LYMPHOCYTE SUBPOPULATIONS IN MALARIA INFECTED INDIVIDUALS LIVING IN AN ENDEMIC AREA

S Srisurapanon[1], S Wiwattanakul[1], S Apibal[2], R Suwannuruk[2], S Sujimanaskul[3], B Petchsuwan[3] and K Pattanapanyasat[4]

[1]Department of Pathology, Faculty of Medicine, Srinakharinwirot University; [2] Department of Pathology, Faculty of Medicine, Ramathibodi Hospital,Mahidol University; [3]Blood Bank Section, Vajira Hospital, Bangkok Metropolitan Administration; [4]Division of Instruments for Research, Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract. Development of partial immunity in people living in malaria endemic area is complex. For better understanding, the lymphocyte subpopulations from infected patients were evaluated by flow cytometer before any antimalarial treatment. In *P. vivax* infection, the frequency of T-helper type 1 (Th1) was decreased significantly (p=0.042). In contrast, the number of T- helper type 2 (Th2) was increased significantly (p=0.001). These trends have also been observed in *P. faciparum* infection. The Th2 predominant response to the natural malaria infection is likely due to persistent stimulation by *Plasmodium* species. In *P. falciparum* infection, CD8+ cytotoxic lymphocytes were significantly reduced (p=0.007). However, such changes were not found in *P. vivax* infection. This might suggest that CD8+ cell responses to different *Plasmodium* spp in a different way. Both Th2 activation and CD8+ cell suppression may reflect less protective effects and chronic malaria infection could be established.

INTRODUCTION

Malaria is responsible for deaths more than any other parasitic diseases in tropical countries. In areas where malaria is endemic, deaths mostly occur in children. With age, partial immunity develops, leading to partial parasite clearance and less symptomatic or asymptomatic parasitemia (Jason *et al*, 2001). In natural infection, available evidence suggest that malaria parasite causes T cell activation, T cell lymphopenia and T-cell dysfunction (Kemp *et al*, 2002). Despite its complexity, data have so far indicated that the T lymphocytes are of major importance for induction of blood stage immunity while the CD8 subset has been shown to have a cytolytic effect against liver stage of the parasite (Winkler *et al*, 1998). Thus both T-helper and T-cytotoxic cells play critical roles in eliminating malaria parasites.

According to previous studies, several groups have suggested the validity of the T-helper type1 (Th1) and T-helper type2 (Th2) models in humans. In defining these T-helper subsets, CD7 and CD57 are two cell surface molecules related to the differentiation or functional stages of CD4+ T cells (Legac *et al*, 1992). By using surface markers, a large number of experiments reported that the CD4+ T cell subset lacking CD7 expression is composed of Th2 differentiated cells as characterized by enhancing IL-4 and lower production of IL-2. In contrast, co-expression of CD4 and CD7 molecules on the surface of T cells are preferential T-helper type1 cytokine producing lymphocytes (Autran *et al*, 1995; Lucey, *et al*, 1996; Reinhold *et al*, 1996; Leblond *et al*, 1997; Ferrante *et al*, 1998). Although the function and differentiation of T-lymphocytes have been investigated intensively, signals responsible for differentiation from the Th0 cell to Th1 or Th2 cells remains poorly understood (Autran *et al*, 1995). In the mouse model, the immune response to some infectious agents may be associated with preferential activation of antigen-specific Th1 or Th 2

Correspondence: Dr Surangrat Srisurapanon, Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Sukumvit 23, Wattana, Bangkok, 10110 Thailand.
Tel: +66 (0) 2243-6510 Ext 29; Fax: +66 (0) 2243-6510 Ext 22
E-mail: ssurangrat@hotmail.com

cells (Del Prete *et al*, 1994). The relative balance between Th1 and Th2 cytokines appears crucial, the palarization of Th1 and Th2 cells determine the presence and outcome of many diseases. In malaria infection, the roles of Th1 and Th2 cytokines in the immune response to uncomplicated *P. falciparum* infection have been investigated (Torre *et al*, 2002). However, Th1 and Th2 cells from malaria infected people who live in endemic areas have not been reported. We therefore, have determined the frequencies of specific T lymphocyte responses to malaria infection and compared the difference in lymphocyte subsets between *P. vivax* and *P. falciparum* infections.

MATERIALS AND METHODS

Study population

A total of 30 malaria-infected patients were enrolled in this study, 18 with *P. vivax* infection and 12 with *P. falciparum* infection. Thirty healthy individuals were included as controls. Malaria infected patients enrolled were checked if they met the following criteria: infection with either *P. vivax* or *P. falciparum*, no recent treatment, no HIV-1 infection. All of them had acquired malaria by residing in malaria endemic areas. The median age was 28 years (range, 16 to 38 years). The malaria infection was diagnosed by microscopic examination of thick and thin blood films. The lymphocyte subpopulations and parasitemia were enumerated in each case at the time malaria was diagnosed.

Immunophenotypic analysis

For enumeration of the percentage of lymphocyte T-helper cells, T-cytotoxic cells, B cells and NK cells, color flow cytometry was applied using mouse monoclonal antibodies conjugated with fluoresceinisothiocyanate (FITC) or phycoerythrin (PE). The following monoclonal antibodies (mAbs) were used: CD3 (UCHT1), CD4 (MT310), CD8 (DK25), CD16 (DJ130C), CD25 (ACT-1), CD45 (TUK4), and CD14 (T29/33). For evaluation of the frequencies of Th1 and Th2, pairs of mAbs were used simultaneously with CD4 (FITC)/CD7(PE). All of the mAbs were purchased from Dako, Glostrup, Denmark. Samples for surface staining were processed on the fresh cells within 6 hours of sample collection. Briefly, 0.1 ml of EDTA peripheral blood was incubated in the dark at room temperature for 30 minutes with 10 μl of fluorochrome-labeled mAb. After incubation, erythrocytes were lysed and washed twice in PBS pH 7.2 and finally resuspended in 0.5 ml of 2% paraformaldehyde (Sigma) in PBS. For negative controls, cells incubated with FITC- and PE-conjugated mouse IgG1/IgG2 (Dako) served as isotype controls. The typical forward and side scatter gate together with a CD14/CD45 gate were set to exclude non- lymphocytic cells. Fifteen thousand events within this gate were acquired per sample. All samples were analyzed on a FACScan flow cytometer (Becton Dickinson). Two parameter histograms demonstrating surface markers were created using Simulset software (Becton Dickinson). The proportions of each T cell subpopulations were expressed as percentages.

Statistical analyses

Data are presented as percentages of lymphocyte subpopulations. The frequencies of lymphocyte subpopulations in each group were compared by one way ANOVA followed by Scheffe's Post-Hoc test. A value of $p<0.05$ was considered significant.

RESULTS

The profound and complex immune responses in malaria infection were T-helper lymphocytes and T-cytotoxic/suppressor lymphocytes. In *Plasmodium* spp infection, induction of an immune response had involved both Th1and Th2 cells activation. The Th2 pathway was preferential in malaria infected patients living in endemic areas whereas inhibition of CD8 T cells response was clearly observed in *P. falciparum* infection. In addition, the frequencies of B cells, NK cells and T cell with activation marker (CD25) in both *P. vivax* and *P. falciparum* did not change significantly when compared with normal subject as shown in Table 1.

Frequency of lymphocyte subpopulations in *P. vivax* infected individuals living in endemic area

In this study, the data showed that in *P. vivax*

infection both Th 1 (CD4+/CD7+) and Th2 (CD4+/CD7-) were shift with statistic significance. The frequency of Th1 cells in infected patients (mean = 24.7) was significantly decreased when compared with that of healthy controls (mean = 29.4, p= 0.042). In contrast, the number of Th2 cells in infected patients (mean =12.1) was higher than those in healthy controls (mean = 7.2) with significant difference (p=0.001), as shown in Fig 1.

Frequency of lymphocyte subpopulations in *P. falciparum* infected individuals living in endemic areas

The frequency of CD8+ cells in *P. falciparum* infection (mean = 23.3) was lower than that of normal healthy subjects (mean = 30.4) and *P. vivax* infection (mean = 29.4) with p=0.007 and p=0.024, respectively, as shown in Fig 1. In addition, the absolute number of lymphocytes in *P. falciparum* infection was significantly lower than that in healthy controls (p=0.009) (data not shown). For Th1 and Th2 in *P. falciparum* infection, the results were remarkably similar to those obtained in *P. vivax* infection; there was no significant difference.

The difference between *P. vivax* and *P. .falciparum* infection

The difference in number of CD8+ T cells between *P. falciparum* infection and *P. vivax* infection was statistically significant (p= 0.024). In this study, a decrease of CD8+ cells was not observed in *P. vivax* infection. In addition, the absolute number of lymphocytes in *P. vivax* infection did not significantly change (data were not shown).

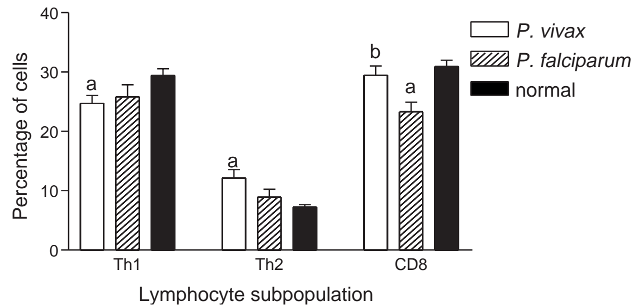

Fig 1–Differences between the percentage of Th1 (left), Th2 (center) and CD8+ cells (right) from malaria infected individuals living in endemic area and normal healthy subjects. The data are presented as means of lymphocyte percentages (horizontal axis) and lymphocyte subpopulations (vertical axis) in each subject group. The error bars indicated the standard deviation and the asterisks signify a p value of less than 0.05.

[a] indicated the statistical difference between Plasmodia species infection and healthy controls (<0.05).

[d] indicated the statistical difference between *P. vivax* and *P. falciparum* infection (<0.05).

Table 1
Percentage of lymphocyte subpopulations expressed by mean ± standard deviation from malaria infected individuals and healthy controls.

Lymphocytes	*P.vivax*	*P. falciparum*	Healthy control	p value
CD4±CD7+(T-helper type 1)	24.7±5.6[a]	25.8±6.9	29.4±6.0	0.042
CD4±CD7-(T-helper type 2)	12.1±6.1[a]	9.0±4.6	7.2±2.3	0.001
CD8+ T-cells	29.4±6.7[b]	23.3±5.4[a]	30.4±5.7	0.007[a]
				0.024[b]
CD25+ cells	4.5±3.3	5.6±3.6	4.7±1.6	0.746
CD56+ (NK cells)	10.83±3.6	10.1±4.2	10.6±2.9	0.873
CD19+ (B-cells)	17.2±5.9	15.7±4.7	16.3±5.4	0.772

[a]indicated the statistical difference between Plasmodia species infection and healthy controls (<0.05).
[b]indicated the statistical difference between *P. vivax* and *P. falciparum* infection (<0.05).
CD = cluster of differentiation.

DISCUSSION

The data presented indicate a shift to Th2 biased response to natural malaria infection in people living in endemic areas. A striking increase in Th2 subgroup was found in both *P. vivax* and *P. falciparum*. In agreement with previous experiments in the mouse model, a Th2 dominated immune response has shown to be essential in preventing recrudescent malaria during the course of disease (Stevenson and Tam, 1993; Taylor-Robinson *et al*, 1993). In fact, the preferential activation of a Th1 response in mice is related to resistance to blood stage *Plasmodium chabaudi* AS infection (Stevenson and Tam, 1993). More recent reports suggested that the protective immunity in malaria is mediated by Th1 cytokine inducing IL-12, IFN-gamma, and tumor necrosis factor (De Souza *et al*, 1997; Torre *et al*, 2002). In supporting this idea, the two different study populations used had significantly different Th1 and Th2 biases in their response to circumsporozoite protein, suggesting that the extent of *P. falciparum* exposure can affect regulation of the immune system (Reece *et al*, 2002). A further complication in high prevalence of malaria, the patients were more pronounced Th2 driven response (a lower ratio of IFN-gamma expression to IL-4 expression) during acute untreated *P. falciparum* malaria. The Th2 response was replaced by a shift towards a Th1-biased response (a higher ratio of IFN-gamma expression to IL-4 expression) (Winkler *et al*, 1998). However, in non-endemic individuals, high IFN-gamma production as part of a Th1-driven immune response has been associated with a more favorable outcome (Winkler *et al*, 1998). In another point of view, Th1 T-cells response needs not be beneficial to the host, and that TNF (alpha) and nitric oxide are critical effector molecules operating downstream of parasite-specific T cells in both immunity and disease (Hirunpetcharat *et al*, 1999). Moreover, other factors that might be involved included infective dose, other cytokine secretion cells such as macrophage and natural killer cells and the time of cytokine responses (Yoshimoto and Paul, 1994). Unfortunately, this study could not define the exact time of infection. In addition, our result did not show any correlation between parasitemia and Th1 or Th2 cells (data not shown). In fact, it confirmed that the polarization of Th2 cells is likely due to the persistent stimulation of *Plasmodium* spp.

Our finding of a significantly lower percentage of CD8+ peripheral blood lymphocytes, indicated that immune suppression is more pronounced in *P. falciparum* than *P. vivax* infection. In consistency with a previous study, during *P. falciparum* infection CD8+ cells were decreased in both percentage and absolute number (Tongtawe *et al*, 1988). However, such change had not been reported in *P. vivax* infection so far. The discordance of CD8+ T cells during the course of *P. falciparum* and *P. vivax* infection suggests a different regulatory mechanism. In addition, we also found that the absolute number of lymphocyte was decreased significantly in *P. falciparum* infection but not in *P. vivax* infection. Lymphocytopenia is a well-established feature of *P. falcipatrum* malaria but is replaced by lymphocytosis a few days after initiation of drug therapy (Hviid *et al*, 1997). Many investigators have proposed that lymphocytopenia results from the reallocation of T lymphocyte to the site of inflammation and from Fas-mediated apoptosis (Hviid *et al*, 2000; Kern *et al*, 2000; Matsumoto *et al*, 2000). The cellular hyporesponsiveness had been associated with the sequestration of activated T cells expressing the adhesion molecule leukocyte function-associated molecule-1 (LFA-1)(Winkler *et al*, 1998). As pointed out by Hviid *et al* (2000) and Kemp *et al* (2002) there has been thus so far no direct evidence that T lymphocytopenia is the consequence of malaria-host induced Fas expression on T cells. However, it was considered that Fas mediated T cell apoptosis is involved in T lymphocytopenia during malaria infection (Hviid *et al*, 2000; Kern *et al*, 2000; Matsumoto *et al*, 2000).

In *P. falciparum* infection, slight increase in T cell activation marker (CD25) in the patients with acute malaria compared with the control donors had been reported (Elhassan *et al*, 1994; Riley *et al*, 1993). In contrast, we did not observe any difference in T- cell activation markers (CD25+) from *P.vivax* and *P. falciparum* infection (Table 1). To explain this observation, persistent stimulation by *Plasmodium* spp might be associated with an anergy or tolerance in response

to antigenic stimulation. Moreover, transient inability of the peripheral blood cells to response to *P. falciparum* during acute infection has also been reported (Elhassan *et al*, 1994).

An earlier study in mice demonstrated that B cells play an essential role in inducing a Th2 response and shutting down a Th1 response. The effective clearance of erythrocyte stage of *P. chabaudi* is dependent on the presence of B cells and/or antibodies (Langhorne *et al*, 1998). However, their role in *Plasmodium* parasite elimination is still unclear.

In conclusion, there appear to be multiple pathways that can be stimulated during *Plasmodium* spp infection. In a malaria endemic area, the susceptibility or resistance to malaria infection might be due to the biases of Th1 and Th2 in the immune response in each individual. These data illustrated the need for more longitudinal studies of natural host-parasite relationships to fully understand the sequence of immune events that take place after malaria infection.

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