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Leukocytes in a *Plasmodium falciparum*-Infected Blood Meal Reduce Transmission of Malaria to *Anopheles* Mosquitoes

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Mosquitoes are infected with *Plasmodium falciparum* by taking a blood meal from a gametocyte carrier. Since a mosquito takes a volume of 1 to 2 μl, a blood meal may contain 1×10^4 to 3×10^4 leukocytes (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 and can contribute to a reduction in the transmission of *P. falciparum* parasites.

Transmission of *Plasmodium falciparum* from man to mosquito is accomplished when a mosquito feeds on a gametocyte carrier. Once inside the mosquito midgut, the intraerythrocytic 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 processes of fertilization and sporogony, and by antibody-mediated complement-dependent lysis of the parasite (13, 15). An additional mechanism may be mediated by activated leukocytes (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 analyzed the influence of phagocytosis and its enhancement by opsonizing antibodies inside the mosquito midgut on malaria transmission.

MATERIALS AND METHODS

WBC. Ten milliliters of heparinized blood of healthy volunteers was added to 2 ml of 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 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 Pfs 48/45 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 by using the semiautomated tipper system (12). After 14 days of culture 10 ml of the parasitized erythrocyte 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 of 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 of 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 by 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 3 days the mice were given sulfadiazine in their drinking water (10 mg/ml) to suppress parasitemia, and another 2 days later the mice were bled. The blood was collected (1:10) in RPMI 1640 (Gibco BRL, Life Technologies, Paisley, Scotland, United Kingdom) without $NaHCO_3$ and containing 5 U of Na-heparin (Leo Pharmaceuticals, Weesp, The Netherlands) per ml. The mixture was kept at 37°C to prevent activation of gametocytes. All the following procedures were performed at 37°C. WBC were removed by rapid filtration through a Plasmodipur filter (Euro diagnostica, Apeldoorn, The Netherlands), and the filtered blood was centrifuged for 5 min at 1,900 × *g*. The pellet was resuspended in RPMI 1640 supplemented with 10% FCS (Integro B.V., Zaandam, The Netherlands) to give a 50% cell suspension. This was layered on a cushion of 48% Nycoprep (Nycomed Pharma AS) in RPMI 1640 with 10% FCS and centrifuged at 1,900 × *g* for 30 min. 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 min 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 μl of heat-inactivated test serum or control serum. A further 100 μl of fresh-frozen control serum was added as a complement source together with 700 μl of 10^{-4} mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma), and the vials were preincubated for 30 min at 37°C. Then, 10^6 gametes in 100 μl of PBS were added, and the CL response (peak value in millivolts) was measured for 30 min at 28°C, to mimic temperature conditions in the mosquito midgut, with a Bio-Orbit 1251-002 luminometer (LKB-Wallac).

The CL index (17) is defined as the ratio of the CL response in the presence of gametes to that in their absence.

Suspension immunofluorescence 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 erythrocytes in 1 ml of PBS. Aliquots of 10 μl of this suspension were incubated with 20-μl 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 μl of PBS followed by 5 min of centrifugation at 200 × *g*. The cell suspension was incubated in fluorescein isothiocyanate-conjugated sheep anti-

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

Serum[a]	Titer
T1	40
T2	320
T3	40
T4	80
T5	40
T6	320
T7	320
T8	160
Co1	<20
Co2	<20
Co3	<20
C1	80
C2	160
C3	40
C4	160
C5	320
C6	80
C7	40
C8	80
C9	320
C10	160
C11	40

[a] T1 to T8, Tanzanian sera; Co1 to Co3, control sera; C1 to C11, Cameroonian sera.

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 with an incident-light fluorescence microscope at a magnification of ×500. The highest dilution of a serum showing fluorescence on the gamete surface was defined as the antibody titer.

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 blood meal were 12,000/μl for WBC and 1,000/μl for gametocytes. For removal of WBC, blood was filtered with a Whatman CF11 cellulose column (11). For the transmission assay, a 14-day-old culture, containing about 0.3 ml of packed cells with 0.5 to 1% mature gametocytes, was centrifuged at 500 × *g* for 2 min. The pellet was carefully mixed with 3.75 ml of 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 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 from each feeder were dissected and oocysts were counted.

Cameroonian sera were analyzed 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 and compared to those of three controls from areas where the disease is not endemic, which each should give at least a 90% mosquito infection. Reductions were calculated by using the log transformed arithmetic mean of oocyst numbers and were expressed as *R* (reduction) values (7). *R* values are classified in three categories, which define sera as either blocking ($R > 0.9$), reducing ($0.3 \leq R \leq 0.9$), or nonblocking ($R < 0.3$).

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

Statistical analysis. The Mann-Whitney U test was used for statistical evaluation of the differences in mean oocyst numbers and CL measurements. The χ^2 test was used for analysis of the percentage of 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 (0.5 mg/ml), the geometric mean for 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 (0.5 mg/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 cytospin 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 titers of the individual sera. All sera from areas of endemicity showed a titer of at least 1:40 and an enhanced CL response compared to sera from regions where the disease is not endemic, indicating the presence of gamete surface-specific antibodies. There is no correlation between the titer and the level of the CL index. 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 a control. Figure 2 shows that only *P. falciparum* gametes were capable of inducing 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 nonblocking in both instances ($R < 0.3$). Sera C2, C3, C4, C5, and C6 changed from nonblocking to reducing ($0.3 \leq R \leq 0.9$) when WBC were present. For the transmission-reducing capacity of 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* was >0.9. The transmission capacity of sera from areas where malaria is not endemic was never influenced by the presence or absence of

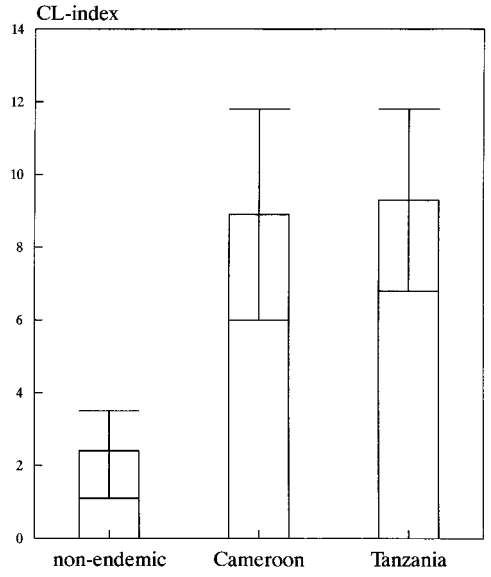

FIG. 1. The CL response of WBC induced by gametes in the presence of control sera or sera from areas of endemicity. Bars show the means ± standard deviations of the CL index. The CL index is the maximum CL response (in millivolts) with gametes/the maximum CL response without gametes. Significance is 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 the Tanzanian sera ($n = 11$).

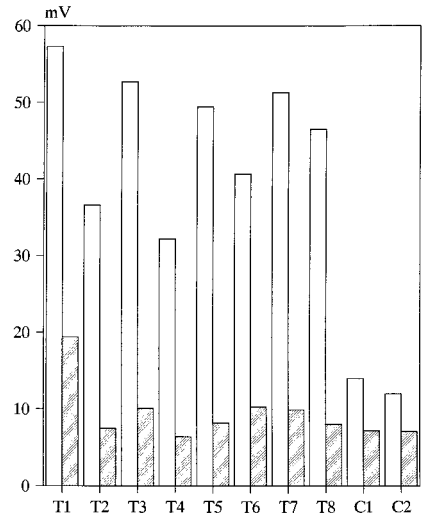

FIG. 2. CL response (in millivolts) of Tanzanian sera induced by *P. falciparum* gametes with *P. berghei* gametes as a control. □, *P. falciparum* gametes; ▨, *P. berghei* gametes. A total of 10^6 purified *P. falciparum* or *P. berghei* gametes were used.

WBC. Although all sera from areas of endemicity showed a significantly enhanced CL response compared to nonblocking control sera, there was no correlation between the level of the CL response and the level of transmission reduction of individual sera.

Table 2 shows the WBC-dependent transmission reduction in Tanzanian sera. 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 of transmission irrespective of the presence of WBC.

DISCUSSION

The principal finding of our study is that the presence of WBC in a blood meal 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 the transmission reduction of

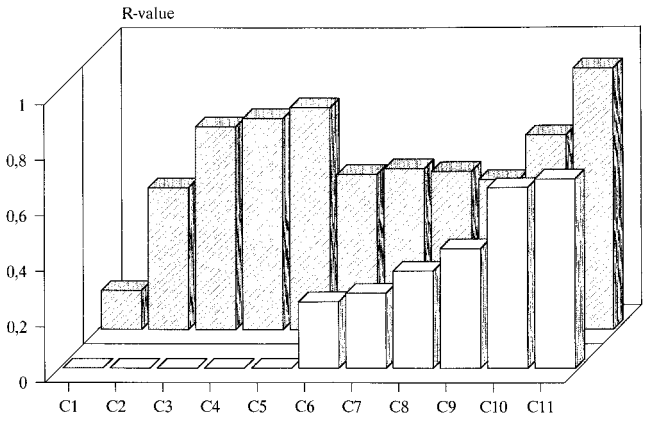

FIG. 3. Effect of WBC present in the mosquito blood meal on the capacity of sera from Cameroon (C1 to C11) to reduce transmission. ▨, WBC present; □, WBC absent. $R = (T_c - T)/T_c$, where T_c = mean oocyst number resulting from a control serum and T = mean oocyst number resulting from a test serum. R (reduction) values are categorized as follows: $R < 0.3$, serum is nonreducing; $0.3 \leq R \leq 0.9$, serum is reducing; $R > 0.9$, serum is blocking.

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

Serum[a]	No. of positive mosquitoes/total no. of mosquitoes dissected (%)	
	Without WBC	With WBC[b]
T1	16/20 (80)	3/20 (15)[c]
T2	11/20 (55)	0/20 (0)[c]
T3	8/20 (40)	2/20 (10)[c]
T4	5/20 (25)	4/20 (20)
T5	3/20 (15)	2/20 (10)
T6	2/19 (11)	1/20 (5)
T7	0/20 (0)	0/20 (0)
T8	0/20 (0)	0/20 (0)
Co1	17/20 (85)	13/20 (65)
Co2	14/20 (70)	11/19 (58)

[a] T1 to T8, Tanzanian sera of clinical malaria patients; Co1 and Co2, control sera.
[b] A total of 10^4 viable WBC/μl were added to the blood meal prior to the membrane feeding.
[c] $P < 0.05$. The χ^2 test was used.

P. falciparum malaria and in phagocytosis inside the mosquito midgut (9, 19). It has been demonstrated that transmission reduction can be obtained by the presence of specific anti-Pfs 48/45 and anti-Pfs 230 MAbs in the mosquito blood meal (13). Moreover, transmission-blocking antibodies are present in human sera from areas where malaria is endemic (4, 16). In the selected sera from two different areas of endemicity, the presence of antibodies recognizing surface antigens was demonstrated by a positive SIFA on living gametes. No correlation could be found between antibody titer and transmission reduction. All tested sera from areas of endemicity 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 of the specificity of this interaction. The lack of correlation between final WBC-dependent transmission reduction, the antibody titer, and the magnitude of the CL response could be explained by differences in affinity and concentrations of specific antibodies. CL measurements reflect WBC-gamete interactions 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 a reduced affinity of antibody is not necessarily less effective. The observed effects of WBC on transmission could be even more pronounced in direct-feeding experiments than in 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, so 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 opsonization have been demonstrated on numerous occasions in sera from areas of endemicity (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 opsonizing 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.

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