Plasmodium falciparum: mechanisms that reduce transmission to mosquitoes

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift
ter verkrijging van de graad van doctor aan de Katholieke Universiteit Nijmegen volgens besluit van het College van Decanen in het openbaar te verdedigen op maandag 23 november 1998 des namiddags om 1.30 uur precies

door

Antonius Henricus Wilhelmus Lensen
geboren op 30 juli 1954 te 's-Hertogenbosch
# CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 1</strong></td>
<td>General introduction</td>
<td>9</td>
</tr>
<tr>
<td>1.1</td>
<td>The malaria problem</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>Approaches to the problem</td>
<td>12</td>
</tr>
<tr>
<td>1.3</td>
<td>Strategies to control transmission</td>
<td>13</td>
</tr>
<tr>
<td>1.4</td>
<td>Outline of this thesis</td>
<td>17</td>
</tr>
<tr>
<td><strong>Chapter 2</strong></td>
<td>Infectivity of malaria parasites to mosquitoes: “The interdependent roles of parasite, vector and host”</td>
<td>25</td>
</tr>
<tr>
<td><strong>Chapter 3</strong></td>
<td>Measurement by membrane feeding of reduction in <em>Plasmodium falciparum</em> transmission induced by endemic sera</td>
<td>35</td>
</tr>
<tr>
<td><strong>Chapter 4</strong></td>
<td><em>Plasmodium falciparum</em>. Infectivity of cultured synchronised gametocytes to mosquitoes</td>
<td>45</td>
</tr>
<tr>
<td><strong>Chapter 5</strong></td>
<td>Transmission blocking antibody of the <em>Plasmodium falciparum</em> zygote/ookinete surface protein Pf25 also influences sporozoite development</td>
<td>55</td>
</tr>
<tr>
<td><strong>Chapter 6</strong></td>
<td>Leukocytes in a <em>Plasmodium falciparum</em> infected bloodmeal reduce transmission of malaria to <em>Anopheles</em> mosquitoes</td>
<td>67</td>
</tr>
<tr>
<td><strong>Chapter 7</strong></td>
<td>Cytokine activated granulocytes reduce transmission of <em>P.falciparum</em> malaria to mosquitoes</td>
<td>81</td>
</tr>
<tr>
<td><strong>Chapter 8</strong></td>
<td>Mechanisms that reduce transmission of <em>Plasmodium falciparum</em> malaria in semiimmune and nonimmune persons</td>
<td>95</td>
</tr>
<tr>
<td><strong>Chapter 9</strong></td>
<td><em>Plasmodium falciparum</em> gametocytes from semiimmunes and nonimmunes differ in innate infectivity to mosquitoes.</td>
<td>111</td>
</tr>
<tr>
<td><strong>Chapter 10</strong></td>
<td>Summary</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Samenvatting</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>List of publications</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Curriculum vitae</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>Dankwoord</td>
<td>135</td>
</tr>
</tbody>
</table>
1.1 The malaria problem

Malaria is one of the leading causes of morbidity and mortality in the world. About 40% of the world population in tropical and subtropical areas is at risk. Up to 500 million people suffer from malaria, it causes 1.5-2.7 million deaths per year, in particular children which die at a rate of four per minute. It is therefore not only responsible for an enormous loss in lives but it has also disastrous social and economic consequences.

Malaria is caused by a parasitic protozoon of the genus *Plasmodium* and can be transmitted by female mosquitoes of the genus *Anopheles* when they take a blood meal. There are four species of *Plasmodium* that can infect man of which *Plasmodium falciparum* is one. This organism has been the major focus of research since infection with this parasite may cause life threatening complications.

The life cycle of *P.falciparum* is shown in figure 1.

Transmission occurs when a female *Anopheles* mosquito takes a bloodmeal from an individual with the sexual stages of the parasite, the gametocytes, in peripheral blood cells. Once inside the mosquito midgut the intra-erythrocytic crescent stages activate, round up and escape from the red blood cell (fig1.A). This happens within minutes after taking the blood meal and the activation is initiated by a drop in temperature from 37° C to ambient, a rise of pH and possibly mosquito factors (Carter & Nijhout, 1977). After escape from the red blood cell the female gametocyte forms a macro-gamete. The activated male gametocyte undergoes the process of exflagellation by which eight microgametes are released by vigorous movements leaving behind a non-functional residual body. Fertilisation takes place and the newly formed zygote develops into an ookinete within the next 20 hours which eventually penetrates the mosquito midgut wall to form an oocyst at the outer surface. Within the oocyst sporozoites are formed and released within 10 to 20 days (depending on temperature and additional blood meals) and they finally find their way to the salivary glands. With a next bite the mosquito injects saliva containing sporozoites in her victim and transmits the parasite (fig1.B). The sporozoites home to the liver, invade hepatocytes and form extra-erythrocytic schizonts in which thousands of merozoites develop. After 5-7 days the schizonts rupture and release the merozoites in the blood stream. The merozoites invade red blood cells and differentiate into erythrocytic schizonts, in 48 hours they mature, then rupture and release ± 30 merozoites which again infect red blood cells (fig1.C). A small proportion of these erythrocytic stages will not develop into blood schizonts but into gametocytes. The gametocytes need approximately ten days to reach maturity and are able to transit the parasite from man to mosquito when a mosquito takes a bloodmeal.
Figure 1. The life cycle of *Plasmodium falciparum*
1.2 Approaches to the problem

In the past malaria control has been based on the world-wide use of chloroquine for treatment and prophylaxis and on attempts to eliminate mosquitoes by massive spraying of insecticides such as DDT. The programme for global malaria eradication, announced in 1955 by the Eight World Health Assembly has been successful in Europe, the USSR, Madagascar and North America but not in tropical areas, for reasons like political instability and compliance. Moreover the strategies applied induced chloroquine resistance and the widespread use of DDT and other insecticides caused resistance in mosquitoes.

Therefore, a WHO study group developed the Global Plan of Action for Malaria Control 1993-2000 resulting in the World Declaration on the Control of Malaria which was adopted by the Ministerial Conference on Malaria in Amsterdam 1992. The ultimate goal of this plan is to prevent mortality and morbidity and thereby social and economic losses. Apart from political commitments there was also consensus on the methods to be followed.

The Global Malaria Control Strategy focuses on early diagnosis, adequate treatment and preventive measures. The latter includes the use of drugs according to a national drug policy and application of insecticide-impregnated materials for individual protection. Therefore primary health services are a prerequisite for successful implementation (WHO Technical Report Series 839, 1993).

Although this approach may result in a decrease of the malaria problem in the near future (Snow et al., 1997), it may prove not to be sufficient in the long run, since it is still based on the use of drugs and insecticides which may induce resistance, as has been shown before. A widely accepted additional and alternative strategy is the development of an effective vaccine.

The major types of vaccines under development are:

1. "Sporozoite vaccines" that can prevent the entry of sporozoites in hepatocytes or inhibit the development of the liver stage of the parasite.

2. "Asexual blood-stage vaccines" and "anti-toxin vaccines" designed to suppress asexual parasitemia or to interfere with the toxic products of the parasites, thereby limiting the clinical manifestations of the disease.

3. "Transmission-blocking vaccines" designed to arrest the development of sporogonic stages inside the mosquito thereby preventing infectivity of the mosquito and thus prohibiting spread of the disease.
A number of sporozoite and blood stage vaccine candidates tested in laboratory and/or field experiments are summarized in Table 1. In 1987 Manuel Pattaroyo described a vaccine consisting of a mixture of three synthetic merozoite peptides that induced protective immunity against *P. falciparum* in Aotus monkeys (Pattaroyo *et al.*, 1987). Further, this vaccine appeared to be protective against experimental infections with *P. falciparum* in 9 out of 13 volunteers (Pattaroyo *et al.*, 1988). Further modifications by addition of anti-sporozoite components achieved the so-called Spf66-vaccine. The Spf66-vaccine was given priority by the WHO and was subject of extensive research and field trials. The initially claimed efficacy of the vaccine varied between 30 and 80% in several trials in Columbia (Valero *et al.*, 1996) but this could not be confirmed in trials in Tanzania (Alonso *et al.*, 1994), Gambia (D’Allesandro *et al.*, 1995) and Thailand (Nosten *et al.*, 1996). Although its efficacy is still highly under debate it is indisputable that the experience with Spf66 has resulted in the acquisition of considerable expertise in conducting malaria vaccine trials (Riley, 1995).

1.3 Strategies to control transmission

Controlling transmission can be achieved by reducing the number of infectious bites by mosquitoes. Insecticides will reduce the total number of mosquitoes but contain the risk of development of resistance. A transmission blocking vaccine which reduces the number of infected mosquitoes is likely to be more effective in the long run. Transmission-blockade (TB) is the arrest of parasite development in the mosquito after transition of the sexual stages (gametocytes) from humans and is considered to be caused by TB-immunity. TB-antibodies can be demonstrated in sera of individuals that experienced a malaria infection (Graves *et al.*, 1988; Roeffen *et al.*, 1996) and can be induced in experimental immunisations with stage specific parasite antigens (Carter and Chen, 1976; Vermeulen *et al.*, 1985; Barr *et al.*, 1991; Kaslow *et al.*, 1994). Mechanisms of transmission reduction include both TB by action of specific antibodies, complement and possible other serum components and by cell-dependent actions. A diagram is given in figure 2. Every vaccine that interrupts the parasites life cycle could be regarded as a transmission blocking vaccine (TBV), however, the term ‘transmission-blocking vaccine’ refers to a vaccine that inhibits the development of the sexual or sporogonic stages.
Figure 2. Summary diagram describing the mechanisms by which malaria development in the mosquito midgut might be regulated. Abbreviations: C3, complement; fab, single chain variable region of immunogloblin G; IFN, interferon-γ; IL-1, interleukin-1; IL-4, interleukin-4; PHAG, phagocytes; pH, extracellular pH; RBC, red blood cell; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; TNF, tumour necrosis factor-α; WBC, white blood cells. Asterix (*) indicates fertilization blocking/enhancing antibody.

Reprinted with permission of Prof. dr. R.E. Sinden.
<table>
<thead>
<tr>
<th>sporozoite vaccine candidates</th>
<th>point of impact</th>
<th>model-system</th>
<th>references</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>asexual blood stage vaccine candidates</th>
<th>merozoite invasion</th>
<th>model-system</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>RESA (erythrocyte surface antigen)</td>
<td>merozoite invasion</td>
<td><em>P. falciparum</em>- Aotus monkeys</td>
<td>Collins <em>et al.</em> 1986</td>
</tr>
<tr>
<td>AMA-1 (apical membrane antigen-1)</td>
<td>merozoite invasion</td>
<td><em>P. knowlesi</em>- Rhesus monkeys</td>
<td>Deans <em>et al.</em> 1988; Perkins 1992</td>
</tr>
<tr>
<td>EBA-175 (erythrocyte binding antigen 175)</td>
<td>merozoite invasion</td>
<td><em>in vitro</em></td>
<td>Sim <em>et al.</em> 1990; Sim <em>et al.</em> 1994</td>
</tr>
<tr>
<td>MSP-1 (merozoite surface antigen-1)</td>
<td>merozoite invasion</td>
<td><em>P. falciparum</em>-Aotus, Saimiri monkeys</td>
<td>Siddiqui <em>et al.</em> 1987; Etlinger <em>et al.</em> 1991</td>
</tr>
<tr>
<td>MSP-2 (merozoite surface antigen-2)</td>
<td>merozoite invasion</td>
<td><em>P. falciparum</em>-Aotus monkeys</td>
<td>Pye <em>et al.</em> 1991</td>
</tr>
</tbody>
</table>
### General introduction

Table 2. The potential *P.falciparum* targets of transmission blocking vaccines

<table>
<thead>
<tr>
<th>Target antigen</th>
<th>expression of antigen</th>
<th>point of impact</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfs 16</td>
<td>sporozoite/gametocyte</td>
<td>?</td>
<td>Moelans <em>et al.</em> 1991</td>
</tr>
<tr>
<td>Pfs 28</td>
<td>oocyst</td>
<td>oocyst maturation</td>
<td>Lensen <em>et al.</em> 1992</td>
</tr>
<tr>
<td>Pfs 28</td>
<td>macrogamete/zygote/ookinete</td>
<td>midgut penetration</td>
<td>Duffy &amp; Kaslow 1997</td>
</tr>
<tr>
<td>Pfs 230</td>
<td>gametocyte/gamete/zygote</td>
<td>fertilisation blockade</td>
<td>Vermeulen <em>et al.</em> 1985</td>
</tr>
<tr>
<td>Pfs 230</td>
<td>gametocyte/gamete/zygote</td>
<td>(1) complement dependent lysis; (2) fertilisation blockade</td>
<td>Rooffen <em>et al.</em> 1995; Heuler <em>et al.</em> 1997; Read <em>et al.</em> 1994; Rener <em>et al.</em> 1980</td>
</tr>
<tr>
<td>Pfs 2400</td>
<td>gametocyte</td>
<td>?</td>
<td>Feng <em>et al.</em> 1993</td>
</tr>
<tr>
<td>Pf chitinase</td>
<td>ookinete</td>
<td>ookinete penetration of perithrophic membrane</td>
<td>Shahabuddin <em>et al.</em> 1993; Shahabuddin &amp; Kaslow 1994; Shahabuddin 1995; Shahabuddin <em>et al.</em> 1996</td>
</tr>
</tbody>
</table>

Table 2 summarises TBV candidates. By blocking transmission, the effect of a TBV is in fact comparable to that of insecticide sprays or impregnated bed nets or other means lowering the parasite inoculation rate (Sinden, 1997). There has been extensive debate on the long-term effects of transmission reducing interventions and thereby also the development of immunity to malaria. In areas with most intense levels of transmission the risk of severe disease is greatest during the first two years of live, at low-to-moderate this period is five years or more. Hence, lowering the transmission intensity by intervention may increase the age at which effective anti-disease immunity is acquired (Snow *et al.*, 1997). Therefore, these intervention measures may be most effective and beneficial in areas with an already low-to-moderate transmission. Combination of a TBV with an anti-sexual or anti-disease vaccine should be useful in places with a high endemicity. Despite, these drawbacks which are shared by any vaccine that does...
General introduction

not eradicate all parasites, a TBV has specific advantages. Firstly, the number of parasites to be eliminated by this vaccine is comparatively small. Secondly, so far sexual stage vaccine molecules show little genetic variation avoiding the risk of selection for resistant parasites. Thus, in combination with another control strategy, a TBV reduces the chances that a variant, non-sensitive asexual stage parasite is transmitted.

1.4 Outline of the thesis

Most studies with TB have concentrated on the role of specific antibodies directly interfering with the development of sporogonic stages inside the mosquito midgut. Not all effects of TB can be explained by the presence of specific antibodies. The main objective of this thesis was to study the possible role of “aspecific” immune factors, which may contribute to the efficacy of TB and are potentially relevant for validation of a future TBV. In order to study transmission reduction one has to understand the details of the processes involved in transmission. Mosquito age, speed of digestion of the blood meal, quantity and quality of gametocytes and the presence of specific antibodies and activated leukocytes in the blood meal may all influence the chance of successful transmission (chapter 2).

For measurement of the TB-capacity, a standardised assay had to be developed, which allowed classification of TB-activity in sera as: blocking, reducing or non-reducing (chapter 3).

Apart from serum factors, it is obvious that both quantity and quality of gametocytes are important factors for transmission capacity. As suggested by James in 1931 “quality” of gametocytes may be important for successful transmission. Quality depends on factors such as genetic background, drug exposure, aberrancy or maturity. Infectious gametocytes in the blood of gametocyte carriers have a half life time of about 2.5 days (Smalley & Sinden, 1977). After appearance in the circulation there might be a non-infectious period of gametocytes for three days (Jefferey & Eyles, 1955), which suggest that age of gametocytes may be an important factor. Synchronisation of gametocyte cultures could contribute to a further standardisation of the standard membrane feeding assay (SMFA). Therefore, the infectious period of mature synchronised gametocytes was studied (chapter 4).

Since Pfs25 is persistently present in the oocyst capsule and human antibodies in a blood meal pass the mosquito midgut wall (Vaughan et al., 1988), the effect of a TB-antibody directed against the gamete/zygote protein Pfs25 on sporozoite development was studied (chapter 5).
In addition to humoral factors also white blood cells may be active inside the mosquito midgut, thus contributing to reduction of transmission. Human white blood cells (WBC) can survive for hours inside a mosquito midgut and are able to phagocytose and kill free gametes (Sinden RE & Smalley MJ, 1976; Naotunne et al., 1993). The contribution to TB of phagocytosis of free gametes of *P. falciparum* mediated by human sera, was studied in chapter 6.

Phagocytosis is influenced by factors that activate WBC and/or opsonise gametes. Therefore, the effects of the cytokines, tumor necrosis factor alpha (TNF-α) granulocyte-monocyte colony-stimulating factor (GM-CSF) and interferon gamma (IFN-γ) that are present in elevated concentrations in the blood of malaria patients (Yamada TMS et al., 1995) were studied in the presence or absence of opsonising antibodies and/or complement (chapter 7).

To elucidate the possible role of WBC and serum factors on transmission capacity of gametocytes maturing in their presence, membrane feeding experiments were performed with naturally infected semiimmune gametocyte carriers in Cameroon. The results of these experiments were compared to those of nonimmune gametocyte carriers with no previous malaria experience (travellers) (chapter 8). The conclusions drawn in chapter 8 may have serious implications for ideas on fitness of gametocytes, their infectiousness to mosquitoes and an epidemiological concept of transmission in endemic areas and are further discussed in chapter 9. A summary of the thesis is given in chapter 10.

References


General introduction


General introduction


Muller HM, Reckmann I, Hollingdale MR, Bujard H, Robson KJH & Crisanti A (1993). Thrombospondin related anonymous protein (TRAP) of Plasmodium falciparum binds specifically to sulfated glycoconjugates and
General introduction


General introduction


General introduction


General introduction


CHAPTER 2

Infectivity of malaria parasites to mosquitoes: "The interdependent roles of parasite, vector and host"

Ton H.W. Lensen

*University of Nijmegen, Department of Medical Microbiology, section Parasitology.*

Annals of Tropical Medicine & Parasitology (1996), 90, 359-365
Chapter 2

It has been possible to infect mosquitoes routinely with cultured gametocytes of *Plasmodium falciparum* since 1980. This has enabled the development of a reliable bio-assay for potential transmission-blocking vaccines and research on the role of specific antibodies from the host on the parasitic stages in the mosquito midgut. After some fine-tuning of the assay, it became apparent that the immune responses of the human host, as well as factors from the parasite and the mosquito, determined the final outcome of mosquito infection. The age of the mosquito, crowding of parasites inside the peritrophic membrane and the quantity and particularly the quality of the gametocytes ingested all influence the chance of successful transmission. Cytokines and/or other mediators of inflammation from the human host can also reduce transmission, probably by promoting phagocytosis of the freshly emerged gametes by leukocytes in the bloodmeal.

*Parasites*

In order to obtain mature gametocytes, fresh isolates of parasites are kept in culture for at least 14 days. After an initial phase of asexual multiplication a peak parasitaemia is reached on days 5-6 followed by a period of massive destruction of parasites during which gametocytogenesis is initiated. Gametocytes need another 7 to 8 day in culture to reach maturity. At present, the real factors inducing gametocytogenesis are unknown.

With subsequent subcultures the infectious isolate adapts further to culture conditions. As a result the multiplication rate increases and the infectiousness of gametocytes produced diminishes. Extended subculturing finally completely eliminates the capacity for gametocytogenesis (Ponnudurai *et al.*, 1982). The infectiousness of isolate NF54 for example wanes after 25 subcultures and a new stablate has to be started from the frozen stock. According to Kemp *et al.* (1992) this loss of infectiousness is related to deletions in the genome coinciding with loss of cytoadherence. Although the adaptation of many parasites isolated from patients to culture conditions and even the production of gametocytes in culture, has been successful, the cultures produced do not always infect mosquitoes. Rapid and continuous proliferation of asexual parasites inhibits gametocyte maturation and infectiousness. The development of mature functional gametocytes may be hampered by competition for nutrients or intoxication by metabolites produced by the fast growing asexual parasites. (Ponnudurai *et al.*, 1988). Rapid asexual multiplication occurs in the initial cultures of some parasite isolates/stablilates and this might interfere with the production of infectious gametocytes. The parasite isolate used to
produce infectious gametocytes is therefore very important. The use of an automatic device for culturing infectious gametocytes (Ponnudurai et al., 1982a) contributes to the quality of gametocytes, since it eliminates variability due to human handling. The serum used in culturing gametocytes is also of great importance. Even sera of normal blood bank donors, without any malaria experience differ in their capacity to support parasite proliferation for as yet unknown reasons. To overcome this source of variation, a large pool of different sera is usually used. Pooling not only dilutes the possible negative effects of an individual serum but also creates a constant supply for a prolonged period. A high standard of culture conditions contributes to the final quality of gametocytes.

The quality of gametocytes can be judged from their morphology and their capacity to form male and female gametes. The mature gametocytes of *P. falciparum* have distinct morphological features which makes it possible to identify immature or aberrant forms; mature gametocytes are crescentic, with rounded ends, whereas aberrant forms tend to have sharp or knob-like ends, even if crescentic. In addition pigment distribution is strongly indicative of quality; the fine granular pigment is located around the nucleus, in functional mature gametocytes, whereas the pigment in aberrant forms is clumped and often localised on one side of the cell. Another criterion for quality is the readiness of gametocytes to activate and exflagellate. Activation *in vitro* can be studied by adding a drop of parasite culture to foetal calf serum (FCS) and incubating for 10 minutes at room temperature in a humid chamber. The fall in temperature of the mixture and the rise in its pH (induced by the escape of CO₂ from the sample), lead to activation of any mature, functional gametocytes. Within minutes the gametocytes start to round up and emerge from their host erythrocyte. Any male gametocytes undergo the process of exflagellation, each producing eight microgametes. Gametocytes that look mature morphologically can fail to activate and/or to exflagellate for yet unknown reasons. Some morphological mature gametocytes seem to round up within the erythrocyte membrane and fail to escape. Although exflagellation is a good indicator for the quality of male gametocytes it still does not guarantee a successful transmission. The same is true for the formation of female gametes. Female gametes in a mosquito bloodmeal can be visualised by reaction with an anti-48/45 kD monoclonal antibody labelled with fluorescein isothiocyanate, 1 h after the infectious feed. However, there does not appear to be a correlation between the numbers of emerged macrogametes and final oocyst loads between different experiments. Such a relationship only exists within an experiment, when dilution series are analysed. Infection experiments using
Chapter 2

synchronised gametocytes reveal that a single crop of cultured gametocytes remains infective for a period of at least 10 days. Maximal infectivity appears to occur 11 days after induction of gametocytogenesis (unpubl. obs.).

Apart from quality, certainly quantity plays an important role in infectiousness to mosquitoes. The number of gametocytes in the infectious blood meal in a laboratory experiment is generally high compared with that in the blood of gametocyte carriers. In mosquitoes infected to obtain sporozoites or in bio-assays to determine the effects of transmission blocking factors, the greater the numbers of oocysts the more sporozoites will generally be produced.

Although, there is an optimum gametocytaemia for subsequent oocyst production, this optimum is unpredictable and may differ considerably between experiments. Too many gametocytes may result in a lower transmission, and the efficiency of infection per gametocyte increases with reduced numbers (Ponnudurai et al., 1987). As few as 10 gametocytes in a total of 2 μl mosquito bloodmeal are still capable of infecting 2 out of 20 mosquitoes, each with one oocyst.

Vector

Apart from parasite factors, mosquito factors also play an important role. Differences have been observed between mosquito species. In comparative experiments, for example, *An. stephensi*, *An. freeborni*, *An. gambiae*, *An. atroparvus* and *Aedes aegypti* were found to have more or less equal numbers of developing ookinetes when examined 24h post-infections. Later, however, all of the surviving *An. stephensi*, *An. freeborni* and *An. gambiae* were found to be infected with large numbers of oocysts, no oocysts could be found in the *An. atroparvus* and only two of 13 *Aedes aegypti* contained oocysts. Apparently susceptibility is determined at the level of ookinete penetration. A specific target receptor might be involved in the interaction between ookinete and midgut epithelium.

The conditions in which mosquito larvae develop, including larval density, food and water source, can also influence infection of the adults. The relative large numbers of oocysts that develop in mosquitoes reared from larvae held at low densities (Table 1) could not be explained by a difference in size of the mosquitoes at the time of feed or the size the bloodmeal they took.
The interdependent roles of parasite, vector and host

Table 1. Effect of larval density on subsequent infection of adult mosquitoes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>0.2 larvae/cm²</th>
<th>0.4 larvae/cm²</th>
<th>p</th>
<th>Degrees of freedom</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>77.9 ± 51.3</td>
<td>49.4 ± 36.7</td>
<td>&lt;0.01</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>81.3 ± 57.4</td>
<td>39.1 ± 35.1</td>
<td>&lt;0.01</td>
<td>116</td>
</tr>
<tr>
<td>3</td>
<td>112.1 ± 54.8</td>
<td>78.9 ± 51.3</td>
<td>&lt;0.01</td>
<td>117</td>
</tr>
</tbody>
</table>

Another interesting observation is the relationship between mosquito age at the time of feeding and the final oocyst load. Mosquitoes aged 3-5 days develop more oocysts when fed on blood containing *P.falciparum* than older ones given similar bloodmeals (Table 2), possibly because the younger mosquitoes digest their bloodmeals more slowly.

Table 2. Effect of mosquito age on infection

<table>
<thead>
<tr>
<th>Experiment</th>
<th>3-5 days</th>
<th>8-10 days</th>
<th>p</th>
<th>Degrees of freedom</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47.8 ± 24.7</td>
<td>0.5 ± 0.9</td>
<td>&lt;0.001</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>5.5 ± 9.9</td>
<td>0.6 ± 1.1</td>
<td>&lt;0.001</td>
<td>38</td>
</tr>
</tbody>
</table>

For unknown reasons, older mosquitoes digest infected bloodmeals much faster, although this difference disappears when no parasites are present in the bloodmeal; the presence of parasites seems to have a direct effect on the speed of digestion. Even among individual mosquitoes of the same age, susceptibility varies considerably and the time taken for digestion may again play a role. By visual examination of the midguts it is possible to distinguish between fast- and slow-digesting mosquitoes at 37-48 h after an infectious bloodmeal. Within an experiment, the speed of digestion appears to correlate with subsequent oocyst load, fast digesting mosquitoes carrying
fewer oocysts than slow ones (Ponnudurai et al., 1987). There may be a delicate balance between the speed of digestion, the availability of haemoglobin [which is necessary for normal ookinete development (Rosenberg et al., 1984)] resulting from red-cell breakdown, the formation of a peritrophic membrane and the possible damage of ookinetes by proteolytic enzymes.

That speed of digestion plays an important role is also demonstrated by the fact that refractoriness of a selected mosquito line correlated with a relative fast of digestion (Feldman et al., 1990). However the same refractory line became less refractory with diminishing numbers of parasites in each bloodmeal. This may again indicate that a parasite factor influences speed of digestion. Disintegrating parasites may release proteolytic enzymes that facilitate digestion, resulting in faster digestion and lower oocyst loads. This could also explain the observation that the number of oocysts developing from each gametocyte ingested increases as the numbers of gametocytes ingested is reduced. However, this observation has only been made when cultured gametocytes were used and the infectious feeds included asexual parasites, of all stages, as well as gametocytes. Experiments using gametocytes depleted of asexual parasites should answer the question whether these stages have an effect on the transmission.

**Hosts**

Transmission-blocking antibodies have been the main subject of research in many malaria laboratories. Natural occurring antibodies have been described on many occasions and research towards the development of a transmission-blocking vaccine is in progress. Several proteins which are capable of inducing transmission-blocking antibodies have been identified on the outer surface of gametocytes and gametes. The three most studied proteins include the PfS 45/48, PfS 230 and the PfS25 (of 45/48, 230 and 25 kDa, respectively). PfS45/48 and PfS230 are present on the surface of gametocyte stages whereas the PfS25 is expressed on the surface of the late gamete/zygote stage and is present on the ookinete and in the oocyst capsule.

Antibodies against each of these proteins have their specific mode of action and these can be elucidated by observing the development of the sporogonic stages inside the mosquito midgut. Transmission-blocking monoclonal antibodies (mAb) raised against the 45 kDa on the gametocyte surface (PfS45/48) protein are believed to interfere with the process of fertilisation as they have no effect on activation or exflagellation. Although the presence of the anti-45/48-kDa mAb in the infectious feed therefore has no effect on the number of newly formed gametes that
The interdependent roles of parasite, vector and host

develop in the mosquito midgut shortly after the feed, it does block further development of the gametes. A remarkable phenomenon with regard to the anti-45/48 kDa mAb is that they may lose effectivity as the density of gametocytes in the bloodmeal is reduced. (Ponnudurai et al., 1987). This observation may indicate that gamete agglutination by the mAb might be involved in fertilisation blocking. However transmission blockade was sustained when Fab fragments were used instead of complete mAb (Carter et al., 1990).

Transmission blocking mAb against Pfs 230 show complement dependent lysis of freshly formed gametes and are consequently restricted to complement-fixing isotypes (Quakyi et al., 1987 and Read et al., 1994). This isotype-specific effect was confirmed by Roeffen et al. (1995) who switched a non blocking anti 230-kDa mAb from isotype IgG1 to a complement-fixing IgG2a which resulted in a transmission-blocking, lytic effect. A relationship has been found in Papua New Guinea between the presence of 230 kD antibodies and transmission blockade (Graves et al., 1988) As there was no evidence of a complement-dependent transmission blockade in blocking human sera from an endemic area in Cameroon (unpubl.obs.), other mechanisms might be involved in transmission blockade by natural, anti-230-kDa antibodies.

The 25-kDa protein (Pfs25) is one of the most promising candidates for use in a transmission blocking-vaccine. So far, recombinant yeast products have shown to be effective in raising transmission-blocking antibodies in mice with alum as adjuvant (Kaslow et al., 1994). The mode of action of mAb against Pfs 25 differs from that of the other mAb described above; although they allow more or less normal ookinete development they apparently either interfere with ookinete penetration or damage late ookinetes. There has been speculation about the functions of Pfs 25; it may be involved in protection against digestion or in their movement or it may have a receptor like function mediating in the parasites’ contact with midgut epithelium. Interference with any of these actions could result in reduced transmission. It has also been shown that Pfs25 kDa persists during oocyst development, ingestion of bloodmeals containing anti-25-kDa antibodies by already infected mosquitoes resulted in reduced sporozoite infection of their salivary glands (Lensen et al., 1992).

Natural transmission blockade may simply be caused by the direct effects of antibodies in the bloodmeal on the sporogonic stages of the parasite inside the mosquito midgut. However, other humoral factors may be of importance. For unknown reasons, sera of healthy individuals who have had no experience of malaria experience may differ considerably in their capacity to
support transmission. Special care therefore needs to be taken in obtaining sera for use in transmission experiments. The serum separators present in blood-collection tubes can abolish mosquito infection completely and use of plasma in membrane-feeding experiments should be avoided. Only fresh, heparinized plasma has been successfully used in membrane feeds; the use of EDTA or citrate-phosphate-dextrose plasma or "overnight" heparinized plasma interferes with successful transmission.

Apart from humoral factors, cell-mediated mechanisms may also be involved. As an Anopheles mosquito takes a bloodmeal of approximately 2 μl it must normally ingest 10,000-20,000 white blood cells (WBC). Gametes of *P. falciparum* may be phagocytosed by some WBC both *in vivo* and *in vitro* (Sinden and Smalley, 1976). Recent findings indicate that WBC-mediated reduction can contribute to the transmission-blocking activity of sera from endemic regions. Such sera probably contain factors that promote phagocytosis, either specific factors, such as antibodies (which may not be transmission-blocking by themselves), or non-specific, such as cytokines and/or other inflammatory mediators. In *P. vivax*, for example, temporary transmission blockade occurs during paroxysms because of the presence of cytokines and/or inflammatory mediators. (Karunaweera *et al.*, 1992). In *P. falciparum* elevated concentrations of tumour necrosis factor (TNF) are associated with transmission reduction but only in the presence of WBC and an as yet unidentified parasitic factor (Naotunne *et al.*, 1993). The role of phagocytosis of freshly emerged gametes inside the mosquito midgut has recently been investigated (unpubl. obs.). Phagocytosis by WBC inside the mosquito midgut occurs despite the probably hostile environment and relative low temperature and can easily be demonstrated in a Giemsa-stained slide of a bloodmeal 20 minutes after the infectious feed. A luminol-dependent chemiluminescence assay may be used to quantify the effects of humoral factors on phagocytosis. Phagocytosis of female gametes in *vitro* is influenced by complement, specific antibodies and cytokines (unpubl. obs.). The presence of complement has a strong synergistic effect, not only in the presence of antibody but also in combination with cytokines, especially TNF. The results indicate opsonization of *P. falciparum* gametes by complement factors such as C3. This would be in agreement with a recent study of Tsubol *et al.* (1995) who detected C3 deposition on zygotes of *P. yoelii*. Transmission experiments have demonstrated that some field-collected sera only show high transmission-blocking activity in the presence of WBC (unpubl. obs.).
The interdependent roles of parasite, vector and host

Preliminary data from experiments in the field, in which WBC are depleted from the blood of gametocyte carriers before membrane feeding indicate that WBC do have a suppressive action on transmission.

Although laboratory experiments on transmission might have specific drawbacks and one has to be careful when extrapolating data to field situations, they are still powerful tools for elucidating the mechanisms of transmission and transmission reduction. Furthermore, membrane feeding assays help to validate alternative tests for detection of transmission blocking activity in field-collected sera.

References:


Cytokine-mediated inactivation of malarial gametocytes is dependent on the presence of white bloodcells and involves reactive nitrogen intermediates. (1993) *Immunology,* 78, 555-562.


CHAPTER 3

Measurement by membrane feeding of reduction in Plasmodium falciparum transmission induced by endemic sera.

A. Lensen\textsuperscript{1}, J. van Druten\textsuperscript{2}, M. Bolmer\textsuperscript{1}, G. van Gemert\textsuperscript{1}, W. Eling\textsuperscript{1} and R. Sauerwein\textsuperscript{1}.

\textit{University of Nijmegen},\textsuperscript{1} Departments of Medical Microbiology, section Parasitology and Medical Statistics, Faculty of Medicine,\textsuperscript{2}.

Transactions of the Royal Society of Tropical Medicine and Hygiene (1996), 90, 20-22
Chapter 3

Abstract

The standard laboratory test for reduction in malaria transmission is based on the measurement of oocyst numbers in mosquitoes fed on blood meals containing test and control sera. Interpretation of the results, however, is often hampered by the large variation in numbers of infected mosquitoes and oocysts. The objective of this study was to compare 3 measures for the assessment of transmission reduction (so-called R values) and to define the experimental criteria that allow interpretation of the results. To determine variability in R values of control sera, a replicate experiment was performed with 10 non-endemic sera of Dutch blood donors. Furthermore, 2 measures for calculation of transmission reduction were compared in a triplicate experiment using, *Plasmodium falciparum*, *Anopheles gambiae* and malaria endemic sera. Calculations using the geometric means of Williams are currently used to identify blocking and non-blocking sera. However, calculations using log-transformed data could distinguish more gradual levels of transmission reduction activity in endemic sera-i.e. blocking, reducing and non-blocking activity. Grading of transmission reduction activity is important for epidemiological studies on transmission immunity and for validation of future transmission-blocking vaccines.

Keywords: malaria, *Plasmodium falciparum*, *Anopheles gambiae*, transmission reduction by serum, measurement

Introduction

Malaria transmission reduction assays are performed by feeding *Anopheles* mosquitoes on cultured fertile gametocytes of *Plasmodium falciparum*, using membrane feeders (Ponnudurai et al. 1987). Reproducible measurement of transmission reduction requires a reliable source of infectious gametocytes and mosquitoes of a standard quality (Ponnudurai et al. 1989). In the assay, test sera are included in the infectious feed and transmission levels are expressed as oocyst numbers in the mosquito. A reduction in the oocyst number can be measured by a so-called R-value which is obtained by comparing the oocyst level resulting from use of a test serum (T) with that obtained with a control serum (Tc):

\[ R = \frac{Tc-T}{Tc} \]

For evaluation of transmission reduction, the R-value then assumes a value between nought (no reduction, \(T=Tc\)) and one, (maximal reduction, \(T=0\)).
Measurement of transmission reduction

There are a number of ways to assess the oocyst numbers as follows.

i) The arithmetic mean:

$$\bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i$$

ii) the geometric mean of Williams:

$$x_g = \sqrt[n]{\prod_{i=1}^{n} (x_i + 1)} - 1$$

iii) the arithmetic mean on a logarithmic scale:

$$\bar{y} = \frac{1}{n} \sum_{i=1}^{n} y_i$$

In all these equations, $x_i$ is the number of oocysts in mosquito $i$, $n$ is the number of mosquitoes in the assay and $y = \ln (x_i+1)$. Each method leads to a different R value for measuring transmission reduction.

Williams's geometric mean is generally used to accomodate the relatively great influence on the mean of individual high oocyst counts (Ichimori et al. 1990). In this study we investigated which type of R value is the most suitable for expressing transmission reduction.

Materials and methods

Study design

The study is based on the data from a replicate experiment using 10 non-endemic sera obtained from Dutch blood donors without malaria experience as test sera. For the determination of the R-values of each serum, a pool made of these sera serves as the control. The transmission capacity of each serum is measured twice in the bio-assay. R-values were calculated using respectively, the arithmetic mean, the geometric mean, and the arithmetic mean on the logarithmic scale.

Cultures

Gametocytes (P.falciparum, strain NF54) were routinely produced in the semi-automated ‘tipper’ system (Ponnudurai et al., 1982a). Gametocytes were harvested for the transmission test after 14 d in culture.
Chapter 3

Feeding procedure

All serum samples were initially inactivated by heating at 56°C for 30 min. In order to restore complement activity, 40 μL of complement rich serum is added to 80 μL of each serum. The culture material of one ‘tipper’ vessel containing about 0.3 mL packed cells with a gametocytaemia of 0.5-1 %, was centrifuged at 500g for 2 min. The supernatant was removed and the pellet, containing the parasitized red blood cells (RBC), was carefully mixed with 3.75 mL freshly washed and prewarmed group O RBC. In the meanwhile 120 μL of test or control serum sample was warmed to 37°C in Eppendorf tubes. Next, 150 μL of the parasitized RBC suspension were quickly added to each serum sample. The mixture (270 μL) was placed in a prewarmed (37°C) ‘minifeeder’ (Ponnudurai et al., 1989), on which fifty female A. gambiae mosquitoes (3-5 d old) were allowed to feed for 15 min. All unfed and partially fed mosquitoes were removed and the fully fed ones kept at 26°C and fed on 5% glucose. Twenty mosquitoes per feeder were examined for oocysts after 6 d.

Results

For standardization purposes we studied the inter- and intra-assay variations using sera from individuals with no previous malaria experience.

The arithmetic means and standard deviations (Sds) of oocyst distributions in the duplicate experiment are shown in Fig 1A. The Sds were greater than half of the mean, strongly suggesting that the distributions are skewed. Oocyst numbers did not differ significantly between the 10 sera in either measurement (Kruskal Wallis test: $P=0.11$ and $P=0.46$). Comparison of oocyst distributions between measurements 1 and 2 for each serum showed that they differed only for serum 3 (Wilcoxon test: $P=0.03$).

Logarithmic transformation is a well-accepted method to reduce skewness and reduce variances. The means and SDs transformed data $y_i = \ln(x_i+1)$, where $x_i$ is the number of oocysts found in mosquito $i$ ($i=1,...,20$), are shown in Fig 1B. Using the log scale, the arithmetic mean of the distributions associated with the pooled sera in experiment 1 and 2 were respectively 3.3 and 3.0 and the Sds were both 1.3. These SDs are less than half the associated means, indicating that the logarithmic transformation has substantially improved the skewness of the oocyst distributions. The geometric (Williams’s) mean and the arithmetic mean on a log scale of the oocyst numbers and their standard errors are shown in Fig.1C and D. The geometric mean accommodates skewed distributions and has been recommended for the calculation of transmission blocking capacity.
Measurement of transmission reduction (Ponnudurai et al., 1989; Ichimori et al., 1990). However, it did not reduce the variation in oocyst numbers (Fig. 1C). The variation was smallest with the arithmetic mean on a log scale (Fig. 1D).

Fig. 1 Numbers of oocysts of *Plasmodium falciparum* developing in *Anopheles gambiae* when the infecting bloodmeal was suspended in one of 10 non-endemic sera and a pool of these sera (1-10) to serve as control. The bars represent means of duplicate experiments plus one standard deviation (A,B) or one standard error (C,D). A, arithmetic mean; B, arithmetic mean on a log scale; C, Williams’s geometric mean; D arithmetic mean on a logarithmic scale. Solid bars indicate the first determination, open bars the second.
The R values for each of the 3 transmission reduction measures are compared in Fig. 2, which clearly shows the relative small distributions of R values obtained when using the log scale. The geometric mean of William's shows the largest variation.

The standard deviations of the R values for non-endemic control sera measured on the log scale were stable (SD=0.12 in both measurements). Therefore we may construct a reference interval for this type of R value. The critical one-sided R value associated with a 0.99 reference interval (normal approximation on log scale) is respectively 0.32 and 0.27. This means that Ry values of test sera above 0.30 indicate a transmission capacity lower than that of non-endemic sera; this contrasts with the R values based on the geometric mean, values of which up to 0.90 may be obtained with the non-endemic sera.

R values based on the geometric means and the means on a log scale were compared in triplicate experiments using endemic sera (Table 1). In the 3 experiments different batches of mosquitoes and parasites were used. The blocking sera (A, B and C) were clearly identified in each experiment using either calculation method. Using the geometric mean all sera, apart from A, B and C, would be regarded as ‘non-blockers’. However, since the log scale suggested a cut-off value
for transmission reduction at 0.30, sera D and E, should be classified as ‘reducers’ and F and G as ‘non-blockers’.

Table. Comparison of two measures of *Plasmodium falciparum* transmission reduction based on Williams's geometric mean and the arithmetic mean measured on a logarithmic scale, in a triplicate experiment using 7 endemic sera.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Inf %</th>
<th>$R_g$</th>
<th>$R_y$</th>
<th>Inf %</th>
<th>$R_g$</th>
<th>$R_y$</th>
<th>Inf %</th>
<th>$R_g$</th>
<th>$R_y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>0.99</td>
<td>0.97</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>D</td>
<td>26</td>
<td>0.92</td>
<td>0.84</td>
<td>95</td>
<td>0.85</td>
<td>0.45</td>
<td>90</td>
<td>0.83</td>
<td>0.46</td>
</tr>
<tr>
<td>E</td>
<td>53</td>
<td>0.74</td>
<td>0.57</td>
<td>95</td>
<td>0.61</td>
<td>0.33</td>
<td>85</td>
<td>0.88</td>
<td>0.54</td>
</tr>
<tr>
<td>F</td>
<td>70</td>
<td>0.49</td>
<td>0.30</td>
<td>95</td>
<td>0.63</td>
<td>0.24</td>
<td>100</td>
<td>-0.21</td>
<td>-0.01</td>
</tr>
<tr>
<td>G</td>
<td>80</td>
<td>0.29</td>
<td>0.15</td>
<td>95</td>
<td>0.59</td>
<td>0.21</td>
<td>100</td>
<td>0.13</td>
<td>0.03</td>
</tr>
</tbody>
</table>

$R_g$= R-value based on the geometric (Williams's) mean

$R_y$= R-value based on the arithmetic mean on a ln-scale

% inf= percentage infected mosquitoes

**Discussion**

There are 2 ways to assess the transmission blocking capacity of sera from the field by membrane feeding. One possibility is to perform the bioassay as described, using cultured gametocytes and a standardized mosquito colony. The other possibility is to feed whole blood of gametocyte carriers by membrane feeding but with serum replacement as described by Mulder et al. 1994. The advantage of the latter method is that transmission reduction is measured under more natural conditions. The complication, however, is that, due to the relatively low oocyst levels and therefore low mosquito infections one has to use sample sizes of at least 50 to 100 mosquitoes (Medley et al. 1993). This makes testing of sera in epidemiological studies extremely difficult. Moreover the intrinsic capacity of gametocytes to infect mosquitoes also influences infectivity and may depend on factors as numbers, maturity, age, viability, the use of
drugs or even the genetic background of the parasite strain. Finally the choice of control serum in the replacement, as shown in our work, influences the final outcome of the test. Therefore, in order to obtain a better standardized control serum, ten sera were pooled. From this pool of non-endemic sera we may expect to obtain an oocyst level, which is in the centre of the range of values obtained with the individual non-endemic sera. The results of the present study were in agreement with this expectation. We consider the bioassay using standardized cultured gametocytes to be preferable. The advantages of the laboratory assay are the relative high oocyst numbers in infected mosquitoes and the possibility of repeating the experiments. The assay is considered successful if at least 90% of the control mosquitoes are infected. Because of this high infection rate, genuine transmission blocking sera (sera A,B,C) can be detected using only 20 mosquitoes. We have always aimed at the highest oocyst level to ensure obtaining reduction over as great a range as possible. In theory this gives the possibility of discriminating between field sera and creating different categories of transmission reduction capacity. However, due to large variations, only R values (based on the Williams geometric mean) of more than 85% gave reliable results—i.e. could be confirmed in repeated experiments. This study showed that this high cut-off level was due to wide distribution of R-values associated with non-endemic control sera. The range of R-values obtained with non-endemic sera provides a basis for classifying the test results of other sera. The R values of control sera should be close to zero: only those values based on a logarithmic scale met this criterion (Fig.2). In fact R-values based on the geometric mean were the least satisfactory for the measurement of transmission reduction. The practical aspects of these results are demonstrated in the Table 1. It has been suggested that only percentage infected mosquitoes should be used for the determination of transmission reduction. However in experiments with high oocyst levels it is possible to have a high mosquito infection rate combined with a high R value as shown in experiment 2 and 3. Since it is still difficult to predict the transmission level in the feeder assay in advance we have to aim at the highest oocyst levels for practical reasons.

In conclusion we would like to suggest to use the log scale in determining R values of endemic sera using feeder assays with high oocyst levels. This enables one to define three categories of sera: blocking (R>0.9), reducing (0.9≥R≥0.3) or non-blocking (R<0.3).

We have compared 3 commonly used measures for transmission reduction. Apart from these measures other measures might be constructed based on other transformations, e.g. the square root transformation, or one might use a parametric approach, e.g. fitting the negative binomial
Measurement of transmission reduction
distribution to oocyst counts (Medley et al., 1994). These approaches will lead to other measures of transmission reduction. Further research should be directed towards these and perhaps other possibilities for constructing R-values.

Acknowledgements
We thank Mrs J. Huisman, Mr J. Hooghof and Mrs J Remmers for technical assistance and Prof. dr J.H.E. Th. Meuwissen for critically reading and discussing the manuscript.
This research was supported by the Dutch Ministry for Development Co-operation (DGIS/-SO) contract nr NL002701.

References:


CHAPTER 4

*Plasmodium falciparum*: Infectivity of cultured synchronised gametocytes to mosquitoes.


*University of Nijmegen, Department of Medical Microbiology, section Parasitology*

Experimental Parasitology, 1998 (in press)
Abstract
Infectivity of *Plasmodium falciparum* gametocytes depends on the quality and maturity of gametocytes. The capacity of gametocytes to infect mosquitoes may depend on the age of the gametocytes. To study the infectiousness of gametocytes in relation to their age, *P. falciparum* gametocytes of two different strains, NF54 and NF134, were synchronised by treatment with N-acetyl-glucosamine and cultured in vitro. This resulted in the production of single crops of gametocytes of the same age. The infectivity of these stages was determined by daily feeds to *Anopheles gambiae* mosquitoes using a membrane feeder system. Crescentic gametocytes were observed from 7 days onwards after initiation of the synchronized gametocyte cultures, 4 days later these stages were most infectious for mosquitoes. Optimum infectivity was restricted to a relative short period of approximately 1 or 2 days, and declined. The results of this study may have implications for field studies on infectivity of gametocyte carriers to mosquitoes.

Introduction
Transmission of *P. falciparum* is influenced by various human, parasite and mosquito factors (Lensen et al. 1996) but effects of human serum and cellular factors have been principle focus of extensive transmission research. Age of the gametocytes may be an important parameter determining the intrinsic infectivity of gametocytes for mosquitoes (Hogh *et al*.1998). Gametocytes develop asynchronously under normal culture conditions since new generations of gametocytes can occur with every schizogony. At day 14 of culture which is the standard time to perform mosquito feeds, this will result in a heterogeneous population of mature, immature and degenerated gametocytes (Ponnudurai *et al*.1989). Studies on gametocyte infectivity in the field or in experimental models may be obscured by asynchrony since different cohorts of gametocytes may be present in the peripheral blood. Moreover, confounding factors influencing transmission are numerous( Sinden *et al*.1996 ), and make assessment of gametocyte infectivity for mosquitoes complicated. To determine the age-dependent infectivity of gametocytes, synchronous cultures were prepared to give rise to single crops of gametocytes of the same age which could be followed longitudinally for their infectiousness to mosquitoes.
Materials and methods

Parasites and synchronisation procedure

Two different strains of *P. falciparum* were used; NF54, the Amsterdam airport strain which has been used in culture experiments for many years (Ponnudurai *et al.* 1989) and strain NF134 which was isolated from a Dutch woman who was infected in Tanzania. Parasites were synchronised and maintained in a suspension culture system as described by Ponnudurai *et al.* (1986). Gametocytes were counted daily in Giemsa stained thin smears. Morphological criteria for assignment of gametocyte stages (I to VB) were used as described (Ponnudurai *et al.* 1986).

Cultures were started in an automated suspension culture system; 10 bottles of 10 ml each at a parasitaemia of 0.5%. Group O red blood cells (RBC) were used from healthy blood bank donors and the starting haematocrit was 5%. Complete medium consisted of RPMI 1640 supplemented with 10% human pooled A serum (NHS) (from Dutch blood bank donors without previous malaria experience), 25 mM Hepes, 20 mM sodium bicarbonate and 50 μg hypoxanthine according to the protocol of Ponnudurai *et al.* (1982). Medium was changed twice daily. After 4 days, when the parasitaemia reached over 10%, the 10 culture bottles were harvested and the parasitized RBC were concentrated to a haematocrit of 0.4 in complete medium in a volume of 12 ml. This suspension was mixed with 6 ml of 3% (w/v) gelatine (300 bloom) in physiological saline and allowed to stand in a 37°C waterbath for 15 min. The supernate, containing the schizont enriched fraction was washed twice in culture medium. Freshly washed RBCs were added to the pellet to reduce the schizontaemia to 5-7% and reintroduced in a culture bottle, adjusted to a haematocrit of 5% with freshly washed RBCs. After 12 hours the complete medium was replaced by complete medium containing 50 mM N-acetyl-glucosamine, which resulted in killing of all asexual stages but no effect on gametocytes (Gupta *et al.* 1985). After 72 hours N-acetyl-glucosamine was omitted from the culture. The result was a synchronised developing gametocyte culture with all parasites in the crescent stage at day 7-8 after the gelatine flotation procedure.

Feeding procedure

From the same crop of synchronised gametocytes feeds were performed daily from day 7 to 17 (NF134) or day 8 to 18 (NF54) after initiation of the culture as follows: 1ml of parasitized RBC suspension was harvested. This was layered over 0.4 ml of washed RBC and centrifuged.
for 2 min 600g at 37° C. The supernate was removed, the pellet suspended in 0.4 ml pooled NHS, brought onto a mini membrane feeder (Ponnudurai et al. 1989) and subsequently fed to 50 female *Anopheles gambiae* mosquitoes of 4 days old. Unfed and not fully engorged mosquitoes were removed. After 6 days stomachs of 20 mosquitoes were dissected and oocysts counted.

In order to keep a constant haematocrit, the volume of medium replacement in the culture bottle was daily reduced by 1 ml, being the volume used for the mosquito feed.

**Gametogenesis**

Exflagellation was induced by mixing a drop of culture material with a drop of foetal calf serum (FCS) on a slide, which was incubated in a humid chamber at room temperature for 10 minutes. Next a coverslip with “Vaseline” edges was placed on the drop which was examined for exflagellation. To quantify macrogamete formation, three hours after the feed 5 mosquitoes were dissected and the blood filled stomachs were gently disrupted by carefully pipetting in 100 μl PBS (phosphate-buffered saline). This was centrifuged for 30 seconds in a Heamofuge® Biofuge A at 8000 g. The supernate was removed and the pellet was gently suspended in 50 μl FITC (fluoresceine-isothiocyanine) labelled anti-Pfs 25 monoclonal antibody 32F81 (Ponnudurai et al. 1987) with 0.05% Evans blue as background stain. After 30 min incubation at room temperature the cells were washed twice with PBS. Fluorescing round forms, (macrogametes/zygotes,) were counted in a Bürker-Türk counting chamber using an indirect fluorescent light microscope.

**Results**

Asexual growth was completely abolished after three days exposure to N-acetyl-glucose-amine, and gametocytes developed synchronously. The results in figures 1(NF54) and 2 (NF134) show a clear optimal period for infection at day 11-12. From day 7 (NF134) or day 8 (NF54) onwards exflagellation was observed and only crescent stage gametocytes were present in culture. However, infections were obtained from feeds performed on day 8 (NF54). The infectivity increased within a few days with a maximum at day 11-12 and then faded away. Fig 1 shows that the maximum of 47 % morphologically mature VB gametocytes coincided with maximal infectivity to mosquitoes. Although all gametocytes were at least
Infectivity of synchronised gametocytes

stage VA at day 7-8, the majority of gametocytes was aberrant which was reflected by clumped, wrongly distributed pigment or pointed/knoblike poles. During development from stage I to stage VA the numbers of gametocytes remained constant. However, from day 7-8 onwards when all gametocytes were crescents, a gradual decline in numbers of gametocytes was observed. Numbers of macrogametes formed were higher one day preceding the highest infectivity (oocyst load) to mosquitoes (fig2). From the mosquito infection rates of feeds it can be concluded that a cohort of gametocytes of the same age can be infective for a total period of approximately 7 days (fig1, fig 2), with enormous differences in infection results. In experiments (n=6) where asexual growth slowly caught up, a similar profile of infectivity was found (data not shown). Comparison of the results of independent experiments revealed that the number of gametocytes in the culture was not predictive for the oocyst load in mosquitoes as described previously (Ponnudurai et al. 1989).

Figure 1. Age dependent infectivity of gametocytes to mosquitoes. Gametocytes of strain NF54 were synchronised and fed daily to mosquitoes by membrane feeding. Age of gametocytes was measured from the day that the schizonts ruptured from which the gametocytes originated. From day 7 onwards all gametocytes were at least stage VA. Bars represent the geometric means of William's of oocyst numbers per mosquito. (n=20) and SE, % mature (stage VB) gametocytes ( ), ND, not done.
Figure 2. Age dependent infectivity of gametocytes. Gametocytes of strain NF134 were synchronised and fed daily to mosquitoes by membrane feeding. Age of gametocytes was measured from the day that the schizonts ruptured from which the gametocytes originated. From day 7 onwards all gametocytes were at least stage VA. Bars represent the geometric means of Williams of oocyst numbers per mosquito (n=20) and SE. Gct ( ), gametocyte counts (from Giemsa stained thin smears) per 1000 red blood cells in the culture before feeding. Gmt ( ), the number of gametes/zygotes in the mosquito midgut per 4.10E3 red blood cells 3 hours after taking a bloodmeal (detection by FITC-labeled anti 25-kD Mab). ND, not done

Discussion
The infectivity of gametocytes to mosquitoes is important in studies of transmission and transmission blockade. Parasite factors, affecting transmissibility, include quantity as well as “quality” of gametocytes. In the laboratory it was shown that gametocyte numbers are an important factor and correlate to oocyst infection within the same experiment. However, this correlation does not exist between different experiments indicating the great variability of gametocyte “quality” (Ponnudurai et al. 1989). Already in older literature (James 1931) the quality of gametocytes was regarded as most important. However, defining “quality” is extremely difficult. First of all, there is the morphological appearance in a Giemsa stained smear which makes it possible to discriminate aberrant and immature forms from mature ones. One step further is to look at the capacity to activate and produce gametes as indicated by
Infectivity of synchronised gametocytes

exflagellation and formation of macrogametes. However, even good exflagellation and macrogamete formation still does not guarantee mosquito infection (Ponnudurai et al. 1989.) In vitro macrogamete formation was higher one day preceding maximum infectivity (fig. 2). In our experiments the percentage of morphological correct stage VB gametocytes corresponded with infectivity (fig 1). There may be a little variation in the time necessary to reach functional maturity of gametocytes which can be explained by the gap in synchronisation of 12 h. At day 8 (fig. 1) a substantial number of gametocytes were still immature in the stage VA. The highest percentage of mature (VB) gametocytes was 47% at day 11, meaning that the majority of gametocytes was either immature or aberrant at any time point measured. The observation that the majority of crescent gametocytes need another 3 to 4 days to reach functional maturity confirms those of Jeffry and Eyles (1955) and Smalley and Sinden (1976). So in natural infections the non-infectious period at the onset of gametocytaemia may be explained by the release of stage VA gametocytes in the peripheral blood.

Mosquito infections from a single crop were obtained for a period of about 7 days. Furthermore, the optimal infectiousness seemed to be restricted to a relatively short period of 1 (NF54) or 2 days (NF134). From the day of initiation of gametocytogenesis approximately 11 days were necessary to reach functional maturity. In “normal” non-synchronised cultures, gametocytes are routinely fed on day 14 after start of the culture (Ponnudurai et al. 1982). If gametocytes need about 11 days to reach functional maturity it means that gametocytes responsible for mosquito infection were initiated only 3 days after the start of the culture. At this time there are no signs of a “stress” situation in these cultures which is often regarded as a major trigger for gametocytogenesis and occurs around day 6 or 7 when the highest asexual parasitaemia’s are reached. Since postponed feeding of these cultures in general does not result in higher levels of mosquito infections it is very likely that mosquito infections from asynchronous cultures are a result of the earliest generated gametocytes. Because these are relatively limited in numbers they must be of good quality regarding the high mosquito infections which can be obtained. The relative short period of high gametocyte infectivity to mosquitoes may have consequences for field studies on the infectiousness of gametocyte carriers and may in part explain the absence of correlation between gametocyte numbers and mosquito infectivity. Due to drug treatment, the majority of gametocytes may appear as a
single crop which reaches full maturity only 11 days later and is maximal infective for a relative short period. So the day of feeding may be critical for mosquito infection. Gametocyte carriers should therefore be tested for several consecutive days when their infectiousness to mosquitoes is to be determined.

Acknowledgements
We thank Prof. Dr. J.H.E.Th. Meuwissen for stimulating discussion and Mrs J. Essong, Mr J. Hooghoof and Mrs J. Remmers for technical assistance. This research was supported by the Dutch Ministry for Development Co-operation (DGIS-SO), contract no. N1002701.

References:


Infectivity of synchronised gametocytes


CHAPTER 5

Transmission blocking antibody of the *Plasmodium falciparum* zygote/ookinete surface protein Pfs25 also influences sporozoite development.


*University of Nijmegen, Department of Medical Microbiology, section Parasitology*

*Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, Maryland 20892 U.S.A.*

Parasite Immunology (1992), 14, 471-479
Summary
The *Plasmodium falciparum* zygote/ookinete surface protein, Pfs25, persists in the oocyst wall throughout its development. Anti-25 kD transmission blocking antibody, given to infected *Anopheles stephensi* or *A.gambiae* mosquitoes in an additional bloodmeal, 3-6 days after being fed gametocyte infected blood, penetrated the oocyst and reacted with the 25 kD protein within it. This reaction caused a significant reduction in the number of developing sporozoites. Mouse serum containing antibodies raised by immunization with a recombinant 25 kD yeast product showed a similar effect.

Introduction
Mature *Plasmodium falciparum* gametocytes are activated by factors such as temperature drop and pH change in the mosquito midgut, to form gametes. Shortly after activation, the 25 kD protein, Pfs25, is expressed on the surface of the gamete, and persists on the zygote and ookinete. It has been suggested that this protein plays a role in the protection of these stages against digestion in the mosquito midgut. It might also contribute to the motility of the ookinete (Ponnudurai *et al.* 1988.) and is likely to be involved in the passage through the peritrophic membrane and in the recognition of receptor sites on midgut epithelial cells (Meis *et al.* 1989, Sieber *et al.*1991). Interference with these functions might influence the transmission of the parasite and it has been demonstrated that some monoclonal antibodies directed against the 25 kD protein, included in the infectious bloodmeal, can completely block transmission (Vermeulen *et al.*1985). The possibility of inducing such potent transmission-blocking antibodies against this protein, makes it one of the major candidate antigens of a transmission-blocking vaccine (Kaslow *et al.*1991). As Pfs25 appears to play such a significant role in transmission, it is important to know whether it persists to the oocyst stage, and if so, whether oocyst development can be affected by antibodies to it. In nature mosquitoes repeatedly take bloodmeals during sporogony. Since anti-circumsporozoite (CS) antibodies in such meals are able to penetrate oocysts (Vaughan *et al.* 1988), we decided to study the effects on sporogony of transmission-blocking anti-25 kD monoclonal and polyclonal antibodies included in a bloodmeal fed to mosquitoes 3-8 days after an infectious feed.
Materials and methods

Mosquito infections.
Three to five day old *Anopheles stephensi* or *A. gambiae* were infected with cultured *Plasmodium falciparum* gametocytes (isolate NF54) as described previously (Ponnudurai *et al.* 1989). Immediately after the bloodmeal unfed or partially fed mosquitoes were removed. A second bloodmeal was given on days 3, 5, 6 or 8 after the infectious one using membrane feeders (Ponnudurai *et al.* 1989).

Detection of the 25 kD protein in the oocyst by immunofluorescence.
Midguts of infected mosquitoes were dissected at various times after infection, and incubated with a fluorescein isothiocyanate (FITC)-labeled anti-25 kD monoclonal antibody in 0.025% Evans blue on a glass slide for 15 min at room temperature. For infections older than 48 hours the midguts were incubated in distilled water for 30 min, prior to the antibody reaction. After incubation the preparations were washed with phosphate buffered saline (PBS, pH 7.4), mounted under a cover glass in 50% glycerine/PBS and viewed with an incident light fluorescence microscope.

Immunoelectron microscopy.
Midguts of mosquitoes infected 5 days previously were dissected in a drop of 0.1% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer and prefixed for 10 min followed by fixation in 2% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C. After extensive washings in 0.1 M phosphate buffer, midguts were embedded in the methacrylate/acrylate mixture HM20 (Biorad, Cambridge). Thin sections were etched for 15 min in drops of a saturated aqueous solution of sodium metaperiodate, rinsed 3 times with distilled water and preincubated for 30 min with 1% bovine serum albumin and 1% cold fish skin gelatin in 0.1 M phosphate buffer (BGP, pH 7.4). Sections were subsequently incubated overnight in a humid chamber at 4 °C with the transmission-blocking anti-25 kD mAb 32F81 (50 mg/ml). After three washes in BGP the grids were incubated for 1 h at room temperature with goat-anti-mouse IgG coupled to colloidal gold (10 nm, Amersham Int.). After three washes in BGP the grids were post-fixed with 1% glutaraldehyde in 0.1 M phosphate buffer for 10 min, rinsed again in distilled water and stained with uranyl acetate. Control sections were incubated with an unrelated mAb or only with the second antibody.

The reaction of antibody with oocysts in *vivo* and its effect on sporogony.
Chapter 5

Three, 6 or 8 days after an infection, infected mosquitoes were given a second human bloodmeal, to which was added a transmission-blocking anti-25 kD mAb (32F81 or 32F61) at a concentration of 0.5 mg/ml (Vermeulen et al. 1985). In control experiments, the second bloodmeal contained either an anti-25 kD non-transmission blocking mAb, 32F72, at the same concentration or no monoclonal antibody at all. Six hours after the second bloodmeal, some of the mosquito midguts were dissected, slit open on one side to wash away the gut contents with an excess of PBS, embedded in Tissue Tek (Miles), and frozen in liquid nitrogen. Cryosections of 6-8 mm were incubated with FITC-labeled goat anti-mouse Ig (Nordic) for 15 min at room temperature and, after washing with PBS, mounted in 50% glycerine/PBS and examined under an incident light fluorescence microscope.

14-16 days after the infectious bloodmeal up to 50 mosquitoes were examined for sporozoites in the following way. Thoraces of pools of 5-10 mosquitoes from each batch were homogenised in a tissue grinder in a minimum volume of PBS. The suspension of mosquito tissue was then transferred to an Eppendorf tube and incubated for 15 min at room temperature with an equivalent volume of a FITC-labeled anti-CS mAb 3Sp2 (Verhave et al. 1988). After incubation, the tubes were filled with PBS and centrifuged in a hemofuge at 10,000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 100 ml PBS. The sporozoites were immediately counted in a Bürker-Türk counting chamber, using an incident light fluorescence microscope with a magnification of x500.

Results

The periphery of two to five day old oocysts reacted readily with FITC-labeled anti-25 kD mAbs, as shown in Fig.1A. Immunoelectron microscopy demonstrated the localisation of the 25 kD antigen in the oocyst capsule, at day 6 after infection (Fig. 1B). Fig.1C and fig.1D show the positive reactions of 9 day old oocysts and a 16 day old mature, ruptured oocyst respectively.

Cryosections of midguts of antibody-fed mosquitoes, 3 and 6 days after infection, showed a positive reaction of the oocysts with FITC- labeled goat anti mouse-Ig, indicating that the anti-25 kD Mab had penetrated the oocyst and reacted with the cyst wall as shown in Fig.1E. On day 8, however, not all the oocysts showed a positive immunofluorescence reaction. Some oocysts, identified by the presence of the typical malaria pigment, did not bind FITC labeled
goat anti mouse-Ig at that time. Five experiments were performed to measure the effect of the second bloodmeal, containing a transmission blocking anti-25 kD mAb, on the number of sporozoites.

Table 1 summarizes 4 experiments in which the second bloodmeal was given on day 3. The first two experiments using two different blocking mAbs 32F81 and 32F61 compared to a normal bloodmeal, demonstrated a significant reduction in the numbers of sporozoites in the experimental groups. The second set of experiments was performed to determine whether these reductions in sporozoite numbers were epitope specific. The normal bloodmeal was replaced by a bloodmeal containing a nonblocking mAb 32F72. The latter had no effect, although mAb 32F72 recognizes the same protein but a different epitope.

Table 2 gives the results of one experiment in which the second bloodmeal was given on either day 3, 6 or 8. Whereas the feeds containing mAb 32F81 given on day 3 and 6 significantly decreased the numbers of sporozoites, the feed of day 8, although still showing a reduction, was not statistically different from the control. Consistent with these findings was the observation that not all of the oocysts in cryo-sections of midguts of day 8 fed mosquitoes reacted with the anti-mouse Ig. Finally on day 7 and 14 of each experiment the numbers of ooyists were counted. There was no significant difference between these numbers on both days in the experimental and control groups, nor was there any evidence of degeneration of oocysts that could account for the differences in the numbers of sporozoites at day 14.

Table 1. Number of salivary gland sporozoites per mosquito in those given an additional bloodmeal on day 3 after infection.

<table>
<thead>
<tr>
<th>Blocking mAb</th>
<th>Control mAb</th>
<th>mAb SE</th>
<th>Control SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>32F81 (40)</td>
<td>NB (45)</td>
<td>16.1 ± 2.7</td>
<td>41.4 ± 4.8</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>32F61 (45)</td>
<td>NB (45)</td>
<td>130.9 ± 9.6</td>
<td>230.2 ± 15.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>32F81 (15)</td>
<td>32F72 (20)</td>
<td>9.5 ± 2.1</td>
<td>36.9 ± 4.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>32F81 (40)</td>
<td>32F72 (40)</td>
<td>14.5 ± 2.8</td>
<td>37.2 ± 4.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NB= Normal bloodmeal; No. in parenthesis gives the total number of mosquitoes examined
SE= standard error; P-Value by two sample T test.
Figure 1. Immunofluorescence and immunoelectron microscopy of oocysts of Plasmodium falciparum using labeled anti-25 kD mAb.

A. 48 hour oocysts (bar=10 mm)
B. Immunogold labeling of oocyst capsule (Oc) at day 6 after infection (bar=0.2 mm)
C. 9 day old oocysts (bar=10 mm)
D. 16 day old ruptured oocyst (bar=10 mm)
anti-Pfs 25 activity

E. Cryosection of 6 day old oocyst 6 hours after a bloodmeal containing anti-25 kD mAb, showing the presence of antibody inside the oocyst using FITC labeled goat anti-mouse conjugate (bar=50mm)

Table 2. Number of salivary gland sporozoites per mosquito in those given a second bloodmeal on day 3, 6 or 8 of the infection.

<table>
<thead>
<tr>
<th>Day</th>
<th>No. sporozoites x 10^3</th>
<th>32F81 SE</th>
<th>NB SE</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5.4 ± 0.7 (50)</td>
<td>10.8 ±2.0</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>9.1 ± 2.7 (50)</td>
<td>24.9 ±6.5</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8.9 ± 2.6 (45)</td>
<td>17.1 ±3.0</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NB= Normal bloodmeal; NS= not significant; No. in parenthesis gives the total number of mosquitoes examined; SE= standard error; P-value by two sample T test.

Table 3. Number of salivary gland sporozoites per mosquito given a second bloodmeal on day 5 after infection containing mouse sera from animals immunised with a recombinant 25 kD yeast product.

<table>
<thead>
<tr>
<th>Serum</th>
<th>sporozoites x 10^3 ± SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IX6</td>
<td>33.7 ±4.1 (45)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>XIV3</td>
<td>45.2 ±4.1 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>XIV6</td>
<td>40.6 ±3.6 (45)</td>
<td>NS</td>
</tr>
<tr>
<td>NB</td>
<td>48.6 ±4.6 (50)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. lists the results of an experiment in which sera obtained from mice after immunization with a recombinant 25 kD yeast product were used. (Barr et al.1991) The sera IX6 and XIV6 had been previously shown to block transmission totally in a transmission-blocking assay whereas the preimmune serum XIV3 failed to do so (Kaslow et al. manuscript in preparation). In our assay, serum IX6 produced a significant decrease in sporozoite numbers compared to the control. Serum XIV6 also caused a reduction but not significantly, and the preimmune serum XIV3 showed no effect at all compared to the normal bloodmeal.
NB= Normal bloodmeal; NS= not significant; No. in parenthesis gives the total number of mosquitoes examined; SE= standard error; P-value by two sample T test.

**Discussion**

We found that the 25 kD protein is present in the oocyst stage throughout its development. The immunofluorescence reaction appeared to be restricted to the periphery of the oocyst and the immunoelectron microscopy clearly showed the localization of the 25 kD protein in the oocyst capsule. This observation strengthens the suggestion of Sinden (1978), that the cyst wall is of parasitic origin. The presence of the 25 kD protein would allow the recognition of very young oocysts of *P. falciparum* in wild caught mosquitoes, using the fluorescence technique.

In the early stages of parasitic development in the mosquito, namely the female gamete, retort form and ookinete, the 25 kD protein is expressed on the parasite surface, making these stages vulnerable to the action of anti-25 kD antibodies. It is possible to block transmission completely by the addition of mAbs like 32F81 to the infectious feed (Vermeulen *et al.* 1985). Therefore it is likely that the 25 kD protein has important functional properties. The nutritional demands of developing oocysts are probably considerable and nutrients have to be transferred across the cyst wall. The 25 kD protein could play a significant role in such transport. Davies & Howells (1973) showed that the cyst wall is readily permeable to $^3$H-adenosine and $^3$H-thymidine and even intact immunoglobulins could pass the oocyst capsule as shown in this study and earlier by Vaughan *et al.*(1988). The reaction of anti-25 kD antibodies with the oocyst was demonstrated using cryosections of infected mosquito midguts. The fact that antibody reacted with the cyst wall in all oocysts on days 3 and 6 and only in some on day 8 shows that the passage of immunoglobulins is likely to be an active process probably correlated with the uptake of nutrients. The variations in the reaction of immunoglobulins with the cyst wall of oocysts on day 8 could be explained by differences in rates of development due to the high oocyst numbers in our experimental infections. The major increase in size of the oocyst is in the first 5 days of development. From day 6, however, instead of enlarging much further the oocyst starts to differentiate and sporoblast formation takes place at day 7 (Posthuma *et al.*1988). The failure of Ig to react with the oocyst wall in a high proportion of the oocysts at day 8 coincides with our observation of the reduced effect on sporozoite development. While feeds containing blocking anti-25 kD
anti-Pfs 25 activity

antibodies given on day 3 and 6, decreased the numbers of sporozoites, the feed on day 8, failed to do so significantly. The mAb 32F72 recognizing a

non-blocking epitope of the 25 kD protein also had no effect on developing sporozoites. The reason for this is unclear and requires further study.

The experiment with the transmission-blocking sera from mice immunized with the recombinant yeast product (Barr et al. 1991) showed that polyclonal antibodies raised by immunisation with a recombinant 25 kD product can also influence sporogony. That the polyclonal antibodies were not as effective as the Mabs could be a matter of concentration and/or affinity.

These experiments demonstrate the possible additional effect of a transmission blocking vaccine even when not all persons are vaccinated. In nature mosquitoes take a blood meal every 2-3 days. A mosquito that feeds on an infective, non-vaccinated individual and develops oocysts on its midgut, still could be affected if its next bloodmeal is on a person who has acquired transmission-blocking immunity by a vaccination with a recombinant 25 kD vaccine. The effect might be even stronger than demonstrated in the present study if also subsequent bloodmeals contained anti-25 kD antibodies. The mechanism of sporozoite reduction could be due to: A patchy total abrogation of sporozoite development in the oocyst or a partial decrease in the number of sporozoites in the oocysts present. If the former explanation is the true one, then in field conditions where light infections are the rule (Pringle, 1966; Muirhead-Thomson, 1957), the application of a 25 kD vaccine could have a additional effect to simply blocking transmission. If however, the latter hypothesis is correct than it is unlikely to have the additional effect, since sporozoite inoculum is independent of salivary gland load (Ponnudurai et al. 1991). Therefore it is important to explore further the mechanisms of the observed reduction, and to study this effect in field conditions.

Acknowledgements
We wish to thank Ms J. Huisman and Mr J.B.A. Hooghof for invaluable technical assistance, Mr T.F.H. Hermsen for cryosections and Ms M. Sieben and Mr W.F.G. Roefhèn for the supply and labeling of monoclonals. This work was supported by grant no. 75396 from the Dutch Ministry of Foreign Affairs, NGO (Educational and Research Department, Section for
Research and Applied Technology). Financial support was also received from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

References


anti-Pfs 25 activity

Pringle G. (1966) A quantitative study of naturally acquired malaria infection in Anopheles gambiæ and Anopheles funestus in a highly malarious area of East Africa. Transactions of the Royal Society of Tropical Medicine & Hygiene 60, 626


Leucocytes in a *Plasmodium falciparum*-infected bloodmeal reduce transmission of malaria to *Anopheles* mosquitoes.

A.H.W. Lensen, M. Bolmer-van de Vegte, G.J. van Gemert, W.M.C. Eling and R.W. Sauerwein

*University of Nijmegen, Department of Medical Microbiology, section Parasitology*

Infection and Immunity (1997), 65, 3834-3837
Chapter 6

Abstract

Mosquitoes are infected with *Plasmodium falciparum* by taking a bloodmeal from a gametocyte carrier. Since a mosquito takes a volume of 1 to 2 μl, a bloodmeal may contain $1 \times 10^4$ to $3 \times 10^4$ white blood cells (WBC). The majority of WBC are composed of neutrophils which may phagocytose and kill developing gametes inside the mosquito midgut. Phagocytosis was measured *in vitro* by a luminol-dependent chemiluminescence (CL) assay. In the presence of *P. falciparum* gametes, sera from areas of endemicity had an increased CL-response compared to controls. In mosquito membrane feeding experiments some such sera showed a transmission reduction which was related to the presence of viable WBC. The results of this study suggest that phagocytosis of opsonized gametes inside the mosquito midgut occurs which can contribute to a reduction in the transmission of *P. falciparum* parasites.

Introduction

Transmission of *Plasmodium falciparum* from man to mosquito is accomplished when a mosquito feeds on a gametocyte carrier. Once inside the mosquito midgut, the intra-erythrocytic gametocytes activate and escape within minutes from their erythrocytes to form gametes. Triggers for this activation are pH rise, temperature drop and possibly, unidentified mosquito factors. Since the freshly formed gametes are no longer protected by the erythrocytic membranes they subsequently become vulnerable to gamete-specific antibodies that may be present in the blood meal. Antibodies have been shown to block transmission, both by interfering with the process of fertilisation, sporogony or an antibody-mediated complement-dependent lysis of the parasite (13, 15). An additional mechanism may be mediated by activated leucocytes (WBC) that phagocytose gametes inside the mosquito midgut shortly after the mosquito takes a blood meal (19). Using a chemiluminescence (CL) assay (3) and a transmission assay (7) we analysed the influence of phagocytosis and its enhancement by opsonizing antibodies inside the mosquito midgut on malaria transmission.

Materials & Methods

**WBC.** Ten ml of heparinized blood of healthy volunteers was added to 2 ml dextran (6% dextran [molecular weight 200,000] in phosphate buffered saline [PBS], pH 7.2. Cells were allowed to settle for 30 min. An equal volume of PBS was added to the supernatant containing
Leucocytes reduce transmission

the WBC-enriched fraction and the sample was centrifuged at 500 × g for 10 min. Two milliliters of distilled water was added to the pellet for 10 s to lyse the remaining erythrocytes. After 10 s, 15 ml of PBS was added and the centrifugation step was repeated. Neutrophils were separated from mononuclear cells by centrifugation through a layer of Ficoll-Paque (density 1.075, Pharmacia) at 700 × g for 30 min at 4°C. After the pellet was harvested and subjected to three washing steps with cold PBS, the neutrophils were kept on ice until used.

Sera and Mab. Control sera were obtained from Dutch blood bank volunteers. Field sera were collected from gametocyte carriers recruited at the Messa dispensary in Yaoundé, Cameroon and from clinical malaria patients recruited at St. Francis Hospital in Ifakara, Tanzania, after all donors had given consent.

Monoclonal antibody (Mab) 32F1, specific for the surface protein Pf48/45 on sexual stages of *P. falciparum* has been described by Vermeulen et al. (20).

Gametes. *P. falciparum* gametocytes of NF54 (Amsterdam, airport strain) were cultured using the semiautomated tipper system (12). After 14 days of culture 10 ml of the parasitized red blood cell suspension was collected. The suspension was centrifuged for 5 min at 500 × g at 37°C. The medium was replaced by an equal volume of fetal calf serum (FCS) and gametocytes were allowed to activate for 1 h at room temperature. The cell suspension was centrifuged at 500 × g for 7 min and the pellet was resuspended in 3 ml PBS and subsequently layered on 12.5% Nycodenz (Nycomed Pharma AS, Oslo, Norway) in PBS. After centrifugation at 2,100 × g for 40 min at 4°C, gametes were collected from the top of the Nycodenz layer. Gametes were resuspended in 15 ml PBS and centrifuged for 5 min at 500 × g. Finally, the pellet was resuspended in PBS to give a final concentration of 10^7 gametes/ml.

*Plasmodium berghei* gametes were obtained using the method described by Beetsma et al. (1). Erythropoiesis in male C57Bl/10 mice was stimulated by bleeding 12 drops of blood (approximately 350 μl). Two days later the mice were infected with 10^7 *P. berghei* parasites (ANKA strain). After three days the mice were given sulfadiazine in their drinking water (10 mg/ml) to suppress parasitaemia and another two days later the mice were bled. The blood was collected (1:10) in RPMI/1640 (Cibco BRL, Life Technologies, Paisley, Scotland, United Kingdom) without NaHCO3, containing 5 U Na-heparine (Leo Pharmaceuticals, Weesp, The Netherlands) per ml and kept at 37°C to prevent activation of gametocytes. All following procedures were performed at 37°C. WBC were removed by rapid filtration through a
Chapter 6

Plasmodipur filter (Eurodiagnostica, Apeldoorn, The Netherlands) and the filtered blood was centrifuged for 5 min at 1,900 \times g. The pellet was resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Integro B.V., Zaandam, the Netherlands) to give a 50% cell suspension. This was layered on a cushion of 48% Nycoprep\textsuperscript{TM} (Nycomed Pharma AS, Oslo, Norway) in RPMI 1640 with 10% FCS and centrifuged at 1,900g for 30 minutes. The gametocytes were collected from the interphase, washed once with RPMI 1640 and resuspended in FCS. This suspension was kept at room temperature for 30 minutes to promote gamete formation and finally was washed twice with PBS before use.

**CL assay.** One hundred microliters of neutrophil suspension in PBS containing $10^6$ cells was introduced into a measuring vial containing 100 \mu l of heat inactivated test serum or control serum. A further 100 \mu l of fresh-frozen control serum was added as a complement source together with 700 \mu l of $10^{-4}$ mM Luminol(5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma), and the vials were pre-incubated for 30 min at 37°C. Then, $10^6$ gametes in 100 \mu l PBS were added, and the CL response (peak value in milliVolts) was measured for 30 minutes at 28°C, to mimic temperature conditions in the mosquito midgut, using a Bio-Orbit 1251-002 luminometer (LKB-Wallac).

The CL-index (17) is defined as the ratio of the CL-response in the presence and absence of gametes.

**Suspension immuno-fluorescence assay (SIFA).** Freshly isolated living gametes of *P.falciparum* were used to detect the presence of gamete-surface specific antibodies in human sera. A suspension was prepared containing $10^7$ gametes and $10^8$ washed red blood cells in 1 ml PBS. Aliquots of 10\mu l of this suspension were incubated with 20- \mu l samples of human sera (serially diluted in PBS) for 30 min at 4° C in a microtiter plate. Next, the cells were washed three times with PBS by adding 150\mu l of PBS followed by 5 minutes centrifugation at 200 \times g. The cell suspension was incubated in fluorescein isothiocyanate-conjugated sheep anti-human Immunoglobulin (diluted 1:100 in PBS, containing 0.05% Evans blue) for 30 min at 4° C. Finally, the cell suspensions were washed twice with PBS and examined using an incident-light fluorescence microscope at a magnification of 500 \times. The highest dilution of a serum showing fluorescence on the gamete surface was defined as the antibody titre.

**Transmission assay.** Prior to use in the transmission assay, WBC were preincubated for 30 min at 37°C in either control or test serum. The final concentrations in the bloodmeal were
Leucocytes reduce transmission

12,000/µl for WBC and 1,000/µl for gametocytes. For removal of WBC, blood was filtered using a Whatman CF11 cellulose column (11). For the transmission assay, a fourteen day old culture, containing about 0.3 ml packed cells with 0.5 to 1% mature gametocytes, was centrifuged at 500 x g for 2 min. The pellet was carefully mixed with 3.75 ml filtered, group O, packed erythrocytes prewarmed at 37°C. Then, 150 µl of this parasite suspension was added to 120 µl of the preincubated test and control sera containing approximately 12,000 WBC/µl or an equal concentration of erythrocytes. Fifty female Anopheles gambiae mosquitoes of 3 to 5 days old were allowed to feed using "mini" membrane feeders as described before (14). Unfed mosquitoes were removed and 6 days later 20 mosquitoes of each feeder were dissected and oocysts were counted.

Cameroonian sera were analysed in the standardized membrane feeding transmission assay (SMFA) as described before (7), in the presence and absence of WBC. In this test the transmission reducing capacity of a serum is measured compared to those of three controls which each should give at least a 90% mosquito infection. Reductions were calculated using the log transformed arithmetic mean of oocyst numbers and were expressed as R(eduction) values (7). R values are classified in three categories which define sera as either: blocking (R>0.9), reducing (0.3≤R≤0.9) or non-blocking (R<0.3).

WBC-dependent transmission reduction by Tanzanian sera was determined by comparing numbers of mosquitoes infected with and without WBC in the same experiment. In experiments with low levels of infections (in which less than 90% of the control mosquitoes are infected) it is more appropriate to compare the percentage of positive mosquitoes (i.e., those with at least one oocyst) than mean oocyst numbers(8).

**Statistic analysis.**

The Mann-Whitney U test was used for statistical evaluation of the differences in mean oocysts numbers and CL measurements. The χ²-test was used for analysis of percentage infected mosquitoes.

**Results**

When mosquitoes were fed with a blood meal containing infectious gametocytes and human serum supplemented with the anti-Pfs-48/45 Mab 32F1 (0.5mg/ml), the geometric mean in
Chapter 6

oocyst numbers was significantly reduced from 10.0 (n=20) when WBC were absent, to 3.4 (n=20) in the presence of WBC (P<0.01). In a large number of control experiments using human sera without malaria antibodies, WBC never had any significant effect on oocyst development. Additionally, control serum supplemented with Mab 32F1 (0.5mg/ml) showed a significantly increased CL-response of 48.5 mV compared to 13.0 mV (n=3) without the addition of Mab (P<0.01). Giemsa stained cytopsin preparations showed phagocytosis of free gametes by neutrophils in the presence of opsonizing Mab 32F1. Sera from areas of endemicity may contain specific antibodies recognizing surface proteins of freshly emerged gametes (2,10).

Table 1 shows the SIFA titres of the individual sera. All endemic sera showed a titre of at least 1:40 and an enhanced CL response compared to sera from areas where the disease is not endemic, indicating the presence of gamete surface specific antibodies. There is no correlation between the titre and the level of the CL index.

Table 1. Reciprocal antibody titres of individual sera against gamete surface antigens as detected by the SIFA

<table>
<thead>
<tr>
<th>serum</th>
<th>titre</th>
<th>serum</th>
<th>titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>40</td>
<td>C1</td>
<td>80</td>
</tr>
<tr>
<td>T2</td>
<td>320</td>
<td>C2</td>
<td>160</td>
</tr>
<tr>
<td>T3</td>
<td>40</td>
<td>C3</td>
<td>40</td>
</tr>
<tr>
<td>T4</td>
<td>80</td>
<td>C4</td>
<td>160</td>
</tr>
<tr>
<td>T5</td>
<td>40</td>
<td>C5</td>
<td>320</td>
</tr>
<tr>
<td>T6</td>
<td>320</td>
<td>C6</td>
<td>80</td>
</tr>
<tr>
<td>T7</td>
<td>320</td>
<td>C7</td>
<td>40</td>
</tr>
<tr>
<td>T8</td>
<td>160</td>
<td>C8</td>
<td>80</td>
</tr>
<tr>
<td>Co1</td>
<td>&lt;20</td>
<td>C9</td>
<td>320</td>
</tr>
<tr>
<td>Co2</td>
<td>&lt;20</td>
<td>C10</td>
<td>160</td>
</tr>
<tr>
<td>Co3</td>
<td>&lt;20</td>
<td>C11</td>
<td>40</td>
</tr>
</tbody>
</table>

T1-T8, Tanzanian sera; C1-C11, Cameroonian sera; Co1 to Co3, Control sera.
Leucocytes reduce transmission

Figure 1 shows the mean gamete-induced CL-indices. Sera from Cameroon and Tanzania where malaria is endemic significantly increased the CL response of gametes as compared to sera from regions where it is not (P<0.005). In order to test the specificity of the CL-responses for *P.falciparum*, purified *P. berghei* gametes were used as control. Figure 2 shows that only *P.falciparum* gametes were capable to induce a significant CL-response when incubated with Tanzanian sera. Next, Cameroonian sera were tested in the SMFA in the presence and absence of WBC (Fig.3). All sera showed an increased R-value if WBC were present. Serum C1 was classified as non-blocking in both instances (R<0.3). The sera C2, C3, C4, C5 and C6 changed

Figure 1. The CL response of WBC induced by gametes in the presence of control or sera from areas of endemcity.

Bars show the means ± standard deviations of the CL-index. CL-index is the maximum CL-response (in millivolts) with gametes/ maximum CL-response (mV) without gametes. Significance is defined by \( P<0.005 \) (Mann-Whitney U test) when comparing the results for sera from areas where malaria is not endemic (n=7) to those for both the Cameroonian sera (n=13) and Tanzanian sera (n=11).
Chapter 6

from nonblocking to reducing (0.3 ≤ R ≤ 0.9) when WBC were present. For the transmission reducing capacity of the sera C7, C8, C9 and C10 the presence or absence of WBC made no significant difference, although R-values were a little enhanced in the presence of WBC. C11 became a blocking serum in the presence of WBC since R > 0.9. The transmission capacity of sera from areas where malaria is not endemic was never influenced by the presence or absence of WBC. Although all endemic sera showed a significant enhanced CL response compared to non-blocking control sera, there was no correlation between the level of the CL response and the level of transmission reduction of individual sera.

Figure 2. CL-response (in millivolts) of Tanzanian sera induced by *P.falciparum* gametes with *P.berghei* gametes as a control.

( ) *P.falciparum* gametes; ( ) *P.berghei* gametes. A total of $10^6$ purified *P.falciparum* or *P.berghei* gametes were used.
Leucocytes reduce transmission

Table 2 shows the WBC-dependent transmission reduction in Tanzanian sera. The sera T1, T2 and T3 showed a WBC-dependent significant transmission reduction (P<0.05), whereas T4, T5 and T6 did not. T7 and T8 were completely blocking transmission irrespective of the presence of WBC.

Table 2. Effect of WBC on the infection of *A. gambiae* in the presence of Tanzanian sera

<table>
<thead>
<tr>
<th>Serum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>without WBC</th>
<th>with WBC&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>16/20 (80)</td>
<td>3/20 (15)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>T2</td>
<td>11/20 (55)</td>
<td>0/20 (0)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>T3</td>
<td>8/20 (40)</td>
<td>2/20 (10)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>T4</td>
<td>5/20 (25)</td>
<td>4/20 (20)</td>
</tr>
<tr>
<td>T5</td>
<td>3/20 (15)</td>
<td>2/20 (10)</td>
</tr>
<tr>
<td>T6</td>
<td>2/19 (11)</td>
<td>1/20 (5)</td>
</tr>
<tr>
<td>T7</td>
<td>0/20 (0)</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>T8</td>
<td>0/20 (0)</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>Co1</td>
<td>17/20 (85)</td>
<td>13/20 (65)</td>
</tr>
<tr>
<td>Co2</td>
<td>14/20 (70)</td>
<td>11/19 (58)</td>
</tr>
</tbody>
</table>

<sup>a</sup>T1 to T<sub>8</sub>, Tanzanian sera of clinical malaria patients; Co1 and Co2, Control sera

<sup>b</sup>A total of 10<sup>4</sup> viable WBC/μl were added to the bloodmeal prior the membrane feeding.

<sup>c</sup>P<0.05, The χ<sup>2</sup>-test was used.

Discussion

The principle finding of our study is that the presence of WBC in a bloodmeal can have a significant effect on malaria transmission. Inside the mosquito midgut WBC remain active and are capable of surviving for several hours. A number of studies suggest a role for WBC in transmission reduction of *P. falciparum* malaria and in phagocytosis inside the mosquito midgut.
Chapter 6

It has been demonstrated that transmission reduction can be obtained by the presence of specific Anti Pf48/45 and Pf230 Mabs in the mosquito bloodmeal (13). Moreover transmission blocking antibodies are present in human sera from areas were malaria is endemic (4, 16). In the selected sera from two different areas of endemity, the presence of antibodies recognizing surface antigens was demonstrated by a positive SIFA on living gametes.

Figure 3. Effect of WBC present in the mosquito bloodmeal on the capacity of sera from Cameroon (C1-C11) to reduce transmission.

\[
R = \frac{(T_c - T)}{T_c},
\]

where \( T_c \) = mean oocyst number resulting from a control serum and \( T \) = mean oocyst number resulting from a test serum. \( R \) (reduction) value are categorized as follows: \( R < 0.3 \), serum is non-reducing; \( 0.3 \leq R \leq 0.9 \), serum is reducing, \( R > 0.9 \), serum is blocking.

No correlation could be found between antibody titre and transmission reduction. All tested sera from areas of endemity enhanced the CL response, demonstrating an interaction between WBC and free gametes due to the opsonizing antibodies. No CL-response was measured with \( P.berghei \) gametes, indicative for the specificity of this interaction. The lack of correlation between final WBC-dependent transmission reduction, the antibody titre and the magnitude of...
Leucocytes reduce transmission

the CL response could be explained by differences in affinity and concentrations of specific antibodies. CL measurements reflect WBC-gamete interaction measured in vitro over a limited period of 30 min. Since WBC may survive for several hours, a delayed action in vivo caused by a lower concentration or affinity of antibody is not necessarily less effective. The observed effects of WBC on transmission could be even more pronounced in direct-feeding experiments compared to experiments using cultured gametocytes. The latter suspensions contain significant amounts of free pigment, originating from asexual multiplication in culture, which is normally absent in whole blood from patients. Phagocytes have a preferential appetite for this material by which it may act as a decoy for gametes. Once ingested malaria pigment decreases or blocks phagocytosis by monocytes (18), the influence of pigment on neutrophil activity is unknown. Antibodies binding to gamete surface proteins and therefore capable of opsonisation have been demonstrated on numerous occasions in endemic sera (2,4,16). So far, emphasis has been laid upon the role of blocking antibodies exclusively. The traditional laboratory membrane feeding assay, which does not include WBC, only accounts for these antibodies. However as shown in this study, there could be a marked difference in the R values of sera tested with and without WBC. In conclusion, WBC-mediated transmission reduction can depend on the presence of opsonising antibodies. Apart from a specific, antibody mediated response, nonspecific immune factors can contribute to an enhanced WBC activity inside the mosquito midgut (6a) It has been described by Naotunne et al. (9) that cytokine-activated WBC together with an unknown parasitic factor could be responsible for transmission reduction. The effects of cytokines and other serum factors present in malarious patients (5,6), on the role of WBC in transmission reduction needs further study. Membrane-feeding experiments in the field, in which WBC are removed should confirm the laboratory data and elucidate the relevance of these observations. An important implication of this study is that the traditional transmission feeder assay (SMFA) can underestimate the transmission-reducing capacity of a serum if no viable WBC are included in the test.
Acknowledgements

We thank Mrs. A.Bril, Mr. J.Hooghof, Mrs. J.Remmers and Mrs. A.Pouwelsen for technical assistance and Dr. J.Curfs for his advise on chemiluminescence measurements. The generous gift of endemic sera by Dr. L.Mulder and Dr. S.Vreden is greatly acknowledged.

This research was supported by the Dutch Ministry for Development Cooperation (DGIS-SO), contract no. NL002701.

References:


6a. Lensen, A. Unpublished data.


CHAPTER 7

Cytokine activated granulocytes reduce transmission of *Plasmodium falciparum* malaria to mosquitoes.


*University of Nijmegen, Department of Medical Microbiology, section Parasitology*
Abstract
Malaria is transmitted from man to mosquito when a mosquito takes a bloodmeal on a person with the infectious sexual stages of the parasite in the blood. Human leukocytes (WBCs) that are present in the bloodmeal may prevent the further development of the malaria parasite inside the mosquito midgut by phagocytosis and the release of toxic substances that kill the parasite. The activity of WBCs is governed by serum factors such as cytokines, opsonising antibodies, complement and possibly other opsonines. In this study the role of tumour necrosis factor alpha (TNF-α), granulocyt-monocyt-colony-stimulating factor (GM-CSF) and interferon-γ (IFN-γ), was studied by both a luminol dependent chemiluminescence (CL) assay and a standardised mosquito membrane feeding assay (SMFA). In the CL assay, TNF-α enhances phagocytosis of the sexual stages of malaria parasites in a complement dependent way whereas GM-CSF and interferon-γ related transmission reduction also depended on the presence of specific antibodies against surface antigens of the malaria parasites. These findings were consistent with the results of the SMFA.

Introduction.
Malaria is one of the major infectious diseases affecting a large part of the human world population especially in tropical and subtropical areas. Increasing anti-malarial drug resistance and insecticide resistant urges the need for alternative anti-malaria interventions.
A transmission blocking vaccine which prevents the transition of the parasites from man to mosquito is in general regarded as an important tool in the control of malaria. However, application of such a vaccine requires knowledge about the inducable and natural occurring mechanisms of transmission reduction.
Initially, the mosquito midgut is not a particular hostile environment since it will take a few hours before digestive enzymes will be released. However, once inside the mosquito the intra-erythrocytic sexual stages of the malaria parasite escape from their red cell within minutes and will be exposed to human immunological defence mechanisms that may be present in the ingested blood meal. The mechanisms that may interfere with normal development of the malaria parasite inside the mosquito midgut can be either specific, particular specific antibodies that prevent normal development of the parasite (Graves et al. 1988; Roelfsen et al. 1996) and/or non-specific by which WBCs interfere with the sporogonic stages in the
Cytokines reduce transmission

mosquito midgut by phagocytosis and the release of toxic substances (Lensen et al. 1997; Naotunne et al. 1993). WBCs remain active for some hours and complement activity has been demonstrated up to 8 hours (Sinden, 1997). Phagocytosis of gametes of *Plasmodium falciparum* has been demonstrated before *in vivo* and *in vitro* and can contribute to transmission reduction (Lensen et al. 1997; Lensen et al. 1998). Experiments, using gametocyte containing blood samples from natural infected patients in the Gambia, revealed that 84% of activated gametocytes were ingested *in vitro*. Neutrophils ingested 94% and monocytes 6% of the total ingested gametes (Sinden & Smalley, 1976). WBCs, activated by malaria schizont extract, may kill gametes by the release of toxic substances (Naotunne et al. 1995), which can be enhanced by the presence of TNF-α and interferon-γ (IFN-γ). Malaria patients show enhanced plasma concentrations of TNF-α, IFN-γ and GM-CSF (Yamada et al. 1995). In this study we investigated the role of TNF-α, GM-CSF, IFN-γ, complement and antibody in reduction of *P. falciparum* transmission.

**Materials and methods**

**Reagens**

Recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) 0.1 mg/mL, (specific activity >1×10^7 units/mL, Pepro Tech EC LTD., London, England)

Recombinant human TNF-α was kindly provided by Prof. Dr J. van der Meer. (Department of Internal Medicine, University Hospital Nijmegen, The Netherlands). Interferon-γ (Immukine 100μg/0.5mL, Boehringer Ingelheim, Germany). Immune serum was obtained from individuals with high titres of antibodies (>1:320) reacting with surface proteins of macro gametes of *P. falciparum*, as detected by an immunofluorescence assay (SIFA) (Roeffen et al. 1995).

Fresh frozen AB serum was used as complement source. Monoclonal antibodies (mAb) 32F1 and 63F2A2 directed against Pfs 48/45 and Pfs230 on gametes of *P. falciparum*, have been described before. (Vermeulen et al. 1982; Roeffen et al. 1996).

**White Blood Cells**

Granulocytes were isolated according to the protocol described before (Lensen et al. 1997). Ten mL of heparinized blood of Dutch blood bank volunteers was added to 2 mL dextran (6% dextran 200,000 in phosphate buffered saline pH 7.2 (PBS)). Cells were allowed to settle for 30 min. An equal volume of PBS was added to the supernatant containing the leukocyte
enriched fraction and centrifuged at 500g for ten min. Two mL of distilled water was added to the pellet to lyse remaining red cells. After 10 seconds, 15 mL of PBS was added and the centrifugation step was repeated. Neutrophils were separated from mononuclear cells by centrifugation through a layer of Ficoll-Paque (density 1.075, Pharmacia) at 700g for 30 min at 4°C. After harvesting the pellet and three washing steps with cold PBS, the neutrophils were kept on ice until used. Viability was in general >99% as detected by trypan bleu exclusion. Monocyte preparations were obtained by counterflow centrifugation as described before (Plas et al. 1988).

*P.falciparum gametes*

*P. falciparum* gametocytes of strain NF54 (Amsterdam, airport strain) were cultured using the semi-automated tipper system (Ponnudurai et al. 1989). After 14 days of culture 10 mL of the parasitized red blood cell suspension was collected. The suspension was centrifuged for 5 minutes at 500g at 37 °C. The medium was replaced by an equal volume of foetal calf serum (FCS) and gametocytes were allowed to activate for 1 hour at room temperature. The cell suspension was centrifuged at 500 g for 7 minutes and the pellet was resuspended in 3 mL PBS and subsequently layered on 12.5 % Nycodenz® (Nycomed Pharma AS, Oslo, Norway) in PBS. After centrifugation at 2100 g for 40 minutes at 4°C, gametes were collected from the top of the Nycodenz layer. Gametes were resuspended in 15 mL PBS and centrifuged for 5 minutes at 500g. Finally, the pellet was resuspended in PBS to give a final concentration of $10^7$ gametes/mL.

*Chemiluminescence assay*

One hundred μL of neutrophil suspension in PBS containing $10^6$ cells was introduced into a measuring vial containing 100 μL of heat inactivated test serum or control serum. Further 100 μL of fresh frozen control serum was added as complement source together with 700 μL of $10^{-4}$ mM Luminol(5-amino-2,3-dihydro-1,4-phtalazinedione; Sigma) and the vials were pre-incubated for 30 min at 37°C. $10^6$ gametes in 100 μL PBS were added and chemiluminescence (CL) response (peak value in mV) was measured for 30 minutes at 28°C, to mimic temperature conditions in the mosquito midgut, using a Bio-Orbit 1251-002 luminometer (LKB-Wallac). CL index (Salmon et al. 1986) is defined as the ratio of the CL response in the presence and absence of gametes.

*Bio-assay for transmission of P.falciparum: standard membrane feeding assay (SMFA)*
Prior to use in the transmission assay, WBCs were preincubated for 30 minutes at 37°C in either control serum or control serum supplemented with cytokine. The final WBC concentration was 12,000/μL and for gametocytes, 1,000/μL. For the transmission assay, a fourteen day old culture, containing about 0.3 ml packed cells with 0.5-1% mature gametocytes, was centrifuged at 500 g for 2 minutes. The pellet was carefully mixed with 3.75 mL filtered, group O, packed red blood cells prewarmed at 37°C. 150 μL of this parasite suspension was added to 120 μL of the preincubated test/control serum containing approximately 12,000 WBC/μL or an equal amount of red blood cells in controls. Fifty female *Anopheles stephensi* mosquitoes of 3 to 5 days old were allowed to feed using "mini" membrane feeders as described before (Ponnudurai *et al.* 1989). Unfed mosquitoes were removed and six days later 20 mosquitoes of each feeder were dissected and oocysts counted.

**Statistic analysis**

The Mann-Whitney U test was used for statistical evaluation of the differences in mean oocysts numbers and CL measurements.

**Results**

Granulocytes pre-treated with increasing concentrations of TNF-α in (not heat inactivated) normal human serum (NHS) induced concentration depended CL responses. However, heat inactivation of the serum completely abolished the TNF-α effect on granulocytes (fig 1). Moreover, granulocytes which were prestimulated with TNF-α (200 pg/mL) and, added to the mosquito bloodmeal, caused a significant reduction of transmission to mosquitoes in the SMFA, compared to controls with non-stimulated granulocytes (fig 2).

The effect of GM-CSF on granulocyte activation was tested in the CL assay in the presence and absence of antibody. In contrast to the observed effect of TNF-α, GM-CSF pre-treatment of granulocytes did not result in an increase of the CL-response in NHS. However, in the presence of an IS, containing opsonising antibodies, GM-CSF pre-treated granulocytes showed a concentration depended increase of the CL response (fig 3) with an optimal Cl response at a concentration of 250 pg/mL.

Next, the transmission reduction activity of granulocytes, pre-treated with 250 pg/mL GM-CSF was studied in the SMFA, in the presence of 50 μg/mL (opsonising) mAb 32F1 (recognizing Pfs 45).
Figure 1. Gamete induced CL indices of TNF-α stimulated granulocytes in the presence and absence of complement.

The CL response of granulocytes induced by gametes, after 30 min pre-treatment with TNF-α in the presence of fresh serum, containing complement ( ), and heat inactivated serum ( ). CL index is the maximum CL response (in millivolts) with gametes/the maximum CL response without gametes.

Significant transmission reduction was observed only with GM-CSF prestimulated WBCs in the presence of opsonising antibody, which is in agreement with the results of the CL assay (table 1). GM-CSF pre-treatment of WBCs or the presence of mAb 32F1 alone, did not affect the transmission. Next, IFN-γ was tested in the CL assay. IFN-γ (100 units/mL) pre-treated granulocytes showed an increased CL response compared to non-stimulated granulocytes but only in the presence of complement. Adding antibody increased the CL response, however, only in combination with complement there was a significant enhancement related to IFN-γ pre-treatment (fig 4).
Figure 2. Transmission reduction by TNF-α stimulated WBCs present in a mosquito bloodmeal, measured in the standard membrane feeding assay (SMFA).

Bars represent the mean (± SE) numbers of oocysts in mosquitoes (n=20) dissected six days after an infectious bloodmeal containing TNF-α stimulated WBCs. Stimulation of WBCs with 200 pg/mL TNF-α resulted in a significant reduction of mean oocyst numbers (P<0.01); GmW, geometric mean of Williams. TNF-α had no effect on transmission results in the absence of WBCs (data not shown).

In the SMFA, IFN-γ stimulated WBCs caused a significant transmission reduction but only in the presence of an opsonising antibody (50μg/mL 32F1). IFN-γ pre-treatment of WBCs or opsonising antibody 32F1 alone did not result in significant transmission reduction. Since both monocytes and neutrophils can phagocytose, an experiment was performed to compare the contribution of each cell type to phagocytosis. Although monocytes readily phagocytosed free gametes, CL indices of monocytes were much lower compared to CL indices of neutrophils (data not shown). Cytospin preparation made after the CL assay revealed that phagocytosis of gametes by monocytes was independent of cytokine stimulation and, occurred also in control experiments without pre-treatment of monocytes. However, phagocytosis by neutrophils was observed only in the presence of specific antibodies or after pre-treatment with cytokines (fig. 5) Since neutrophilic cells greatly outnumber monocytes in human blood, and monocyte CL responses were low, experiments were carried out with purified granulocytes.
Figure 3. Gamete induced CL indices of GM-CSF stimulated granulocytes in the presence and absence of immune serum.

The CL response of granulocytes pretreated with GM-CSF (30 min), in the presence of normal human serum (NHS) and in the presence of a serum from a malaria endemic area containing antibodies that recognise surface proteins of *P. falciparum* gametes (as detected by an immuno fluorescence assay). CL index is the maximum CL-response (in millivolts) with gametes/ the maximum CL response without gametes.
Cytokines reduce transmission

Figure 4. The CL response of interferon-γ stimulated granulocytes in the presence and absence of complement and/or antibody

Bars represent the CL response (in millivolts ± standard deviation) of granulocytes induced by gametes, after 30 min pre-treatment with IFN-γ (100 units/mL), in the presence and absence of complement and/or mAb 63F2A2 (50μg/mL) (anti-Pfs 230, triplicate experiment)

Figure 5. Giemsa stained cytospin preparations of monocytes and granulocytes showing phagocytosis of *P.falciparum* gametes.

Picture A shows a monocyte which has ingested gametes and made contact with others. Picture B shows granulocytes which have ingested gametes. Phagocytosis of monocytes occurred without pre-treatment with cytokines or the presence of antibodies. Phagocytosis by granulocytes only occurs after cytokine pre-stimulation and/or the presence of antibody and complement.
Table 1. Effect of GM-CSF and IFN-γ stimulated WBC present in a mosquito blood meal on transmission of *P. falciparum* malaria measured in the SMFA.

<table>
<thead>
<tr>
<th></th>
<th>mean oocysts (SE)</th>
<th>% infection</th>
<th>significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.9 (3.3)</td>
<td>90</td>
<td>NS</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>10.8 (3.0)</td>
<td>90</td>
<td>NS</td>
</tr>
<tr>
<td>Mab 32F1</td>
<td>11.3 (2.8)</td>
<td>95</td>
<td>NS</td>
</tr>
<tr>
<td>GM-CSF + mAb 32F1</td>
<td>5.4 (1.9)</td>
<td>75</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.1 (0.9)</td>
<td>80</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.7 (0.6)</td>
<td>80</td>
<td>NS</td>
</tr>
<tr>
<td>Mab 32F1</td>
<td>2.3 (0.9)</td>
<td>75</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-γ + mAb 32F1</td>
<td>1.1 (0.3)</td>
<td>50</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

NS = not significant; P-value by Mann-Whitney U test; SE, Standard Error; mean oocysts, geometric means of William’s oocysts from 40 mosquitoes; mAb 32F1, 50μg/mL; GM-CSF, 250U/mL; IFN-γ, 500 U/mL.

**Discussion**

It has been shown both *in vitro* as well as *in vivo* that WBCs in a bloodmeal can have a significant effect on transmission of malaria from man to mosquito (Naotunne *et al.* 1993; Lensen *et al.* 1997; Lensen *et al.* 1998). Using the CL assay, it has been demonstrated that *in vitro* phagocytosis of gametes by neutrophils is enhanced in the presence of sera from individuals living in malaria endemic areas (Lensen *et al.* 1997). This may be explained by the presence of opsonising antibodies and/or other serum factors that stimulate phagocytosis, however, there was no direct correlation between antibody titres and the level of transmission reduction as observed in the SMFA. In addition immunoglobulin classes of antibody are a crucial factor, which was illustrated by comparing CL responses by switch variants of the same
mAb 63F2A2, whereby the IgG2a variant, which fixes complement, showed a CL response, which was 3 times higher than the IgG1 (data not shown). In this study we investigated the role of non specific immune factors. WBCs can be activated by cytokines. TNF-α, GM-CSF and IFN-γ, which mediate different effects on WBCs, have shown to be at elevated levels in malaria patients, (Yamada et al.1995). TNF-α enhances the expression of CR3 on neutrophils and the oxidative burst (Curfs et al.1997) which explains the complement depended TNF-α mediated effects in the CL assay. Furthermore, the results implicate that free gametes of *P.falciparum* are opsonised by C3. C3 deposition on free gametes of *P.falciparum* could be observed using immuno-fluorescent techniques (data not shown) which is in agreement with the observation of Tsubol et al. (1995) who demonstrated the deposition of complement factor C3 on zygotes of *P.yoelii*. However, this was only possible by keeping gametes at 4°C thereby lowering their metabolism. Shedding of deposed complement factors could be part of an immune evasion mechanism of the parasite at normal temperatures.

Karanuweera et al. (1992 a,b,c) studied the temporary loss of infectivity of gametocytes during paroxysms in *P.vivax*, which was associated with a rapid increase and decline of TNF-α. Naotunne et al.(1993) demonstrated the possible effects of enhanced TNF-α levels on malaria transmission in *P.falciparum*. They showed that the transmission reducing effect of TNF-α was mediated by the release of nitrogen intermediates from WBCs. Enhanced plasma levels of TNF-α correlated with parasitaemia and severity of the diseases and were observed in patients with symptomatic malaria (Yamada et al. 1995). GM-CSF levels, however, were enhanced in malaria patients compared to controls but did not correlate with parasitaemia or severity of disease. Furthermore, GM-CSF levels were sustained for a much longer period compared to TNF-α levels (Yamada et al. 1995). Enhanced CL responses caused by pre-treatment of granulocytes with GM-CSF *in vitro* relied on both the presence of opsonizing antibodies and complement. This observation is in agreement with the described effect of GM-CSF on phagocytosis of asexual parasites of *P.falciparum* and is caused by upregulating the expression of FcyRII and FcyRIII receptors as well as CR3 on neutrophilic granulocytes (Kumuratilake et al.1996). Although complement is needed for a significant response the major effect of GM-CSF is antibody mediated. The phagocytic activity of WBCs mediated by IS could be further enhanced by GM-CSF with an optimal concentration of 250pg GM-CSF/ml.
Chapter 7

This concentration can be easily demonstrated in malaria patients (Yamada et al. 1995) which implicates that enhanced GM-CSF concentrations in sera from gametocyte carriers may contribute to transmission reduction. Significant GM-CSF related reduction of transmission was obtained in the presence of opsonising antibody (table 1). Also IFN-γ has profound effects on WBC activity. Treatment of normal human subjects resulted in a 4.7 fold increase in neutrophil expression of the high affinity FcγR1-receptor. Furthermore phagocytosis of Staphylococcus aureus significantly improved. (Schiff et al. 1997) The influence of IFN-γ, produced by the host as a result from malaria infection, on transmission has been demonstrated before in Plasmodium cynomolgi (Naotunne et al. 1991) but was related to the death of intra-erythrocytic gametocytes mediated by crisis serum and dependent upon the presence of additional unidentified factors. In our experiments IFN-γ had a direct effect on WBC activity as it occurred in the mosquito midgut and did not rely on additional factors other than complement and specific antibody.

Conclusively, cytokines can contribute to transmission reduction by different mechanisms, affecting either intra-erythrocytic gametocytes as was shown before, or by activating granulocytes, which affect the development of gametes/zygotes in the mosquito midgut, as shown in this study. Whereas TNF-α mediated transmission reduction was depended on the presence of complement only, GM-CSF and IFN-γ related transmission reduction could only be demonstrated in the presence of opsonising antibody.

Acknowledgements

We thank Mrs J. Bril, Mrs K. Teelen, Mr J. Hooghof, Mrs J. Remmers and Mrs Pouwelsen for technical assistance and J.Curfs for advise on CL measurements. This research was supported by the Dutch Ministry for Development Co-operation (DGIS-SO), contract no. N1002701.

References:


Cytokines reduce transmission


Chapter 7


CHAPTER 8

Mechanisms that reduce transmission of *Plasmodium falciparum* malaria in semiimmune and nonimmune individuals

Lensen A*, Mulder L*0, Tchuinkam, T-0, Willemsen, L*, Eling W*, Sauerwein R*

*University of Nijmegen Department of Medical Microbiology, section Parasitology; 0Malaria Department, Organisation de Coordination pour la lutte contre les Endemies en Afrique Centrale/ Organisation de Recherche Scientifique et Technique Outre-Mer, Yaoundé, Cameroon

The Journal of Infectious Diseases 1998; 177:1358-63
Abstract

Transmission of *Plasmodium falciparum* can be reduced by immune factors present in the mosquito bloodmeal. Specific antibodies and white blood cells (WBCs) can interact with the sexual stages of the parasite inside the mosquito midgut. The relative contribution of serum factors and WBCs, on transmission reduction in gametocyte carriers from an endemic area in Cameroon and in travelers with a first malaria experience was studied. Blood from these gametocyte carriers was fed to mosquitoes through membrane feeders after serum replacement, WBC depletion, or both. In most imported malaria cases, serum factors, WBCs, or both showed a significant effect on transmission reduction, while infectiousness of gametocyte carriers from Cameroon was reduced by humoral plasma factors only. In addition, the infectivity of gametocytes from semiimmune carriers was significantly lower compared with that of nonimmune carriers and infectivity was independent of gametocyte density and the presence of WBCs or plasma factors (or both) in the bloodmeal.

Introduction

One of the possible strategies in diminishing the numbers of infectious mosquitoes and thus the transmission intensity in endemic areas may be the use of a transmission blocking vaccine [1]. However, implementation of such a vaccine requires knowledge about the major transmission reservoir within a population and the existence of natural transmission blocking mechanisms. Most studies concerning natural transmission blockade have concentrated on the role of specific antibodies interfering with the development of the sporogonic stages inside the mosquito midgut [2,3]. The lower temperature and higher pH in the mosquito midgut stimulate the intraerythrocytic gametocytes to remove their protective red cell membrane and form gametes. As a result these stages become susceptible to antibodies. Antibodies binding to free gametes can directly prevent fertilization but may also stimulate white blood cell (WBC)-mediated phagocytosis and killing of the parasite in the mosquito bloodmeal [4]. Furthermore, WBC-activating factors such as cytokines, may reduce transmission [5]. WBC-dependent reduction of *Plasmodium falciparum* transmission mediated by factors present in sera of
Mosquito infectivity of gametocyte carriers

Gametocyte carriers from an endemic area was recently shown in a standardized membrane feeding assay (SMFA) using cultured infectious gametocytes and laboratory-reared mosquitoes [4].

In this study, we investigated the relative contribution of serum factors and WBCs to the reduction of transmission of *P. falciparum* by gametocyte carriers from an endemic area in Cameroon and imported malaria cases using the direct membrane-feeding assay (DMFA). This assay, in which autologous gametocytes of carriers are fed to mosquitoes allows the manipulation of cells and plasma independently, creating the possibility of determining the mechanisms of transmission reduction.

**Materials and methods**

**Nonimmune gametocyte carriers**

Travelers with imported *P. falciparum* malaria were recruited at either the Academic Medical Center, Amsterdam or the University Hospital Nijmegen, Nijmegen, The Netherlands. Blood samples were obtained by venipuncture. The heparinized blood for feeding experiments was carefully kept at 37 °C in a water filled isothermal container until use. Gametocyte-containing blood of patient Ha was fed to mosquitoes on three occasions (with intervals of 2 and 6 days) and that of patients Ro and Hi was fed twice (with intervals of 4 and 7 days respectively). Patients Ha, Ro and Hi were symptomatic and febrile (temperature >37.5°C) when they participated in the initial experiment but they were without complaints in subsequent experiments. Other patients participated a few days after treatment and were afebrile.

**Semiimmune gametocyte carriers**

Twenty-four *P. falciparum* gametocyte carriers were recruited at the Messa dispensary in Yaoundé, Cameroon. Yaoundé is situated in an area endemic for malaria, with transmission rates of between 2 and 45 infective bites per person per year [6, 7]. Body temperatures varied between 36.2 °C and 37.8 °C with a mean of 36.8°C. Two of the 24 patients were febrile (temperature of 37.8°C). Asexual parasites were present in the circulation of 6 subjects; however, none of these patients had an increased body temperature indicating malaria (disease)
Chapter 8

immunity. Gametocytes were counted per 200 WBCs in Giemsa stained thick smears. The number of WBCs per microliter of blood was counted by the use of a CBC Coulter Counter (Coulter Electronics, Luton, UK)

Mosquitoes

A laboratory-adapted, *Anopheles gambiae* mosquito strain described by Ponnudurai et al. [8] was used for feeding experiments that were carried out in the laboratory of the department of Medical Microbiology in Nijmegen. The feeding experiments in the Organisation de Coordination pour la Lutte contre les endemies en Afrique Centrale (OCEAC) laboratory in Yaoundé used a local strain of *A. gambiae* (described by Tchuinkam et al.[9]). To exclude differences, we compared the susceptibility for *P. falciparum* infection by feeding mosquitoes of both strains simultaneously with aliquots of the same batch of cultured gametocytes of *P.falciparum* strain NF-54.

Mosquito feeding experiments

*DMFA*. Blood (5 mL) was collected from a gametocyte carrier into a lithium heparinized tube by venipuncture. The tube was kept at 37°C until use. This temperature was maintained during all manipulations in order to avoid accidental activation of the gametocytes. The heparinized blood was centrifuged (4 min, 770g, 37°C) and the plasma was removed and stored at 37°C. A Plasmodipur filter (Euro-diagnostics B.V., Arnhem, The Netherlands) that removes WBCs with > 99% efficiency without affecting gametocytes [10] was wetted with warmed (37°C) RPMI 1640, supplemented with 20mM HEPES and 0.2% bicarbonate. The blood pellet was diluted in 10 mL of RPMI, and half of the cell suspension was quickly passed through the filter, using a syringe, and collected in a 50-mL tube. The filter was rinsed with 20 mL of RPMI, and medium was added to the filtered blood to give a final volume of 50 mL. The remaining unfiltered blood was suspended in 50 mL of RPMI and both tubes were centrifuged (7 min at 770g, 37°C). From the pellets, 200-µL samples were mixed with 200 µL autologous plasma or 200 µL of a control serum (pooled AB serum from Dutch
Mosquito infectivity of gametocyte carriers

blood donors without malaria experience). The suspensions were fed immediately to 50 female A. gambiae mosquitoes using “mini” membrane feeders as described [8]. After the feed, unfed mosquitoes were removed. The mosquitoes were kept at 26° C at maximum humidity, and six days later, at least 20 mosquitoes were dissected, and their oocysts counted.

SMFA. Infective gametocytes of P. falciparum strain NF 54 were cultured using a “Tipper” culture system [11]. This automated system guarantees a constant temperature and gas condition (even during twice daily medium exchanges) during the 14 days of culture. Constant temperature and gas conditions are required to obtain infectious P. falciparum gametocytes. Membrane feeding experiments in the presence of viable WBCs were performed and R(reduction)-values calculated as previously described [4,12].

Tumor Necrosis Factor-α (TNF-α) Measurements

TNF-α was measured using a radio-immuno-assay (RIA) as described [13].

Results

Infection of mosquitoes by non-immune gametocyte carriers. Whereas the DMFA uses autologous gametocytes from the gametocyte carrier, the SMFA uses culture-obtained gametocytes of P. falciparum. Table 1. shows the effect, as determined by the DMFA, of WBC depletion and plasma replacement on the number of oocysts in mosquitoes that received a blood meal from Dutch patients with imported malaria. Mean oocyst numbers in experiments using autologous plasma without WBC depletion varied between 0 and 238.7. WBC depletion resulted in an increase of mean oocyst numbers in 4 of 8 experiments. Replacement of autologous plasma by a control serum showed an increase in mean oocyst numbers in 8 of 11 experiments. The experiments with Ro1, Ro2 and Uu demonstrated a synergistic effect of WBC and serum factors on oocyst reduction since an increase in oocyst numbers was obtained by the combination of WBC depletion and serum replacement. Blood from one patient was fed on two occasions (Ro1 and Ro2), with an interval of 4 days and gametocyte densities of
Chapter 8

240/µL and 260/µL, respectively. Patient Uu had a gametocyte density of 50/µL at the time of feeding. Maximum percentages of infected mosquitoes (with the exception of Hi1) varied between 87% and 100% (data not shown) with highest mean oocyst numbers between 3.2 and 238.7. The transmission efficacy of patient Hi1 was clearly lower than that of the others, with a maximum of 35% of the mosquitoes infected and a mean of 0.3 oocysts.

Table 1. The effect of white blood cell (WBC) depletion and plasma replacement on the number of oocysts in mosquitoes that received a meal of gametocyte infected blood from Dutch patients with imported malaria, as determined by direct membrane-feeding assay.

<table>
<thead>
<tr>
<th>Mean nos. of oocysts*</th>
<th>Autologous plasma</th>
<th>Control serum†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+WBC</td>
<td>-WBC</td>
</tr>
<tr>
<td>Patient serum</td>
<td>No. of gametocytes/µL of blood</td>
<td>+WBC</td>
</tr>
<tr>
<td>Ha1</td>
<td>12,000</td>
<td>238.7</td>
</tr>
<tr>
<td>Ha2</td>
<td>8760</td>
<td>14.2</td>
</tr>
<tr>
<td>Ha3</td>
<td>4080</td>
<td>54.5</td>
</tr>
<tr>
<td>Ro1</td>
<td>240</td>
<td>11.3</td>
</tr>
<tr>
<td>Ro2</td>
<td>260</td>
<td>7.1</td>
</tr>
<tr>
<td>Hi1</td>
<td>300</td>
<td>0.3</td>
</tr>
<tr>
<td>Hi2</td>
<td>280</td>
<td>26.2</td>
</tr>
<tr>
<td>Py</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Uu</td>
<td>50</td>
<td>1.0</td>
</tr>
<tr>
<td>Me</td>
<td>180</td>
<td>3.4</td>
</tr>
<tr>
<td>Bo</td>
<td>70</td>
<td>0.1</td>
</tr>
</tbody>
</table>

NOTE. +WBC= WBC present; -WBC= WBC absent; ND= not done; * Geometric means of Williams of no. of oocysts per dissected mosquito (n= 20); † From Dutch blood bank donors.
Subsequently, graded numbers of WBCs were added to an infectious blood meal, in the SMFA containing Ro1 or control serum (figure 1). In the absence of WBCs there was no significant difference in the mean number of oocysts between serum Ro1 or control serum. However, at a concentration of 8000 WBCs/μL a significant reduction in oocyst numbers could be observed ($P < .03$, Mann-Whitney $U$ test). Figure 2 shows the effect of serially diluted Ro3 serum in the SMFA in the presence and absence of WBCs. Serum Ro3 was taken 21 days after the first feeding experiment. In a 1:9 dilution, serum Ro3 alone almost completely blocked transmission. However, at a serum dilution of 1:27 transmission blockade depended on the presence of WBC ($P < .003$, Mann-Whitney $U$ test). In control experiments using sera of Dutch blood bank donors without malaria experience no effect of the presence of WBC could be found.

**Figure 1.** Effect of presence or absence of white blood cells (+WBC and -WBC), respectively) in mosquito blood meal on oocyst development. In a standardized membrane-feeding assay, mosquitoes were fed with cultured gametocytes of *P. falciparum* strain NF-54 in presence of patient serum Ro1 and graded numbers of WBC * P < .03 (Mann-Whitney test). Control serum = pooled AB serum of healthy Dutch donors. GmW = geometric means of Williams.
**Figure 2.** Effect of white blood cells (WBC) in mosquito bloodmeal on oocyst development. In standardized membrane feeding assay, mosquitoes were fed with cultured gametocytes of *P. falciparum* strain NF-54 and serial dilutions of patient serum Ro3 in presence (+WBC) and absence (-WBC) of WBC. *P<0.003* (Mann-Whitney test). Control = pooled AB serum of healthy Dutch donors. GmW = geometric means of Williams.

*Infection of mosquitoes by gametocyte carriers from an endemic area*

Next, we studied the effects of serum factors and WBCs in gametocyte carriers from an endemic area. The blood of 24 *P. falciparum* gametocyte carriers from Cameroon was tested by DMFA. A second, and in some cases, a third feeding experiment was done with 4 gametocyte carriers resulting in a total of 29 feeding experiments. The median gametocyte density was 330 gametocytes/μL (25th-75th percentile, range 164-510), and the mean number of WBCs was 6641/μL (range 2100-12,400). In 12 out of 29 experiments there was no infection in any experimental group.

Table 2 shows the mean percentage of infection and the mean number of oocysts in the remaining successful transmissions. As determined by DMFA, only 6% of the mosquitoes became infected from blood with WBCs; the mosquitoes had a mean of 1.2 oocysts per midgut. WBC depletion did not increase the mosquito infection rate or the mean number of oocysts. However, when autologous plasma was replaced by a control serum there was an
increase in mosquito infection to 19.5 % with a mean of 3.3 oocysts per midgut. A synergistic effect of WBC depletion and plasma replacement was not observed.

Table 2. The mean percentage of infection and the mean number of oocysts on mosquitoes that received a gametocyte-infected blood meal from 17 patients living in P. falciparum-endemic areas of Cameroon as determined by direct membrane feeding.

<table>
<thead>
<tr>
<th></th>
<th>Autologous plasma</th>
<th>Control serum*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+WBC</td>
<td>-WBC</td>
</tr>
<tr>
<td>% mosquito infection</td>
<td>6.0 (2.7)</td>
<td>5.4 (2.0)</td>
</tr>
<tr>
<td>No. of oocyst</td>
<td>1.2 (0.5)</td>
<td>0.6 (0.3)</td>
</tr>
</tbody>
</table>

NOTE. Data in parentheses = SE. WBC = white blood cells present (+) or absent (-).
* From Dutch blood bank donors.

Because low infectivity of gametocytes from Cameroon may have obscured possible WBC effects, sera of twenty one Cameroonian gametocyte carriers were tested in the SMFA using highly infectious cultured gametocytes in the presence and absence of WBCs. Figure 3 shows a significant increase of R values in most sera when WBCs were present (Wilcoxon, P < .001).

Two of 14 sera were reducing while 12 were nonblocking in the absence of WBCs. However, 6 of these 12 sera could be reclassified as reducing in the presence of WBCs. The remaining 7 sera had a R value of ≥ 0.9 and completely blocked transmission independently of WBCs. There was no WBC effect on transmission in combination with control sera of Dutch blood bank donors without malaria experience.

Comparison of mosquito infection results for Cameroonian and imported malaria cases showed remarkable differences (see tables 1 and 2). Percentages of mosquito infection and mean oocyst numbers were much higher in imported malaria cases, despite similar gametocyte numbers in the blood. To exclude a low susceptibility of the mosquito strain used in Cameroon, comparative experiments were performed. Simultaneous feedings of both mosquito strains on the same batch of cultured gametocytes (table 3) showed an equal susceptibility for P. falciparum.
Chapter 8

Since an increased TNF-α concentration may enhance WBC-mediated transmission reduction [5], TNF-α concentrations in the plasma of gametocyte carriers were measured. From the nonimmune donors, only plasma samples of patients Hi1 and Py showed a significant enhanced TNF-α concentration (855 pg/mL and 230 pg/mL, respectively) by RIA. All Cameroonian donors had TNF-α concentrations of < 100 pg/mL which is compatible with concentrations in healthy Dutch donors [13].

Figure 3. Transmission reduction values (R-values) of individual serum samples from subjects in Cameroon in absence and in presence of white blood cells, as measured in a standardised membrane-feeding assay. B= transmission blocking R> 0.9; R = transmission reducing R>0.3 and ≤ 0.9; N = no transmission blocking, R-value ≤ 0.3.

Discussion

This study shows that WBCs in a mosquito blood meal can significantly reduce transmission of *P.falciparum* by nonimmune donors as determined by two bioassays for transmission. WBCs in the bloodmeal induced partial reduction in transmission efficiency, which never resulted in a complete blockade. In contrast, there was no effect of WBCs in the DMFA using gametocytes from semiimmune individuals from Cameroon. However, a WBC-dependent transmission reduction was clearly found in the SMFA with cultured gametocytes upon
addition of sera from Cameroonian. This suggests that WBC-activating factors are indeed present in the endemic sera. In contrast with the DMFA results in nonimmune donors, the overall infection rates in the Cameroonian gametocyte carriers were always very low which has been found before in endemic patients [6,9]. The absence of a WBC effect in the DMFA with Cameroonian gametocyte carriers may be real but could also be due to the low number of oocysts not allowing the detection of the partial effects of WBCs on transmission. WBC-dependent effects can be mediated by nonspecific factors that enhance WBC-activity such as cytokines and/or specific Abs that opsonize free gametes. All sera from travelers as well as Cameroonian donors showed a positive reaction in a surface IFA using purified live gametes of *P. falciparum* indicating the presence of specific antibodies (data not shown). However, there was no correlation between WBC-dependent transmission reduction in the SMFA or DMFA and the titer of specific antibodies as measured by surface IFA. It has been demonstrated, using cultured gametocytes of *P. falciparum*, that TNF-α together with WBCs can promote transmission reduction, [5].

Table 3 Comparison of the susceptibility for *P. falciparum* infection of *Anopheles gambiae* mosquitoes of the Nijmegen and the Yaoundé strains after feeding them the same cohorts of cultured infectious gametocytes in two separate experiments.

<table>
<thead>
<tr>
<th></th>
<th>Mosquitoes from Yaoundé</th>
<th>Mosquitoes from Nijmegen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
</tr>
<tr>
<td>% infected mosquitoes</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>Mean no. of oocysts*</td>
<td>45.7 (18.7)</td>
<td>34.7 (10.6)</td>
</tr>
</tbody>
</table>

NOTE. data in parentheses = SE. Exp = experiment.

*Geometric means of Williams of 20 dissected mosquitoes
Chapter 8

Among the nonimmune donors only patients Hi1 and Py had significantly enhanced plasma TNF-α concentrations (855 pg/mL and 230 pg/mL, respectively). Hence, the low transmission efficacy of patient Hi1 could be explained by a low infectious period at the early onset of gametocytaemia as described before [14], and by the increased plasma TNF-α concentration (855 pg/mL). After 1 week the TNF-α concentration was within the normal range (90 pg/mL) and a high rate of mosquito infection was obtained, with a similar gametocyte density (patient sample Hi2, table 1). TNF-α concentrations in the Cameroonian plasma samples were always <100 pg/mL which is compatible with concentrations in healthy Dutch donors [13]. Therefore, a more complex interaction between WBCs, specific Abs, and nonspecific factors may be responsible for the observed WBC effects. In conclusion, the results of this study emphasize that reduced WBC-dependent transmission reduction has to be taken into account when interpreting results of both the SMFA and DMFA in epidemiological studies.

The next important finding was the lower infectiousness of gametocyte carriers from Cameroon compared with that of nonimmune donors with a similar gametocyte density and corrected for WBC and serum (antibody) effects. This could be explained by a number of reasons, the first one being the different colonies of mosquitoes which were used at the two locations. However, comparative experiments with the SMFA showed that both mosquito colonies were equally susceptible to \textit{P.falciparum} infection (table 3). Second, the assays were done in different laboratories. Since the methodology, materials, and personnel involved were the same, we do not favor this explanation. In our opinion, the most likely explanation is the difference in previous exposure to \textit{P.falciparum} in travelers and Cameroonian donors. Evidence for very efficient mosquito infection from “non-immune” gametocyte carriers is available from older literature [14]. This increased infectivity has been explained by higher gametocyte densities[15]. However, gametocyte densities of the travelers were comparable to those in the Cameroonian subjects. Because infections in Cameroon were low even after removal of both possible interfering humoral and cellular factors, it is feasible that an immune-based mechanism affecting gametocyte infectivity is involved. This immunity should be directed against the gametocyte inside the human host and distinct from transmission-blocking
Mosquito infectivity of gametocyte carriers

Immunity in sensu stricto, which acts in the mosquito midgut, affecting the extra-erythrocytic gamete and zygote stages of the parasite. The explanation of the mechanism can only be speculative. Gametocytes are intraerythrocytic meaning that they are not directly accessible for specific antibodies. However, there is some controversial evidence that transmembranal ducts would allow the passage of macromolecules, like specific antibodies, into the parasitophorous vacuole [16]. Alternatively, antibody-dependent cellular inhibition (affecting gametocyte infectiousness) could play a role, as has been described for asexual blood stages of P. falciparum [17]. Finally, antigens expressed on the infected erythrocyte membrane could be targets for specific antibodies. These antibodies might for example disturb the homing towards the specific sequestration sides for young gametocytes inside the human body thereby affecting normal maturation.

It is generally accepted that the major gametocyte reservoir consists of young children aged 0-5 years [18]. It would be interesting to see whether the infectivity of gametocytes, tested in the DMFA after WBC depletion and plasma replacement, is higher in endemic areas before the onset of immunity in young children or in gametocyte carriers from an area with low or seasonal endemicity. Such experiments could further elucidate the possible involvement of immune factors on the infectivity of P. falciparum gametocytes.

Acknowledgments:
The co-operation of gametocyte carriers in Cameroon and the Netherlands is greatly acknowledged. This research would not have been possible without the excellent technical assistance of the personnel from both the entomological units of the O.C.E.A.C in Yaoundé, Cameroon and from the University Hospital of Nijmegen. We are grateful for the collaboration with the parasitology department of the Academic Medical Center, Amsterdam, for recruitment of gametocyte carriers. We thank Mrs T. Verver-Jansen and Mrs. L. Jacobs for TNF-α measurements.
Chapter 8

References:


Plasmodium falciparum gametocytes from semi-immunes and non-immunes differ in innate infectivity to mosquitoes.

Ton Lensen¹, Bert Mulder¹,² and Robert Sauerwein¹

¹University of Nijmegen Department of Medical Microbiology, section Parasitology; ²Malaria Department, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale/ Organisation de Recherche Scientifique et Technique Outre-Mer, Yaoundé, Cameroon
Plasmodium falciparum immunity is generally referred to as immunity to asexual stages with protection against malaria morbidity and mortality. Immunity against sexual stages that interferes with transmission is considered secondary to asexual immunity. To study the impact of immunity to sexual stages on transmission it is necessary to know the transmission capacity of these stages.

Transmission of *P. falciparum* from man to mosquitoes can be studied in the field by either direct skin feeding on the gametocyte carrier or by membrane feeding of the *Anopheles* vector on blood taken by venapuncture. The first method gives information about the actual transmission capacity of such a patient whereas membrane feeding creates the possibility to manipulate the infectious bloodmeal and to investigate factors such as antibodies that may affect transmission. Most of our knowledge on the effects of antibodies on transmission has been obtained with the standard membrane feeding assay. Cultured gametocytes are added in a membrane feeder to natural or “experimental” sera and transmission is compared to feeders with control sera. Although the value of this test is beyond any doubt it does not necessarily reflect the conditions in the field. One of the criteria for a successful assay is that control sera should at least give 90% mosquito infection to allow appropriate statistical analysis but this figure is rarely found in the field.

Epidemiological studies on gametocyte infectivity are rare. Moreover, in about half of the studies either the number of subjects and/or the mosquitoes dissected seem to small to allow more than an anecdotal interpretation. Table 1 shows that mosquito infections are low from gametocyte carriers in endemic populations in contrast to infections from non-immune travellers and neurosyphilis patients undergoing therapeutic malaria therapy that show a much higher efficiency of transmission.

What could be the explanation for this striking difference? The outcome of transmission may be influenced by methodological as well as interdependent factors of host, parasite and vector. Direct- as well as membrane feedings were applied in experiments with semi-immune and non-immune gametocyte carriers and can not account for the observed differences.

Another variable is the source of mosquitoes but they were obtained from various origins and contained both wild and laboratory adapted strains.
**Table 1.**

Efficiency of transmission in semi-immune and non-immune gametocyte carriers

<table>
<thead>
<tr>
<th>Direct or Membrane feed</th>
<th>positive experiments (percentage)</th>
<th>% positive mosquitoes per positive experiment</th>
<th>mean number of oocysts/positive mosquito</th>
<th>mean dissected mosquitoes per experiment</th>
<th>country</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>semi-immunes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5/12 (47)</td>
<td>47</td>
<td>?</td>
<td>10</td>
<td>Nigeria</td>
<td>22</td>
</tr>
<tr>
<td>D</td>
<td>5/5 (51)</td>
<td>51</td>
<td>?</td>
<td>?</td>
<td>Ghana</td>
<td>23</td>
</tr>
<tr>
<td>D</td>
<td>12/28 (43)</td>
<td>36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22</td>
<td>Ghana</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>15/42 (36)</td>
<td>10</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;-7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ghana</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>9/37 (24)</td>
<td>2.5</td>
<td>&lt;6</td>
<td>Nigeria</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>M&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12/27 (44)</td>
<td>16</td>
<td>11</td>
<td>Pnguinea</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1/4 (25)</td>
<td>23</td>
<td>9</td>
<td>Cameroon</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>10/27 (37)</td>
<td>14</td>
<td>20</td>
<td>Cameroon</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>non-immunes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5/5 (100)</td>
<td>&gt;50</td>
<td>&gt;30</td>
<td>?</td>
<td>therapeutic malaria</td>
<td>3</td>
</tr>
<tr>
<td>D</td>
<td>391/682 (57)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>42.3</td>
<td>22.7</td>
<td>21</td>
<td>therapeutic malaria</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>504/674 (75)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>72.0</td>
<td>56.2</td>
<td>15</td>
<td>malaria</td>
<td>4</td>
</tr>
<tr>
<td>M</td>
<td>10/11 (91)</td>
<td>70.1</td>
<td>46.8</td>
<td>20</td>
<td>imported malaria</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> based on gland infections  
<sup>b</sup> in low infections  
<sup>c</sup> in high infections  
<sup>d</sup> one extreme infectious carrier was excluded  
<sup>e</sup> South Carolina strain  
<sup>f</sup> Panama strain
Only laboratory adapted strains were used for the non-immunes, but so far there is no documented evidence that this may be an explanation for the gross differences in infection rates. Parasite and host factors are well known variables in the efficiency of transmission. Increased gametocyte densities have been shown to be associated with higher infection rates\textsuperscript{1-6} and, indeed, high densities of gametocytes were found in neuro-syphilics. In a recent review Taylor and Read\textsuperscript{7} mentioned a maximum of 68000 /µl gametocytes in neurosyphilis patients whereas in the field the maximum is approximately 1200/µl. However, there is evidence that high gametocyte density is not necessarily the most likely explanation for this high infection rate. Shute and Maryon\textsuperscript{3} stated that, "Experience has thought us that 50 to 100 male and female crescents per c.m.m. are necessary in order to get the majority of a batch of mosquitoes infected heavily (30 or more oocysts per mosquito) after one or two blood meals". So despite the modest or even low numbers of gametocytes the observed infection rate was high and would be extremely rare under field conditions. Also the experiments from Jeffery and Eyles\textsuperscript{2} confirm the high efficacy from gametocytes from non-immunes. With densities as low as less than 10 gametocytes per µl, they still obtained infection rates of 14.6-17.8% with a mean of 1.2 oocysts, which are figures that would be quite common under field conditions with much higher gametocyte densities. A recent study investigating gametocyte infectiousness in imported malaria cases confirms the older data\textsuperscript{4}. Gametocytes of people with a first malaria experience (non-immunes) are magnitudes more infectious per gametocyte than those of people living in endemic areas (semi-immunes).

So if density is not the principle explanation for the difference, infectiousness of the gametocyte itself has to be addressed. This infectiousness is determined by the "quality " as was suggested by S.P. James in 1931\textsuperscript{8} and by the presence of transmission inhibiting serum factors. The existence of transmission-blocking antibodies in field sera has been demonstrated in numerous studies\textsuperscript{9-10-11}. These antibodies bind to gametocytes as soon as they emerge from red cells and form gametes inside the mosquito midgut. Experimental immunisations with gamete-specific antigens can induce these transmission-blocking antibodies\textsuperscript{12}. However, if these antibodies would play such an important role in the field one would expect that removal of plasma by replacement with non-immune serum would result in an increased infectivity. Indeed, it was shown by Mulder et al.\textsuperscript{11} that infectivity increased but only moderately.
Innate infectivity of gametocytes

Significant transmission reduction was present in only 30% of the gametocyte carriers in Cameroon.

White blood cells (WBC) in the bloodmeal may also be responsible for transmission reduction, either by the induction of reactive radicals which are harmful for freshly emerged gametes or by phagocytosis of (opsonized)-gametes inside the mosquito midgut. The evidence for the effect of WBC’s was supported in a recent study in non-immunes showing a significant enhancement of transmission upon removal of WBC from the infectious feed. However, similar experiments performed with semi-immunes failed to do so.

Thus, the presence of specific antibodies and/or phagocytes in the bloodmeal of non-immune individuals has a significant impact on the infection rate of the mosquitoes, whereas the effect of these factors is limited in (semi)-immune individuals from malaria endemic areas.

The immune status is the most obvious difference between the neurosyphilis /imported malaria patients and the patients from endemic areas. In the review of Taylor and Read, it was argued that there might be an immune mechanism which could remove gametocytes before entering the peripheral blood thereby diminishing transmission. The existence of this immune mechanism may explain the relative low numbers of gametocytes compared to asexual forms and the extremely high densities as often found in non-immunes. Although not supported by some authors, the concept of an immunity directly acting against gametocytes inside the human host is attractive to others. MacDonald concluded that the infection produces a immune reaction which reduces gametocytogenesis and thereby transmission. He suggested that the most important manifestation of immunity is probably the restriction of gametocyte output which protects the community, whereby antiparasitic and antitoxic features of malaria immunity protects the individual. This gametocyte-specific immunity may occur very early, even before any reduction of asexual parasites is observed. Nedelman calculated the ratio’s of age-specific gametocyte- versus total parasite-prevalences of several epidemiological studies and found that the prevalence of gametocytes decreased more rapidly with age than the prevalence of asexual parasites. The overall ratio in these studies decreased from 0.5 in new-borns to less than 0.2 in adults. Thus, the evidence for gametocyte-specific immunity is based
on a reduction of the numbers and infectiousness (fitness, quality) of gametocytes. This
immunity is directed against the gametocyte inside the human host and distinct from
transmission-blocking immunity *in sensu strictu* which acts in the mosquito midgut.
Discrepancies between gametocyte density and infectiousness occur more frequently in
people with a history of several infections\(^{19}\) and may be explained by the presence of
transmission-blocking immunity. However, plasma replacement studies clearly show that the
innate infectivity of gametocytes in non-immunes is much higher than in semi-immunes. The
lower infectivity in the latter group can not be explained by the presence of transmission-
blocking antibodies which can be washed away in replacement experiments. Figure 1
demonstrates the difference in innate infectivity of gametocytes from carriers in an endemic
area in Cameroon and imported malaria cases.

One can only speculate on the possible mechanisms involved. It is very unlikely that
antibodies will interfere directly with the developing intra-erythrocytic gametocyte, although
there is some controversial evidence that transmembranal ducts connect the parasitophorous
vacuole with the exterior of the red blood cell by which passage of macro molecules as
specific antibodies into the parasitophorous would be possible\(^ {20}\). Alternatively, antigens
expressed on the erythrocytic membrane could be targets for specific antibodies. It is
possible

that early gametocytes share common antigens on the red blood cell with asexual forms. They
could be the cause for diminishing numbers of gametocytes in a similar way as occurs with
asexual parasites. But how to explain reduced infectivity per gametocyte? These antibodies
may be responsible for changes in sequestration sites for example, thereby influencing final
maturity. There are distinct sequestration sites for both asexual as well as sexual forms.
Whereas developing asexual parasites sequester in organs as muscle, hearth, and adipose
tissue, immature gametocytes are confined to the blood spaces of the spleen and bone
marrow.\(^ {21}\) Sequestration sites of gametocytes in semi-immunes may be different from non-
immunes.

Some field sera are capable to completely block infection in mosquitoes in membrane feeding
experiments with even more than 100 oocysts in the control. The mechanism causing the
extreme low innate infectivity of gametocytes in semi-immunes is at least from the same magnitude but seems to be present in almost all individuals since high mosquito infections are extremely rare in the field. It is evident that a mechanism with such an important impact is worthwhile intensive research since it could be an alternative target for malaria control.

Figure 1. Innate infectivity of gametocytes from semi-immune (+) and non-immune (□) gametocyte carriers. Semi-immune gametocyte carriers were recruited from an endemic area in Cameroon. Non-immune gametocyte carriers were recruited from Dutch travellers with no former malaria experience. Blood of gametocyte carriers was membrane fed to mosquito after depletion of leukocytes and replacement of plasma by control serum from a healthy donor without malaria experience. Six days after the feed midguts were dissected from 20 mosquitoes and oocysts counted. mean* = geometric mean of William’s.

References:


CHAPTER 10

Summary
Samenvatting
Summary

The discovery that mosquitoes transmitted malaria was made by Ronald Ross in 1897, in Secunderabad, in India. Although our understanding of malaria transmission has increased since then, some major mechanisms involved in transmission are still poorly understood. In this thesis we studied some of these mechanisms to get a better understanding of transmission and transmission reduction, as a contribution towards the development of a transmission blocking vaccine.

The WHO policy of malaria control today focuses on the use of permethrine-impregnated bed nets and a strictly regulated drug application. However, an effective malaria vaccine would be more desirable. The types of vaccines that are currently under study are: sporozoite (liver stage), asexual, anti-toxin and a transmission-blocking (TB) vaccine. A TB-vaccine has the distinct advantage above anti-sporozoite and anti-sexual vaccines that it is based on non-polymorphic molecules and thereby less sensitive to failure due to variation of parasites (Chapter 1). Studying transmission and TB it is important to know that mosquito infection depends on numerous factors of parasitic, vectorial and host (human) origin. Quantity and quality of gametocytes, the age of the mosquitoes and the immune status of the host appear very important factors for transmission in general (Chapter 2).

To analyse natural and experimentally induced TB, an assay was developed for comparing TB-capacity of test sera with control sera. In this assay, the standard membrane feeding assay (SMFA), cultured infectious gametocytes of *P. falciparum* were mixed with test or control sera and fed to mosquitoes through membrane feeders and TB-activity was expressed as a R(eduction)-value (the percentage reduction in the mean number of oocyst compared to controls). However, control sera showed also considerable variation in their capacity to support transmission. R-values were calculated using the geometric means of William’s, but this method did not discriminate between reducing and non-blocking sera. Therefore we developed a calculation method using the arithmetic mean of the log-transferred data to calculate R-values which makes it possible to define different categories of sera: non-blocking, reducing and blocking. (Chapter 3)

One of the problems with bio-assays is the variability between experiments. The SMFA uses gametocytes which are cultured according to a standard protocol. Attempts to standardise
these cultures further resulted in a method to make asexual stage free synchronised gametocyte cultures by the use of gelatine flotation of schizonts and killing of asexual forms by N-acetyl-glucose-amine which is toxic to mature schizonts and prohibits invasion of merozoites in red blood cells. Two different strains of parasites were used, NF54 and NF134, and from the moment exflagelation was observed these cultures were fed to mosquitoes for 10 consecutive days. We found that the majority of gametocytes were mature and infectious to mosquitoes for a relative short period, around 11 days after initiation of gametocytogenesis. The results indicate that it might be necessary to test gametocyte carriers for several days when their infectiousness to mosquitoes has to be assessed. (Chapter 4)

One of the promising TB-vaccine candidates is based on Pfs25 antigen. This protein is expressed on the surface of the midgut stages of the parasite a few hours after ingestion of the blood meal. We demonstrated by immuno-fluorescence and immuno-EM techniques that this protein remains present in the oocyst capsule during oocyst development. Furthermore, when mosquitoes with developing oocysts were fed extra blood meals containing anti-Pfs25 TB-antibodies there was a significant reduction in the number of sporozoites that finally developed. When this effect is due to elimination of individual oocysts it will contribute to the efficacy of a Pfs25-based vaccine. (Chapter 5).

Although antibodies against sexual stage antigens have been the main subject of research on mechanisms of TB, it has been demonstrated before that human phagocytes, present in the mosquito blood meal, can interfere with further development of the parasite (phagocytosis, killing) inside the mosquito midgut. We showed that this interference is mediated by serum factors (Chapter 6). In a chemiluminescence (CL) assay for phagocytosis, white blood cells (WBC) incubated with malaria endemic sera showed an increased phagocytosis of gametes as compared to control sera. The SMFA revealed that transmission reduction of some of these sera was related to the addition of WBC. The results of this study indicate that phagocytosis of opsonised gametes inside the mosquito midgut can contribute to a reduction in the transmission of *P.falciparum* parasites.

The activity of human WBC inside the mosquito midgut is not only influenced by opsonisation but also by the presence of WBC-activating cytokines. Cytokines such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and granulocyte-monocyte-colony-stimulating-factor
(GM-CSF) are present at elevated concentrations in the plasma of malaria patients. In the CL-assay with WBC and gametes, TNF-α activity depended completely on the presence of complement, whereas IFN-γ and GM-CSF related effects were only observed in the presence of opsonising antibodies. The transmission reducing activity of WBC stimulated by these cytokines was confirmed in mosquito membrane feeding experiments. (Chapter 7).

The relative contribution of WBC and plasma factors on transmission reduction was studied in gametocyte carriers from an endemic area in Cameroon (semiimmunes) and travellers with a first malaria experience (non-immunes). In nonimmune gametocyte carriers plasma factors and WBC showed a (synergistic) transmission reducing effect while in semiimmune gametocyte carriers from Cameroon there was a moderate effect of humoral plasma factors only. In addition, the infectivity of gametocytes of nonimmune carriers was far superior compared with that of semiimmune carriers. This difference was independent of gametocyte density and observed in the absence of WBC and/or plasma factors. (Chapter 8).

In Chapter 9 we discussed the difference in innate infectivity of gametocytes from nonimmune gametocyte carriers compared to that of semiimmune gametocyte carriers. Although experiments reported in literature on transmission are difficult to compare since they were performed at different locations with different mosquito and parasite strains and with different intentions, they support the observation that infectivity of nonimmune carriers is superior to that of semiimmune gametocyte carriers. This difference could not be explained by the difference in gametocyte numbers.

The major conclusion of this study is that transmission reduction is not only governed by antibodies or/and WBC acting against the sporogonic stages in the mosquito. Additional mechanisms in immune individuals from endemic areas exist which diminish gametocyte infectivity. If such an effect is of immunological origin it is worthwhile investigating this further since it might be an alternative target for interruption of the *Plasmodium* lifecycle.
Samenvatting

Malaria is een parasitaire ziekte die met name in de tropen voorkomt en een bedreiging vormt voor 40% van de wereldbevolking. Malaria wordt veroorzaakt door een parasitair protozo van het geslacht *Plasmodium*. Er zijn 4 typen Plasmodia die de mens kunnen besmetten, waarvan er drie min of meer goedaardig zijn en zelden leiden tot levensbedreigende situaties. Dit in tegenstelling tot *Plasmodium falciparum*, de veroorzaker van malaria tropica, die vele dodelijke slachtoffers eist.

Per jaar worden 500 miljoen mensen besmet en overlijden er 1,5-2,7 miljoen, met name jonge afrikaanse kinderen. Behalve dat malaria veel menselijk leed teweegbrengt, heeft de omvang van het malaria probleem enorme gevolgen voor de sociaal-economische ontwikkeling van "derde wereld" landen. Plasmodia worden overgebracht door "malaria" muggen van het geslacht *Anopheles* die besmet worden wanneer zij een bloedmaal nemen van een malaria patiënt met geslachtelijke stadia (gametocyten) van de parasiet in het bloed. De parasiet vermenigvuldigt zich in de mug en komt terecht in de spekselklieren. Bij een volgende beet wordt de parasiet overgebracht naar een nieuw slachtoffer. Na een eerste fase in de lever infecteert de parasiet rode bloedcellen waarbinnen asexuele vermenigvuldiging plaatsvindt. De parasieten komen vrij uit de rode bloedcellen en infecteren opnieuw rode bloedcellen. Een kleine minderheid ontwikkelt zich tot gametocyten waardoor de patiënt infectieus wordt voor muggen.

In het verleden is malaria vooral bestreden door massaal gebruik van anti-malaria middelen (met name chloroquine), ter voorkoming en genezing van de ziekte. Bovendien werd getracht het aantal muggen te reduceren door grootschalige bespuitingen met insekticiden, zoals DDT. Hierdoor is op grote schaal resistentie van de parasiet tegen malaria middelen ontstaan en ongevoeligheid van de mug voor insekticiden. De hedendaagse malaria bestrijding is gebaseerd op het gecontroleerd gebruik van anti-malaria middelen en persoonlijke bescherming door met insekticide-geïmpregneerde bedden. Alhoewel deze benadering op de korte termijn succesvol is blijft het gevaar van resistentie onverminderd aanwezig.

Een alternatieve benadering is de toepassing van een malaria vaccin. Door vaccinatie wordt een immuun respons geïnduceerd in de mens waardoor de parasiet in zijn verdere ontwikkeling
geblokkeerd wordt. Er kunnen een drietal typen malaria vaccins worden onderscheiden, afhankelijk van het aangrijpingspunt in de levenscyclus:

- Pre-erythrocytaire vaccins verhinderen het binnendringen of uitgroeien van de parasiet in de levercel.
- Vaccins tegen asexuele stadia onderdrukken de vermenigvuldiging van de parasiet in het bloed of neutraliseren de ziekmakende produkten van de parasiet.
- Transmissie-blokkerende vaccins verhinderen de overdracht van de parasiet van de mens naar de mug door de ontwikkeling van de parasiet in de muggemaag te blokkeren en voorkomen daarmee de verspreiding van de ziekte.

Het malaria onderzoek van de afdeling Medische Parasitologie richt zich op de ontwikkeling van een transmissieblokkerend (TB) vaccin.

In de in dit proefschrift beschreven studies is gekeken naar immuun mechanismen die, naast blokkerende antistoffen, verantwoordelijk kunnen zijn voor een verminderde transmissie. Hoofdstuk 2 geeft een samenvatting van de factoren die, naast antistoffen, een rol spelen bij de transmissie; het betreft eigenschappen van de mug, de parasiet, de gastheer en omgevings factoren zoals de leeftijd van de mug, de snelheid van digestie van het bloedmaal, kwaliteit en kwantiteit van de gametocyten en geactiveerde witte bloed cellen (WBC).

Mensen die in een malaria endemisch gebied leven kunnen antistoffen ontwikkelen die in staat zijn de overdracht van de parasiet van mens naar mug te reduceren of zelfs te blokkeren. Voor de toepassing van een toekomstig vaccin is het belangrijk een idee te krijgen van het vóórkomen van deze antistoffen in de populatie en hun TB vermogen. Om het TB vermogen van veld sera te kunnen meten in het laboratorium werd een gestandaardiseerde test ontwikkeld. In deze test worden gekweekte gametocyten gemengd met test en controle sera en aan muggen gevoed via een membraan voeder. Na 7 dagen worden oöcysten geteld en de resultaten van test sera vergeleken met die van controle sera. De TB activiteit wordt uitgedrukt in een R(eductie)-waarde (het percentage reductie in gemiddelde oöcysten aantallen t.o.v. controles). Voor de berekening van R-waarden werd oorspronkelijk het geometrisch gemiddelde van William’s gebruikt, maar deze methode maakte geen onderscheid tussen reducerende en niet-blokkerende sera. Door gebruik te maken van het rekenkundig gemiddelde van log-getransformeerde data is het mogelijk sera te classificeren in 3 categorieën: niet-blokkerend ($R<0,30$), reducerend ($0,30 \leq R \leq 0,90$) en blokkerend ($R>0,90$).
Een dergelijke classificatie is van belang voor veldonderzoek naar natuurlijk verworven TB-immuniteit (hoofdstuk 3). Een belangrijk probleem met de TB-test is de variabiliteit van de kwaliteit van de gametocyten tussen verschillende experimenten. De gedachte was dat deze mogelijk veroorzaakt werd door asexuele stadia van de parasiet die ook aanwezig zijn in de gametocytenkweken. Er werd een methode ontwikkeld deze te verwijderen m.b.v N-acetylglycerine-amine, wat uiteindelijk resulteerde in synchrone gametocyten zonder andere bloedstadia die bovendien in staat waren muggen te infecteren. Alhoewel dit niet direct het kwaliteitsprobleem oploste gaf het wel de mogelijkheid de infectiviteit van gameten in relatie tot hun leeftijd te bestuderen. Twee verschillende P.falciparum isolaten, NF54 en NF134 werden gesynchroniseerd en vanaf het moment dat de gametocyten konden activeren werden dagelijks muggen gevoed. Het bleek dat na 11 dagen de meeste gametocyten “rijp” waren en in staat muggen te infecteren. De resultaten laten zien dat voor het bepalen van de infectiviteit van gametocyten-dragers voor muggen, het wenselijk zou zijn deze meerdere dagen achtereen te voeden. (hoofdstuk 4).

Een TB-vaccin dat in een ver stadium van ontwikkeling is, is het TBV25H, dat is gebaseerd op het Pfs25 eiwit (dat aanwezig is op late stadia van de sexuele ontwikkeling in de muggemaag). Aangetoond werd dat dit eiwit ook nog aanwezig is in de cyste wand van jonge oöcysten. Als reeds geïnfecteerde muggen, met jonge oöcysten op de maagwand, een bloedmaal nemen waarin antistoffen tegen dit eiwit, vermindert het aantal sporozoieten in de speekselklieren. Het is niet ondenkbaar dat bij lage oöcysten aantallen de ontwikkeling van sporozoieten totaal geblokkeerd wordt, waardoor dit een additief effect zou zijn van dit type TB-vaccin. (hoofdstuk 5).

Behalve antistoffen kunnen ook humane WBC bijdragen tot transmissie reductie. Humane WBC overleven een aantal uren in de muggemaag en kunnen de extra-cellulaire parasieten fagocyteren en doden. Met behulp van een fagocytose test werd aangetoond dat sommige endemische sera fagocytose stimuleren met een reductie van transmissie als gevolg. Aldus laten resultaten van deze studie zien dat fagocytose van geopsoniseerde gameten in de muggemaag bijdraagt tot transmissie reductie (hoofdstuk 6).

De activiteit van WBC in de muggemaag wordt niet alleen bepaald door opsonisatie van gameten maar ook door de aanwezigheid van cytokinen. De concentratie van TNF-α (tumor necrosis factor-α), granulocyte-monocyte-colony-stimulating factor (GM-CSF) en IFN-γ
(interferon-γ) is dikwijls verhoogd in het bloed van malaria patiënten. In een fagocytose test was de activiteit van TNF-α gestimuleerde WBC volledig afhankelijk van de aanwezigheid van complement, terwijl de activiteit van WBC gestimuleerd door GM-CSF of IFN-γ vooral bepaald werd door de aanwezigheid van gameet opsoniserende antistoffen. De transmissie reducerende activiteit van cytokine gestimuleerde WBC werd bevestigd in membraan voeding experimenten. (hoofdstuk 7).

De bijdragen van WBC en plasmafactoren aan transmissie reductie werden bestudeerd in (semi-immune) gametocytendragers in een malaria endemisch gebied in Kameroen en (niet-immune) reizigers met een eerste malaria infectie. Door het vervangen van plasma door een controle serum en/of het verwijderen van WBC uit het bloedmaal konden de afzonderlijke bijdragen aan reductie van de transmissie bepaald worden. In niet-immune gametocytendragers bestond een synergie in transmissie reducerend effect tussen WBC en plasmafactoren, terwijl in semi-immune gametocyten dragers uit Kameroen slechts een beperkt reducerend effect werd gevonden van plasma factoren. Wat opviel in de studie was dat gametocyten van niet-immune gametocytendragers vele malen infectieuser waren dan die van semi-immune gametocytendragers. Dit verschil was onafhankelijk van de dichtheid van de gametocyten en de aanwezigheid van zowel plasma factoren als WBC (hoofdstuk 8).

In (oudere) literatuur wordt reeds beschreven dat transmissies met gametocyten van niet-immune dragers buitengewoon efficiënt verlopen in vergelijking met die van semi-immune dragers. Het verschil in transmissie succes van semi-immune en non-immune gametocyten dragers wordt niet alleen verklaard door het verschil in dichtheid van gametocyten, antistoffen en/of WBC. De gegevens suggereren dat er een additionele factor bestaat in semi-immune dragers die de kwaliteit van de gametocyt beïnvloedt en die zich uit in een vermindere ontwikkeling in de mug. Dit zou een alternatief kunnen zijn om de levenscyclus van *Plasmodium* te onderbreken (hoofdstuk 9).
LIST OF PUBLICATIONS


List of Publications


De schrijver van dit proefschrift werd geboren op 30 juli 1954 te ’s-Hertogenbosch.
Na de middelbare school volgde hij een HBO-opleiding analytische chemie gevolgd door een HBO-opleiding zoölogie (fysiologisch, farmacologische richting) waarvan hij in 1979 het diploma haalde. In het kader van deze opleiding werd een stage verricht op de afdeling Nefrologie van het Academisch Ziekenhuis “St Radboud” te Nijmegen, bij het onderzoek van Prof. dr. J. Berden. In 1979 werd hij aangesteld als analist op de afdeling Medische Parasitologie en werkte onder leiding van Dr. T. Ponnudurai aan de ontwikkeling van kweeksystemen voor de kweek van de malaria parasiet, P.falciparum. De mogelijkheid fertiele geslachtelijke stadia van deze parasiet te kunnen kweken was de basis voor het transmissie onderzoek zoals dat tot op heden plaats vindt. In 1991 behaalde hij het HLO-diploma Biologie aan de Hogeschool van Amsterdam. Aanvankelijk onder leiding van Prof. dr. J.H.E.Th. Meuwissen en Dr. T. Ponnudurai en later onder leiding van Dr. R. Sauerwein en Dr. W Eling werd het onderzoek uitgevoerd zoals dat hier beschreven staat.
Dankwoord

Een proefschrift schrijf je welliswaar grotendeels alleen maar de inhoud weerspiegelt het resultaat van het werk van velen. Daarom wil ik iedereen die op enigerlei wijze heeft bijgedragen aan het tot stand komen van dit proefschrift bedanken. Graag wil ik enkele personen met name noemen:

Prof. Dr. J.H.E.Th. Meuwissen die mij indertijd de gelegenheid heeft gegeven dit onderzoek te starten en mijn promotor Prof. Dr. J.A.A. Hoogkamp-Korstanje voor de begeleiding bij de afronding ervan. Grote waardering heb ik ook voor mijn beide co-promotores Dr. R.W. Sauerwein en Dr. W.M.C. Eling voor hun opbouwende discussies, ideeën, geduld en inzet bij het gereedkomen van de wetenschappelijke publicaties.

Uiteraard was dit werk niet mogelijk geweest zonder de inbreng van de mensen van de malaria-kweek en muggenunit. Marga van de Vegte-Bolmer, Geert-Jan van Gemert, Arianne Bril, Jo Hooghof, Jolanda Remmers en Astrid Pouwelsen bedankt voor alle hulp.

De studenten Karlijn Baert, Linette Willemsen en Martijn van Kruistum wil ik bedanken voor hun enthousiasme en bijdrage aan het onderzoek. Veel waardering heb ik voor de morele en praktische ondersteuning, die ik de afgelopen jaren mocht ontvangen, van de medewerkers en ex-medewerkers van de afdeling Medische Parasitologie, in het bijzonder die van mijn “lotgenoten”, Rob, Will, Bert en Annette.

Op deze plaats zou ik ook Thivi Ponnudurai willen gedenken met wie ik een groot aantal jaren mocht samen werken en die mij enorm gestimuleerd heeft met zijn enthousiasme voor het transmissie onderzoek. Vrienden en familie wil ik bedanken voor hun belangstelling, met name mijn ouders die mij altijd alle steun hebben gegeven.

Last but not least, het “thuisfront”, Francis, Joyce en Simone, zonder jullie was dit niet mogelijk.