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Antiparasite Adherence Activity in Thai Individuals Living in a *P. falciparum* Endemic Area

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SUMMARY **Two types of antimalaria antibodies in the serum of 54 villagers living in a malaria endemic area of Thailand were determined by indirect immunofluorescence assay in order to define the status of malaria immunity within the group. Antibodies to parasite-derived antigens in the membrane of ring stage-infected erythrocytes were very high (≥ 1:1,250) in 44%, moderate to low (≤ 1:250) in 37% of the sera, and the rest did not have the antibody. However, all the sera had antibodies to antigens of the intraerythrocytic mature parasites, showing a very high level in 65%, and moderate to low levels in 37% of the sera. Sera with high antibody titers to either type of antigen significantly inhibited cytoadherence of *P. falciparum*-infected erythrocytes. All the sera variably inhibited rosette formation of the parasites but showed no association with the antibody titers. These results suggest that the antibodies to cytoadherence and rosette formation can be elicited and sustained in the malaria experienced host while living in the endemic area. This may be a natural preventive mechanism against the severity of *P. falciparum* infection in the infected host. How long the antiparasite adherence activity will last remains to be investigated.**

Plasmodium falciparum infection elicits both protective and nonprotective antibodies in the malaria experienced host. A high level of antimalaria antibodies produced by the host living in a malaria endemic area has been shown to correlate with reduced clinical manifestations but not with disease protection.[1,2] These antibodies can be characterized by immunofluorescence assay into 2 types i.e., erythrocyte membrane immunofluorescence assay (EMIF) and indirect immunofluorescence assay (IFA) which recognise ring stage antigens (RESA) and mature stage antigens of the parasite, respectively. Antimalaria antibodies against several major malaria antigens are shown to prevent merozoite invasion in *in vitro* studies, and thus terminate parasite multiplication.[3-5] Some antimalaria antibodies interfere with adherence properties of the infected erythrocytes in the *in vitro* assay,[6-8] the effects which may facilitate parasite clearance *in vivo*. The antiadherence antibodies are shown to have restricted activity to autologous parasite strain due to a high diversity of the adherence molecules.[9,10] However, a number of malaria antibody positive adults may acquire cross reaction against the diversed adherence molecules, provided they have been exposed regularly to malaria. We therefore investigated the prevalence of antiparasite adherence activity in villagers living in a small district of malaria endemicity of Thailand.

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MATERIALS AND METHODS

Study population

Blood samples were collected from 54 villagers between 15-55 years old living in a small district of Trad province on the eastern border of Thailand. Thick blood films were examined by light microscopy for different plasmodia species, and the number of parasites per 200 leukocytes was then determined. Sera were separated, heat-inactivated at 56°C and stored at -70°C until assay. Five normal sera obtained from healthy persons who had never had experience of malaria infection were used as control.

P. falciparum culture and antigen preparations

A Thai *P. falciparum* isolate having both cytoadherence property and rosette formation was maintained under *in vitro* culture conditions[11] at 37°C, 5% CO_2 and air mixture, in human group O erythrocytes, using RPMI-1640 medium at pH 7.4 containing 40 μg/ml of gentamicin, supplemented with 25 mM HEPES, and 10% heat-inactivated human serum. Synchronized culture was prepared by lysis in 5% sorbitol.[12]

Ring stage-infected erythrocytes were used to prepare thin blood films for determination of antibody to ring-infected erythrocyte surface antigen.[13] Infected erythrocytes with mature stage parasite were used to prepare thin blood films for determination of antibody to late stage malaria antigen. Both types of blood films were prepared from phosphate-buffered saline (PBS)-washed infected erythrocytes, air dried, fixed with methanol, and stored at -20°C until used.

Immunofluorescence assay

An erythrocyte membrane immunofluorescence assay (EMIF)[13] using blood film of ring-stage parasites, and a conventional immunofluorescence assay (IFA) using blood film of mature-stage parasites were performed by incubating each serum at 1:50 dilution on a demarcated area of the blood films for 30 minutes at room temperature. After washing with PBS, goat antihuman immunoglobulin conjugated with fluorescein isothiocyanate was added onto each spot on the blood films for 30 minutes. Stained blood films were rinsed in PBS and the parasites were counterstained with ethidium bromide, mounted with 50% glycerol, and examined under ultraviolet light microscope. All sera showing positive parasite staining were titrated to determine the titer of antimalaria antibodies in both EMIF and IFA.

Target cells

A C32 melanoma cell line was maintained at 37°C, 5% CO_2 and air mixture in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 40 μg/ml gentamicin. To prepare for cytoadherence assay, 10^5 melanoma cells in 0.5 ml were cultured on sterile cover glasses for 15 hours, rinsed and fixed with 1% formalin in PBS for 1 hour, then stored in PBS under refrigeration until used for no longer than one month.

Cytoadherence inhibition assay

Cytoadherence assay was performed by incubating the formalin-fixed C32 melanoma cells with 1 ml of 1% mature stage infected erythrocytes under cell culture conditions for 1 hour with a gentle agitation at every 15 minutes. Unbound erythrocytes were rinsed off with PBS and the erythrocytes bound on C32 melanoma cells were fixed with 1% glutaraldehyde in PBS, washed with water, stained with Giemsa, and examined with a light microscope (x 1,000 magnification).

Cytoadherence inhibition assay was performed by preincubating an equal volume of the pack erythrocytes from the parasite cultures and the malaria immune serum for 30 minutes at 37°C. The serum-treated erythrocytes were then resuspended in 1 ml RPMI-1640 medium and cytoadherence with C32 melanoma cells was performed as described above. The number of infected erythrocytes bound to 100 melanoma cells was counted, and the percentage of inhibition was determined by comparing with the cytoadherence of the infected erythrocytes preincubated with normal human serum as control.

Inhibition of rosette formation

Ring stage parasites cultured at 3% parasitemia and 2% hematocrit was added into a 96-well flat-bottomed plate for 100 μl per well. To enumerate the number of rosettes, an infected erythrocyte binding to two or more uninfected erythrocytes, acridine orange was added into each well at a final concentration of 10 μg/ml.[14] One drop of the culture from each well was then placed on a glass slide, mounted with a cover slip, and 500 infected erythrocytes were examined with an ultraviolet light microscope. To inhibit rosette formation, an equal volume of the sera was added in each well, mixed, and incubated under parasite culture conditions for 24 hours. A pool of serum from

individuals who never had malaria was used as control. The number of rosettes was expressed as a percentage of mature stage parasites. The percentage of rosette formation inhibition was then determined by comparing with the number of rosettes in the control cultures.

Statistical analysis

To determine the correlation between each group, the statistical *t*-test was used. Significant difference between the group was analysed by SPSS and GLIM software packages. Percentage inhibition of cytoadherence and rosette formation in each group of EMIF and IFA were tested for homogeneity by analysis of variance (F-test) with Bartlett-Box F and showed no homogeneity. The data were then transformed by inverse sine transformation (arcsin square root) in order to obtain homogeneity of the variances. This transformation is applicable to binomial data expressed in percentages.[15] The number of parasites in each group of EMIF and IFA were compared by analysis of variance as above, and show no homogeneity. The data were therefore logarithmically transformed, which then equalised the variances for an appropriate analysis.

RESULTS

Incidence of parasitemia and prevalence of antimalaria antibodies in Thai villagers

Parasitemia was found in 35 of 54 villagers ranging from 1 to 500 parasites per 200 leukocytes. Blood films from 19 villagers showed negative parasitemia by the microscopic determination. Of the 35 parasite positive villagers, 31 had *P. falciparum* infection, 2 had *P. vivax* infection, and the other 2 had mixed-infection with *P. falciparum* and *P. vivax*. All villagers had lived in the malaria endemic area and had had a history of *P. falciparum* infection.

Table 1 summarizes different types of malaria infection among villagers, and the number of villagers having antimalaria antibodies to *P. falciparum* antigens. Normal sera tested at 1:50 were negative on both EMIF and IFA. Lower dilution of these normal sera gave a low background of red cell staining. All the sera were then studied at 1 in 50 dilution. Forty-four of the 54 villagers (81%) had antibody to the ring-infected erythrocyte surface antigen (RESA) of *P. falciparum* as determined by EMIF assay. Twenty of these villagers (37%) had low to moderate EMIF titer (≤ 1:250), and the other 24 villagers (44%) had high EMIF titer (1:1,250 to 1:6,250). The rest of the villagers (19%) had no antibodies to RESA. On the other hand, all villagers had antimalaria antibodies to mature stage *P. falciparum* parasite as determined by IFA assay. High IFA titers (1:1,250 to 1:6,250) were shown in 35 of 54 (65%) villagers, and the rest (35%) had low to moderate IFA titer (≤ 1:250). Association between the prevalence of both antibody types within each villager was analysed. There was no correlation in the level of each antibody ($r = 0.16$, $p = 0.26$). The number of infected erythrocytes per 200 leukocytes and the level of EMIF or IFA titers was not correlated ($r = -0.22$, $p = 0.12$, and $r = -0.20$, $p = 0.14$, respectively).

Table 1 Incidence of parasitemia and prevalence of antimalaria antibodies in the villagers

Antimalaria antibody titers	Number of the villagers infected with			
	P. falciparum	*P. vivax*	Coinfection	Nonparasitemia
EMIF ≥ 1:1,250	14	1	1	8
EMIF ≤ 1:250	9	1	0	10
EMIF negative	8	0	1	1
IFA ≥ 1:1,250	20	1	2	12
IFA ≤ 1:250	11	1	0	7

EMIF: antibodies to ring-infected erythrocyte surface antigen of *P. falciparum*

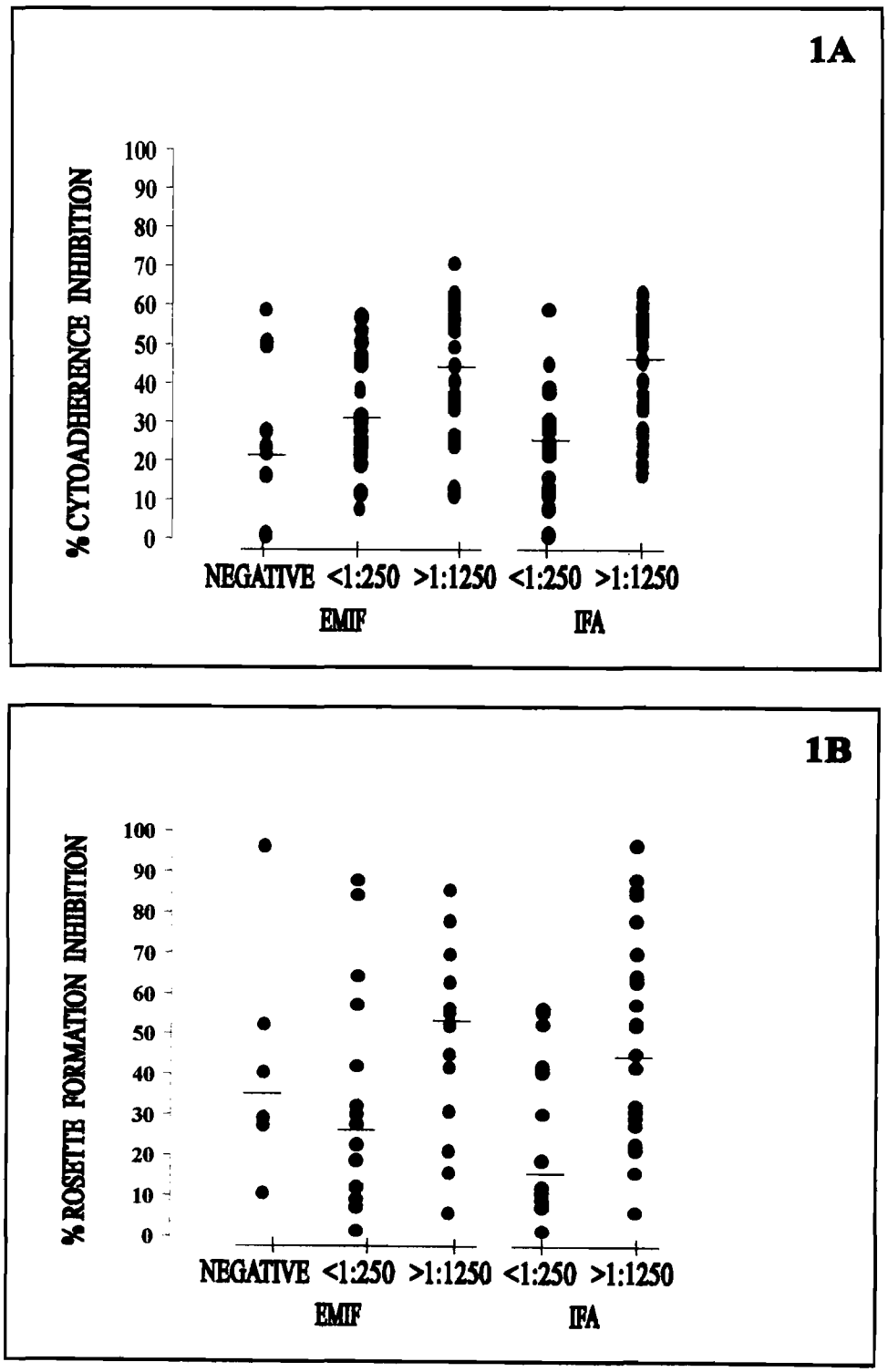

Fig. 1 Inhibition of (A) cytoadherence to C32 melanoma cells, and (B) rosette formation by sera of 54 villagers shown in relation to the titers of antibody to ring stage (EMIF titer), and to mature stages (IFA titer) of *P. falciparum*. Each symbol (•) represents one individual. Median percentage of inhibition (–) is shown in each group.

Anti-parasite adhesion activity

These sera showed a wide range of inhibition on cytoadherence (Fig. 1A) and rosette formation (Fig. 1B) of the parasites. The median percentage (range) of cytoadherence inhibition in groups of high EMIF titer (n = 24), low to moderate titer (n = 20), and EMIF negative (n = 10) were 47 (11, 70), 30 (8, 57), and 23 (1, 58), respectively. Median percentage (range) of cytoadherence inhibition in groups of high IFA titer (n = 35) and low to moderate IFA titer (n = 19) were 47 (16, 63) and 25 (1, 58), respectively. Whereas, the median percentage (range) of rosette formation inhibition in the group of high EMIF titer (n = 14), low to moderate titer (n = 16), and EMIF negative (n = 6) were 52 (6, 85), 25 (1, 88), and 35 (11, 96), respectively. Those for high IFA titer (n = 23) and low to moderate IFA titer (n = 13) were 45 (6, 96) and 19 (1, 56), respectively.

Inhibition of parasite adherence but not that of rosette formation corresponded with the levels of antibody titers. Analysis of variance to test between the titers of EMIF positive sera and the levels of cytoadherence inhibition showed a significant association (p = 0.0249, Table 2). In contrary, these EMIF positive sera showed no significant inhibition (p = 0.9957, Table 2) of rosette formation.

The same analysis was performed and it was similarly observed that there was a strong association (p = 0.0003, Table 2) between the level of IFA titers and cytoadherence inhibition. The effect of IFA antibody on rosette formation was lower than that observed by the EMIF antibody, and showed no significant effect (p = 0.5927, Table 2) in the inhibition of rosette formation.

DISCUSSION

Sera from Thai villagers having either acute *P. falciparum* or *P. vivax* infection or no infection were studied for the levels of antimalaria antibodies to both antigens of ring stage and mature stage *P. falciparum*. Using immunofluorescence assay technique, it showed that all villagers with or without acute infection had antibodies to mature stage *P. falciparum* antigen, indicating that all villagers had had experiences of *P. falciparum* infection in the past. Only about 80% of these villagers had antibodies to the ring stage antigen, suggesting that most of the villagers had recently been exposed to *P. falciparum* infection.[16] Previous history of the villagers having acute *P. vivax* infection showed that they also had had *P. falciparum* infection in the past. This verified the presence of antibodies to *P. falciparum* antigens in the *P. vivax* infected villagers. Cross reaction between the antigens of both malaria species could not be excluded.

Recent work has shown that plasma from malaria infected African children can agglutinate the infected red cells from heterologous African parasite isolates.[17] We therefore investigated the relevance of different antibodies that possibly interfere with parasite adhesion properties. The inhibitory effects of these Thai sera were investigated by *in vitro* assays with a Thai *P. falciparum* strain. Both antibody types, to the ring stage membrane antigen such as RESA, and to the mature stage antigens, similarly inhibited cytoadherence of the parasites. The inhibitory activity of these antibodies related with the antimalaria titer. Involvement of RESA or other ring stage antigen in adhesion of the parasite has not been confirmed. However, sera of the *P. falciparum*-experienced individuals have been reported previously to inhibit cytoadherence of the parasites.[18] The efficiency of inhibition was associated with the titers of the antibodies to the ring stage membrane antigen and to the mature stage parasites. Whether or not the effects are due partly to the existence of the antibodies to the same adhesion molecule of the parasite remains to be investigated. That the inhibition

Table 2 Analysis of variance between the levels of EMIF and IFA versus the inhibition of parasite adherence

Variables	F	p-value	Bartlett-Box F	p-value
EMIF group				
% CI	3.0627	0.0249	1.008	0.402
% RI	0.0469	0.9957	0.158	0.960
IFA group				
% CI	7.5500	0.0003	0.227	0.877
% RI	0.6403	0.5927	0.801	0.493

CI: cytoadherence inhibition, RI: rosette formation inhibition

levels never reached 100% may imply that i) the specific antibody to the parasite adhesion molecules is present at low levels among these Thai villagers, ii) the high antigenic diversity[19] of the parasite adhesion molecules reduced the antibody binding on infected red cells. Antibodies to *P. falciparum* in adults living in the endemic area have shown cross reactivity to aggregate mature stage-infected erythrocytes of different parasite strains leading to termination and clearance of the parasites.[20-21] Previous reports[6, 8,21-23] and our results here imply that immunity against malaria infection currently exists in other forms, i.e. anticytoadherence and antirosetting activities, besides the inhibition of merozoite invasion.[3-5] This small study group showed that the presence of antibodies (EMIF or IFA) did not correlate with the level of parasitemia, meaning that the effect of antibody in the inhibition of parasite multiplication was not consistent. This suggests that the mechanism which prevents the severity of malaria among the villagers is due to the interference of antibody in parasite sequestration. Although the effect on parasite adhesion by these sera was not 100% inhibition, the presence of these activities may be of importance as anti-disease activity which reduces severity of the malaria infection.

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