

IgG ANTIBODY PROFILE TO C-TERMINAL REGION OF *PLASMODIUM VIVAX* MEROZOITE SURFACE PROTEIN-1 IN THAI INDIVIDUALS EXPOSED TO MALARIA

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Abstract. Naturally acquired immune response to C-terminal region of *Plasmodium vivax* merozoite surface protein1 (PvMSP1) in 200 individuals with recent clinical episodes of malaria from malaria endemic areas along Thai-Myanmar border in the west and Thai-Cambodia border in the east of Thailand was evaluated by enzyme-linked immunosorbent assay (ELISA). The anti-PvMSP1-IgG antibody was observed in 110 individuals (55%). Among IgG responders, IgG1 coexpressed with IgG3 were the predominant subclasses. The levels of anti-PvMSP1 total IgG, IgG1 and IgG3 antibody response seem to be increased with age although no detectable significant correlation was found ($r=0.004$, $p=0.484$ for total IgG; $r=0.035$, $p=0.386$ for IgG1; $r=-0.600$, $p=0.142$ for IgG2; $r=0.077$, $p=0.227$ for IgG3; $r=0.664$, $p=0.051$ for IgG4). However, the mean level of specific total IgG was highest in the age group of >40 years. These levels of either specific total IgG or each IgG isotype did not vary among individuals with different malaria episodes. A higher level of specific total IgG, IgG1 and IgG3 antibody response related with the lower of parasitemia density was observed although no significant correlation was found. Our data indicate that individuals exposed to vivax malaria in Thailand developed antibodies to the potential candidate vaccine antigen, PvMSP1 (C-terminal).

INTRODUCTION

Malaria remains one of the major public health problems in Thailand. It is forest-related with the disease being prevalent along the international borders, especially on the Thai-Myanmar border in the west and Thai-Cambodia border in the east with 51% being *P. falciparum* and 48% *P. vivax* (Roll Back Malaria, 2005). Although the mortality rate of *P. vivax* is considerably lower than that of *P.*

falciparum infection, its morbidity is significant. Because *P. vivax* forms hypnozoites and might relapse many years after apparent cure (Stevenson and Riley, 2004), this indicates the need of malaria vaccine as one of the control measures and prevention.

Several antigens from different stages of the *Plasmodium* species have been identified and could be considered as potential vaccine antigens against the parasites. Among the proteins of blood stages of *Plasmodium*, the merozoite surface protein 1 (MSP1) has been the most intensively studied as a potential target for protective immunity. In *P. falciparum* infection, this protein is proteolytically cleaved during the invasion process. Only the glycosylphosphatidylinositol-anchored MSP1 (C-terminal) is maintained on the surface of merozoite that

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invades red blood cell (Blackman *et al*, 1990). The induction of protective immunity in rodent infected with *P. yoelii* requires both epidermal growth factor (EGF)-like motifs present in MSP1 (C-terminal), which is mediated predominantly by antibodies (Daly and Long, 1995). A certain degree of protective immunity is achieved against *P. falciparum* infection by vaccinating Aotus monkeys with either a baculovirus-derived recombinant protein containing a 42 kDa fragment of the C-terminal region of *P. falciparum* MSP1 (PfMSP1) (Chang *et al*, 1996) or a yeast derived MSP119 recombinant protein (Kumar *et al*, 1995). Similarly, humans living in an area where malaria is endemic also develop antibody to both N- and C-terminal region of PfMSP1 (Holder and Riley, 1996) that reduce susceptibility to clinical disease in some studies (Riley *et al*, 1992; Egan *et al*, 1996; Shi *et al*, 1996).

In vivax infection, studies in Brazil demonstrated that individuals who are exposed to *P. vivax* have antibody response to PvMSP1 (C-terminal) and the levels of IgG1 and IgG3 subclasses are predominant during patent infection as after treatment (Soares *et al*, 1997, 1999a). Furthermore, individuals who are exposed to malaria since birth in Brazil reveal a highly statistically significant association of antibodies against *P. vivax* MSP1 (PvMSP1)(N-terminal) with clinical protection and reduced risk of *P. vivax* infection over the 1-year follow-up period and the IgG3 response is the predominant subclass, whereas the predominant subclass in response to PvMSP1 (C-terminal) is IgG1 (Nogueira *et al*, 2006). However, these predominant IgG subclasses did not vary among individuals with different numbers of malaria episodes (Soares *et al*, 1997, 1999a). Nevertheless, the mechanisms of the secretion of an IgG subclass that confers protection are still unclear. It may depend upon many factors including antigenic variation, different levels of malaria endemicity in each location chosen for study, and the host genetic

background.

It has been previously shown that individuals naturally exposed to vivax malaria in some endemic areas in Thailand can develop the specific antibodies IgG, IgM or both against *P. vivax* crude extract blood stage antigens (Sirikajornchaksakul, 2003). However, the antibody profile against specific blood stage antigen PvMSP1 has never been reported. In this study, we evaluated antibody response to PvMSP1 (C-terminal) in individuals who have been living in malaria endemic areas along Thai-Myanmar border in the west and Thai-Cambodia border in the east of Thailand. The effects of age, parasitemia, and malaria exposure on antibody response and IgG isotypes were also evaluated.

MATERIALS AND METHODS

Blood sample collection

A total of 200 blood samples in 0.5 M ethylenediaminetetraacetic acid (EDTA) were obtained from patients diagnosed with *P. vivax* infection with informed consent at the outpatient clinic, Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok from May, 2002 to June, 2004. They have been living along the Thai-Myanmar border in the west and Thai-Cambodia border in the east of Thailand where malaria is endemic. There were 128 males and 72 females, aged between 13-66 years. Thick and thin blood smears were examined throughout the study period. Parasite counts per microliter were determined by counting the number of asexual parasites per 200 white blood cells (WBC) in thick films or the number of parasite-infected red blood cells (RBC) per 1,000 RBC in thin films and calculated by using the formula: (Number of parasites count x WBC ($10^3/\mu\text{l}$) x 1,000)/200 or Number of parasites count x RBC ($10^6/\mu\text{l}$) x 1,000.

Blood from thirty healthy individuals living in Bangkok where malaria is not endemic

were used as controls. They had not traveled to any malaria endemic area during the past two years and hence would be most unlikely to have been exposed to malaria during the time of the study. This study has been approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University.

Blood samples were centrifuged at 5,000 rpm for 5 minutes and plasma were collected and stored at -20°C.

***Plasmodium vivax* merozoite surface protein-1 (PvMSP1) (C-terminal) antigen**

Recombinant PvMSP1 (C-terminal) antigen, expressed as a glutathione S-transferase (GST) fusion protein produced as described previously (del Portillo *et al*, 1991) was used. In brief, the gene encoding amino acid 1615 to 1726 was amplified by PCR and the amplified fragment was cloned by standard method into pGEX-3X expression vector. As control, GST was produced alone. Recombinant protein and GST were affinity purified on glutathione-Sepharose 4B columns (Pharmacia, Uppsala, Sweden) and their purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration was measured by dye-binding method (Bradford, 1976).

Determination of anti-PvMSP1 (C-terminal) total IgG and IgG isotypes

Anti-PvMSP1 (C-terminal) total IgG and IgG isotypes in plasma were determined by ELISA. Tests were done in duplicate. In brief, wells of microtiter plates (Costar, USA) were coated with 50 µl of 0.5 µg/ml of PvMSP1 (C-terminal) antigen suspended in 0.1 M carbonate-bicarbonate buffer pH 9.6. Plates were incubated at 37°C until dry and washed three times with phosphate-buffered saline (PBS) plus 0.05% Tween-20 (PBS-T). The unbound sites were blocked for 2 hours at 37°C with blocking buffer containing 2.5% powdered-milk in PBS-T. After being washed, plates were added with 50 µl of 1:5 diluted plasma in

1.25% powdered-milk in PBS-T and incubated for 1 hour at 37°C. Plates were washed and incubated with 1:1,000 peroxidase-conjugated rabbit anti-human IgG (Dako A/S, Denmark) in 1.25% powdered-milk in PBS-T for 1 hour at 37°C for IgG antibody detection. After washing, the color reaction was developed by using *o*-phenylenediamine (OPD) substrate (Dako A/S, Denmark). The optical density (OD) was measured at a wavelength of 492 nm by an ELISA reader (Titertex Multiskan® PLUS, Finland).

For determining the anti-PvMSP1 (C-terminal) IgG isotypes, appropriate dilutions of plasma at 1:5 were used for IgG1, IgG2, IgG3 and IgG4. After incubation at 37°C for 1 hour, mouse anti-human IgG1, -IgG2, -IgG3, and -IgG4 monoclonal antibodies [clone NL16 (Boehringer), HP6002 (Sigma), Zg4, and R14 [both from Immunotech]] at dilution of 1:1,000, 1:2,000, 1:1,000, and 1:500, respectively in 1.25% (wt/vol) powdered-milk in PBS-T were added. After incubation for 1 hour at 37°C, peroxidase conjugate rabbit anti-mouse IgG (Dako A/S, Denmark) at dilution of 1:1,000 in 1.25% powdered-milk in PBS-T was added and the plate was incubated for 1 hour at 37°C. After washing, the color reaction was developed by using OPD substrate (Dako A/S, Denmark) and OD measured as described above.

Statistical analysis

Statistical analysis was performed by using Microsoft Excel (OfficeXp) and SPSS 11.5 for Window. The relationships between age, parasitemia, malaria exposure and the anti-PvMSP1 (C-terminal) antibody level were determined using Spearman's rank correlation test.

RESULTS

Anti-PvMSP1 total IgG, IgG isotypes and their patterns of expression

The levels of specific total IgG or IgG isotypes greater than the mean optical density of

controls plasma plus two standard deviation (OD + 2SD) were considered as positive. The anti-PvMSP1 (C-terminal) antibodies were detected in 110 out of 200 malaria individuals (55%). All 110 plasma samples that contained anti-PvMSP1 (C-terminal) IgG were reactive to at least one subclass. The anti-PvMSP1 (C-terminal) IgG1 coexpressed with IgG3 was the most predominant subclasses (59%). IgG2 coexpressed with IgG3 and the IgG3 coexpressed with IgG4 was 3% and 3% respectively, while the IgG1 coexpressed with IgG4 was 1%. IgG1 was coexpressed with IgG3 and IgG4 was 1%. The rest (34%) contained only one anti-PvMSP1 IgG subclass, which was 4% for IgG1, 2% for IgG2, 24% for IgG3 and 4% for IgG4. The results are summarized in Fig 1.

Relationship of anti-PvMSP1 (C-terminal) total IgG, IgG subclass expression and age

In this study, four different age groups were defined: 13-17 years, 18-25 years, 26-40 years, and >40 years of age. We observed that the percentage of anti-PvMSP1 (C-terminal) total IgG responders was the highest in age group of >40 years. In all age groups, the IgG isotypes responses consisted mainly IgG1 and IgG3 whereas IgG2 and IgG4 were rarely detected (Table1). The mean level of specific total IgG tended to increase with age group

and the mean OD of 0.206 was highest in age group of >40 years. The mean level of IgG1 was highest among four age groups. However, no significant correlation between the levels of either specific total IgG or IgG isotype and age was found ($r = 0.004$, $p = 0.484$ for total IgG; $r = 0.035$, $p = 0.386$ for IgG1; $r = -0.600$, $p = 0.142$ for IgG2; $r = 0.077$, $p = 0.227$ for IgG3; $r = 0.664$, $p = 0.051$ for IgG4).

Relationship of total IgG and IgG subclass expression to PvMSP1 (C-terminal) with parasitemia

When the relationship between the level of either specific total IgG or IgG isotypes and parasitemia in individuals infected with vivax malaria was evaluated, it was found that a higher level of specific total IgG, IgG1 and IgG3 antibody response related with lower parasitemia density (Fig 2). However, no significant correlation was found ($r = -0.089$, $p = 0.177$ for total IgG; $r = -0.072$, $p = 0.289$ for IgG1, $r = -0.099$, $p = 0.217$ for IgG3). Only 5 plasma samples contained specific IgG2 or IgG2 coexpressed with IgG3 while only 9 plasma samples contained specific IgG4 or IgG4 coexpressed with IgG1 or IgG3.

Relationship of total IgG and IgG subclass expression to PvMSP1 (C-terminal) with malaria exposure

The level of specific total IgG was signifi-

Table1
Number of total IgG and IgG isotypes responders among 200 vivax individuals in different age groups.

Age groups (years)	Number of subjects	Number of specific total IgG responders/number tested (%)	Number of IgG subclass responders/number tested (%)			
			IgG1 ^a	IgG2 ^b	IgG3 ^c	IgG4 ^d
13-17	37	16/37 (43)	8/37 (22)	-	15/37 (40)	1/37 (3)
18-25	84	43/84 (51)	28/84 (33)	1/84 (1)	40/84 (48)	4/84 (5)
26-40	67	40/67 (60)	26/67 (39)	3/67 (5)	34/67 (51)	3/67 (4)
>40	12	11/12 (92)	8/12 (67)	1/12 (8)	9/12 (75)	1/12 (8)

^ainclude IgG1 only and IgG1 coexpressed with other subclasses

^binclude IgG2 only and IgG2 coexpressed with other subclasses

^cinclude IgG3 only and IgG3 coexpressed with other subclasses

^dinclude IgG4 only and IgG4 coexpressed with other subclasses

IgG ANTIBODY PROFILE TO C-TERMINAL OF PvMSP1

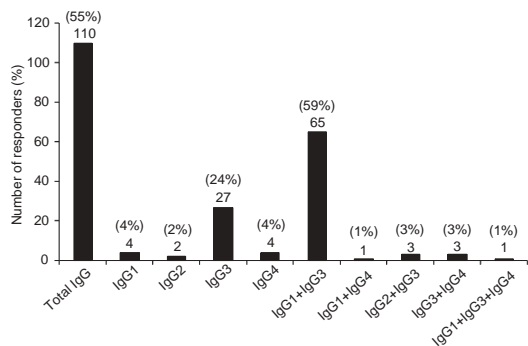
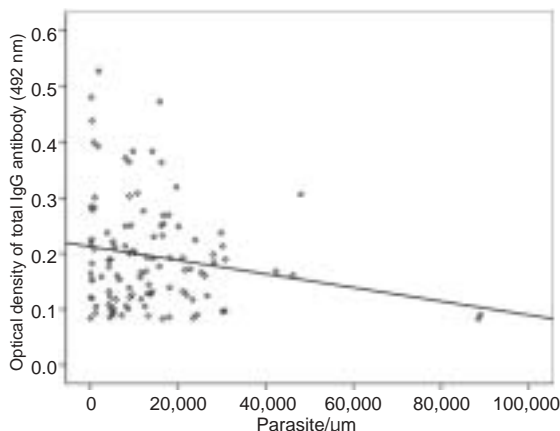


Fig 1—Number of immunoglobulin class and subclasses responders to PvMSP1 (C-terminal) antigen among 200 vivax individuals. Each IgG subclass responders and percent indicated on top was calculated from the 110 IgG responders.

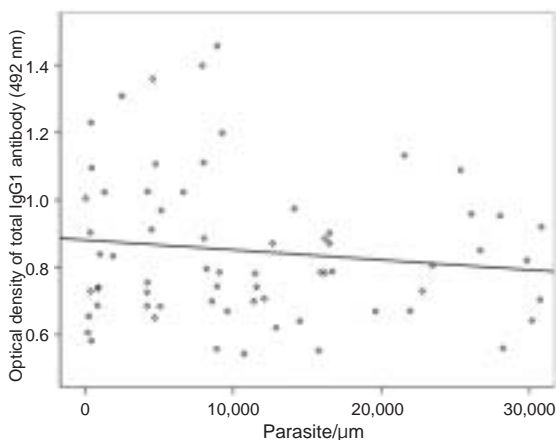
cantly correlated with the number of malaria exposures ($r = -0.230$, $p = 0.008$) but the level of each IgG isotype was not correlated with the number of malaria exposures ($r = -0.004$, $p = 0.486$ for IgG1; $r = -0.224$, $p = 0.359$ for IgG2; $r = 0.021$, $p = 0.418$ for IgG3; $r = 0.147$, $p = 0.353$ for IgG4).

DISCUSSION

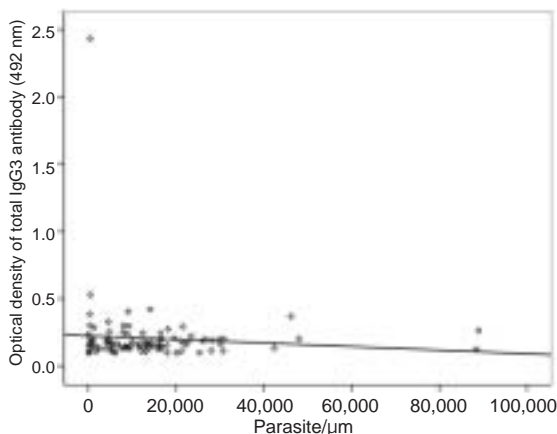
In the present study, we investigated antibody levels and their isotype response to PvMSP1 (C-terminal) in individuals who have been exposed to *P. vivax* infection. Our data showed that 55% of individuals who were exposed to *P. vivax* infection had total IgG response to PvMSP1 (C-terminal) antigen. Among the IgG responders, IgG1 and IgG3 isotypes as well as IgG1 coexpressed with IgG3 were predominant over the IgG2 and IgG4 isotype responses. A higher level of specific total IgG, IgG1 and IgG3 antibody response was related with lower parasitemia density, suggesting that higher levels of IgG1 or IgG3 antibody response may play a role in protection against malaria. Our finding in Thai individuals infected with *P. vivax* was in accordance with the recent study in Brazilians in that the main isotypes in response to



a



b



c

Fig 2—Correlation between parasitemia and optical density of anti-PvMSP1 (C-terminal) antibodies. (a) 110 total IgG responders ($r = -0.089$, $p = 0.177$), (b) 70 IgG1 responders ($r = -0.072$, $p = 0.289$), (c) 98 IgG3 responders ($r = -0.099$, $p = 0.217$).

PvMSP1 (C-terminal) recombinant protein were specific IgG1 and IgG3, while smaller proportions were specific IgG2 or IgG4 antibodies (Soares *et al*, 1997, 1999a). These specific antibody profiles in response to vivax infections were more or less the same as reported in falciparum infection which showed that IgG1 and IgG3 isotypes response to PfMSP119 antigens are predominant over the IgG2 and IgG4 isotype responses and correlate with protection against malaria (Riley *et al*, 1992, 1993).

In the present study, the levels of anti-PvMSP1 IgG1 and IgG3 antibody response seem to be increase with age although no significant correlation was found. However, the mean level of specific total IgG was highest in the age group of >40 years, while the highest mean levels of IgG1 were found in all age groups. Similar finding was reported in Cotijuba, Northern Brazil, in that no association between the prevalence of anti-PvMSP1 (C-terminal) antibody and age was found. However, the highest prevalence of antibodies to PvMSP1 (C-terminal) occurs in the group of individuals with 31-40 years old (Soares *et al*, 1999b). The variations observed within each age group may reflect either different individual ability to response to the antigen or different degrees of exposure to malaria, or may simply depend on the delay between the last antigenic restimulation and the blood stage sampling (Druihe and Khusmith, 1987).

The level of either specific total IgG or each IgG isotype did not vary among individuals with different malaria episodes. This is possibly due to such factors as the lack of antigenic restimulation (Druihe and Khusmith, 1987), since the majority of individuals who were recorded to have multiple exposures of vivax malaria had the last malaria exposure of more than one year, while the rest varied up to eight years, and some individuals had low levels of antibody beyond the detection limit. However, a recent study in persistence of spe-

cific antibodies in Brazilian living in a rural community briefly exposed to a *P. vivax* outbreak outside the endemic area showed that the anti-PvMSP119 IgG antibodies are highly prevalent (40%) in subjects who have had malarial symptoms 8 months before and decrease after 7 years (28%), and these antibodies persist only among subjects who have clinical malarial symptoms (Morais *et al*, 2005).

In summary, our data indicate that individuals exposed to vivax malaria in Thailand developed antibodies to the potential candidate vaccine antigen, PvMSP1 (C-terminal) and IgG1 coexpressed with IgG3 were the most predominant subclasses. To properly address the question of whether the anti-PvMSP1 immune response is protective in humans, longitudinal immuno-epidemiological studies to evaluate the association of the immunity to PvMSP1 and resistance to *P. vivax* infection in individuals who have been living in malaria endemic area should be further performed.

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