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Infectivity of malarial parasites to mosquitoes: 'the interdependent roles of parasite, vector and host'

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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 development and 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 the 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 leucocytes in the bloodmeal.

Since 1980 it has been possible to infect mosquitoes routinely with cultured gametocytes of *Plasmodium falciparum* by membrane-feeding. Mosquito infection in the laboratory not only provided a source of infective sporozoites of *P. falciparum* but also boosted the research towards a transmission-blocking vaccine. It became possible to evaluate the effects of antibodies, either raised in the laboratory or present in endemic sera, in a functional bio-assay. In this assay, mosquitoes are membrane-fed cultured gametocytes of *P. falciparum* in the presence of control and test sera. Oocyst counts, performed 7 days after the infectious feed, indicate transmission capacity and any reduction in it compared with that with control sera. However, developing and fine tuning of this assay made it apparent that, besides antibodies, other factors from the vertebrate host and factors from the parasite and mosquito play crucial roles in mosquito infection.

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 or 6, followed by period of massive destruction of parasites during which gametocytophagocytosis is initiated. Gametocytes need another 7 or 8 days in culture to reach maturity. At present, the real factors inducing gametocytophagocytosis are unknown.

With subsequent subcultures, the infectious isolate adapts further to culture conditions. As a result, the multiplication rate increases and the infectiousness of the gametocytes produced diminishes. Extended subculturing finally completely eliminates the capacity for gametocytophagocytosis (Ponnudurai *et al.*, 1982b). The infectiousness of isolate NF54, for example, wanes after 25 subcultures and a new stabilitate has to be started from the frozen stock.

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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 appears to inhibit 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., 1989). Rapid asexual multiplication occurs in the initial cultures of some parasite isolates/stablets 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 automated device for culturing infectious gametocytes (Ponnudurai et al., 1982a) can also contribute 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 from normal, blood-bank donors who have never been exposed to Plasmodium spp. 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 possibly 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 make 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, the distribution of pigment 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 localized 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 (PCS) and incubating for 10 min 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 erythrocytes. Any male gametocytes then undergo the process of exflagellation, each producing eight microgametes. Gametocytes that look mature morphologically can fail to activate and/or to exflagellate for as yet unknown reasons. Some morphologically mature gametocytes seem to round up within the erythrocytic membrane and fail to escape. Although exflagellation is a good indicator of the quality of male gametocytes, it still does not guarantee successful transmission. The same is true for the formation of female gametes; female gametes in a mosquito bloodmeal can be visualized, by reaction with an anti-48/45-kDa 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 synchronized 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, quantity certainly plays an important role in infectiousness to mosquitoes. The number of gametocytes in the infectious bloodmeal 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
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>Degrees of freedom</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>77.9 (51.3)</td>
<td>49.4 (36.7)</td>
<td>98</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2</td>
<td>81.3 (57.4)</td>
<td>39.1 (35.1)</td>
<td>116</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
<td>112.1 (54.8)</td>
<td>78.9 (51.3)</td>
<td>117</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

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>Degrees of freedom</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47.8 (24.7)</td>
<td>0.5 (0.9)</td>
<td>21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>5.5 (9.9)</td>
<td>0.6 (1.1)</td>
<td>38</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

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 lower transmission and the efficiency of infection per gametocyte increases with reduced numbers of gametocytes (Ponnudurai et al., 1987). As few as 10 gametocytes in a 2-μl mosquito bloodmeal are still capable of infecting two out of 20 mosquitoes, each with one oocyst.

VECTORS

Apart from parasitic 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 24 h post-infection. 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 *Ae. 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 relatively large numbers of oocysts that developed 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 of bloodmeal they took.

Another interesting observation is the relationship between mosquito age at the time of feeding and the final oocyst load. Mosquitoes aged 3–4 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. 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 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 to 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 was associated with a relatively fast rate 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 parasitic 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 number 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 separated from asexual parasites should determine whether the asexual stages have an effect on transmission.

HOSTS

Transmission-blocking antibodies have been the main subject of research in many malaria laboratories. Naturally-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 surfaces of gametocytes and gametes. The three most studied proteins are Pfs45/48, Pfs230 and Pfs25 (of 45/48, 230 and 25 kDa, respectively); Pfs45/48 and Pfs230 are present on the surface of gametocyte stages whereas 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 specific modes 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 45/48-kDa protein on the gametocyte surface (Pfs45/48) are believed to interfere with the process of fertilization, 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 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 effectiveness 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 the blocking of fertilization. However, transmission blockade was sustained when Fab fragments were used instead of complete mAb (Carter et al., 1990).

Transmission-blocking mAb against Pfs230 show complement-dependent lysis of freshly formed gametes and are consequently restricted to complement-fixing isotypes (Quakyi et al., 1987; 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 had a transmission-blocking, lytic effect. A relationship has been found in Papua New Guinea
between the presence of anti-230-kDa 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 been 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 Pfs25 differs from that of the other mAb discussed 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 function(s) of Pfs25; it may be involved in protecting the parasites against digestion or in their movement or it may have a receptor-like function, mediating in the parasites' contact with the midgut epithelium. Interference with any of these actions could result in reduced transmission. It has also been shown that Pfs25 persists during oocyst development, ingestion of bloodmeals containing anti-25-kDa antibodies by already infected mosquitoes resulting 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 from healthy individuals who have had no experience of malaria 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 in gamete numbers within the mosquito midgut 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 malaria, 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 malaria, 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 relatively low temperature and can easily be demonstrated in a Giemsa-stained slide of a bloodmeal smeared 20 min 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 the results of a recent study by 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.).

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 the results 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


INACTIVITY TO MOSQUITOES


