria parasites. *Plasmodium* has shown itself to be a most elegant, resourceful and downright devious cell. To reveal any of its manifold secrets is a hard-won privilege. The thrill of this intellectual endeavour, however, has to be tempered by the realism that we have made unremarkable progress in attacking malaria in the field, where it remains almost as omnipresent as it ever was in the 19th and 20th centuries, and both the parasite and vector have become more difficult to control than ever before. This personal view looks back at the significant progress made, and forward to the challenges of the future, focusing on work on sexual development.

Key words: malaria, development, transmission, *Plasmodium* spp.

LOOKING BACK

The current malaria research community surely recognizes that we are in a land of fabulous riches. Never have we had more comprehensive resources at our disposal. When looking at the parasites, experimental technologies, reagents and databases, it is difficult to comprehend the barriers to research that preceded the current environment. Despite these riches, current well-meaning legislation has not enhanced our capacity to work on the malaria parasites either in man or animal models. Of the mammalian malarias the rodent parasites, and *Plasmodium berghei* in particular, offer systems in which to explore plausible hypotheses, hypotheses that nonetheless require validation in the human parasites, not because *P. berghei* in the laboratory mouse, or its laboratory vectors *Anopheles stephensi*/*A. gambiae* is atypical of the majority of *Plasmodium* spp., but because the biology of most important human pathogen, *P. falciparum* is, in key aspects of its development, distinct from most other *Plasmodium* species.

How has our understanding of malaria biology changed in the lifetime of this journal? By the middle of the last century detailed light microscopic studies had described and classified the majority of malaria-like species known today, in hosts ranging from living fossils e.g. the Tuatara (*Sphenodon*) and crocodilians, to the most recent mammals (man). These researches are comprehensively recorded in a series of seminal publications by Maegraith (1948), Boyd (1949), Garnham (1966), Coatney *et al*. (1971), Bruce-Chwatt (1985), Wernsdorfer and McGregor (1988) and Valkiunas (2005) – texts that should be compulsory reading for any malaria researcher today.

Studies on the parasite life cycle continued well into the 1980s when P.C.C. Garnham, the joint discoverer of the liver stages, promoted a successful international effort to search for the elusive relapse form of *P. vivax*, now termed the hypnozoite (see Krotoski, 1985). Likewise, the enigmatic journey of the sporozoite from the skin to liver provoked widespread microscopic and physiological studies in the 1970s. The disparate observations reported were only 'recently' resolved with the advent of fluorescent parasites and *in situ* multiphoton confocal microscopy (Pradel and Frevert, 2001; Baer *et al*. 2007). The major question was whether the Kupffer cell was a protagonist or an antagonist of sporozoite sequestration in the liver. It turns out to be the former – prompting interesting studies as to how the parasite avoids destruction (Brown and Kreier, 1986). Important though remaining questions may be (e.g. what is the role of lymphatic system in infection biology), the key pieces of the morphological jigsaw puzzle are clearly in place ..... or so we believe (Landau *et al*. 1999; Amino *et al*. 2008).

A deeper appreciation of malaria organization was provided by the application of electron microscopy (scanning, transmission and high voltage), approaches initiated by Garnham, Trager and Rudzinska and subsequently applied widely by many others of whom Masamichi Aikawa set a technical standard that few could match. These studies, while still classically descriptive, nonetheless revealed the amazing, indeed unique, subcellular structures mediating the parasites' complex life cycle, structures that led to the revision and re-naming of the phylum (Apicomplexa). In the author's opinion perhaps the most important accomplishment of this

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phase of malaria research was the recognition that the parasite alternates 2 key 'conserved' strategies, invasion (merozoite, sporozoite and ookinete); and growth (erythrocytic schizont, liver schizont, oocyst), with a single excursion into sexual development (gametocytes). This unique phase of development, as a sheer spectacle of what a eukaryotic cell can do is, in the eyes of the writer, one of the wonders of biology. The male gametocyte undergoes 3 mitotic divisions and the assembly of 8 axonemes in 5–20 min. These spectacular capacities should have forewarned investigators that novel processes were afoot, making their analysis not so much an examination of the parasite, but more of the investigators' imaginations. But, as a deeply committed zoologist, it is with a sense of sadness that I have to recognize the profound 'challenge' *Plasmodium* inflicted, when the multi-laminate organelle (an ill-defined series of concentric cytoplasmic membranes) was finally revealed to be a degenerate chloroplast! – respectfully renamed the apicoplast (Sullivan *et al.* 2000). *Plasmodium*…. a plant! No wonder the work on the yeasts had been useful in guiding analysis of the parasite's mitotic divisions.

The current molecular revolution in malaria research was made possible by the development of methods for the culture of the parasite. The substantive early efforts of Bass and Johns (1912) to culture blood stages and of Ball, Chao and Schneider to culture the sporogonic forms (Chao and Ball, 1964; Schneider and Vanderberg, 1980) were rewarded when Trager and Jensen (1976) successfully cultured the asexual blood stages of *P. falciparum*. This was rapidly complemented by methods for the culture of gametocytes (Smalley, 1976), pre-erythrocytic schizonts (Strome *et al.* 1979) and ookinetes (Yoeli and Upmanis, 1968; Janse *et al.* 1985), but only much later by the culture of the oocyst (Al-Olayan *et al.* 2002) – the last method remains difficult to reproduce even today.

Without doubt, it is the genomic revolution, by now founded in a largely secure understanding of parasite ultrastructure, that has transformed our capacity to analyse the molecular cell biology of the parasite. Previously, molecules of interest were often identified because they were significantly immunogenic. First, a monoclonal or mono-specific antibody had to be generated and bacterial colonies screened and parasite genes cloned and sequenced by individual researchers. Each identified gene represented many months of work. The hard-won monoclonal antibodies, however, had significant benefit, permitting not only identification of the encoding gene, but also the subcellular location of the immunogen; its expression profiling, and where relevant its evaluation as a target for immune intervention. These diverse opportunities have prompted some to suggest that a new 'omics' approach could be the generation of monoclonal antibodies to every protein expressed by the parasite. The benefits that have accrued from the sequencing of even one malarial gene are many, but the projected ability to sequence in their entirety, genomes of many thousands of malarial parasites will permit a deep understanding of the evolution of these organisms, and additionally promises powerful new insights into potential drug resistance mechanisms.

Perhaps the greatest contribution of genomic era to the understanding of malaria cell biology, is the new-found ability to detect coordinated patterns of gene regulation (the whole question of how does a genome make an organism?). The low-hanging fruit of this analysis has been transcriptomics, which spawned the elegant 'just-in-time' hypothesis of gene product synthesis (Hayward *et al.* 2000; Bozdech *et al.* 2003; Le Roch *et al.* 2004; Silvestrini *et al.* 2005). The limitations of this conclusion are immediately apparent when considering how far removed the mRNA transcript is from the final/active gene product. An early indication of this distance was forecast by the observation that expression of the ookinete protein P28 was subject to translational repression in the mature female gametocyte (Paton *et al.* 1993). With the advent of high-throughput proteomics (only possible following genome sequencing), it was possible to ask more directly 'What proteins are expressed at each accessible stage of development?' This has resulted in a deeper, but still far from complete, understanding of the parasite's biochemical pathways, and molecular machines (Florens *et al.* 2002; Lasonder *et al.* 2002, 2008; Hall *et al.* 2005; Khan *et al.* 2005; Lal *et al.* 2009*a*, *b*; Talman, personal communication).

Where does HTP analysis go from here? Existing datasets are incomplete and incompletely analysed. If we are to understand the parasite's regulatory networks, not just within the parasite, but between the parasites and their hosts we must apply Systems Analysis to openly shared/integrated datasets. Malaria cell biology has made amazing progress, and it might be argued that some *Plasmodium* species (notably the rodent malarias) are close to being model systems, but to analyse their biology requires diverse and unique resources simply to permit experimentation, not least amongst these resources are biologists who understand the organisms themselves.

BIOLOGY OF SEXUAL DEVELOPMENT

Whereas in Haemoproteids all bloodstage parasites are 'by default' gametocytes, in *Plasmodium* a small percentage of asexual bloodstages are, it is believed, induced by ill-defined 'stress' factors at each round of schizogony to form gametocytes in the next generation. Not only are all merozoites from a single schizont committed to the sexual pathway (Bruce *et al.* 1990), but all become either male or females (Silvestrini *et al.* 2000; Smith *et al.* 2000). In most

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species gametocytes mature only fractionally slower than that of the asexual parasite; however, in 2 species *Plasmodium falciparum* and *Plasmodium reichenovi* maturation is dramatically extended to 8–12 days, compared to the asexual maturation period of just 48 h. Early studies demonstrated that the immature gametocyte during its first 3 days of development was susceptible to bloodstage schizonticides (reviewed by Butcher, 1997). However, from stage III and beyond these gametocytes became progressively insensitive to many drugs, with the exception of inhibitors of energy metabolism e.g. the 8-aminoquinolines and artemisinin which therefore have the critical potential not only to provide direct protection to the patient, but, recognizing the local patterns of transmission (Carter *et al*. 2000) also protect the immediate community from transmission.

Mature gametocytes are morphologically distinct from the asexual parasite and must therefore be expected to contain a significantly different repertoire of proteins. Transcriptomics (Bozdech *et al*. 2003; Le Roch 2003, 2004; Young *et al*. 2005; Silvestrini *et al*. 2005) and proteomics (Florens *et al*. 2002; Lasonder *et al*. 2002, 2008; Hall *et al*. 2005; Khan *et al*. 2005; Patra *et al*. 2008; Tarun *et al*. 2008; Talman, personal communication) have confirmed this obvious conclusion. Notable amongst the proteins found in the mature female gametocyte are proteins Pfs230 and Pfs48/45, in the male gametocyte tubulin is amongst the most abundant of the cytoplasmic proteins. These and other proteins are required for sexual development, not only for the gametocyte itself but in the ensuing gametes which develop in the mosquito midgut.

The development of the gametocytes in the mosquito bloodmeal is without doubt one of the most dramatic developmental events in the parasite life cycle. In just a matter of minutes, both male and female parasites emerge from the red-cell, a process mediated by the exocytosis of vesicles (osmiophilic bodies) containing Pfg377 (Alano *et al*. 1995) and Mdv-1 (Lal *et al*. 2009), which may mediate dissolution and disruption of the enveloping erythrocyte. The now-naked female cell is fertilization competent; males by contrast, additionally undergo three rounds of DNA replication, each round followed by endomitotic genome segregation. Prior to activation of the microgametocyte 1 amorphous MTOC lies in the cytoplasm adjacent to a nuclear pore, it is connected to the genome through the pore. It rapidly transforms into 2 orthogonal tetrads of basal bodies, which are then segregated at each mitotic division such that at the third division 1 basal body is bound to each of the resultant 8 haploid genomes. The basal bodies act as the nucleation centres for the formation of the axonemes that will drive the flagella of the male gamete when released from the parental cell. All these events occur with incredible rapidity, microgametes are released typically within 15 min of bloodfeeding. The signalling processes regulating this dramatic development are now extensively characterized (see Billker *et al*. 2004). It is known that the primary inducers of the process are a fall in temperature of 5 °C in concert with presence of raised concentrations of the mosquito excretory product xanthurenic acid (Billker *et al*. 1997, 1998). Together, these events activate the phosphoinositol pathway resulting in the release of calcium from cytoplasmic stores. The downstream activation of CDPK and thereafter MAPkinase, organize all the events of DNA replication, axoneme assembly, nuclear division and expulsion of the gametes.

It is now recognized that the most abundant proteins (e.g. tubulin, P230 etc) required for gamete formation are pre-synthesized in the terminally arrested mature gametocytes. Enigmatically many of these early proteins (e.g. the LAP/CCP family) could be knocked out with no immediate phenotype. The first detectable morphological change is the failure of the oocyst to undergo cytokinesis when sporozoites are normally formed some 10 days later (Raine *et al*. 2007). An elegant analysis (Mair *et al*. 2006) identified in the female gametocyte a large number of mRNA species, that are under translational control (> 370 species to date, including the ookinete surface proteins P25 and P28) that are translated only following activation of the gametocyte in the mosquito gut. Key motifs were identified either the 5′ or 3′ UTR's of these inhibited messages. A critical component of the regulatory machinery was the DDX6-class RNA helicase DOZI. Subsequently protein expression in the developing zygote/ookinete (e.g. CTRP) is controlled by transcription control factors including AP2-O (Yuda *et al*. 2009). Both gamete and ookinete surface proteins have been shown to be extremely immunogenic, if antibodies to these molecules are ingested in the bloodmeal they have been shown to inhibit fertilization (P230, P48/45, HAP2) or ookinete development (P25, P28) most effectively (Carter *et al*. 2000).

The properly formed male gamete is one of the simplest eukaryotic cells. It is composed solely of a nucleus, an axoneme (attached to its basal body) and a plasmalemma (Sinden *et al*. 1976). Proteomic analysis has confirmed the simplicity of this organization (Talman, personal communication). Unsurprisingly mitochondrial and apicoplast genomes were subsequently shown to be maternally inherited (Creasey *et al*. 1993; Vaidya, 1993). The characteristic stop-go motility of the male gamete is exclusively energized by glycolysis, a process that can be readily inhibited with appropriate compounds in the surrounding medium – thus offering a very accessible and novel target for intervention (Talman, personal communication).

Fertilization of the female by the male gamete is now known to be mediated by a number of surface

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molecules. Pfs230 and Pfs48/45 are believed to act in a multi-molecular complex and are perhaps fertilization receptors, P47 is female specific (van Schaijk *et al*. 2006); Pfs48/45, although present on both male and female gametes, is essential only to the fertility of the male (van Dijk *et al*. 2001). Following molecular recognition of male and female, fusion of the gamete membranes is mediated by HAP2/GCS (Liu *et al*. 2008). This is followed by the movement of the entire male gamete into the cytoplasm of the female. Nuclear fusion follows within a few hours, forming the now diploid zygote. The parasite immediately returns to a haploid organization, meiosis being the first 2 divisions of the zygote genome (Sinden *et al*. 1985). Development of the ookinete from the zygote is analogous to the formation of either the merozoite or sporozoite with the exception that there is only 1 daughter cell. Proteomic analysis of the ookinete, and of its isolated secretory organelles suggests, very strongly, that ookinetes unlike the sporozoite and merozoite, contain micronemes but not rhoptries (Lal *et al*. 2009*b*). The presence of this single class of secretory organelle is reflected in a dramatically different invasive biology. The ookinete, on reaching the midgut epithelial cell, ruptures the plasma membrane and migrates directly into the cytoplasm of the now-dying host cell (Han *et al*. 2000). Invasion provokes a series of well-characterized molecular responses in susceptible mosquitoes, including synthesis of reactive nitrogen intermediates (Luckhart *et al*. 1998) and peroxidase (Kumar and Barillas-Mury, 2005). Should the ookinete survive this assault, it will, on leaving the epithelial cell, meet the mosquito haemolymph within which lie many immune effector molecules including the lethal complement-like molecule TEP1 (Levashina *et al*. 2001), the activity of which is regulated by molecules including LRIM 1 and 2 (Povolones *et al*. 2009). In refractory mosquito genotypes all ookinetes may be killed, either by lysis (Vernick *et al*. 1995) or by melanization (Collins *et al*. 1986).

The few ookinetes that survive this assault will, on meeting the basal lamina of the mosquito midgut, transform into oocysts, which over a period of 9–20 days undergo approximately 11 synchronous endomitotic divisions to form 2000–8000 sporozoites. We still know surprisingly little of these events at the molecular level, but we should anticipate that future transcriptomic and proteomic analyses will provide fascinating insights into these processes which, at the molecular level, may be expected to reiterate, many of the events observed in both blood- and liver-stage schizonts.

POPULATION DYNAMICS OF DEVELOPMENT IN THE MOSQUITO

Whereas the explosive expansion of knowledge on parasite molecular development reflects the rational application of the new and diverse technologies, other, less glamorous approaches have revealed fascinating insights into the dynamics of parasite development in the mosquito vector. Substantive studies in the rodent malaria parasites revealed massive sequential density-dependent losses as the parasite transformed from the gametocyte to oocyst, such that the ingestion of even many thousands of pre-committed sexual cells in the blood often yields, as few as 5 surviving oocysts, revealing the most critical population bottleneck in the parasite's life cycle (Alavi *et al*. 2003; Vaughan, 2006; Poudel *et al*. 2008). Not only will this significantly influence the genetic structure of endemic parasite populations, it provides an attractive focus for effective intervention. The ensuing increase in parasite number, as sporozoites are formed within the oocyst, is temporary respite, as few as 10% of the sporozoites successfully enter the mosquito salivary glands, and of these only the sporozoites residing in the duct of the gland are likely to be injected by the mosquito into the skin of the next host. These two population bottlenecks resulting from the obligatory jump of the parasite from host to host offer the most attractive targets for attacking the parasite as part of any strategy looking to control or eliminate malaria endemic populations.

Further observations on the population structure of *Plasmodium berghei* in *Anopheles stephensi* have emphasized the non-linearity of the relationship between successive developmental stages (Sinden *et al*. 2008). All developmental transitions of the parasite in the mosquito exhibit a hyperbolic saturation curve i.e., developmental transitions become less efficient at progressively higher parasite densities. Uniquely, at *very* low ookinete numbers, increasing parasite density initially results in an increase in transmission efficiency. This is followed by a short, approximately linear relationship, and then at high intensities the transition saturates commonly at $\sim$100 oocysts. The mechanisms by which these relationships are achieved at present remain unknown, but clearly a major focus of interest is on innate immune system of the mosquito (Dimopoulos *et al*. 2001; Blandin and Levashina, 2004; Osta *et al*. 2004). A further dynamic that has been examined for many years, and is now the subject of renewed interest, asks whether the parasite places an evolutionary burden on the mosquito. Past studies suggested that flight performance and fecundity are adversely affected (Schiefer *et al*. 1977; Rowland and Boersma, 1988; Ahmed *et al*. 1999). Recent studies (Dawes *et al*. 2009), suggest that the survival of the mosquito vector (and hence its reproductive potential) is both age- and parasite density-dependent. It is obvious that we have a great deal to learn about the parasite/vector relationship and how this will impact on the transmission of parasite. Mathematical models, securely based in this new understanding of parasite biology, will have an

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important role to play in attempting to predict the impact of any transmission blocking measures.

THE FUTURE

It is right that the malaria research community has recently been challenged as to whether it is feasible to reconsider malaria eradication (Greenwood, 2009). Re-assessment of global research objectives naturally recognizes the need to treat the infected individual – a focus, which has dominated malarial research for the past 40 years, but it also appreciates that we have failed to address adequately the need to suppress the numbers of new cases of malaria. This latter objective brings into acute focus the need, in endemic populations, to suppress transmission. Objective evaluation of past eradication campaigns, of current interventions based on bed nets or the use of drugs with transmission-blocking activity (e.g. artemisinin; 8-aminoquinolines), and the rational analysis of parasite biology, strongly suggests that attacking transmission in the mosquito vector is likely to be one of the more effective control strategies in the future. Additional new concepts to achieve this objective through reduction or modification of mosquito populations are being entertained, and include the development of new fungal (Thomas and Read, 2007; Read *et al*. 2009), bacterial (Geissbühler *et al*. 2009) and microsporidan (Hulls, 1971) biocides, or the use of genetic manipulation technology (Terenius *et al*. 2008) to modulate mosquito breeding habits, olfaction or susceptibility to the parasite. Perhaps one of the most powerful mechanisms, previously underappreciated, remains the application of transmission blocking vaccines (Carter *et al*. 2000).

Without doubt, research over the past century has generated numerous and exciting concepts to attack *Plasmodium* – a parasite of truly horrifying global impact. The careful re-evaluation of well-established concepts, together with the rational prioritization and careful implementation of additional new interventions in the field has the potential to reduce malaria burden within the foreseeable future. Without doubt, to engage in new campaigns to achieve local or national elimination or global eradication will require that the scientific community works together more effectively than ever before to understand the optimal implementation of appropriate interventions in diverse endemic situations. Then 'all' we will require is the political and moral integrity to see the campaigns to completion.

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