IDENTIFICATION OF HLA-A24 RESTRICTED PRE-ERYTHROCYTIC STAGE SPECIFIC T-CELL EPITOPES USING *PLASMODIUM FALCIPARUM* SYNTHETIC PEPTIDES: A PRELIMINARY STUDY

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Abstract. Fourteen (9 amino acids) peptides of *Plasmodium falciparum* pre-erythrocytic stage antigens, namely, TRAP, CTRP, LSA-1, STARP and MSP-1, restricted to HLA-A24 and specific to T-cell response were identified. The antigen-specific IFN-γ responses of these synthetic peptides in malaria exposed and non-malaria exposed healthy adult volunteers were detected using the *ex vivo* ELISPOT assay. Five peptides from TRAP and CTRP antigens significantly increased IFN-γ responses of 1/9 in malaria-exposed volunteers. There is no statistically significant difference in positive T-cell response induced by any peptides in malaria exposed volunteers when evaluated as a group. The frequency of expressed HLA-A24 in malaria-exposed and non-malaria-exposed healthy adults living in northwest and central Thailand was 90% (27/30) and 100% (12/12), respectively. Although no association between positive T-cell response and HLA-A24 was found, due to the low number of positive responders achieved, one positive responder in malaria- exposed group was presented as HLA-24.

INTRODUCTION

Secretion of interferon-gamma (IFN-γ) in response to stimulation of *Plasmodium falciparum*-primed T-cells by specific antigens has been used as a useful indicator of cellular immunity to malaria infection. IFN-γ is believed to eliminate directly or represent a molecular correlation of T-cell toxicity against liver-stage *P. falciparum* (Miyahira *et al*, 1995; Doolan *et al*, 1996; Sedegah *et al*, 1998). Potential malaria vaccine antigens should therefore be assessed for their ability to induce secretion of IFN-γ from malaria-specific T-cells (Kabilan *et al*, 1988; Versteegen *et al*, 1988), and great interest has been focused on assessing the level of IFN-γ in response to the pre-erythrocytic stage (P-ES) antigens using an *ex vivo* IFN-γ ELISPOT assay (Stoute *et al*, 1997; Wang *et al*, 2001).

$CD8^+$ T-cells recognize parasite-derived peptides that are presented in association with class I human leukocyte antigens (HLA) on the surface of infected hepato-

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cytes. Consequently, genetic polymorphism of HLA molecules represents a major obstacle to the development of an epitope-based vaccine designed to induce protective T-cell immune response. It has become apparent that different HLA molecules may overlap in their peptide binding specificities, leading to the definition of HLA supertypes (Sidney *et al*, 1996). Understanding HLA-restricted adaptive host immunity to defined epitopes of malarial antigens is necessary for the development of successful malaria vaccines.

In the present study, we analyzed HLA-A24 specific T-cell response to the *P. falciparum* P-ES antigens in malaria exposed and non-malaria exposed healthy adult Thai subjects living in Northwest and Central Thailand. A total of 14 peptide sequences containing the HLA-A24 binding motifs of *P. falciparum* P-ES were selected for evaluating the T-lymphocyte IFN-γ response. These peptides consisted of the following antigens: thrombospondin-related anonymous protein (TRAP), circumsporozoite (CSP) and TRAP related protein (CTRP), liver-stage antigen-1 (LSA-1), sporozoite treonine and asparagine-rich protein (STARP), and merozoite surface protein-1 (MSP-1). Peripheral blood mononuclear cells (PBMCs) obtained from each volunteer were stimulated with these peptides and IFN-γ productions were determined by *ex vivo* ELISPOT assay. In addition, the association between the prevalence of HLA-A24 gene allele and positive T-cell response was also investigated.

MATERIALS AND METHODS

Study subjects

A total of 42 healthy adult Thai volunteers were included in the study (30 and 12 subjects in experiment and control group, respectively). The experiment group (malaria exposed group) consisted of subjects who had previously been infected with *P. falciparum* and lived in malaria endemic area of Mae Sot District (Tak Province, Northwest Thailand) during the period from 1999 to 2005, whereas the control group (non-malaria exposed group) consisted of those who lived in non-malaria endemic area (Pathum Thani Province, Central Thailand) and had never been exposed to malaria infection, and with no previous history of anemia, HIV nor hepatitis-B virus infection. Ethical approval of the study protocol was obtained from the Ethics Committee of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Blood sample collection

Venous blood samples (25 ml each) were collected from all subjects by venipuncture. An aliquot of 20 ml was collected into a 10 ml heparinized tube for *ex vivo* ELISPOT assay, and another aliquot of 5 ml was collected in a cryotube for the determination of HLA-A24 typing.

Ex vivo IFN-γ ELISPOT assay

Preparation of PBMCs. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood samples by density gradient centrifugation using Lymphoprep® solution (Sigma). The cells were washed three times in phosphate-buffered saline containing 0.05% Tween 20. The numbers of cells were counted by an automated cell counter and resuspended in R10 medium (RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, antibiotics and 20 mM HEPES) at a final concentration of $5x10^6$ cells/ml.

Synthesis of peptides. Fourteen peptide sequences (9 amino acids) of *P. falciparum* P-ES antigens (TRAP, CTRP, STARP, LSA-1 and MSP-1) consisting of the binding motif to the HLA-A24 class I molecule were synthesized based on the peptide motifs of the HLA mol-

ecules, MHC ligands, secondary anchor residue, as well as data from gene bank (http: www.ncbi.nlm.nih.gov/sites/entrezdb =pubmed) and free software BIMAS at (http:/ /bimas.dcrt.nih. gov/molbio/hla_bind) (Kubo *et al*, 1994; Kondo *et al*, 1995; Rammensee *et al*, 1995). To preliminarily investigate their stimulatory effects on the effector cells, these synthetic peptides were randomly pooled into 3 peptide groups: peptide pool group I (antigens No. 1-5), peptide pool group II (No. 6-10), and peptide pool group III (No. 11-14) (Table 1). Stock solutions of the peptides were stored at -20ºC and were reconstituted in urea (8 M) solution before use. Phytohemagglutinin (PHA, 1 μg/ml) was used as a positive mitogen control and R10 medium was used as negative control. All the PBMC samples (30 malaria exposed and 12 non-malaria exposed) were tested for their response to stimulatory effect of the three groups of pooled synthetic peptides in parallel with the positive and negative controls.

***Ex vivo* IFN-γ ELISPOT assay.** IFN-γ productions following stimulation of PBMCs obtained from all volunteers were assessed using ELISPOT assay. The assay was carried out using a 96-well human IFN-γ microplate and human IFN-γ ELISPOT kits (R&D Systems). The number of synthetic peptide-specific IFN-γ-producing cells was determined by ELISPOT assay after 36-hour stimulation of PBMCs *in vitro* in the presence of 10 μM of each peptide. Nitrocellulose bottom plates coated with anti-IFN-γ monoclonal antibody as the solid phase capture were blocked with R10 medium for 20 minutes at room temperature. Fresh PBMCs at 100 μl/well ($5x10^6$ cells/ml) were plated onto the microplate. Cells were stimulated (in duplicate) with 5 μl of peptide pool of group I, II and III, PHA and R10 medium. Following 16 hours of incubation (at 37ºC, under 5% CO_2 atmostphere), PBMCs were washed with phosphate-buffered saline and a second detecting antibody (biotinylated polyclonal antibody specific for human IFN-γ) was added. The plates were incubated overnight and then washed 6 times with phosphate-buffered saline before streptavidin alkaline phosphatase conjugate was added. The plates were subsequently incubated at room temperature for 2 hours, washed 6 times and developed with chromogen [5-bromo-4-chloro-3' indolyphosphate p-toluidine salt and NitroBlue tetrazolium chloride (BCIP/NBT chromogen)]. The plates were then washed and reaction was stopped after 1 hour by flicking off the liquid and washing the plate with deionized water. Each IFN-γ -producing cell leaves a single spot in the ELISPOT assay well. The wells were scored visually using a stereomicroscope for the numbers of purple spots [spot forming cells (SFCs)] per well. Spots were enumerated by two independent readers. Experiments were performed in duplicate.

The numbers of responder cells in the stimulated wells were compared with the background negative well and results were expressed as the mean number of SFCs per $5x10^6$ PBMCs. The response was considered significant if a minimum of 5 SFCs were present per well and if the number of SFCs in each tested well was at least twice of that found in the negative control well.

HLA genotyping

Molecular subtyping for HLA-A24 was performed following amplification of the DNA sequence by PCR using sequence-specific primers. Total DNA was extracted from blood samples collected from volunteers using RNeasy kit (Qiagen®, USA), and served as a template for PCR amplifications using the HLA-A24 specific primers CCAGGGCCC AGCATCTCAGA and GGG CCGGAGTATT GGGACG (Ishikawa *et al*, 1997). A relative quantification of HLA-A24 by PCR was done

on a Thermocycle (Eppendrof®, German) using the TagMan PCR kit (Takara Bio, Japan) according to the manufacturer's instructions. Thermal cycling conditions were 5 minutes at 96ºC, 20 seconds at 64ºC and 1 minute at 72ºC, followed by 40 cycles of 1 minute at 96ºC, 20 seconds at 64ºC and 1 minute at 72ºC. The final cycle was followed by an extension step at 72ºC for 5 minutes. The amplicon was separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide (1.0 μg/ml) and observed under ultraviolet trans-illumination (Gel Doc®, USA). The sample was considered to be HLA-A24 positive when the 1,250 bp specific band was detected.

Statistical analysis

Quantitative differences in positive response in malaria exposed (experiment group) and non-malaria exposed (control group) subjects were evaluated using non-parametric Mann-Whitney *U* test at the statistical significance level of $\alpha = 0.05$.

RESULTS

IFN-γ response following synthetic peptide stimulation

Fourteen peptides of *P. falciparum* P-ES (Table 1) were tested in an overnight *ex vivo* ELISPOT assay for their ability to induce IFN-γ production of the PBMCs obtained from 42 healthy adult subjects. Out of the 30 samples in the experiment group (malaria exposed group), the assay was valid only in 9 samples (collected from 1 female and 8 males aged between 29 and 73 years, with the range of duration of previous malaria infection varying between 3 to 9 years).

Table 1
Amino acid sequences of fourteen synthetic peptides (9 amino acids) specific to HLA-A24 derived from pre-erythrocytic *Plasmodium falciparum* antigens.

Peptide	Reference	Antigen	Sequence	Position (amino acid)
Peptide pool Group I				
1	X87840	TRAP	YYCGGGTKI	93-1011
2	U34363	CTRP	CYCKDFYDI	562-570
3	U34363	CTRP	KYGSSCMEL	1555-1563
4	U34363	CTRP	GYSTRSRTF	1654-1662
5	AB006332	TRAP	KYLVIVFLI	8-16
Peptide pool Group II				
6	AF086802	LSA-1	LYISFYFIL	5-13
7	AF086802	LSA-1	FYFILVNLL	9-17
8	AF209925	STARP	FYKTAIFTL	5-13
9	AF325919	MSP-1	KYKSDLDSI	129-137
10	AF325919	MSP-1	LYKTVNDKI	161-169
Peptide pool Group III				
11	AF062349	MSP-1	SYCQIPFNL	199-207
12	AF062349	MSP-1	YYNGESSPL	1129-1137
13	AF286876	MSP-1	FYNESFTNF	61-69
14	AJ131294	MSP-1	YYKNLISKI	566-574

TRAP, Trombospondin-related anonymous protein; CTRP, CSP (circumsporozoite) and TRAP related protein; LSA-1, Liver stage antigen-1; STARP, Sporozoite threonine and asparagine-rich protein; MSP-1, Merozoite surface protein-1.

However, only group I peptide pool was found to induce a significant increase in IFN-γ responses in 1 (case number 3) out of the 9 of malaria exposed subjects. It is noted that sample from 1 (case number 5) out of 12 non-malaria exposed subjects was also found to induce a significant increase in IFN-γ responses against all the three groups of peptide pools (Fig 1).

There is no significant difference in the IFN-γ responses following stimulation of PBMCs by the three peptide pool groups, both in malaria exposed and non-malaria exposed groups. The median (range) of SFCs subtracted from the background wells in the stimulated wells tested in all the three groups of peptide pools in the experiment or control group were also comparable [group I: 0 (0-6); group II: 0 (0-4), and group III: 0 (0-4)].

Prevalence of HLA-A24

The prevalence of HLA-A24 molecules in the study population was found to be 93% (39/42), with the prevalence in the malaria exposed group and non-malaria exposed group of 90% (27/30) and 100% (12/12), respectively. A positive specific response to group I peptide pool was observed in malaria exposed subjects who also expressed HLA-A24. The PCR product obtained for HLA-A24 was 1,250 bp in size (Fig 2).

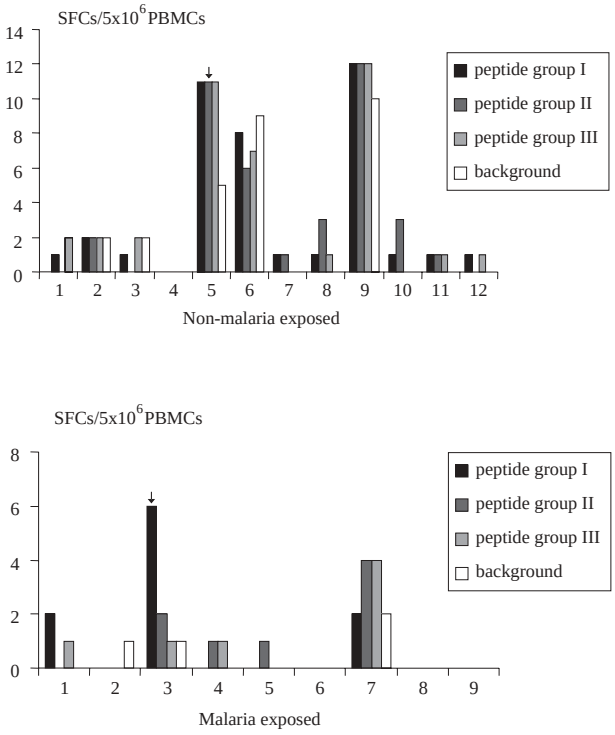

Fig 1–Spot forming cells (SFCs) of each volunteer in stimulated and background wells. PBMCs (5 x 10^6) from 12 non-malaria exposed and 9 malaria exposed volunteers were exposed to 3 groups of peptide pools. Arrow indicates positive responder.

DISCUSSION

This study directly characterized peptide-specific T-cells induction of PBMCs obtained from healthy Thai subjects with and without past exposure to falciparum malaria infection using single cell IFN-γ release as a measure of effector function. A positive response to group I peptide pool consisting of TRAP and CTRP antigens (No. 1-5) was observed in one out of the nine cases of the malaria exposed group (experiment group). In the non-malaria exposed group (control group), one positive response to all 14 synthetic peptides tested (group I, II and III peptide pools) was obtained. This phenomenon could be due to a non-specific immune response of Th-1 exposed to other epitopes or antigens, which were similar to these synthetic peptides.

HLA molecules are considered to play a crucial role in the defense of the host against malaria infection, and different HLA class I and II alleles have been reported to be associated with reduced susceptibility to severity of malaria in different populations in Gambia (Hill *et al*, 1991). The common Thai alleles did not show this association in

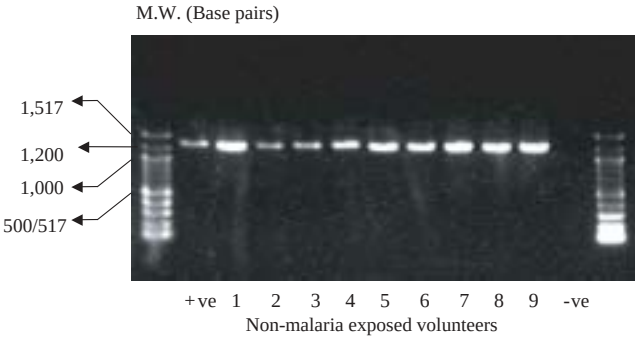

Fig 2–Detection of HLA-A24 by PCR. Total DNA was extracted from blood samples collected from HLA-A24 positive, HLA-A24 negative and non-malaria exposed healthy volunteers using Rneasy kit (Qiagen®, USA) and served as a template for PCR amplification using the HLA-A24 specific primers. Amplication of HLA-A24 by PCR was conducted in a Thermocycler (Eppendrof®, German). The amplicon was separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide and observed under ultraviolet transillumination. HLA-A24 specific band is 1,250 bp. Lanes 1 and 13, molecular weight marker (MW); lane 2, HLA-A24 positive volunteer (+ve); lanes 3-11, 9 non-malaria exposed volunteers; lane 12, HLA-A24 negative volunteer (-ve).

severe malaria patients (Hananantachi *et al*, 2005). Several studies have identified *P. falciparum* $CD8^+$ T-cell epitope on P-ES antigens that bind multiple HLA supertype alleles, and induce recalled T-cells and cytokine responses in malaria patients and healthy controls (Robson *et al*, 1988; Doolan *et al* 1997; Gonzalez *et al*, 2000; Flanagan *et al*, 2003; Lyke *et al*, 2005). The phenotype frequencies of HLA class I have been studied in healthy Thai population living in the northeastern part of the country (100 cases) and the alleles commonly found are HLA-A2 (49%), A24 (42%), A11 (40%), B46 (27%), B15 (26%) and B22 (16%) (Romphruk *et al*, 1996). In native northern Thai population (146 cases), the common alleles A2, A11 and A24 were reported at frequency of 36.4, 35.4 and 15.6%, respectively, whereas the allele frequency of B46, B40 and B13 was reported as 21.1, 15.7 and 8.6%, respectively (Fongsatikul *et al*, 1997). HLA-A24 is the most common allele in Japanese (more than 60%) and it is also present in European (nearly 20%) (Rammensee *et al*, 1995). Interestingly, the frequency of HLA-A24 found in the current study in subjects living in northwestern and central part of Thailand was as high as 90% (27/30) and 100% (12/12), respectively. Due to a limited sample size, association between the HLA-A24 genotype and response to peptide-restricted P-ES specific T-cell epitopes could not be definitely concluded. Nevertheless, it is noteworthy that 1 out of 9 subjects in the malaria-exposed group who expressed HLA-A24 genotype induced positive T-cell response to peptide pool group I (TRAP and CTRP antigens).

Based on this preliminary data, peptides from TRAP and CTRP antigens (No.1-5) might be considered for use as candidate peptides for testing a malaria vaccine in the Thai population. Further study in a larger number of malaria-exposed subjects is required in order to ascertain the activity of group I peptide pool in malaria exposed group, including the efficacy of HLA-A24 restricted T-cell response to these antigens, and finally, the association between positive response and HLA-A24.

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