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# SOME ASPECTS OF TRANSMISSION BLOCKING IMMUNITY REVIEWED

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**Introduction** – Ten years have passed since Gwadz (1976) and Carter & Chen (1976) published their papers on the induction of transmision blocking immunity in chickens immunized with microgametes of *Plasmodium gallinaceum*. These experiments followed a line of studies by Huff, Marchbank & Shiroishi (1958) in avian malaria models.

Subsequently these studies have been extended to experiments in monkeys (Gwadz & Green, 1979) and in rodents (Mendis & Targett, 1979). Studies on the sexual stages of the human parasite *P. falciparum* have been initiated following the development of methods for the *in vitro* production of fertile gametocytes (Campbell et al. 1980; Meuwissen, Ponnudurai & Leenwenberg, 1980; Ifediba & Vanderberg, 1981; Ponnudurai et al., 1982a). More recently the development of small – and large-scale *in vitro* cultivation systems for the production of fertile *P. falciparum* gametocytes (Ponnudurai et al. 1982b, 1983, 1986) have contributed to biochemical, immunochemical and molecular biological studies of the sexual stages of this most important malaria parasite of man.

The subject of transmission blocking immunity has been reviewed by Carter & Gwadz (1980), Carter et al. (1984), Targett & Sinden (1985) and most recently in a scientific working group of the World Health Organization (1985). For that reason the following will briefly summarize the present state of the art, it will emphasize studies related to *P. falciparum* and it will indicate areas of special interest for further studies on account of results of recent experimental observations. Developments with regard to transmission blocking immunity in *P. vivax* infections will be briefly reviewed also.

The targets of transmission blocking immunity are the extracellular sporogonic stages of parasite development. These are: – *the male and female gametes* – These stages are released from the micro – and macrogametocytes following their activation which is due to a fall in temperature and a decrease in the $CO_2$ tension of the blood as a result of its transfer from the vertebrate host to the mosquito midgut; – *the zygote* – This is formed in the mosquito midgut by fertilization of the macrogamete; – *the ookinetes* – This is the motile stage which penetrates the midgut and becomes a sporozoite forming oocyst on the mosquito stomach wall.

The effector mechanism of transmission blocking immunity depends on antibodies but Harte, Rogers & Targett (1985) obtained evidence in a *P. yoelii nigeriensis* rodent model that passive transfer of immune T-cells resulted in reduced transmission of the parasite infection by 95%, which effect was partly due to a significant reduction in the number of circulating gametocytes. So far this is the first and only communication on the involvement of cellular immune mechanisms. Therefore this summary will be restricted to the humoral aspects of transmission blocking immunity.

Transmission blocking antibodies induced in the vertebrate host are ingested as part of the bloodmeal of the malaria mosquito. Fertile gametocytes that are present also in the blood and which become the extracellular sporogonic stages are inhibited in their development in the mosquito midgut by these antibodies. Thereby they suppress the transfer of the infection from man to man via the mosquito. It should be well understood that this form of stage specific, "protective" immunity does not prevent the immunized host in becoming infected by sporozoites, nor does it influence the asexual parasites of the pathogenic erythrocytic cycle. Transmission blocking immunity does not act at all at the level of the individual person, but only at the level of the community. It could reduce the local level of transmission intensity and in this way a transmission blocking malaria vaccine might become a welcome additional tool in malaria control operations.

Since it is impossible to apply *in vitro* cultivated stages of the parasite for use as a vaccine it is necessary to use biochemical, immunological and molecular biological methods for the identification of specific immunogens or their relevant epitopes for the envisaged production of malaria vaccines by biochemical or recombinant DNA techniques. Some of the results of these experimental efforts will be discussed below.

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**Target antigens of transmission blocking antibodies** – Target antigens of transmission blocking antibodies are surface proteins of the sporogonic stages. Following surface labelling of these parasites they have been identified by precipitation with monoclonal antibodies (MoAbs) against *P. falciparum* macrogametes. Individual MoAbs have been classified as transmission blocking or non-blocking on account of their ability to suppress transmission to anopheline vectors when the former are included in an infective bloodmeal. The relative number of oocysts present in the mosquito midgut compared to that in control mosquitoes seven days after the feed provided a relative measure of the transmission blocking capacity.

Under non-reducing conditions the three major surface proteins of gametes have molecular weights of 230, 48 and 45 kD (Carter et al. 1984; Vermeulen et al. 1985a). By the use of biosynthetic labelling of synchronized gametocyte cultures it could be shown that these proteins are synthesized simultaneously early during gametocyte development (Vermeulen et al. 1986). The 48/45 kD proteins – a doublet – are not products of processing of the hydrophilic 230 kD protein as was shown in pulse chase experiments. They react with non-blocking and blocking MoAbs. In view of the latter they are considered as target antigens of transmission blocking immunity.

The role of the 230 kD proteins has not been demonstrated yet. There is one non blocking MoAb, which recognizes only the 230 kD protein and there are several other non-blocking MoAbs that recognize this protein in association with the 48/45 kD doublet.

Other non blocking MoAbs react with the 45 kD protein in association with molecules of 10 kD. The role of the low molecular weight proteins has not yet been studied in detail. They have not yet been shown to react with blocking MoAbs. Finally there is a fourth protein, with a relative molecular weight of 25 kD, that reacts both with blocking and non blocking MoAbs. This protein cannot be labelled by surface iodination but can only be detected in immunoblotting experiments. It is also synthesized from day 2 onwards by developing gametocytes, but its synthesis is boosted dramatically following gametocyte activation. This 25 kD protein replaces all the other surface proteins and remains expressed in zygotes, ookinetes and even in oocysts.

Table I summarizes the main characteristics of these major surface proteins.

TABLE I

Potential target proteins of transmission blocking immunity in *Plasmodium falciparum*.

| | *Mr of the protein* | |
|---|---|---|
| 1. | 230 kD | – surface protein of gametes (and newly formed zygotes?)<br>– absent on developing ookinetes<br>– synthesis starts by day 2 of gametocyte development<br>– not glycosylated, not hydrophobic<br>– role in transmission blocking immunity unknown |
| 2/3. | 48/45 kD | - doublet surface proteins of female gametes<br>– absent on developing ookinetes<br>– synthesis starts by day 2 of gametocyte development<br>– isoelectric point: pI = 6.0 ± 0.1<br>– hydrophobic glycoproteins<br>– target of transmission blocking immunity<br>– at least three different non-repetitive epitopes are recognized by MoAbs<br>some evidence of antigenic diversity of one of the epitopes |
| 4. | 25 kD | – surface protein of activated female gametocytes, zygotes, ookinetes and oocysts<br>- synthesis starts by day 2 of gametocyte development, but production is largely predominant following gametocyte activation<br>- isoelectric point: pI = 5.6 ± 0.1<br>- glycosylated, acylated<br>– target of transmission blocking immunity<br>– at least two different non-repetitive epitopes |

(mr = relative molecular mass – apparent molecular weight on SDS-PAGE under non reducing conditions).

**Epitopes of the target proteins** – The 48/45 kD and 25 kD proteins contain at least *one* non-repetitive epitope reacting with blocking MoAbs and another epitope also non-repetitive reacting with non-blocking MoAbs. This has been demonstrated with a two-site solid phase immunoradiometric assay (IRMA) (Vermeulen et al. 1986) and a competitive inhibition immonoradiometric assay (CI-IRMA) (our unpublished data). In contrast to a polyvalent rabbit anti-macrogamete serum, these MoAbs reacted with the proteins under non-reducing conditions only. This might indicate that these epitopes have a conformational nature, but this point has still to be confirmed.

Studies have been initiated in our group on the presence of disulphide bridges in these epitopes. Following reduction by DTT and alkylation with iodoacetamide of NP40 extracts of biosynthetically labelled gametes, the proteins were incubated with blocking anti 48/45 and anti 25 kD MoAbs and the immune complexes analyzed. The anti 48/45 kD MoAbs reacted but the anti 25 kD MoAbs did not, which would indicate that S-S bridges are not a part of the 48/45 kD epitope. In this same series of experiments we obtained the first evidence that removal of sugar from the glycoproteins by treatment with tri-fluoromethanosulfonic acid did not influence the interaction with MoAbs. This suggests that sugars are not a part of the epitopes.

These studies have to be extended and pursued since one of the options in the field of malaria vaccine development might be a synthetic vaccine which is specifically based on immunization with conformationally normal epitopes that are linked to a suitable carrier.

**Mechanism of transmission blocking immunity** – It has been reported before (Vermeulen et al., 1985a) that there is a difference in the target of the anti 48/45 and anti 25 kD blocking MoAbs. Both block the formation of oocysts but the former interferes with ookinete formation and the latter does not. This result has been interpreted as a likely interference by the anti 48/45 MoAb in the process of fertilization of the macrogamete and as an interaction of the 25 kD MoAb with a ligand on the ookinete for receptor on the mosquito midgut cell wall and so interfering in the penetration by this stage. The latter should be reconsidered, in the light or our recent studies on biological aspects of oocyst formation following an infectious bloodmeal by the mosquito. These will be reported first in some greater detail.

On account of experience with routine *in vitro* production of fertile gametocytes of *P. falciparum* we report here that more gametocytes are produced by a fresh stabilate of the isolate NF54 of this parasite species than when the same stabilate has been in continuous culture for a period of 1-2 months. Not only is the number of gametocytes greater, but this applies also to the number of exflagellations. When these cultures are fed to mosquitoes almost 100% of the mosquitoes become infected. It is peculiar, however, that the average number of oocysts that is produced in the mosquitoes is lower than with the later harvests whilst at that moment the number of gametocytes has been reduced from about 2% to 0.5%. In order to explain this apparently contradicting phenomenon we performed the following experiment. The early gametocyte-rich cultures have been diluted 3x, 9x, 18x and 36x with a 50% suspension of normal erythrocytes, keeping the haematocrit of the starting material and the dilutions constant. The suspensions were fed to mosquitoes and the average number of oocysts was compared.

The results of repeated experiments show that in contrast to what one would expect – the threefold dilution produces significantly more oocysts than the undiluted culture ($p = 0{,}005$). The further dilutions show a statistically significant decrease ($p = < 10^{-5}$). We have no explanation for this phenomenon. However, the results indicate that there is no linear relationship between the number of oocysts and the number of mature gametocytes in the bloodmeal. There are two different aspects to this: in the first place the increase in the number of oocysts following the initial dilution of the culture and secondly the absence of a direct linear relationship between dilution and resulting number of oocysts. The relative decrease of the number of oocysts is less with increasing dilution. That means that the efficiency of gametocytes in diluted cultures relatively increases with regard to their ability to develop into oocysts. Table II gives an example of such an experiment. Clearly this unexplained biological phenomenon is of significance with regard to the quantitative assessment of the transmission blocking capacity of antisera and especially monoclonal antibodies. So far it has been assumed that a fixed quantity of purified monoclonal antibody, added to a decreasing number of gametocytes would result in a correlated decrease of the number of oocysts. This has been separately studied for blocking anti 25 kD and anti 48/45 MoAbs. For the assessment of this effect the dosage of the anti 25 kD MoAb (32F 81) was reduced ro 0.25 mg since 1 mg protein per ml of the bloodmeal in undiluted cultures blocked oocyst development up to 99%. The resulting number of oocysts was compared with control cultures without MoAbs. Table III gives the results of one of the repeated experiments.

TABLE II

Relationship between the number of infectious gametocytes and number of developing oocysts.

| Fertile gametocyte culture | No. of fed mosquitoes | Range oocysts | Mean no. oocysts | |
|---|---|---|---|---|
| undiluted | 14 | 0-139 | 38.1 ± 39.3 | * |
| 1:3 | 13 | 2-160 | 92.1 ± 46.7 | |
| 1:9 | 18 | 0-142 | 57.3 ± 36.7 | |
| 1:18 | 10 | 4-120 | 35.0 ± 37.7 | ** |
| 1:36 | 19 | 0.44 | 16.3 ± 11.5 | |

*Test of Wilcoxon p = 0.005; **Trend test of Terpsta p= $<10^{-5}$.

TABLE III

The effect of gametocyte numbers on transmission blocking activity by anti 25 kD MoAb (32F81).

| | Dilution of gametocyte suspension: | | | | | |
|---|---|---|---|---|---|---|
| | 1x | | 10x | | 70x | |
| | Exp. | Control | Exp. | Control | Exp. | Control |
| No. of examined mosquitoes | 22 | 19 | 20 | 20 | 19 | 18 |
| Range of oocysts | 0-315 | 33-237 | 0-20 | 50-184 | 0-1 | 1-18 |
| Mean no. of oocysts | 51.4± 96.1 | 155.4± 68.2 | 3.9± 5.4 | 115.5± 39.7 | 0.1± 0.3 | 8.1± 4.9 |

According to expectations the percentage of infected mosquitoes decreased from about 50% to 0% in the test population and the inhibition in the oocyst production increased from about 50% to 100% when compared with the controls.

However, the results with the anti 48/45 kD MoAb (32F3) were completely different. Table IV shows the results.

TABLE IV

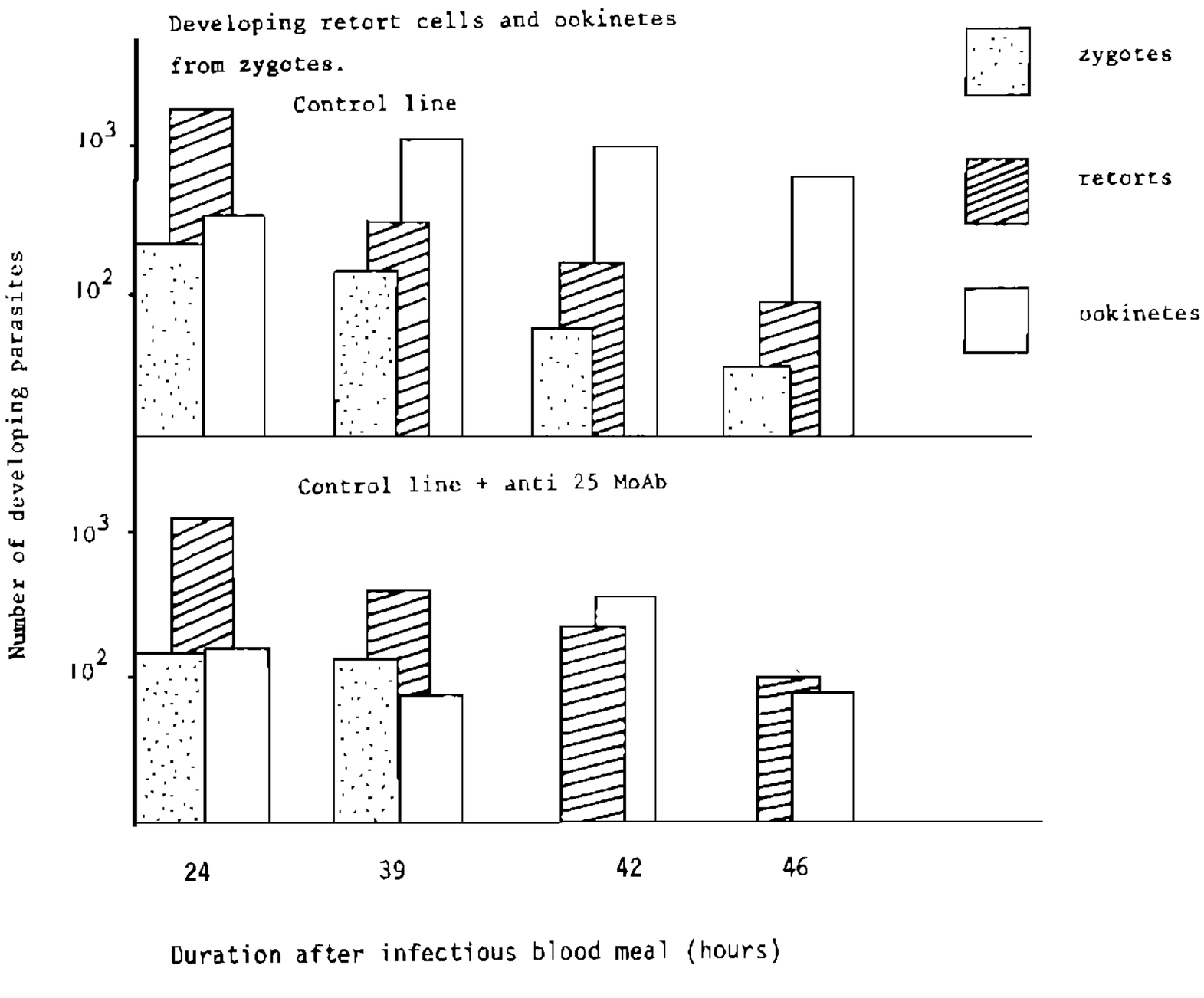
The effect of gametocyte numbers on transmission blocking activity by anti 48/45 kD MoAb (32F3).

| | Dilution of gametocyte suspension: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1x | | 3x | | 9x | | 36x | |
| | Exp. | Contr. | Exp. | Contr. | Exp. | Contr. | Exp. | Contr. |
| No. of examined mosquitoes | 20 | 20 | 19 | 19 | 18 | 20 | 19 | 23 |
| Range of oocysts | 0-197 | 3-278 | 3-198 | 1-197 | 5-172 | 4-183 | 0-43 | 0-49 |
| Mean no. of oocysts | 33.3 ± 48.2 | 85.5 ± 62.8 | 66.0 ± 52.9 | 101.1 ± 69.2 | 58.2 ± 50.5 | 80.4 ± 48.1 | 10.5 ± 12.7 | 14.1 ± 15.2 |
| p | 0.008 | | 0.10 | | 0.13 | | 0.38 | |

Here we found again a significant increase in the number of oocysts in mosquitoes fed with 3x diluted culture with added MoAbs (Wilcoxon, p = 0.008). A significant decrease was seen between the three times and 36 diluted cultures with added MoAbs. The differences between experimental and control mosquitoes were not significant except for the group fed with undiluted suspensions.

These two results indicate that there is an essential difference between the effector mechanism of both types of transmission blocking antibodies. It is supposed that the anti 48/45 kD antibodies interfere with the fertilization of macrogametes, but it is difficult to see how the MoAbs become less efficient in the presence of a lower number of parasites. On the other hand, according to expectations, the anti 25 kD antibodies acting on ookinetes appear more efficient blockers when the number of gametocytes is low. The 25 kD protein has to be preferred if a choice has to be made between these two proteins as potential immunogens.

In order to get a better understanding of the effector mechanism of the protective anti-25 kD MoAbs, we examined with the indirect immunofluorescent antibody technique the unfixed suspensions of the infective bloodmeals present in the mosquito guts 24, 39, 42 and 46 hours after the feed. In cohorts of 8-10 mosquitoes we estimated in a counting chamber the number of zygotes, developing ookinetes – so called retort cells – and mature ookinetes. In order to prove the effectiveness of the blocking MoAb, samples of cohorts of the experimental mosquitoes fed with the 25 kD MoAb and controls were examined after seven days for the presence of oocysts. The controls were 100% infected and in mosquitoes from the experimental population oocyst production was almost completely inhibited. In the following diagram the number of the various parasite classes over the first two days after the feed are indicated (on a logarythmic scale).




In the controls the number of zygotes decreases over a period of 48 hours; only some degenerate zygotes remained. At 24 hours the number of retort cells is at its maximum, and it decreases later on as expected along with the increase of the number of ookinetes; the number of the latter decreases also since they leave the midgut lumen.

In the experimental group the results are different. It is clear that the transition of retort cells to ookinetes is inhibited. Morphologically these parasites showed changes i.e. the "neck" of some of these stages appeared broken off, the cell walls were irregular and seemed fluffy. This indicated that the anti 25 kD blocking MoAbs might cause cell lysis of ookinetes in the lumen.

**Cloning of genes coding for target antigens of transmission blocking antibodies** – Genomic and cDNA libraries have been constructed in phage gt11 and in a so called secretion vector in an attempt to isolate DNA sequences encoding target antigens. The real breakthrough in this field seems not yet achieved. However clones have been identified which produce fusion proteins that react on immunoblots with polyvalent rabbit antisera to macrogametes and zygotes. The fusion proteins induced antibody production in mice but the mouse antisera did not react with gamete proteins by immunoblotting. Moreover transmission blocking MoAbs have not identified bacterial clones coding for parts of the 48/45 kD and 25 kD proteins. Most fusion proteins are aggregates with intermolecular disulfide bonds and this causes a technical problem with regard to the recognition by MoAbs. As indicated above MoAbs recognize only epitopes with the correct conformational expression. Recently the screening of a secretion library with MoAbs was also not successful. Alternative methods of producing sera that will be used for screening the libraries are being investigated, especially immunization with antigens purified by immunochromatography and encapsulated in liposomes.

In addition target proteins have been coupled to a carrier for affinity purification of polyvalent sera (antibody select method). Clones have been identified by these polyvalent monospecific antibody probes, but definitive evidence that they contain the genetic code for the relevant epitope has still to be obtained.

**Natural occurrence of transmission blocking antibodies in *P. falciparum* and *P. vivax* infections** – Sera of *P. falciparum* patients in New Guinea, tested in membrane feeding experiments were shown to block transmission. The reduction in infectivity was correlated with a positive IFA test with macrogamete surface antigens. The blocking sera reacted with the 230 kD antigen and one also with the 48/45 kD doublet. In a serum sample collected from a patient returning from Africa, were he lived for over 25 years, transmission blocking antibodies could be demonstrated in a membrane feeding experiment. The purified antibodies from the sample reacted predominantly with the 48/45 kD proteins and inhibited the interaction of radiolabelled anti 48/45 MoAbs in a two dimensional immunoblotting experiment.

Mendis (personal communication) reported recently about a study on *P. vivax* malaria in Sri Lanka. She demonstrated in membrane feeding experiments that sera of patients with an acute *P. vivax* infection contain transmission blocking antibodies. Most sera reacted both with homologous and heterologous strains of parasites but there was evidence for some antigenic diversity. Repeated attacks boosted the transmission blocking immunity but this boosting was possible only within a period of about four months following an attack and the duration of effective blocking immunity remained for about 3 months. The transmission blocking capacity of a serum sample correlated with IFA titers with gametes as the antigen but not with the titers against asexual blood stage antigen. Sera with high anti-gamete IFA titers suppressed transmission by over 95%. Surprisingly she found that transmission blocking antibodies applied in membrane feeding experiments at a subneutralizing concentration as well as sera collected from immune patients later than three months after the last attack of *P. vivax* enhanced the infectivity of the parasite of mosquitoes.

**Conclusion** – Results of recent studies indicate that just a start has been made with the assessment of the various factors that are involved in the biological process of sporogony. Various aspects need much more attention and might appear to be conditional for a successful achievement of the development of transmission blocking vaccines. The molecular genetics of gametocytogenesis and its induction remain to be studied. More exact information about the duration of the fertility of male and female gametocytes will be needed. Such studies are complicated by the effect of the occurrence of the recently demonstrated naturally occurring transmission blocking antibodies. Moreover there is evidence that there exists an inverse relationship between the number of developing oocysts as well as a similar inverse relationship between the number of oocysts and their capacity to produce sporozoites (J.P. Verhave, personal communication). This points to the existence of a balance of parasitologic as well as entomological factors which regulate transmission.

Finally, besides the biological aspects of the parasite-vector relationships, epidemiologic field studies should be initiated in order to assess in a quantitative manner the impact of transmission blocking immunity on the prevalence of malaria in the population. This should be considered as essential for the local application of transmission blocking vaccines in malaria control operations, which might be anticipated in the future.

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