HUMORAL IMMUNE RESPONSES AGAINST *PLASMODIUM VIVAX* MSP1 IN HUMANS LIVING IN A MALARIA ENDEMIC AREA IN FLORES, INDONESIA

Mucide Ak[1,2], Trevor R Jones[2], Yupin Charoenvit[2], Sanjai Kumar[2], David C Kaslow[3], Dorina Maris[2], Harijani Marwoto[4], Sofyan Masbar[5] and Stephen L Hoffman[2]

[1]Ege University, Medical Faculty, Department of Parasitology, Bornova, Izmir, Turkey; [2]Malaria Program, Naval Medical Research Center, 12300 Washington Avenue, Rockville, MD 20852, USA; [3]National Institute of Allergy and Infectious Diseases, Building 4, National Institutes of Health, Bethesda, MD 20892, USA; [4]National Institutes of Health Research and Development, Jl Percetakan Negara No. 29, Jakarta, Indonesia; [5]US Naval Medical Research Unit No. 2, Jl Percetakan Negara No. 29, Jakarta, Indonesia

Abstract. The aim of this study was to evaluate the relationship among age, parasitemia status, spleen size, hematocrit, and antibody levels to *Plasmodium vivax* merozoite surface protein 1 (MSP1) in individuals chronically exposed to *P. vivax*. Subjects were recruited from the population of three adjacent villages on the Island of Flores in Indonesia where malaria transmission is hyperendemic and tropical splenomegaly syndrome is highly prevalent. Subjects were evaluated for spleen size, hematocrit, presence of parasitemia, and presence of antibodies to a recombinant peptide consisting of 90 amino acids from the carboxy terminus of MSP1. Fifty-seven percent of 2-4 year olds, 45% of 5-9 years old, and 7% of ≥ 15 years old were parasitemic; 99% of the ≥ 15 years old had splenomegaly, and 31% of them had Hackett 4 or 5 spleens. The frequency of antibody positivity to MSP1 antigen in ELISA increased with age reaching a maximum of 89% in ≥ 20 years old. The frequency of antibody positivity to MSP1 also increased with spleen size, and with a decline in the prevalence of parasitemia.

INTRODUCTION

Plasmodium vivax is one of the two most common causes of human malaria worldwide. Analysis of the immune response in subjects undergoing natural exposure to parasite antigens is useful in identifying factors involved in the development of naturally acquired immunity to both infection by *Plasmodium* and resistance to malarial disease. Additionally, antibodies may be helpful in the development of better diagnostic techniques. Over the past few years, considerable progress has been achieved in the molecular characterization of some of the constituent proteins of *P. vivax* merozoites.

Merozoite surface protein 1 (MSP-1, also referred to as Pf195, PMMSA or MSA-1) is one of the most studied of all malaria proteins (Holder *et al*, 1992), particularly MSP1 in *P. falciparum*, and is one of the most promising candidates for inclusion in a malaria vaccine directed against erythrocytic stages. The sequence of an MSP1 analog in *P. vivax* has already been described (del Portillo *et al*, 1988, 1991). This protein may be involved in the attachment to and invasion of red blood cells by merozoites (Barnwell *et al*, 1984).

In this study, we have evaluated the association among age, spleen size, hematocrit, parasitemia status, and the level of antibody to MSP1 $Pv\ 200_{19}$ in the sera of subjects living in Flores, Indonesia, an area with endemic malaria transmission.

MATERIALS AND METHODS

Study site

The study was performed on samples and data collected in Robek, Gincu and Golo, which are subvillages of a larger village called Robek (population approximately 1,400) located at 8° 17′S and 120° 24′E in the Lesser Sunda Islands of Indonesia. The inhabitants are primarily farmers and fishermen. Malaria transmission is stable in Robek, and

Correspondence: Dr Trevor Jones, NMRI Malaria Program, 12300 Washington Avenue, Rockville, MD 20852. Tel: (301)-295-5637; Fax: (301)-295-6171

considered to be meso- to hyper-endemic (Hoffman *et al,* 1984a; 1986). At the time blood samples were drawn no vector control measures were being carried out in Robek.

Study population

The study population consisted of 169 volunteers who lived in the three adjacent villages, Robek, Gincu and Golo. The subjects ranged in age from 0 to 73 years. Other demographic data are presented in Table 1. The spleen sizes of 144 of the subjects were scored by the method of Hackett, with normal spleens scored as 0 and enlarged spleens scored as 1 through 5 (Hackett, 1944). The spleens were measured while the subjects were supine with legs flexed. The protocol upon which this study was based was approved by officials of the National Institutes of Health Research and Development of the Republic of Indonesia, and conformed to the US Navy regulations governing the use of human subjects.

Specimen collection

In September 1981 venous blood samples were obtained from the subjects. Giemsa-stained thick and thin blood films were prepared, and read at 1,000x oil immersion. Two hundred fields were evaluated before a slide was designated as negative for parasites. The remainder of the blood samples were allowed to clot and then centrifuged. Sera were separated and stored in liquid nitrogen in the field, and in -70°C freezers thereafter.

Antigen

Sera from the subjects were tested by ELISA for antibody activity to MSP1 $Pv200_{19}$, a recombinant product consisting of 90 amino acids (Leu_{1639}-Ser_{1729}) from the carboxy terminus of MSP1. MSP1 is produced and expressed by merozoites (Kaslow and Kumar, 1996).

ELISA

MSP1 $Pv200_{19}$ was used as solid phase antigen in the ELISA. Briefly, 50 µl of antigen (0.0125 µg/ml) were incubated in 96 well plates for 6 hours at room temperature. After washing and overnight incubation with 5% nonfat dry milk (blocking buffer), the wells were washed, then incubated for 2 hours with 50 µl of different dilutions (range = 1:200 to 1:204,800) of the human sera. After washing, secondary antibody (peroxidase-labeled goat anti-human IgG and IgM, Kirkegaard and Perry, Gaithersburg, MD) was added for 1 hour. Reactivity was visualized using ABTS substrate [2,2'-azinodi (3-ethyl-benzthiazole sulfonate) and hydrogen peroxide, Kirkegaard and Perry, Gaithersburg, MD] and measured at 410 nm. Mean + 2SD of the OD readings of quadruplicate assays were calculated. All sera were also tested in antigen-free wells. The cutoff for positivity was set at two standard deviations above the mean OD_{410} value obtained with sera from eight healthy individuals with no history of malaria exposure.

Table 1

Demographic data and parasitemia status of 169 subjects from the villages of Robek, Gincu and Golo, Flores, Indonesia, in September 1981.

Age Groups (yrs)	0-1	2-4	5-9	10-14	15-19	≥20	Total
Subjects (n)	6	14	29	21	11	88	169
% male	33	50	48	52	45	44	46
n Parasitemia (%)	1(17)	8(57)	13(45)	3(14)	1(9)	6(7)	32(19)
n *P. falciparum* (%)	1(17)	6(43)	5(17)	2(9)	1(9)	2(2)	17(10)
n *P. vivax* (%)	0(0)	2(14)	6(21)	1(5)	0(0)	2(2)	11(7)
n *P. malariae* (%)	0(0)	0(0)	1(3)	0(0)	0(0)	2(2)	3(2)
n *P. ovale* (%)	0(0)	0(0)	1(3)	0(0)	0(0)	0(0)	1(<1)

Data analysis

Chi-square, and chi-square for trend tests were performed in the Statcalc module of EpiInfo 6.04b; Student's *t*-tests and logistic regression analyses were performed in SPSS for Windows version 6.1.4.

RESULTS

Prevalence of parasitemia

Malaria parasites were found in 19% of the 169 blood films examined. *P. falciparum* was the predominant species (10%), followed by *P. vivax* (7%), and *P. malariae* (2%). *P. ovale* was identified in one blood film from a 6 year old. Age-specific parasite prevalence is shown in Table 1. The highest prevalence of *P. vivax* occurred in the 5-9 years age group (14%) while *P. falciparum* prevalence was greatest in the 2-4 years age group (43%). Overall, 57% of the 2-4 years old subjects and 45% of the 5-9 years old subjects were parasitemic.

Spleen rates and hematocrits

Table 2 shows the age-specific spleen rates (prevalence of enlarged spleens) in this population. Age-specific spleen rates ranged between 75% (2-4 years) and 100% (0-1 years and 15-19 years) with a significant increase in prevalence with age (chi-square for trend, p = 0.009). The age-specific hematocrit values ranged between a low of 34.7% (0-1 year) and a high of 38.8% (2-4 years) with no apparent association between mean hematocrit and age (Table 3).

Antibody correlations with age

Subjects with ELISA optical densities greater than the mean plus two standard deviations of the control (nonexposed) volunteers were designated as positive for antibodies to MSP1 $Pv200_{19}$. Seventy percent of all subjects were positive, and the frequency of antibody positivity to MSP1 $Pv200_{19}$ increased significantly with age; percent positivity ranged from 17% in the youngest subjects to 89% in the adults (chi-square for trend, p<0.00001, Table 4). The geometric mean titer for positive subjects (stratified by age) was calculated. Among subjects designated positive for antibodies to MSP1 Pv 200_{19}, the geometric mean OD for 2-4 years old was lower than the means of other groups, but the difference was not significant (unpaired Student's *t*-test, p = 0.09) (Table 4). Fig 1 illustrates data on twelve positive subjects plotted with the results from 8 nonexposed controls. The positive samples required dilution to 1:102,400 before approximating the mean OD plus two standard deviations of the 8 negative control sera.

Antibody correlations with spleen size

There was an association between spleen size and frequency of positivity in ELISA for antibody

Table 2

Spleen rates and mean spleen size by age of 144 subjects from the villages of Robek, Gincu and Golo, Flores, Indonesia.

Age groups (yrs)	0-1	2-4	5-9	10-14	15-19	≥20	Total
Subjects (n)	2	8	26	19	9	80	144
Spleen rate*	100	75	92	89	100	99	95
Average enlarged spleen**	1.5	2.2	2.8	2.5	2.7	2.8	2.7
Number of subjects with Hackett 4 or 5 Spleens (%)	0(0)	1(13)	7(27)	2(11)	1(11)	27(34)	38(26)
Antibody positive (%)***	17	29	48	71	55	89	70

*Percent of subjects with a Hackett spleen score greater than 0.

**Mean Hackett spleen for subjects with a positive Hackett score (1 to 5) spleen size.

***Serum samples producing optical densities in the anti-MSP1 $Pv200_{19}$ ELISA that were greater than the mean plus two standard deviations of the control sera (8 samples) from volunteers never exposed to malaria transmission were designated as positive.

Table 3

Mean hematocrits by age of 145 subjects from the villages of Robek, Gincu and Golo, Flores, Indonesia.

Age groups (yrs)	0-1	2-4	5-9	10-14	15-19	≥20	Total
Subjects (n)	3	8	24	18	9	83	145
Hematocrit (%)	34.7	38.8	34.5	35.0	38.6	35.6	35.6

Table 4

Geometric mean optical densities in ELISA against MSP1 $Pv200_{19}$ in 169 subjects from the villages of Robek, Gincu and Golo, Flores, Indonesia.

Age Groups (yrs)	0-1	2-4	5-9	10-14	15-19	20	Total
Subjects (n)	6*	14	29	21	11	88	169
Antibody positive(%)	17	29	48	71	55	89	70
+ Geo mean OD(n)*	1.33(1)	0.54(4)	0.81(14)	0.69(15)	0.77(6)	0.86(78)	0.82(118)
- Geo mean OD(n)	0.06(5)	0.08(10)	0.13(15)	0.15(6)	0.2(5)	0.15(0)	0.12(51)
Total Geo mean OD	0.096	0.14	0.32	0.44	0.42	0.71	0.46

* Serum samples (1:200) producing optical densities in the anti-MSP1 $Pv200_{19}$ ELISA that were greater than the mean plus two standard deviations of the control sera (8 samples) from volunteers never exposed to malaria transmission were designated as positive. Geometric means were calculated for the optical densities of samples designated as positive (+Geo mean OD) and for samples designated as negative (- Geo mean OD).

for MSP1 $Pv200_{19}$; the higher the Hackett spleen score, the greater likelihood that the subject was ELISA-positive (chi-square for trend, p = 0.018) (Table 2). The association among ELISA outcome, spleen size and age was examined in unconditional logistic regression. In a model containing only spleen size as an independent variable, spleen size was a significant predictor of ELISA outcome (p = 0.02) but when age was added as a second independent variable, the effect of spleen size was reduced (p = 0.09). This indicates that age is artificially enhancing the effect of spleen size, indicating that it confounds the effect that spleen size has on ELISA outcome.

Antibody correlations with parasitemia status

Subjects positive for antibody to MSP1 $Pv200_{19}$ were less likely to be parasitemic with any *Plasmodium* species (17% *vs* 41%, p = 0.03). This effect was also noted with *P. vivax* parasitemia alone (5% *vs* 16%, p = 0.052). The association among ELISA outcome, parasitemia status and age was examined in unconditional logistic regression. In a model containing only parasitemia status as an independent variable, parasitemia status was a significant predictor of ELISA outcome (p = 0.03) but when age was added as a second independent variable, the effect of parasitemia status was reduced (p = 0.78). This indicates that age is artificially enhancing the effect of parasitemia status, indicating that it confounds the effect that parasitemia status has on ELISA outcome.

DISCUSSION

MSP1 is a leading candidate for inclusion in multivalent vaccines directed against *Plasmodium*. Immunization of mice with either full length native *P. yoelii* MSP1 or recombinant C-terminus product protects mice from death when infected with a

lethal strain of *P. yoelii* (Daly and Long 1993; Tian *et al,* 1997), and immuniqation with MSP1 DNA vaccines also produces protechtion against P. yoelii spcrozoite challenge (Becker et al, 1998). A C-terminus recombinant product has also been shown effective in the induction of protective immunity to lethal challenge of *Aotus* monkeys with *P. falciparum* (Kumar *et al,* 1995). A 42-kDa C-terminus fragment of MSP1 produced by recombinant baculovirus also protected *Aotus* monkeys against subsequent challenge with *P. falciparum.* Most of the antibody against the fragment was directed against the 19-kDa C-terminus part of the 42-kDa recombinant product (Chang *et al,* 1996). As efforts to develop *P. vivax* vaccines intensify, the relationship between the immune response to *P. vivax* MSP1, and other factors related to malaria illness is of increasing interest.

This study is the first to evaluate *P. vivax* MSP1 antibodies in an Asian population. Other studies carried out in South America also showed antibody reactivity to MSP1 fusion proteins in persons exposed to *P. vivax*. Sixty-four percent of the subjects tested (most were adults) had antibodies that reacted with a C-terminus recombinant protein (Soares *et al,* 1997). They also reported that in persons treated for vivax malaria, the prevalence of antibody to the C-terminus of MSP1 increased from 46% after one episode of malaria to 84% after four episodes. Antibody to the N-terminus protein did not increase. This suggests that C-terminus proteins such as that used here may better reflect the boosting caused by natural infection.

In this study, the mean optical density in ELISA for samples designated as negative or as positive do not change significantly (Table 4), but there is a

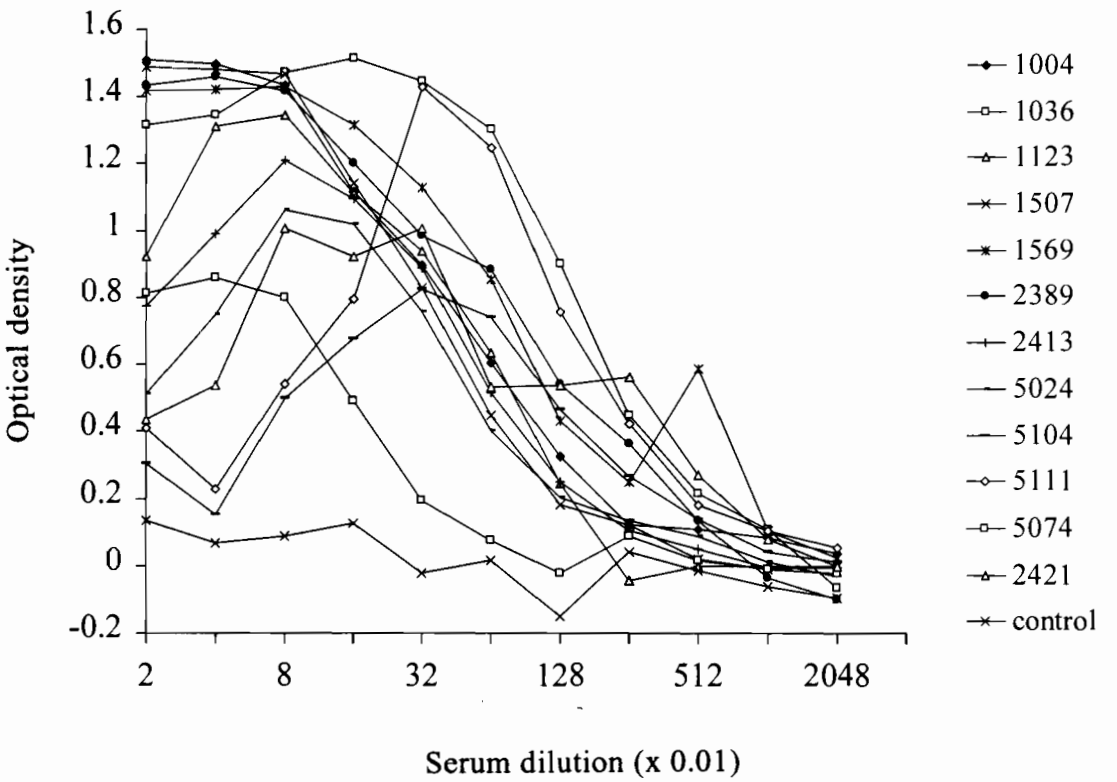

Fig 1–IgG antibody levels to $Pv200_{19}$ in 12 subjects exposed to malaria transmission, Flores Indonesia. The x line represents the mean optical density plus two standard deviations of sera taken from eight control subjects never exposed to malaria transmission. A cut-off value specific for each dilution was calculated; these cut-off values had a range of 0.133 to -0.15, and a mean ± SD of 0.011 ± 0.09.

striking increase in the frequency of positive samples as age increases. Age may be a surrogate marker for increased numbers of *P. vivax* episodes, and, therefore, degree of exposure to *P. vivax* MSP1. This view is supported by the apparent confounding by age of spleen size effect and parasitemia status effect on ELISA outcome. In other words, spleen size, probability of not being parasitemic, and probability of having a positive ELISA are probably all positively associated with or dependent on the number of exposures to *P. vivax*. Age, on the other hand, is an indirect measure of the real effector, the number of *P. vivax* episodes.

There was no association between age or antibody status and hematocrit. Mean hematocrit values for the age groups ranged between 34.5% and 38.8%. Normal value ranges for different ages in normal Americans are 28-42% for 2 month old infants, 35-45% for 6-12 year old children, 37-49% for 12-18 year old males, 36-46% for 12-18 year old females, 41-53% for 18-49 year old males, and 36-46% for 18-49 year old females (Behrman *et al,* 1965). Spleen rate and average enlarged spleen rate were highest in adults even though the rates of parasitemia decreased with age. This is a reflection of the high prevalence of tropical splenomegaly syndrome (hyperreactive malaria splenomegaly) in these villages (Hoffman *et al,* 1984b).

When sera from 12 antibody-positive subjects were diluted serially and tested in ELISA (Fig 1), six of the subjects showed a noticeable prozone effect. Although this prozone effect never caused any of the positive sera to appear negative at low dilution, this demonstrates the importance of testing sera at several different dilutions.

These data show that the presence of antibody to a C-terminus sequence of *P. vivax* MSP1 increases with age in people who are subjected to *P. vivax* infection. In addition, MSP1 antibody prevalence to the C-terminus of MSP1 increases as average enlarged spleen sizes increase, and as people become more resistant to parasitemia.

ACKNOWLEDGEMENTS

This study was supported by funds from Naval Medical Research and Development Command workunits #1432-62787A00101EFX and #1433-63002AA010HFX. The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Government of Indonesia.

REFERENCES

Barnwell JL, Galinski MR. *Plasmodium vivax:* a glimpse into the unique and shared biology of the merozoite. Ann Trop Med Parasitol 1995; 89, 113-20.

Becker SI, Wang R, Hedstrom *et al.* Protection of mice againmst *Plasmodium Yoelii* sporozoite challenge with *P. yoelii* Merozoite Surface Protein 1 DNA vaccines. *Infect Immun* 1998; 66: 3457-61.

Behrman RE, Nelson WE, Vaughen VC, eds. Nelson's Textbook of Pediatrics, 14th ed. W B Saunders 1995: p 1812.

Chang SP, Case SE, Gosnell WL, *et al.* A recombinant baculovirus 42-kilodalton C-terminal fragment of *Plasmodium falciparum* Merozoite Surface Protein 1 protects *Aotus* monkeys against malaria. *Infect Immun* 1996; 64: 253-61.

Daly TM, Long CA. A recombinant 15-kilodalton carboxyl-terminal fragment of *Plasmodium yoelii yoelii* 17XL Merozoite Surface Protein 1 induces a protective immune response in mice. *Infect Immun* 1993; 61: 2462-7.

del Portillo HA, Gysin J, Mattei DM, *et al. Plasmodium vivax:* Cloning and expression of a major blood-stage surface antigen. Exp Parasitol 1988; 67, 346-53.

del Portillo HA, Longacre S, Khouri E, David PH. Primary structure of the merozoite surface antigen 1 of reveals sequences conserved between different *Plasmodium* species. *Proc Natl Acad Sci USA* 1991; 88: 4030-4.

Hackett LW. Spleen measurement in malaria. *J Natl Malaria Soc* 1944; 19: 184-5.

Hoffman SL, Masbar S, Hussein PR, *et al.* Absence of malaria mortality in villagers with chloroquine-resistant *Plasmodium falciparum* treated with chloroquine. *Trans R Soc Trop Med Hyg* 1984a; 78, 175-8.

Hoffman SL, Piessens WF, Ratiwayanto S, *et al.* Reduction of suppressor T lymphocytes in the tropical splenomegaly syndrome. *N Engl J Med* 1984b; 310: 337-41.

Hoffman SL, Wistar R, Ballou WR, *et al.* Immunity to malaria and naturally acquired antibodies to the circumsporozoite protein of *Plasmodium falciparum. N Engl J Med* 1986; 315: 601-6.

Holder AA, Blackman MJ, Burghaus PA, *et al.* A malaria merozoite surface protein (MSP1) - structure, processing and function. *Mem Insti Oswaldo Cruz* 1992; 87(suppl III): 37-42.

Kaslow DC, Kumar S. Expression and immuno- genicity of the C-terminus of a major blood-stage surface protein of *Plasmodium vivax*, $Pv200_{19}$, secreted from *Saccharomyces cerevisiae*. *Immunol Lett* 1996; 51: 187-9.

Kumar S, Yadava A Keister DB, *et al.* Immunogenicity and *in vivo* efficacy of recombinant *Plasmodium falciparum* Merozoite Surface Protein-1 in Aotus monkeys. *Molec Med.* 1995; 1: 325-332.

Soares IS, Levitus G, Souza JM, del Portillo HA, Rodrigues MM. Acquired immune responses to the N- and C-terminal regions of *Plasmodium vivax* Merozoite Surface Protein 1 in individuals exposed to malaria. *Infect Immun* 1997; 65: 1606-14.

Tian JH, Kumar S, Kaslow DC, Miller LH. Comparison of protection induced by immunization with recombinant proteins from different regions of Merozoite Surface Protein 1. *Infect Immun* 1997; 65: 3032-6.