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Malaria transmission-blocking activity in experimental infections of *Anopheles gambiae* from naturally infected *Plasmodium falciparum* gametocyte carriers

Bert Mulder[1,2], Timoléon Tchuinkam[2,3], Koen Dechering[1,2], Jan Peter Verhave[1], Pierre Carnevale[2], Joep H. E. Th. Meuwissen[1] and Vincent Robert[2] [1]*University of Wageningen and University of Nijmegen, Department of Medical Parasitology, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands;* [2]*Medical Entomology Service, ORSTOM/OCEAC, Yaoundé, Cameroon;* [3]*Faculty of Sciences, University of Yaoundé, Cameroon*

Abstract

Experimental infections of anopheline mosquitoes were carried out with *Plasmodium falciparum* gametocytes from 65 naturally infected patients in Cameroon. A comparison was made between infections with blood containing autologous plasma and blood in which the plasma was replaced with plasma from a donor without previous malaria exposure. A lower infection rate was observed in 50 of 65 autologous plasma samples. Transmission was significantly blocked in 3 infections. This indicates that, in a population living in an area endemic for malaria, blood plasma factor(s) can reduce the transmission capacity of gametocyte carriers to mosquitoes.

Introduction

Gametocytes, the sexual erythrocytic stages of *Plasmodium* species, can become apparent in the bloodstream during malaria infection. Transmission of the parasite depends on ingestion of gametocytaemic blood by the *Anopheles* vector. In the mosquito macrogametes are fertilized by microgametes, form zygotes and develop via intermediate stages to ookinetes, oocysts and sporozoites successively. Transmission can be inhibited by the reduction of the number of fertile gametocytes or by factors in the blood meal which react with gamete surface antigens and prevent fertilization or interfere with the development of zygotes into oocysts (CARTER *et al.*, 1988). Factors involved in natural transmission blocking may include antibodies (MEUWISSEN *et al.*, 1985; GRAVES *et al.*, 1988b), phagocytic leucocytes (SINDEN & SMALLEY, 1976), T cells (HARTE *et al.*, 1985), and cytokines and other serum factors (NAOTUNNE *et al.*, 1991). With *P. falciparum*, gametocytocidal drugs like primaquine and sporontocidal drugs like proguanil and pyrimethamine also prevent sporozoite development in the mosquito (COVELL *et al.*, 1955).

With the aid of monoclonal antibodies (Mabs) which are able to block transmission, several important target antigens of sexual stages of *P. falciparum* have been identified. These target antigens have molecular weights of 25, 48/45 and 230 kDa (RENER *et al.*, 1983; KUMAR & CARTER, 1984; VERMEULEN *et al.*, 1985). The mechanism of transmission blocking by antibodies directed to the 230 kDa antigen is unknown, whereas the mechanisms of transmission blocking by Mabs directed to the 48/45 kDa doublet and the 25 kDa antigen are partially identified. Both block the formation of oocysts; however, the former probably interferes with fertilization of the macrogamete, while it has been suggested that the latter interacts with a ligand of the ookinete for a receptor on the mosquito midgut wall, preventing penetration of the ookinete (VERMEULEN *et al.*, 1985; MEUWISSEN & PONNUDURAI, 1986; MEUWISSEN, 1989).

A striking difference between the mechanism of action of both antibodies was found in the transmission blocking capacity with progressively reduced numbers of parasites. As would be expected, inhibition by the anti-25 kDa Mab is strengthened when there is a lower number of gametocytes in the blood meal. The transmission blocking capacity of the anti-48/45 kDa Mab appears to be relatively reduced when the number of gametocytes in the blood meal is low (PONNUDURAI *et al.*, 1987). Transmission-inhibiting Mabs against Pfs2400, recently described as a sexual stage specific antigen, would interfere with parasite development before gamete fertilization and are supposed to inhibit the formation of extracellular gametes (FENG *et al.*, 1993).

Transmission blocking immunization with parasite antigens has been achieved in chicken, monkey and rodent models (CARTER & CHEN, 1976; GWADZ, 1976; MENDIS & TARGETT, 1979). Immunization with a recombinant 25 kDa yeast product has resulted in the production of specific *P. falciparum* transmission blocking antibodies in mice and monkeys (BARR *et al.*, 1991). In humans, transmission-blocking immunization has not yet been achieved, although the 25 kDa protein, a neoantigen of *P. falciparum* gametes, remains a major candidate antigen for a transmission blocking vaccine (KASLOW *et al.*, 1991).

While much progress has been made in immunological and molecular biological laboratory studies, a great need for specific epidemiological studies in endemic malarious areas remains (MEUWISSEN, 1989). The development of natural transmission blocking immunity in endemic populations may be important in malaria transmission and its understanding will be critical for transmission blocking vaccine studies. Field studies have been conducted to estimate infectivity of *P. falciparum* gametocytes to the vector in West Africa (MUIRHEAD-THOMSON, 1957), Papua New Guinea (GRAVES *et al.*, 1988a) and East Africa (LINES *et al.*, 1991; GITHEKO *et al.*, 1992).

Transmission blocking antibodies against *P. falciparum* were demonstrated in serum from a missionary returning from Africa, where he had lived for 28 years. Purified antibodies reacted with blocking epitopes of the 48/45 kDa proteins (MEUWISSEN *et al.*, 1985). Sera from individuals in Papua New Guinea have been examined for the presence of anti-gamete antibodies and transmission blocking properties. In about one-third of the sera, antibodies to gamete surface antigens were observed and half of these sera produced significant reduction in infectivity of *P. falciparum* gametocytes to mosquitoes, which correlated especially with the presence of precipitating antibodies against the 230 kDa protein (GRAVES *et al.*, 1988b).

Studies in Sri Lanka by MENDIS *et al.* (1987) showed that transmission blocking with *P. vivax* was correlated with titres of antibodies against air-dried gametes and that heat-labile factors, presumably complement, were involved. Transmission blocking immunity was boosted by frequent reinfection (RANAWAKA *et al.*, 1988). ZOYSA *et al.* (1988) compared the observed parameters of a *P. vivax* epidemic with those calculated in a mathematical model and concluded that an effect of transmission blocking immunity was essential to describe this malaria epidemic.

Here we report on the natural occurrence of factors that reduce transmission in an African population. Experimental infections of *Anopheles gambiae* with *P. falciparum* were performed by membrane-feeding of blood collected from local gametocyte carriers. Simultaneously, feeds were carried out using the same (autologous) blood in which the plasma had been replaced by heterologous plasma from donors without previous exposure to malaria. Infectivity of gametocytes was compared between feeds with autologous and heterologous plasma.

Materials and Methods

Gametocyte carriers

Each morning thick films were prepared from blood of patients reporting at the dispensary in Messa, an urban quarter of Yaoundé, the capital of Cameroon. After a rapid 20 min 8% Giemsa staining procedure, slides were examined for the presence of gametocytes.

Experimental infections

A strain of *A. gambiae s.s.*, originating from Yaoundé, was infected by membrane feeding with blood of the *P. falciparum* gametocyte carriers. In each experiment 2 feedings were performed: (i) feeding on blood with autologous plasma (OWN) and (ii) feeding on blood, the plasma of which had been replaced by universal donor plasma from Dutch donors without any malaria exposure (AB). Donor plasma was obtained in Holland and stored at −20°C until use.

Two lots of 2 mL of venous blood from each gametocyte carrier were collected in heparinized tubes. To avoid activation of gametocytes, the tubes, the AB plasma and the syringes used to fill the membrane feeders were kept at 37°C during all manipulations. The tubes were centrifuged for 5 min at 540 *g*. In one of the tubes the plasma was replaced by AB plasma, which had been tested previously in transmission blocking assays at the University of Nijmegen (PONNUDURAI *et al.*, 1989) and found to have no transmission blocking or enhancing activity. Membrane feeders were filled with blood from each tube and the mosquitoes were allowed to take a blood meal for 15 min. After feeding, blood-fed mosquitoes were counted and kept at 26–28°C with permanent access to a 10% sucrose solution without further blood meals.

The remaining heparinized blood was diluted 1:10 in phosphate-buffered saline and stored at −20°C for evaluation of chloroquine levels by enzyme-linked immunosorbent assay (ELISA) as described by WITTE *et al.* (1990). At the moment of venous blood collection another thick film was made, stained for 45 min with 4% Giemsa's stain and examined. Gametocyte density was estimated from the parasite:leucocyte ratio by counting against 1000 white blood cells and assuming an average number of 8000 leucocytes/μL.

Detection of oocysts

After 7 d, the surviving mosquitoes were dissected and their midguts were stained with 2% mercurochrome in distilled water to facilitate examination for the presence and number of oocysts by light microscopy.

Enrolment criteria

Experimental infections were included in the study if the following enrolment criteria were met: samples of at least 20 surviving mosquitoes in both the OWN and AB groups could be dissected on day 7 and at least one mosquito with oocyst(s) was observed in either group.

Statistical analysis

Data were analysed using the SPSS® statistical software. A monofactorial analysis of the percentage of infected mosquitoes in both groups was performed with gametocyte density or age of the gametocyte carrier as independent variables. The Wilcoxon two-sample test was used for statistical analysis of the differences between the experimental groups. Differences in group means were analysed according to distribution by the *t* test or the non-parametric test (Mann-Whitney *U* test). Differences in proportions were determined by χ^2 with Yates's correction.

Results

Gametocyte carriers

Sixty-five *P. falciparum* gametocyte carriers met the enrolment criteria. Their mean age was 18·9 years (range 6–36) and their mean gametocyte density was 212/μL (range 8–1208).

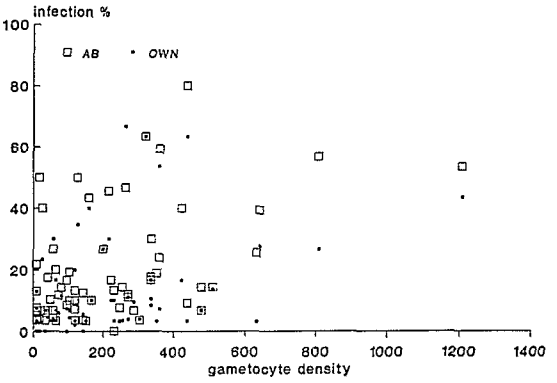

Fig. 1. The relationship between *P. falciparum* gametocyte density and the percentage of infected *A. gambiae* mosquitoes in 65 experimental infections in autologous plasma (OWN) and after plasma replacement with plasma from Dutch blood bank donors (AB).

Experimental infections

A comparison of the relationship between gametocyte density and the percentage of infected mosquitoes in the OWN and AB experimental infections is shown in Fig. 1. The gametocyte density correlated significantly with the percentage of infected mosquitoes in both the OWN (r=0·41, P<0·001) and the AB (r=0·47, P<0·001) groups. There was no correlation between the age of the gametocyte carrier and the percentage of infected mosquitoes.

Mosquitoes fed and survived equally well in both groups. 2173 mosquitoes in the OWN group and 2159

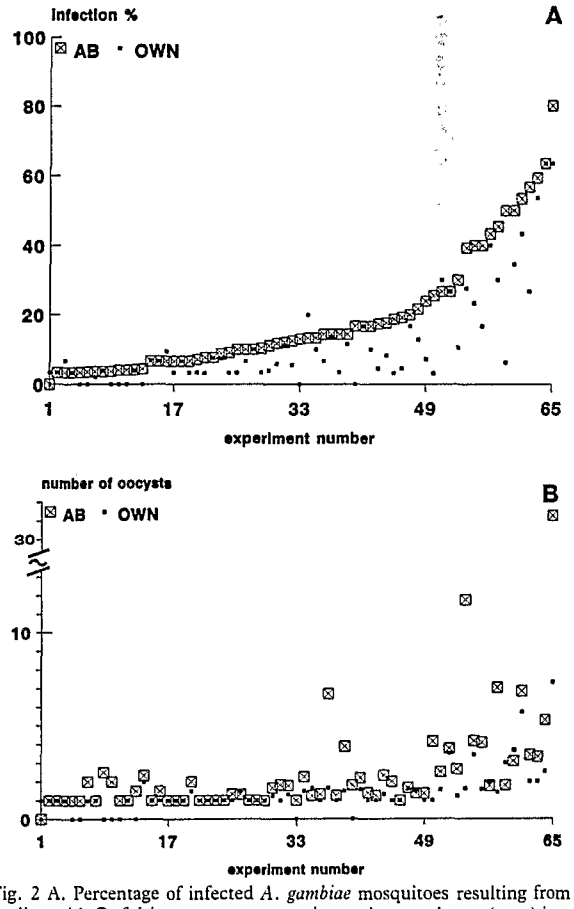

Fig. 2 A. Percentage of infected *A. gambiae* mosquitoes resulting from feeding with *P. falciparum* gametocytes in autologous plasma (OWN) in a malaria endemic area or feeding with gametocytes in replaced AB group plasma from European blood bank donors without malaria exposure; 65 experiments ranked in increasing order of infection percentage in the AB group. B. Mean number of *P. falciparum* oocysts per infected *A. gambiae* gut in 65 experiments, ranked in the same order as in A.

mosquitoes in the AB group were dissected. The difference between the mean numbers of examined mosquitoes in these groups (33·43 and 33·21, respectively) was not significant (paired samples t test: $t=0·19$, degrees of freedom (df)=64, $P=0·853$). There were 244 mosquitoes with oocysts in the OWN group and 387 in the AB group. The difference in the proportion of success in the 2 groups of infections was statistically significant ($\chi^2=28·66$, $P<10^{-7}$). The overall mean percentages of infected mosquitoes were 12·10 and 18·88, respectively, and the overall mean numbers of oocysts per midgut were 1·41 and 2·64, respectively.

The percentage of infected mosquitoes in the OWN and AB groups is shown in Fig. 2 A. A higher percentage of infected mosquitoes in the AB than in the OWN group was found for 50 of the gametocyte carriers (77%). While 8 feedings on OWN blood did not give rise to infected mosquitoes, all but one of the AB feedings infected at least one mosquito. The mean oocyst number per infected gut per experiment is shown in Fig. 2 B. A significant difference in the percentage of infected mosquitoes, as well as in the mean oocyst number per infected gut, was found between the OWN and the AB groups (Wilcoxon matched pairs test, $P<10^{-5}$ in both cases).

The relationship between age and reduction in infection, expressed as the difference between the AB group and the OWN group, is shown in Fig. 3. In 3 experiments

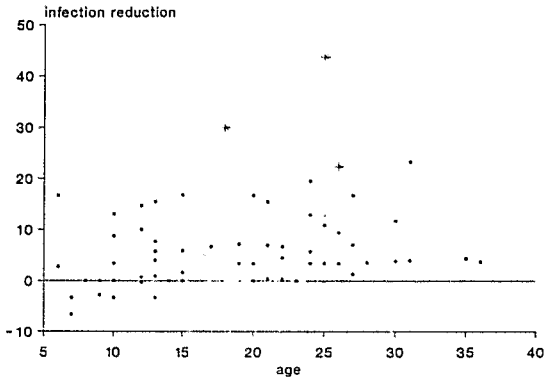

Fig. 3. The relationship between the age of the *P. falciparum* gametocyte carriers and the observed reduction in *A. gambiae* infection rate. Reduction in each experimental infection has been expressed as the percentage of infected mosquitoes in the replacement plasma (AB) group minus the percentage of infected mosquitoes in the autologous plasma (OWN) group. The correlation coefficient was 0·3 ($P<0·05$). Asterisks (*) indicate individual transmission experiments with significant differences (Yates' corrected χ^2, $P<0·05$) in the proportion of infected mosquitoes between the AB and OWN groups.

the reduction in the proportion of infected mosquitoes was significant. Based on the OWN/AB ratio, the mean reduction in the percentage of infected mosquitoes was 36·8%. There was a highly significant difference in mean reduction of percentage with age. In the age group ≤15 years the mean reduction percentage was 17·6, while in the age group >15 years it was 51·4 (Mann-Whitney U test, $P<0·005$). The reduction in the mean number of oocysts per infected gut was not significantly different in these 2 age groups (Mann-Whitney U test, $P>0·9$); this could be expected since the overall median oocyst number in infected mosquitoes was only 1 (Fig. 2b).

High transmission reducers (more than 50% reduction in OWN infection in comparison with AB infection) appeared not to differ from the other gametocyte carriers in mean gametocyte density, prevalence of trophozoites, or prevalence of fever (i.e., body temperature ≥38°C) (Table).

Chloroquine (15–600 ng/mL) was detected by ELISA in the blood of 24 (44%) of 55 gametocyte carriers. There was no relation between infectivity in the OWN group and chloroquine levels, neither could a correlation be demonstrated between chloroquine levels and reduction percentage in the 2 groups (OWN and AB).

Table. Characteristics of high transmission reducers (>50%) and low transmission reducers among 65 carriers of *P. falciparum* gametocytes

	Low reducers	High reducers	Significance of differences
Number	36	29	–
Average age (years)	15·7	22·6	$P<0·005$*
Average gametocyte density (per μL)	219	204	NS
No. with trophozoites	25 (69%)	20 (69%)	NS
No. with fever	7 (19%)	4 (14%)	NS

*Mann-Whitney U test; NS=not significant.

Discussion

Replacing the autologous plasma by non-immune plasma resulted in a higher percentage of infected mosquitoes and a higher mean oocyst load, indicating that blood plasma of naturally infected gametocyte carriers contains factors that reduce transmission or is deficient in nutrients that promote oocyst development.

The rate of occurrence of this factor(s) or deficiency in individuals from an endemic area appears to be high. In 65 experimental feeds with autologous plasma, 50 resulted in a lower percentage of infected mosquitoes compared to feeds with control plasma. In only 7 experiments was the infection percentage higher with autologous plasma, and in 8 others it was similar. The control plasma was obtained from Dutch donors and had been shown not to modulate the infectivity to mosquitoes of cultured *P. falciparum* gametocytes (PONNUDURAI *et al.*, 1989); it did not contain any chloroquine whereas plasma of 44% of the gametocyte carriers did so. However, chloroquine does not influence the infectivity of gametocytes (WILKINSON *et al.*, 1976; SMALLEY, 1977; PONNUDURAI *et al.*, 1989; CHUTMONGKOKNKUL *et al.*, 1992). The number of mosquitoes obtained for dissection in both groups was comparable, indicating no difference in preference or survival of mosquitoes given fresh autologous or frozen and thawed donor plasma, even though mosquitoes in the colony had been maintained on human blood <7 d old.

The first factor considered as a possible mediator of the transmission blocking was circulating antibody, which has been shown to block transmission of *P. falciparum* by MEUWISSEN *et al.* (1985). However, the replacement procedure did not result in the reacquisition of infectivity in every non-infective gametocyte carrier; therefore, factors other than those in the plasma must be considered. Gametocyte density is another factor which determines whether or not a gametocyte carrier is capable of infecting mosquitoes (BOYD, 1949; TCHUINKAM *et al.*, in press). Entomological factors such as differences in the speed of digestion of the blood meal in individual mosquitoes (PONNUDURAI *et al.*, 1989) and the fact that partially fed mosquitoes were not discarded in our infection experiments may also have contributed to low infection rates. The effects of ageing of gametocytes also should be considered. Treatment of gametocyte carriers with drugs that will kill only asexual stages or the early gametocyte stages prevent the formation of new generations of gametocytes. Consequently the existing gametocyte population becomes senescent and within days these gametocytes show reduced intrinsic physiological activity (PONNUDURAI *et al.*, 1986) and signs of degeneration such as coalescent pigment.

The observed increase in the extent of reduction of infectivity with age suggests that transmission-reducing activity depends on the host's total experience of *P. falciparum* malaria; this may differ from the situation described for *P. vivax* in Sri Lanka by RANAWAKA *et al.* (1988).

PEIRIS *et al.* (1988) reported enhancement of transmission of *P. vivax* malaria by low concentrations of antibody, which was also dependent on the intrinsic infectivity of the parasite isolate (GAMAGE-MENDIS *et al.*, 1992). In *P. falciparum* infections we never observed significant enhancement of transmission in the OWN group compared to the AB group. Also, in transmission experiments with cultured *P. falciparum* enhancement was never observed (PONNUDURAI *et al.*, 1987).

In order to investigate the role of antibodies, we are conducting experiments to compare the extent of transmission blocking, assessed by bio-assay (PONNUDURAI *et al.*, 1989), induced by whole sera and isolated immunoglobulin G fractions of these sera. In these experiments we hope also to determine whether the factors involved influence the fertilization of gametes or the development of zygotes into oocysts.

Not only antibodies, but also cell-mediated immunity, have been reported to play a role in transmission blocking. Immune T cells can reduce gametocyte numbers (HARTE *et al.*, 1985) and with *P. cynomolgi* it has been reported that loss of infectivity of gametocytes during the crisis is due to the presence of cytokines and crisis factors in the serum (NAOTUNNE *et al.*, 1991). Cytokine levels are correlated with parasitaemia (KWIATKOWSKI *et al.*, 1990) and have pyrogenic activity (LE & VILCEK, 1987). Our observation that high transmission reducers, although recruited from patients who had experienced malaria-like complaints, did not differ from low transmission reducers in body temperature or prevalence of trophozoites, suggests that 'crisis' serum factors and/or cytokines may not play an important role in transmission blocking in *P. falciparum* infections. Because *P. falciparum* gametocytes appear in the blood stream about 10 d after the initial onset of parasitaemia, it is not likely that factors appearing in the acute phase of the attack have an epidemiologically significant influence on the transmission capacity of circulating gametocytes.

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Corrections

F. Pratlong *et al.* (1993). Characterization of *Leishmania* isolates from two AIDS patients originating from Valencia, Spain. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87**, 705–706.

The international code numbers of 2 of the strains of *Leishmania infantum* isolated from these patients were incorrectly printed on p. 705 (3 lines from the bottom of column 2) and p. 706 (line 13 of column 1); the correct numbers are MHOM/ES/91/LEM2298 and MHOM/ES/91/LEM2361, respectively. The editor apologizes for these errors.

M. Corcos and C. Corcos (1993). A transposon in Hansen's bacillus? [Correspondence]. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87**, 708.

The authors have pointed out that the word 'its', in line 4 of paragraph 5 of their letter, appeared as 'whose' in the original typescript, and that this more clearly indicates their meaning, that it is the replication of the *plasmid* which is an epiphenomenal self-perpetuating feedback process.