LOW CD8+T LYMPHOCYTE RESPONSE TO *P. FALCIPARUM* CIRCUMSPOROZOITE PROTEIN IN NATURALLY-EXPOSED MALARIA ENDEMIC POPULATIONS IN THAILAND

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Abstract. Cytotoxic T lymphocytes (CTLs) specific for epitope(s) within the circumsporozoite (CS) protein of malaria sporozoite have been shown to play an important role in protective immunity against malaria. Human CTLs against the potential epitope at the carboxy terminal region of CS protein of *Plasmodium falciparum* 7G8 strain (Pf7G8CS 368-390) were determined in thirty-six falciparum malaria patients and ten healthy controls. Four of 36 individuals and none of the healthy controls developed Pf7G8CS 368-390 specific CTL activity. The CTL activity was antigen specific and CD8+ T cell dependent. Although low CTL response has been determined, the study suggested that there was a correlation between initial parasitemia and the specific Pf7G8CS 368-390 CTL activity. A correlation between such CTL activity and anti-R32tet32 antibody levels among individuals with previous malaria experience was found, which was in contrast to those among individuals with recent malaria infection. All these 4 CTL positive individuals had at least two episodes of clinical malaria experience while all 25 individuals who were exposed to malaria for the first time did not have such a specific CTL response. These results showed that individuals with a history of natural endemic exposure to *P. falciparum* sporozoite developed low specific CTL responses to Pf7G8CS 368-390, so that previous but recent sporozoite exposure might be a prerequisite for generation of such CS protein specific CTL response.

INTRODUCTION

One important malaria vaccine development program is aimed at sporozoites, the stage inoculated by infectious anopheline mosquitos. Although antibodies to circumsporozoite (CS) protein can be demonstrated to protect against malaria, work in rodent models has clearly demonstrated a role of CD8+ T cells in protective immunity to malaria (Kumar *et al*, 1998; Romero *et al*, 1989; Weiss *et al*, 1990; Rodrigues *et al*, 1991). It has been reported that proliferation of peripheral blood mononuclear cells from Kenyans to peptides 361-380 and 371-390 of the 7G8 *Plasmodium falciparum* CS protein (Pf 7G8CS 361-380 and Pf 7G8CS 371-390) correlated with resistance to malaria infection (Hoffman *et al*, 1989). Another study showed that some individuals with a history of extensive malaria exposure did contain peripheral blood CTL specific for the *P. falciparum* CS protein and the epitope was located to the sequence PfCS 351-395; 3 of 4 volunteers immunized with radiation attenuated *P. falciparum* sporozoites were shown to have T lymphocytes that lysed autologous Epstein Barr virus (EBV) transformed lymphoblasts transfected with the gene encoding the Pf7G8C 368-390, and the CTL activity is antigen specific, genetically restricted and dependent on CD8+ T cells (Malik *et al*, 1991). Three of eleven life-long Kenyan residents highly selected for their resistance to infection with malaria developed CTL against peptide Pf7G8CS 368-390 (Sedegah *et al*, 1992). However, there was no conclusive evidence of CS protein specific CTL in either Karen Thai or Papua New Guinea populations. Recently, in a study using a reverse immunogenic approach, CTL lines and clones from one responder in Burkina Faso were shown to be HLA - A2.1 restricted and specific for peptide 334-342 of the CS protein (Blum-Tirouvanzian *et al*, 1995). Therefore, if this is a crucial mechanism for protecting individuals against malaria, further investigation regarding the characteristics of CTL responses in people of varied genetic backgrounds and varied exposure to transmission of malaria is needed.

The objectives of this study were to determine if Thai individuals naturally exposed to malaria develop CTL against peptides of 7G8 strain of *P. falciparum* CS protein (Pf 7G8CS 368-390) and to identify the prevalence of such response in relation to different extent of malaria exposure.

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MATERIALS AND METHODS

Subjects

Thirty-six patients aged 14 to 49, who had been naturally exposed to falciparum malaria, were admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Bangkok, Thailand. Blood samples were obtained after treatment for one week, 3 weeks (P2, P5) or 4 weeks (P1, P12). These patients had been exposed to falciparum malaria in a malaria endemic area of Thailand along the Myanmar border in western Thailand, along the border between eastern Thailand and Cambodia, whereas the exact origin of one patient was not known. Twelve of the subjects had been exposed to malaria more than one time and the remainder only once. One subject was reported to have a mixed infection of *P. falciparum* and *P. vivax* (P3). Ten healthy individuals residing in Bangkok where malaria is not endemic were also studied. They had no history of malaria exposure, denied traveling to any endemic area in the past 2 years and hence would be most unlikely to have been exposed to malaria during the time of study.

Synthetic peptides

Oligopeptides representing amino acid residues 368-390, a potential epitope in the carboxy terminal of the CS protein of 7G8 strain of *P. falciparum* were synthesized according to published amino acid sequences (Dame *et al*, 1984). Peptide purity was checked by reverse phase high-performance liquid chromatography and amino acid composition analysis (Houghten, 1985).

Peptide stimulated effector cells

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized venous blood of immune individuals and healthy controls by density gradient centrifugation. From each subject 3 x 10^6 PBMCs were stimulated *in vitro* for 6 days in 24 well plates (Costar, USA) in a final volume of 2 ml of RPMI 1640 medium containing 10% heat inactivated human AB serum, in the presence of 10 mg/ml peptide. Two days after initiation of the culture, 50 U/ml of human recombinant interleukin-2 (rIL-2) (Genzyme, USA) was added to stimulate T cells. The culture plates were incubated at 37°C in the presence of 5% CO_2 for 6 days.

EBV transformed B cells

Lymphoblastoid B cells from immune and non immune individuals were transformed with Epstein Barr virus (EBV) and maintained as long term culture (Sugden and Mark, 1977).

Peptide and ^{51}Cr labeling of target cells

Eighteen hours before an assay, 1 x 10^6 transformed B cells incubated with synthetic peptides in RPMI 1640 medium (25 mg/ml) were washed twice with RPMI 1640 medium and further incubated with 20 μCi of radioactive chromate (^{51}Cr) for 1 hour. The labeled cells were washed twice with RPMI 1640, counted and used as target cells.

CTL assay

CTL activity was measured by ^{51}Cr release assay according to the method described previously (Malik *et al*, 1991). Effector cells (100 μl) were plated at appropriate effector cell to target cell ratio of 100:1, 30:1, 10:1, 1:1 in 96 well U bottom plates (Costar, USA). The tests were done in triplicate. After exposure to the peptide, 1 x 10^3 ^{51}Cr labeled target cells (100 μl) were added. The supernatants were collected after 6 hours of incubation at 37°C, 5% CO_2 by using the Skatron SCS system (Skatron Inc Sterling, VA, USA) and the released ^{51}Cr was counted using a gamma counter (Beckman Gamma 5500). The percent specific ^{51}Cr release was [100 x (experimental release - spontaneous release / maximum release - spontaneous release)]. Maximum release was determined by lysing target cells with 5% Triton X-100. Spontaneous release was determined from the labeled target cells incubated without effector cells.

Depletion of CD8$^+$ and CD4$^+$ effector cells

After 6 days of *in vitro* stimulation with synthetic peptide, CD8$^+$ and CD4$^+$ T cells were depleted from effector cells using Dynabeads (Dynal Inc, Great Neck, NY, USA) coated with anti-CD8$^+$ (M-450 CD8) and anti-CD4$^+$ (M-450 CD4) monoclonal antibodies (MAbs), respectively. Briefly, 1x10^7 effector cells were incubated with 2x10^8 magnetic beads coated with anti- M-450 CD8 or anti-M-450 CD4 MAbs for 1 hour in an ice bath, and then the beads were removed with a magnet (MPC-1). The depleted cells were washed with RPMI 1640 medium and then used for CTL assay. Fluorescence activated cell sorter (FACS) analysis indicated depletion of 98% of CD8$^+$ and of 99% of CD4$^+$ T cells.

Detection of anti-CS protein antibody by ELISA

The plasma was removed from heparinized blood of immune and healthy controls and stored at -20°C until the assay. Antibody to CS protein was measured by ELISA using a peptide construct

Table 1
Demographics of patients with falciparum malaria who developed Pf7G8CS 368-390 specific CTL.

Subject	No.of infections (last attack)	Parasitemia (parasites/μl of blood)	% Specific lysis (E:T=100:1)	Anti-R32tet32 antibody[a] (OD 414 nm)
P3	>5 (NR)	135,520	29.69	0.918
P9	2 (6 yrs)	780	28.76	1.480
P10	>5 (9 mo)	28,700	27.82	0.626
P11	2 (1 yr)	9,100	27.55	0.212

P = patient, NR = not report
[a] = Antisporozoite antibodies to the R32tet32, reported as units of optical density from ELISA value > 0.140 OD were considered positive.

R32tet32 as described (Webster, 1992). In brief, 96 well Immulon 2 "U" plates (Dynatech laboratories, USA) were coated overnight with 50 μl of synthetic peptides (4 μg/ml in boiled casein buffer), and blocked with 200 μl of casein blocking buffer for 1 hour. Fifty microliters of diluted plasma (1:100 in blocking buffer) were added to two positive and two negative wells. The wells were washed twice after 2 hours of incubation at room temperature. Peroxidase-conjugated rabbit anti-human IgG (Miles-Yeda Ltd, Naperville, Il USA) diluted to 1:2,000 was added and incubated for 1 hour with peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) before the optical density (OD) at 414 nm was determined. Known positive and negative control sera were assayed in parallel with each run. A value of 0.14 OD unit (mean + 2 SD) was taken as the cutoff level.

RESULTS

Thirty-six male and female Thai adults naturally exposed to *P. falciparum* malaria were found to develop CTL activity against peptide 368-390 of 7G8 *P. falciparum* CS protein. Patient demographics including parasitemias are shown in Table 1. These individuals were estimated to be naturally exposed to malaria 10 to 14 days before presentation at the clinic and experienced symptoms for about 3 days on the average before seeking treatment. It is not known whether any of the patients had parasitemia but were asymptomatic before their clinical episode.

Antigen specific and CD8⁺ dependent CTL

PBMCs from falciparum malaria patients were tested 1 week, 3 weeks (P2,P5) and 4 weeks (P1,P12) after treatment for their ability to generate specific CTL against Pf7G8CS 368-390 *P. falciparum* CS protein. Four of 36 individuals did produce Pf7G8CS 368-390 specific CTL activity. At an effector to target cell ratio of 100:1, the cytolytic activity ranged from 27.55% to 29.69%. CTL response to this specific epitope could not be detected in any of 10 non-malaria exposed individuals (Fig 1).

The CTL activity was eliminated or reduced by depletion of $CD8^+$ T cells (P3,P11), but was not effected by depletion of $CD4^+$ T cells (P11) (Fig 2). The subjects P9 and P10 had not been detected their CTL phenotypes due to the limitation of the cell numbers. The specific CTL responses were dependent on $CD8^+$ T cells.

All malaria subjects who had Pf7G8CS 368-390 specific CTL response (4 of 36) had a history of previous malaria experiences as shown in Table 1. Two subjects had been exposed twice (P9, P11),

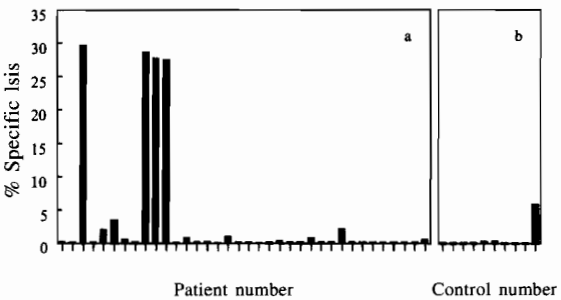

Fig 1–CTL activity against Pf7G8CS 368-390 peptide. The PBMCs were stimulated *in vitro* with Pf7G8CS 368-390 peptide and the cells were tested as effector cells against autologous EBV transformed target pulsed with Pf7G8CS 368-390 peptides. (a) CTL activity of 36 individuals naturally exposed to falciparum malaria. (b) CTL activity of non exposed controls.

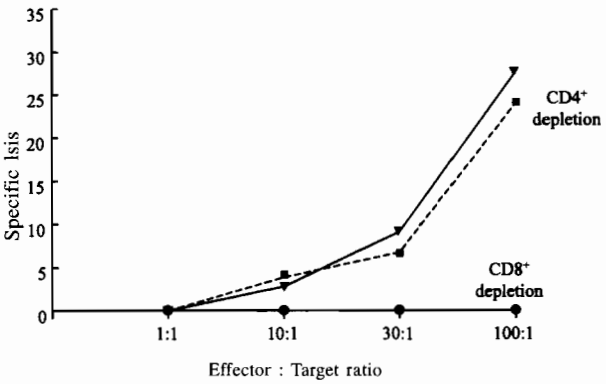

Fig 2–CTL activity of subject P3, P9, P10, and P11. The percent specific lysis shown here is the average of triplicate tests as describe in materials and methods. ▼–▼ represent effector cells lysed antologous EBV transformed B cells pulsed with Pf7G8CS 368-390 peptide. ■–■ represent CTL activity after depletion of CD4+ cells. ●–● represent CTL activity after depletion of CD8+ T cells. CTL activity was not affected by depletion of CD4+ cells (P11), but was reversed by depletion of CD8+ T cells (P3.P11). P9 and P10 were not determined the effection of CD4+ or CD8+ T cells.

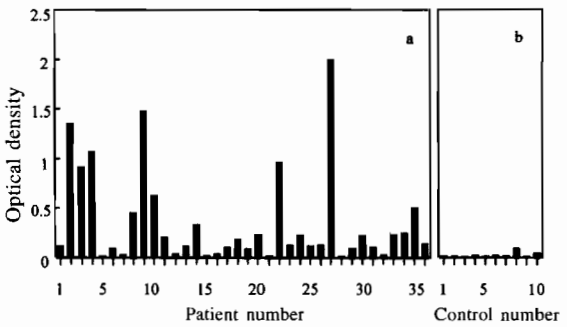

Fig 3–Anti-R32tet32 antiboby response in 36 natural malaria patients (a) and non exposed controls (b). Plasma was diluted 1/100 and determined by ELISA test. The optical density indicate the absorbance measurement at a wavelength of 414 nm. The value >0.14 OD were considered positive.

while the other two individuals had been exposed several times (P3, P10). The previous malaria experiences may exert higher CTL precursor frequency. However, the duration between the recent infection and last attack was different, ranged from 9 months to 6 years. Interestingly, three of them had been exposed from the west of Thailand (Kanchanaburi) (P3, P10, P11) and the other in the east (Cambodia) (P9). The results demonstrate that Pf7G8CS 368-390 specific CTL can be generated after at least 2 natural exposures.

Antibodies to CS protein repeat

The plasma of 36 malaria patients and 10 healthy donors were determined for IgG antibodies to the *P. falciparum* CS protein repeat (R32tet32). The frequency distribution of R32tet32 specific IgG level of 36 malaria individuals and ten healthy controls (non-exposed) are shown in Fig 3. Among 36 malaria positive individuals, the range of antibody levels was 0.012 to > 2 (OD). The value for each individual was taken from the sample point 7 days, 21 days (P2,P5) and 28 days (P1,P12) after treatment. An antibody response to the immunodominant epitope of the CS protein of *P. falciparum* was observed in 50% of patients with natural acquired infections. A relationship was observed between the level of antibody and the number of previous attacks of malaria. Individuals who had previous malaria experience had higher frequency of these antibodies (58.33%, n=12) than those who had first attack (45.83%, n=24). In comparison, a group of ten individuals, residing in a malaria-free area, who had no history of clinical malaria infection had a mean anti-R32tet32 levels of 0.028±0.017 (OD) range from 0.010 to 0.099 (OD).

Parasitemia, CTL activity and R32tet32 antibody

All 4 individuals detected for Pf7G8CS 368-390 specific CTL had anti-R32tet32 IgG ranging from 0.212 to 1.480 (mean OD = 0.806±0.532) which values were considered as positive (positive OD = >0.14), whereas those who could not produce Pf7G8CS 368-390 specific CTL had anti-R32tet32 ranging from 0.012 to > 2 (mean OD = 0.297±0.157). On statistical analysis, there was a significant correlation between CTL activity and antibody levels among individuals with previous malaria experience (r=0.733, p=0.014, n=12), which is in contrast to those among individuals with recent malaria infection (r=–0.050, p=0.408, n=24) as determined by Pearson's correlation.

The relationship among parasitemia CTL and R32tet32 antibody was examined (Table 1). Three of the four individuals with falciparum infection who were found to generate specific CTL against Pf7G8CS 368-390 peptide had low parasitemia of 780(P9), 9,100(P11), 28,700(P10), respectively. The another one who induced positive CTL activity but had high parasitemia (P3) of 135,520 was found to have a mixed infection of *P. falciparum* and *P. vivax*. All these four individuals had high anti-R32tet32 antibody level of 0.626(P10), 0.918(P3), 1.480(P3)

and 0.212(P11), respectively.

DISCUSSION

In this study, we were able to show that 4 of 36 falciparum malaria patients naturally exposed to sporozoites had CTL precursors that recognized epitopes within the carboxy terminal domain of the *P. falciparum* CS protein (Pf7G8CS 368-390) in their peripheral blood. The CTLs were antigen specific and $CD8^+$ T cells dependent. The finding that only 4 malaria positive individuals had CTL against peptide Pf7G8CS 368-390 was not surprising. It has been shown that among volunteer responders, CTL cannot be identified each time when the assay is run (Malik *et al*, 1991). The reasons for this phenomenon have not been defined but may reflect a low circulating precursor frequency since antigen-specific T cells are likely to be sequestered in the appropriate lymphoid organ, *eg* spleen (Langhorn and Simon-Haarhaus, 1991). Class I major histocompatibility molecules have been shown to bind short peptides in the range of 8 to 10 residues (Schumacher *et al*, 1991). However, longer peptides may still bind as indicated by the activity of the 23 mers Pf7G8CS 368-390. Perhaps the use of shorter peptides could increase our capacity to identify CTL in the non responders and might show an increase in cytolytic activity of the responders.

In Kenyans, proliferation of PBMCs in response to Pf CS 361-380 and Pf CS 371-390 of *P. falciparum* correlated with resistance to malaria infection (Hoffman *et al*, 1989). Subsequently, 4 of 11 lifelong Kenyan residents highly selected for their resistance to infection with malaria developed CTL against peptides Pf7G8CS 368-390. Moreover, none of the 4 individuals who were shown to have cytolytic activity had asexual *P. falciparum* parasitemia (Sedegah *et al*, 1992), which is similar to our findings that 3 of 4 individuals in whom we detected specific CTL activity had low initial parasitemias. However, the fourth such individual had a rather high parasitemia; this individual was found to have a mixed infection of *P. falciparum* and *P. vivax*, whereas *P. falciparum* parasitemia itself was low. The correlation between parasitemia and CTL activity may reflect a degree of protective immunity, but the prevalence of CTL response and protection requires to be studied more specifically. However, although low CTL responsiveness was found in 4 malaria positive individuals who had Pf7G8CS 368-390 specific CTLs, they were found subsequently to have had preexisting naturally acquired malaria immunity. In this study, in all 4 individuals who had previous malaria experience, this varied from two to several times and the period reported since the last malaria attack was 9 months to 6 years. If this is the case, it seems to be that CTLs persisted for up to 6 years. Although the persistence of CTL in our study varied, they all had similar levels of cytolytic activity, which reflected the specific CTL precursor frequency of individuals naturally exposed. In contrast, individuals who were recently exposed to malaria were not found to generate this specific CTL response. This finding may suggest that specific memory CTLs were required for induction of CTL or may be due to a very low circulating precursor frequency of specific CTL in a primary response. Naive or resting T cells seem to have very stringent activation requirement, probably best activation in lymphoid tissue. Further study on the prevalence, preexisting, and/or lasting of circulating CTL response against the CS protein and other proteins are required.

The findings that 3 of 4 Thais from malarious areas in the east and west of Thailand and 4 of 11 Kenyan residents of a highly malarious area induced circulating CTL against peptide 368-390 (Malik *et al*, 1991; Sedegah *et al*, 1992), suggests that the response to peptide Pf7G8CS 368-390 may not to be under strong genetic restriction. The issue of genetical restriction of both HLA type and TCR V gene usage, and polymorphism within the epitope of *P. falciparum* CS protein or other proteins in relation to antigenic recognition remain to be examined in depth.

In the present study, 45.58% (n=24) of the patients had detectable levels of anti-R32tet32 antibodies after their initial exposure to *P. falciparum*. These results are comparable to the 48% antibody (Anti - $NANP_{40}$) prevalence in 51 Caucasian travelers with falciparum malaria reported by Del Guidice *et al* (1987) and 62% prevalence in individuals from Sri Lanka with primary *P. falciparum* attack (Wijesundera *et al*, 1990). The prevalence of anti-R32tet32 in individuals who had previous malaria experience was 58.33% (n=12) which was a little higher than in individuals who had recent malaria infection (45.83%). A boosting anti-CS protein antibody response was observed in only 6% of individuals reinfectied with *P. falciparum* suggested that previous malaria experiences or recent malaria infection was not associated with protective levels of anti-CS antibodies (Webster *et al*, 1988). Thus, the prevalence of anti-R32tetR32 in individuals who had previous malaria experience may be more indica-

tive of individual level of ewhosure to infection than of protective immunity.

The Pf7G8CS 368-390 specific CTL was detected in subjects with detectable anti-R32tet32 antibodies. There was a significant correlation between Pf7G8CS 368-390 specific response and anti-R32tet32 antibody response in individuals who have had preexisting naturally acquired malaria immunity (r=0.733, p=.014, n=12). It has been shown that among children with high titers of antibody to $(NANP)_{40}$, the mean lymphocyte stimulation index (LSI) for Th3R peptide was somewhat higher in children who remained free of infection during the ensuing malaria season than in children who became infected (Reiley *et al*, 1990). Concerning the 4 subjects who had this specific CTL, 3 of them had high level of anti-R32tet32 antibodies. Interestingly, the subject who had the highest anti-R32tet32 had the lowest parasitemia. It might be possible that the effects of antibody and cellular immunity to sporozoite are synergistic and that protection is evident only when both mechanisms operate together. Indeed, antibodies, $CD4^+$ T cells and $CD8^+$ T cells participate in the over all protective effect induced by sporozoite immunization (Rodrigues *et al*, 1993). Further studies with multiple peptides or transfected target stimulation will be required to determine whether the responders produce CTL against this specific epitope and/or other epitopes on the *P. falciparum* CS protein or whether additional responders can be identified.

Both experimental and natural exposure to *P. falciparum* sporozoites have been shown to induce specific CTL against the *P. falciparum* CS protein in several areas of the world (Malik *et al*, 1991; Doolan *et al*, 1991; Sedegah *et al*, 1992; Blum-Tirouvanzia *et al*, 1995). The present preliminary study in Thai individuals has demonstrated that CTL against an epitope(s) including amino acid 368-390 of the 7G8 *P. falciparum* circumsporozoite protein are present in the circulating blood of individuals naturally exposed to *P. falciparum* sporozoites in this country. This Pf7G8CS 368-390 specific $CD8^+$ CTL activity correlated with the antibody response to the repeat region of the CS protein in the presence of low parasitemia as well as being associated with the preexisting naturally acquired malaria immunity.

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