Original Article

Natural Infection of *Plasmodium falciparum* Induces Inhibitory Antibodies against Gametocyte Development in Human Hosts

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SUMMARY: We identified naturally induced antibodies from malaria patients in Thailand and clarified the effect of the antibodies on gametocyte development. Fifty-nine percent of the *Plasmodium falciparum*-infected blood samples (17 of 29) fed to female *Anopheles* mosquitoes showed no oocyst infection. Seventeen percent of the samples (5 of 29) distorted the morphology and hampered the maturity of the gametocytes. A possible mechanism for the gametocyte inhibitory activity was shown by the binding of the plasma antibodies to live, immature, intraerythrocytic gametocytes during the incubation period. One hundred fifty-seven proteins specific to different gametocyte stages were explored to find the targets of the antisera that bound to the live gametocytes. However, no additional gametocyte transmission- blocking vaccine candidate was detected. Therefore, the development of alternative transmission-blocking vaccines in high-transmission areas should focus on the identification of more gametocyte antigens-inducing inhibitory antibodies that reduce gametocytemia.

INTRODUCTION

Gametocytes, sexual stages of the *Plasmodium* parasite, are essential for the transmission of malaria from humans to *Anopheles* mosquitoes and the subsequent transmission of malaria from the mosquitoes to other human hosts. Blocking of gametocyte development in the human hosts or blocking of gamete fertilization in the mosquito vector will prohibit the spread of malaria. Transmission-blocking vaccine (TBV) candidates target proteins at the gametocyte and mosquito stages (1). The gametocyte antigens reported to date as TBV candidates, i.e., Pfs230 (2) and Pfs48/45 (3), are also expressed in gametes. These antigens can induce antibody formation during a natural infection, and the acquired antibodies show transmission-reducing activity (TRA) in malaria-infected patients in endemic regions (4,5).

Most studies have focused on the TRA of the antibodies that function in the mosquito midgut (6). However, antibodies with TRA that inhibit gametocyte development could present an alternative strategy for TBV development. In this study, we identified antibodies against gametocytes in *Plasmodium falciparum*-infected humans, identified the target proteins of the antibodies, and determined the effect of the antibodies

on the maturation of gametocytes. In addition, this study confirmed the possibility to intervene across a wider range of gametocyte developmental stages and thus eradicate malarial transmission.

MATERIALS AND METHODS

Gametocyte culture and gametocyte enrichment: *P. falciparum* strain AMB47, isolated in Thailand, was maintained under in vitro culture conditions as described by Trager and Jensen (7). AMB47 continuously produced gametocytes in in vitro cultures upon induction as described by Ifediba and Vanderberg (8). Briefly, a schizont-predominant culture was diluted with 0.2% parasitemia and 5% hematocrit using O-positive erythrocytes in RPMI 1640 medium (Gibco, Grand Island, N.Y., USA) supplemented with 25 mM HEPES (Sigma, St. Louis, Mo., USA), 0.36 mM hypoxanthine (Sigma), 40 μg/ml gentamicin (Gibco), and 10% non-inactivated human serum. The medium was changed daily without the addition of fresh erythrocytes. Gametocytogenesis appeared within 1 week, and after 2 weeks, mature gametocytes in a Giemsa stained blood smear were examined under a microscope. Enrichment of the gametocytes was performed by gradient centrifugation (1,500 g for 15 min) on 47% Percoll at 30°C.

Blood samples: (i) Parasite collection: *P. falciparum*-infected blood was collected in a heparinized tube from malaria patients (n = 31) at the Malaria Clinic, Mae Sot, Tak province. The infected blood was individually fed to *Anopheles* mosquitoes by replacing the plasma

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with normal AB serum as described previously (9). Nine to 11 days post feeding, oocyst production was evaluated for infectivity of the gametocytemic blood. All participants provided informed consent according to the approved protocol of the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army.

(ii) Plasma collection: Plasma samples from the patients identical to the parasite donors described above ($n = 31$) were collected at Malaria Clinic, Mae Sot, Tak province, and before the plasma was used, it was heat inactivated at 56°C for 30 min. Plasma samples from healthy malaria-naive donors that live in malaria-free regions were used as controls ($n = 10$).

Detection of anti-gametocyte antibodies: (i) Antibodies that bind to air-dried gametocytes: The anti-gametocyte antibodies in the plasma samples were quantified by the immunofluorescence assay (IFA). The enriched gametocytes prepared according the above-mentioned procedure were collected and washed twice with warm incomplete medium and were then adjusted to a concentration of 2×10^8 gametocytes/μl in phosphate-buffered saline (PBS). One microliter of the gametocyte suspension was spotted in each well of multi-well slides, air-dried, acetone-fixed, and stored at -20°C for the IFA. The slides were blocked with 5% skim milk and incubated with plasma from the *P. falciparum*-infected donors that had been serially diluted 4-fold in PBS (1:25 to 1:25,600). The anti-gametocyte antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG antibodies (1:200) (Dako, Glostrup, Denmark). The stained slides were then examined under a fluorescent microscope. Each incubation step was performed at 37°C for 1 h.

(ii) Antibody binding to live gametocytes: Gametocyte cultures (days 9 or 14) were incubated with a Pfs230-specific rabbit antiserum (at a 100-fold final dilution) at 37°C for 24 h. The erythrocytes were harvested from the cultures and washed twice with 10 ml PBS. The erythrocyte pellet was suspended as a 4% hematocrit solution, and 1 μl of this solution was spotted in each well of a multi-well slide. The slide was rapidly dried, and stored at -80°C. For antibody staining, the multi-well slide was dried and fixed with cold acetone for 10 min, and then blocked by incubation with 5% skim milk at 37°C for 1 h. The blocking buffer was removed and the slides were incubated for 30 min with a 500-fold diluted Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Molecular Probes, Eugene, Oreg., USA) and 4′,6-diamidino-2-phenylindole (hydrochloric acid salt, 1 μg/ml; Wako Pure Chemical, Osaka, Japan). The slides were washed and mounted with anti-fade (Prolong Gold; Invitrogen, Carlsbad, Calif., USA) and examined under a confocal scanning laser microscope (LSM5 PASCAL; Carl Zeiss Micro Imaging, Thornwood, N.Y., USA).

Incubations of the gametocytes with the patient plasma or the healthy donor plasma at final ratios of 1:25 were performed as described above. The antibodies bound to the live gametocytes were detected using a one-step staining with FITC-conjugated rabbit anti-human IgG antibodies (1:200) (Dako), and then detected under a fluorescent microscope.

Inhibition of gametocyte maturation: Seven-day-old gametocyte cultures were co-cultivated with the patient plasma or the healthy donor plasma (10% final concentration). The cultures were prepared in duplicate on two independent occasions. The cultures were maintained by daily changing the medium containing the patient plasma until day 15. Thin smears were prepared on day 15, and the smears were stained with Giemsa stain for microscopic examination. Parasitemias were evaluated, and 400 gametocytes were counted and classified into stages I to V. The results were expressed as percentages for various stages. The number of gametocytes with an abnormal morphology or a crisis form was recorded. The number of stage I to V gametocytes was statistically compared for the cultures containing the malaria patient plasma and the normal plasma.

Characterization of gametocyte-specific proteins: One hundred fifty-seven gametocyte genes, specifically or highly expressed at the gametocyte stage (10), were cloned. To produce gametocyte-specific proteins, small-scale transcription and translation were performed with a bilayer wheat germ cell-free expression system as described elsewhere (11). The synthesized proteins were used in an enzyme-linked immunosorbent assay (ELISA) with oocyst inhibitory plasma (OIP), oocyst non-inhibitory plasma (ONP), and normal plasma. Briefly, each of the 157 gametocyte-specific proteins produced with the wheat germ system was diluted 50-fold with 1 × PBS at pH 7.4, coated in duplicate wells of the ELISA plate (Maxi; Nunc, Roskilde, Denmark), and incubated overnight at 4°C. The plates were blocked with 0.5% gelatin in PBS-Tween (PBS-T). This blocking step was followed by the addition of the patient plasma or the normal plasma (diluted 100-fold in PBS-T) and incubation for 1 h at 37°C. The reaction was detected by the addition of a 2,000-fold dilution of the rabbit anti-human IgG/HRP (Dako) followed by incubation for 1 h at 37°C. The plates were washed and subsequently incubated with the ABTS® peroxidase substrate (KPL, Gaithersburg, Md., USA) at room temperature for 30 min, and the optical density (OD) at 405 nm was measured with a microplate reader (Wallac Victor; Perkin-Elmer, Rodgau-Jugesheim, Germany). Negative control wells coated with non-gametocyte proteins were included in each plate. The OD of each well was divided by the OD of the negative control well to provide the fold increase. The increase of each protein was compared for the three groups of plasma (OIP, ONP, and normal plasma) using the Kruskall-Wallis H test. Protein tests were considered positive when the number of fold increase was significantly different for the three plasma groups.

Data analysis: The quantified gametocyte and asexual stage parasites that had been co-cultivated with normal plasma or malaria patient plasma were analyzed with the nonparametric Kruskal-Wallis H test using the GraphPad Prism software. A significant difference has a P value ≤ 0.05.

RESULTS

Quality of the blood specimens: Microscopy showed that 29 of the 31 blood samples contained gametocytes. Twelve of the 29 gametocytemic blood samples infected

the mosquitoes and produced 1 to 209 oocysts per mosquito (mean number of oocysts = 45) in the membrane-feeding assay. Plasma from these patients was denoted ONP. Conversely, 17 of the 29 gametocytemic blood samples did not infect the mosquitoes and could therefore not form oocysts. Plasma from these patients was denoted OIP. The other two blood samples had no gametocytes and did not infect the mosquitoes. Because we hypothesized that the mosquitoes could not be infected because of gametocyte defects, we excluded these two non-gametocytemic blood samples from further experiments.

Antibodies to gametocytes of *P. falciparum*: All of the 12 ONP plasma samples had anti-gametocyte antibodies with IFA titers from 1:100 to 1:6,400 (median = 1:400). Fifteen of the 17 OIP samples had lower anti-gametocyte IFA titers from 1:25 to 1:400 (median = 1:100). Two of the OIP plasma samples had no detectable anti-gametocyte antibody.

Binding of antibodies to live-intraerythrocytic gametocytes: We hypothesized that antibodies can intervene with gametocyte development during gametocyte maturation in human hosts. To support this hypothesis, preliminary experiments were performed to elucidate the mechanism of transmission blockage during natural infections. Live gametocytes were incubated with rabbit anti-Pfs230 antibodies for 24 h before IFA staining. The rabbit anti-Pfs230 antibodies specifically bound to the surface of the live-intraerythrocytic gametocytes
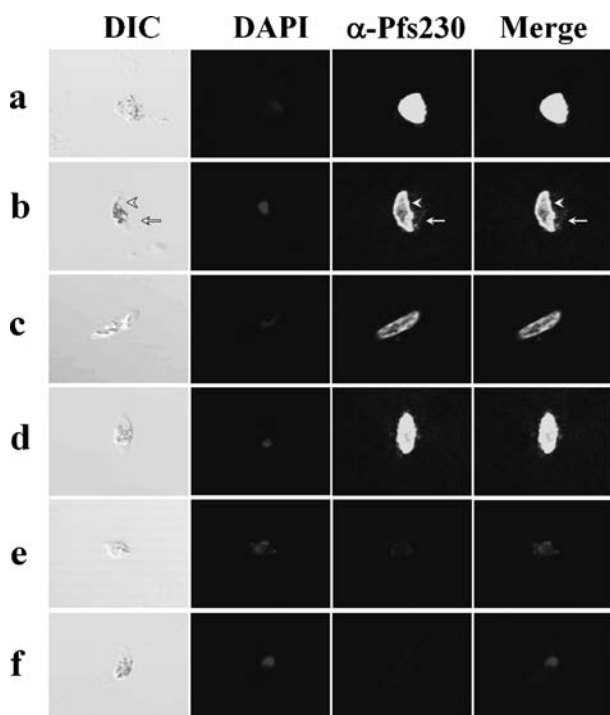

Fig. 1. Indirect immunofluorescence assay of live intraerythrocytic gametocytes of *Plasmodium falciparum* incubated with rabbit anti-Pfs230 antibody. DIC, morphology of gametocyte under different interface contrast; DAPI, parasite nuclei shown with DAPI staining. (a) stage II. (b) stage III. (c) stage IV. (d) mature stage V gametocyte, reacted with anti-Pfs230 immune rabbit serum. (e) stage II. (f) mature stage V gametocyte, reacted with preimmune rabbit serum. Arrow head, intraerythrocytic gametocyte; arrow, gametocyte-infected erythrocyte membrane.

(Fig. 1) and did not bind to proteins in asexual stage parasites in the same culture (data not shown). Moreover, the antibodies bound to stage II, III, and IV immature gametocytes (Fig. 1a, b, and c, respectively) and to mature gametocytes (Fig. 1d). No binding was observed for uninfected erythrocytes. Preimmune rabbit serum did not show any binding when incubated with gametocytes at various developmental stages (Fig. 1e and f).

Antibodies in the malaria patient plasma also bound to proteins present at all of the stages of the live-intraerythrocytic gametocytes. The OIP antisera reacted more strongly than the ONP antisera (Fig. 2a and b) and the schizonts in the infected erythrocytes (data not shown). Immunoglobulin was not detected in the gametocytes incubated with normal human plasma (Fig. 2c).

Effects of antibodies on gametocyte maturation: To support our hypothesis that antibodies that target live gametocytes can effectively intervene with the development of gametocytes in humans, gametocyte maturation was examined in vitro during co-cultivation of stage I gametocytes with OIP, ONP, or normal plasma. The results showed that 5 of the 29 (17.2%) *P. falciparum*-infected plasma samples reduced the number of gametocytes and inhibited gametocyte maturation in the cultures. These five plasma samples belonged to the OIP group, and they contained a significantly reduced number of immature and mature gametocytes (stage II, $P = 0.03$; stage III, $P = 0.03$; stage IV, $P = 0.01$; and stage V, $P = 0.01$) (Fig. 3a). Moreover, the number of gametocytes of the crisis form ($P = 0.004$) was significantly higher in the OIP group than in the normal plasma group and the ONP group (Fig. 3b). In addition, the number of asexual stage parasites (mean = 0.03%; $P = 0.02$) was significantly lower in the OIP group than the ONP group (mean = 0.13%) and the normal plasma group (mean = 0.88%) (Fig. 3c). The number of gametocytes was also significantly lower in
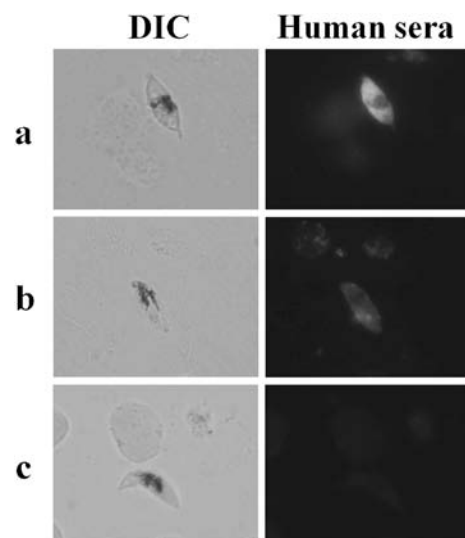

Fig. 2. Indirect immunofluorescence assay of live intraerythrocytic gametocytes of *Plasmodium falciparum* incubated *P. falciparum*-infected patients. Mature stage V gametocytes reacted with oocyst inhibitory plasma (a), oocyst non-inhibitory plasma (b), and malaria naive plasma (c).
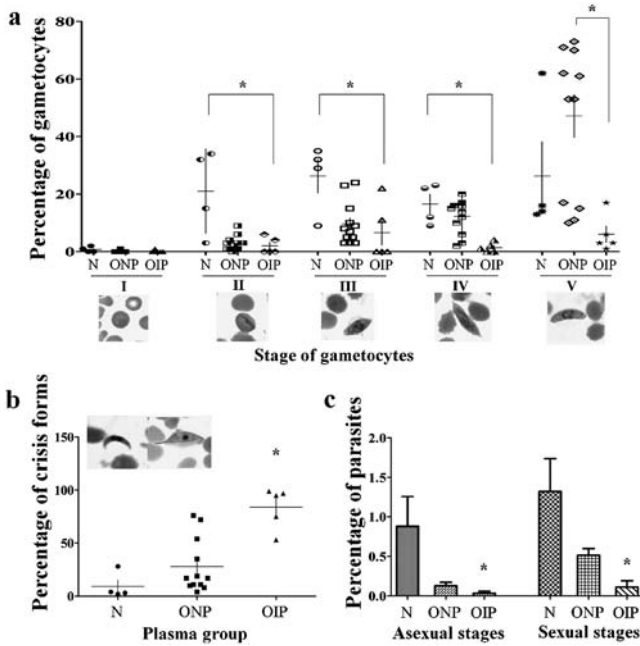
Fig. 3. The effects of human plasma from malaria patients on maturation of *Plasmodium falciparum* gametocytes. Plasma with various activities, malaria naive plasma (N) ($n = 4$), oocyst non-inhibitory plasma (ONP) ($n = 12$), or oocyst inhibitory plasma (OIP) ($n = 5$), co-cultivated with 7-day-old gametocyte cultures until day 15 and examined by microscopy of Giemsa stained blood films. The data are shown in mean percentages of gametocytes from stage I to V (a), mean percentages of crisis form gametocytes (b), and number of asexual and sexual stage parasites (c). * indicates significant difference at $P \leq 0.05$ by Kruskal-Wallis H Test. Bars, mean percentages of parasites; vertical line, standard error of the means; inset photos, stage I to V gametocyte (a) and various crisis form gametocytes (b).

the OIP group (mean = 0.11%; $P = 0.01$) than in the ONP group (mean = 0.51%) and the normal plasma group (mean = 1.32%) (Fig. 3c).

Screening for gametocyte-specific proteins with human antisera: To identify the target proteins of human antisera that inhibited gametocyte maturation, 157 genes encoding gametocyte proteins were expressed and tested with the malaria patient plasma. Ninety-one percent of the genes (143 of 157) could be transcribed and translated in the wheat germ cell-free system. The mean values of the increased number of individual proteins were compared for the OIP, ONP, and normal plasma groups; however, there was no significant difference between the antibodies of the OIP and the ONP groups.

DISCUSSION

It is unclear why the rate of gametocytemia is higher in people that live in areas with a high malaria endemicity than in people who live in areas with a low endemicity. Naturally induced antimalarial antibodies against *P. falciparum* gametocytes may be a contributing factor to this phenomenon. Studies in high-transmission regions have revealed that *P. falciparum* patients produce anti-gametocyte antibodies (4,5). These studies have also described antibodies that developed against immature and mature gametocytes during natural infections. Thailand is considered a low-transmission area where game-

tocytemic and non-gametocytemic patients elicit naturally induced antimalarial antibodies against immature and mature gametocytes. The gametocytemic rate in the present study was high because we deliberately collected *P. falciparum* samples for mosquito feeding; therefore, gametocytemic blood samples were required. This criterion limited us to a very small sample size and led to the overestimation of the gametocytemic rate, which was lower in other studies that used all *P. falciparum* samples (12–14).

This study aimed to investigate the effects of antibodies on gametocyte development. The recent notion that immunity to sexual stages is short-lived and thus requires a repeated exposure to the gametocyte antigens supports the association between anti-gametocyte antibodies and gametocytemia (15). Possible causes for antibody elicitation in intraerythrocytic gametocytes include: (i) gametocyte-infected erythrocytes are acquired by phagocytes (16) that eventually activate the cell-mediated immunity response resulting in the production of antibodies, and (ii) intraerythrocytic gametocytes produce and release exoantigens (17) in the blood circulation that then induce the host immune response.

In this study, the anti-gametocyte antibodies might reduce fertilization and oocyst production by affecting the maturation of young gametocytes. We showed for the first time that the antibodies permeated the gametocyte-infected erythrocytes and bound to intraerythrocytic gametocytes at an early developmental stage (stage II). This permeation may occur because of passive diffusion through an impaired erythrocyte membrane (which might result from extended presence of erythrocytes in the blood circulation) or perhaps due to the previously described parasite-modified transportation network (18).

On the basis of our in vitro gametocyte co-cultivation experiments, gametocyte-specific antibodies in the OIP group may reduce gametocytogenesis, thereby reducing the number of stage II gametocytes. In contrast, the ONP group did not reduce gametocytogenesis. Therefore, the co-cultivation assay is a suitable pretest to estimate the transmission inhibitory activity of a test serum and can be used in laboratories that lack an *Anopheles* insectary. Blocking of gametocytogenesis should be evaluated properly on the basis of the number of stage I gametocytes. However, this could not be performed because the antisera was tested when stage I gametocytes had appeared in the cultures. In addition, asexual stage parasites were also affected during the co-cultivation in the presence of OIP. Antibodies to asexual stage parasites may regulate the number of asexual parasites, the precursors of the gametocytes, and consequently reduce gametocytemia (19). Moreover, other circulating plasma components, including complement, tumor necrosis factor, and interferon-γ kill blood-stage parasites in monkey malaria (20) and human malaria (*P. vivax* and *P. falciparum*, respectively) (21,22).

Because of the absence of the autologous antimalarial plasma in the blood-feeding assay (using gametocytes from patient blood samples), the lack of mosquito infections could be explained in two ways. First, the defect in the gametocytes might appear before the membrane-feeding assay because of the binding of the antibodies to the developing intraerythrocytic gameto-

cytes, resulting in oocyst inhibition. Second, the innate immune system of *Anopheles* vectors (by encapsulation and melanization of *Plasmodium* ookinetes) could result in the inhibition of oocyst development (23). We concluded that oocyst inhibition resulted from a gametocyte defect because the mosquitoes in the assays were from the same batch. Based on the membrane-feeding assay and the maturation-inhibition assay, the anti-gametocyte antibodies and other immune system components destroyed the immature gametocytes residing in the human host during the long maturation process.

The OIP inhibited gametocyte development more effectively than the ONP. Contrary to our expectations, the IgG antibody IFA titer of the gametocyte native proteins in the OIP sample was lower than the IFA titer with the ONP sample because the malaria parasites can induce the production of polyspecific antibodies during natural infections. The human antisera used in this study contained polyspecific antibodies against native proteins in the malaria parasite. Although the OIP sample might contain lower levels of polyspecific antibodies, these antibodies might bind specifically to essential proteins involved in gametocyte development or transmission. To detect specific gametocyte antigens, 157 gametocyte-specific proteins were screened with ELISA; however, no proteins were differentially detected with the OIP and ONP samples. More than 300 proteins are expressed exclusively in the gametocyte (10); thus, screening with a larger number of proteins may help in identifying specific target proteins for the inhibitory antibodies. Therefore, the gametocyte proteins that influence the transmission blocking activity remain to be discovered. The characterization of antimalarial antibodies that bind to large number of gametocyte antigens will facilitate the development of TBVs.

Conflict of interest None to declare.

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