

# Lymphocyte Subpopulations during Acute and Convalescence Phases of Malaria

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Malaria is associated with an alteration in the distribution of lymphocyte subpopulations in the circulation, spleen and lymph nodes during infection. Wyler<sup>1</sup> studied children with *P. falciparum* infection and found no change in B lymphocytes but an increase in null (non-T, non-B) cells in peripheral blood. Greenwood *et al.*<sup>2</sup> found a decrease in B cells, an increase in null cells and an increase in K cell activity (antibody dependent cell-mediated cytotoxicity; ADCC). Wells *et al.*<sup>3</sup> did not find an absolute change in either B or null cells in adults with malaria while Strickland *et al.*<sup>4</sup> found reduction of B cells during acute phase. In monkeys with lethal *P. knowlesi* infection, both T and B cells (especially the former), were reduced.<sup>5</sup> These results were similar to those of Krettli and Nussenzweig<sup>6</sup> who reported a loss of T cells from the thymus and of both T and B cells, but with a rise in null cells, in lymph nodes of *P. berghei* infected rodents.

Increased NK cell activity in the peripheral blood, assessed against K562 target cells, has been observed in children with falciparum malaria.<sup>7</sup> This increase was shown to be correlated with degree of parasitaemia and interferon level. Chaicumpa

**SUMMARY** Lymphocytes of normal healthy persons were separated from blood by Ficoll-Hypaque gradient centrifugation and iron-magnet application. peripheral blood lymphocytes (PBL) were stained by various dye-labeled monoclonal antibodies. Cells positive for specific surface markers were enumerated by a fluorescence activated cell sorter (FACS) and fluorescence microscope (FM). The results revealed that the percentages of cells positive with one monoclonal antibody counted by these two techniques were similar while the percentages of cells with double staining were higher when counted by FACS than by FM.

Lymphocyte subpopulations of 18 patients infected with *Plasmodium falciparum* during acute and convalescence period were studied. Lymphocytopenia occurred during the acute infection while total white blood cell counts were normal. PBL of the patients were stained with OKT<sub>3</sub>, OKT<sub>4</sub>, OKT<sub>8</sub>, Leu-11 and a combination of Leu-7, Leu-1 monoclonal antibodies. The absolute numbers of all lymphocyte subpopulations were decreased during the acute infection while T8 positive cells were decreased in both percentage and absolute number. Thus T4:T8 ratio (1.7:1) became higher than normal (1.3:1) at this period. During convalescence phase, absolute numbers and percentages of Leu-7<sup>+</sup>, Leu-1<sup>+</sup> and perhaps Leu-7<sup>+</sup>, Leu-11<sup>-</sup> cells which had low NK cell activity were significantly higher than during acute illness. The finding might explain why the NK cell activity was low during the convalescence period.

*et al.*<sup>8</sup> found that null cells in Thai adults with falciparum malaria had normal lytic activity against K562 during acute phase of the illness but during convalescence the lytic activity was markedly decreased, although there was a pronounced increase in the null cell population during the later period.

Experiments in this report were designed to study variation of subpopulations of lymphocytes especially T and null cells in peripheral blood

of patients during acute and convalescence phases of malaria in comparison with the normal controls.

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Comparative studies on peripheral blood lymphocyte (PBL) subpopulations of normal donors using the fluorescence activated cell sorter (FACS) and conventional fluorescence microscopy (FM) were also performed.

## MATERIALS AND METHODS

### Blood specimens

Twenty-five ml of peripheral blood from normal healthy donors (Bangkok residents who have never been in malaria endemic areas) and patients with malaria at acute and convalescence periods were collected in 1% EDTA anticoagulant. Patients with acute phase of malaria were those who had signs and symptoms of the illness and asexual forms of *P. falciparum* were found in their peripheral blood. They did not receive any anti-malarial drug prior to taking blood samples. After the blood sample at acute phase was taken, anti-malarial drugs were then given to the patients. Three and seven days later, their blood samples were retaken. Neither sign of illness nor parasites (except one patient which will be mentioned later) were found in the patients during these convalescence periods.

### Monoclonal antibodies (MAbs)

MAbs used in this study were OKT<sub>3</sub>, OKT<sub>4</sub>, OKT<sub>8</sub> (Ortho pharmaceuticals, Raritan, NJ) which were specific to pan T cells, helper/inducer T cells and suppressor/cytotoxic T cells, respectively and Leu-1, Leu-7 and Leu-11a (Becton-Dickinson Co., Mountain View, CA.) which were specific to T and B cell subsets, K and NK cells, and Ig Fc receptor on PMN and NK cells, respectively. These MAbs were used to stain white blood cell preparations by direct immunofluorescence method and subsequently the stained cells were analysed by either fluorescence activated cell sorter (FACS) or conventional fluorescence microscopy (FM).

### Isolation of mononuclear cells

White blood cell differential

counts were performed on blood specimens before isolation of peripheral blood mononuclear cells was carried out by density gradient centrifugation.<sup>9</sup> The mononuclear cells contaminated with few granulocytes were collected and washed once in a large volume of Selegmar's balanced salt solution (SBSS). The cells were resuspended in RPMI 1640 medium supplemented with 3% fetal bovine serum (FBS) to a density of  $5 \times 10^6$  cells per ml.

Carbonyl iron was added to the mononuclear cell suspension at the ratio of 20 mg per 1 ml of the cells. The preparation was incubated at 37° C for 30 minutes without disturbance. A magnetic bar was applied to remove phagocytic cells which had ingested the iron and the excess carbonyl iron. The mononuclear cells were washed once again and adjusted to  $5 \times 10^6$  cells per ml. They were labeled "peripheral blood lymphocytes; PBL". An aliquot of the PBL was set aside for direct immunofluorescence staining while the rest were proceeded to the steps of null cell preparation.

### Removal of T-cells from PBL

One volume of PBL was mixed with two volumes each of FBS and 0.5% sheep red blood cells (SRBC) in RPMI 1640 medium. The mixture was incubated at 37° C for 5 minutes then it was centrifuged at  $450 \times g$  for 10 minutes. The preparation was kept at 4° C for 1 hour then it was recentrifuged. Most of the supernatant was discarded. An appropriate volume of RPMI 1640 medium was gently added to suspend the cells. The preparation was overlaid onto Ficoll-Hypaque (FH) and centrifuged at  $450 \times g$  for 30 minutes. T cells which formed E-rosettes with SRBC and free SRBC settled to the bottom of the tube. B cells and null cells appeared as a white ring at the interface of the medium and the FH. These cells (labeled PBL-T) were collected, washed once and resuspended in

RPMI 1640 medium + 3% FBS to the concentration of  $5 \times 10^6$  cells per ml.

### Preparation of null cells

Equal volumes of PBL-T and 2.5% 19S-EAC (see below) were mixed and incubated at 37° C for 30 minutes. The mixture was agitated gently every 10 minutes. After the incubation, it was overlaid onto FH and centrifuged at  $450 \times g$  for 30 minutes. B cells which formed rosettes with 19S-EAC and free 19S-EAC sank to the bottom of the tube. Null cells at the interface of the medium and FH were collected, washed and counted.

Preparation of 19S-EAC: Equal volumes of a minimum subagglutinating dose of rabbit 19S antibody to human group O red blood cells (1:10,000) in RPMI 1640 containing 3% FBS and 2.5% human group O red blood cells were mixed. An appropriate volume of fresh, non-haemolytic mouse complement was added to the mixture to give a final dilution of 1:20. The mixture was incubated at 37° C for 30 minutes with gentle mixing. After incubation, the cells were washed twice and resuspended to 2.5% in RPMI 1640 medium.

The percentage of T cells, B cells and null cells in PBL were calculated from the following formulas:

$$\% \text{ null cells} = \frac{\text{No. of total null cells}}{\text{No. of total PBL}} \times 100$$

$$\% \text{ B cells} = \frac{\text{No. of total PBL-T}}{\text{No. of total PBL}} \times 100$$

$$\% \text{ T cells} = 100 - \% \text{ B cells} - \% \text{ null cells}$$

### Direct immunofluorescence assay

Aliquots of PBL in 0.2 ml volumes were placed in tubes. Ten  $\mu$ l of either OKT<sub>3</sub>, OKT<sub>4</sub>, OKT<sub>8</sub> or 5  $\mu$ l of Leu-7 or Leu-11a conjugated to fluorescein isothiocyanate (FITC) were added to appropriate tubes. The tubes were placed in ice for 30 minutes. Three ml of cold haemaline

(Baker Ins. Co., Allentown, PA) containing 0.1%  $\text{NaN}_3$  was added to each tube and the preparations were centrifuged at  $800 \times g$  for 5 minutes at  $4^\circ \text{C}$ . The cells were washed with the haemaline solution and then resuspended in 0.5 ml of 0.5% formaldehyde and 1.5 ml of haemaline. These fixed cells were stored at  $4^\circ \text{C}$  in the dark until analysis.

#### Two-color direct immunofluorescence assay

For staining of cells with a combination of Leu-1 and Leu-7 MAbs, (markers of NK cells with low activity), the two-color immunofluorescence staining method was used. Five  $\mu\text{l}$  of Leu-7-FITC conjugate and 10  $\mu\text{l}$  of Leu-1-phycoerythrin (PE) conjugate were added to 0.2 ml of  $5 \times 10^6$  cells per ml in a tube. The cells were kept on ice for 30 minutes, then they were washed, fixed and kept as above.

Null cells were stained by the same methods as for PBL but OKT<sub>3</sub>, OKT<sub>4</sub> and OKT<sub>8</sub> were omitted.

#### Analysis of stained cells by FM

The cell suspension was centrifuged, the supernatant was discarded and 1-2 drops of mounting medium were added to the cell pellet. The preparation was mixed well, dropped on a microscopic slide and a total of 300 cells per one sample were counted for positive and negative cells through a Zeiss fluorescence microscope. FITC positive cells (T3<sup>+</sup>, T4<sup>+</sup>, T8<sup>+</sup>, Leu-7<sup>+</sup> and Leu-11<sup>+</sup>) emitted bright green color and PE positive cells (Leu-1<sup>+</sup>) expressed red color. The numbers of two-color stained cells (Leu-7<sup>+</sup>, Leu-1<sup>+</sup> cells) were counted also among the 300 total cells. The percentages of positive cells were then calculated.

#### Analysis of stained cells by FACS

For this type of analysis, 10,000 stained cells from each sample were analysed at the rate of 200-300 cells per second. Fluorescence emissions

for FITC and PE were collected by a  $530 \pm 15 \text{ nm}$  (for FITC) and  $575 \pm 12.5 \text{ nm}$  (for PE) band pass filters, respectively<sup>18,19</sup> and then passed into the photomultiplier tubes for green and red fluorescence. The green and red fluorescences were measured and stored in the list mode for re-analysis by a Consort 30 computer system (Becton Dickinson, FACS Division, Mountain View, CA). The computer collected 4-parameter data (volume; fluorescence-1, FL-1; fluorescence-2, FL-2; and side scatter or ratio) in a list mode, and could also do up to 4 single parameter histogram as well as dot, contour and gated analysis.

In determining the single color immunofluorescence, the results were shown as histograms with negative and positive peaks. The percentage of positive cells was calculated and the control percentage which was measured from an unstained sample was subtracted from this value. For two-color immunofluorescence, two parameters were used (FL-1 and FL-2) and displayed as a contour map. The contour map expressed 4 populations of cells (FL-1<sup>-</sup>, FL-2<sup>-</sup>; FL-1<sup>+</sup>, FL-2<sup>+</sup>; FL-1<sup>+</sup>, FL-2<sup>-</sup>; and FL-1<sup>-</sup>, FL-2<sup>+</sup>) and the percentage of each population was calculated by the computer system.

#### Statistical analysis

Analysis of results obtained from patients at acute and convalescence phases in comparison with the data from normal donors was performed by two-tailed unpaired *t*-test. A significant difference (*p*-value) of less than 0.05 was considered.

## RESULTS

Peripheral blood lymphocytes (PBL) from 17 healthy Thai donors who lived in Bangkok which is a malaria free area and who did not have history of illness with malaria, were isolated and stained with various monoclonal antibodies by the direct immunofluorescence method. A representative result of analysis by FACS is shown as histograms (for single staining) of negative (unstained sample) peak in Figure 1A, and positive and negative peaks in Figure 1B, respectively. The percentage positive peak (Figure 1B) was corrected for the percentage of the background of the unstained control (Figure 1A) before the end result was recorded as shown in Table 1. Figure 2 shows a representative result of analysis for double staining. The machine expressed a contour map with 4 quadrants as well as the percentage of each quadrant (Figures 2A and 2B). The percentages in quadrants 1, 2, 3 and

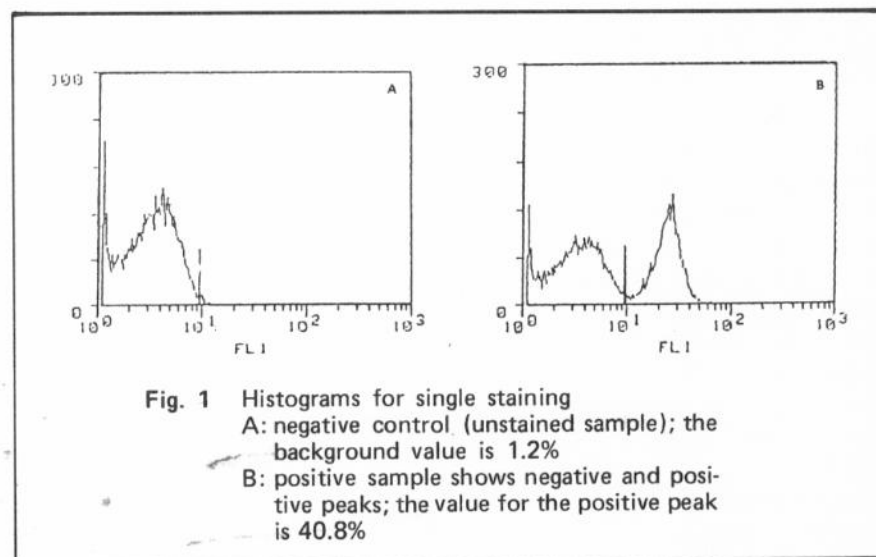


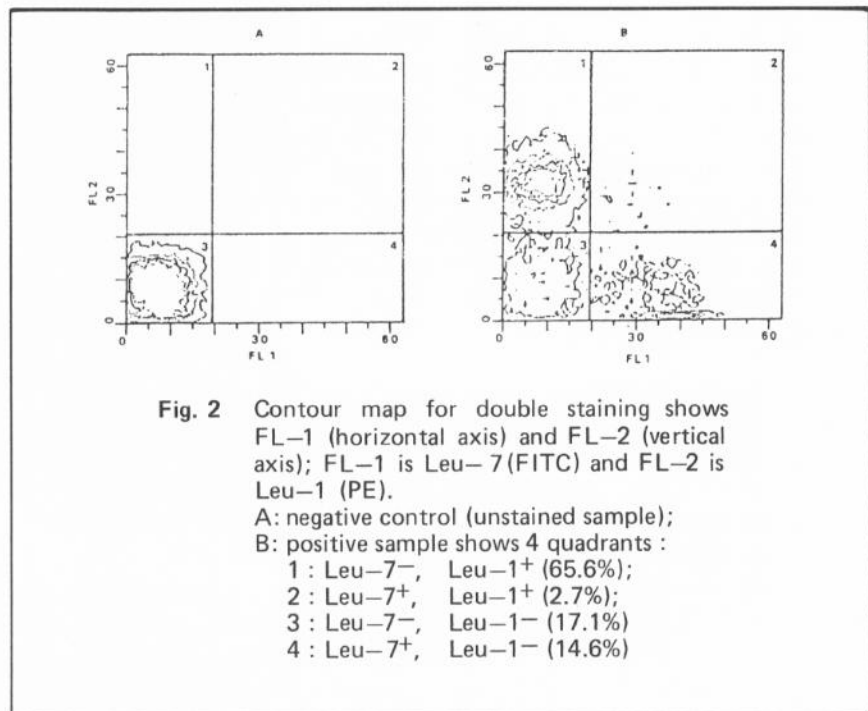
Fig. 1 Histograms for single staining  
A: negative control (unstained sample); the background value is 1.2%  
B: positive sample shows negative and positive peaks; the value for the positive peak is 40.8%

4 in Figure 2B were the percentages of Leu-7<sup>-</sup>, Leu-1<sup>+</sup>; Leu-7<sup>+</sup>, Leu-1<sup>+</sup>; Leu-7<sup>-</sup>, Leu-1<sup>-</sup> and leu-7<sup>+</sup>, Leu-1<sup>-</sup> cells, respectively. The control values (Figure 2A) of the corresponding quadrants were subtracted from these percentages and the end values are recorded in Table 1. Although the percentage of Leu-1<sup>+</sup> cells (pan T cells and B cell subset) can be obtained from the contour map (quadrants 1 and 2; Figure 2B), the results were not recorded because the population of pan T cells was also determined by OKT<sub>3</sub> which is more specific for T cells than the Leu-1.

Three hundred lymphocytes from each sample were deliberately counted by conventional fluorescence microscopy. The cells with bright green color and/or red color were enumerated. The mean percentages of positive cells are recorded in Table 1. It was found that the percentages of cells with various surface markers stained by single MAbs as determined by FACS and FM were not significantly different. However, FACS analysis gave higher percentage of cells with double markers (Leu-7<sup>+</sup>, Leu-1<sup>+</sup> cells) than when analysed by FM.

The percentages of T cells, B cells and null cells in the PBL of 10 normal Thai donors as calculated from the formula mentioned earlier were  $66.7 \pm 2.8$ ,  $19.5 \pm 1.6$  and  $13.8 \pm 1.7$ , respectively.

Cells in PBL and the null cell fraction of the same individual were stained by Leu-7, Leu-11 and Leu-7, Leu-1 combinations. The mean percentages of cells positive with these monoclonal antibodies are shown in Table 2. The percentages of Leu-11 and Leu-7 positive cells in null cell fractions (22.2% and 20.7%, respectively) were significantly higher than those in the PBL fraction (12.3% and 13.0%, respectively). The higher percentages indicate that Leu-11<sup>+</sup> and Leu-7<sup>+</sup> cells were enriched by the null cell purification



**Table 1.** Means of percentages of positive cells defined by various monoclonal antibodies in peripheral blood of 17 healthy donors as determined by FACS and FM and T4 : T8 ratio.

MAbs	FACS	FM
T3	$61.7 \pm 2.4$	$59.9 \pm 1.9$
T4	$40.2 \pm 2.4$	$40.7 \pm 1.8$
T8	$34.6 \pm 1.6$	$32.5 \pm 1.0$
T4 : T8	1.2 : 1	1.3 : 1
Leu-11	$12.9 \pm 1.2$	$13.5 \pm 1.1$
Leu-7	$14.2 \pm 1.9$	$12.8 \pm 1.5$
Leu-7 Leu-1	$*4.5 \pm 0.9$	$*2.8 \pm 0.5$

\*significant difference in comparison between FACS and FM

**Table 2.** Means of percentages of Leu-11<sup>+</sup>, Leu-7<sup>+</sup> and Leu-7<sup>+</sup>, Leu-1<sup>+</sup> cells in PBL and null cell fractions of 10 normal donors

	PBL	Null cell fraction
Leu-11 <sup>+</sup>	$12.3 \pm 1.4$	$*22.2 \pm 2.9$
Leu-7 <sup>+</sup>	$13.0 \pm 2.3$	$*20.7 \pm 2.9$
Leu-7 <sup>+</sup> , Leu-1 <sup>+</sup>	$3.1 \pm 0.7$	$2.0 \pm 0.5$

\*significantly higher ( $p < 0.05$ ) than the respective counterparts in PBL fraction



process after T and B cells were removed. However, the mean percentage of Leu-7<sup>+</sup>, Leu-1<sup>+</sup> cells of the null cell pools (2.0%) was arbitrarily, although not statistically, lower than those of the PBL which is not an expected result. Because of these results, the PBL, and not the null cell fraction, was used throughout the remaining part of the study.

Peripheral blood samples were taken from normal Thai donors at day 0 (D<sub>0</sub>), day 3 (D<sub>3</sub>) and day 7 (D<sub>7</sub>). When the blood was taken, white blood cell (WBC) counts and differential leukocyte counts were performed, then absolute lymphocyte numbers were calculated. Thereafter, the PBL were isolated from the blood and stained with various monoclonal antibodies. It was found that there was no significant change either in the number of circulating white blood cells or lymphocytes, percentages of lymphocytes or the percentages of lymphocyte subpopulations during D<sub>0</sub>, D<sub>3</sub> and D<sub>7</sub>. Thus, frequent bleeding had no effect on the WBC and lymphocytes of the normal healthy donors.

Eighteen patients with malaria were included in the study. Four patients were females while the rest were males. Their age range was 5 to 50 years with a mean of 27 years. They had had fever for 2 to 10 days before admission (D<sub>0</sub>). At D<sub>0</sub>, the range of asexual forms of *P. falciparum* per mm<sup>3</sup> of blood was between 1,333 to 649,276 (mean = 169,792). After treatment (D<sub>3</sub> and D<sub>7</sub>), the presence of parasites in blood was checked by thick smears and found that only one patient still had the parasite on D<sub>3</sub> (0-1 asexual from/oil field). There were no detectable parasites in the blood of this patient at D<sub>7</sub>. The mean values of leukocyte counts and lymphocyte densities and the percentages of lymphocytes of the patients at D<sub>0</sub>, D<sub>3</sub> and D<sub>7</sub> as well as those of 17 healthy donors are shown in Table 3. At acute phase (D<sub>0</sub>), the mean value of the white blood cell count

of the patients (6119/mm<sup>3</sup>) was not different from the normal value (6456/mm<sup>3</sup>). However, the percentage of lymphocytes as well as the lymphocyte density at this period were markedly decreased (22% and 1426 cells/mm<sup>3</sup>, respectively) when compared with the control group (38% and 2453 cell/mm<sup>3</sup>, respectively).

During convalescence phases (D<sub>3</sub> and D<sub>7</sub>), the numbers of absolute lymphocytes as well as the percentages of lymphocytes in the blood returned to normal levels. The average number of white blood cells of the patients at D<sub>3</sub> was not different to that of D<sub>0</sub> but the number at D<sub>7</sub> was higher than that of D<sub>0</sub> although statistically equal to that of the normal control.

The absolute numbers of T cell and null cell subpopulations of the normal donors and the patients are shown in Table 4 while the percentages as well as T4:T8 ratios are in Table 5. It was found that absolute numbers of all of the lymphocyte subpopulations of the malaria patients were markedly decreased during the acute phase of illness (D<sub>0</sub>). However, the numbers of all kinds of the lymphocytes returned to normal values at D<sub>3</sub> except the T8 which did not become normal until D<sub>7</sub>. Although the absolute numbers were reduced at D<sub>0</sub>, the percentages of most of the lymphocyte subpopulations were unchanged except those of Leu-7<sup>+</sup> and Leu-1<sup>+</sup>, Leu-1<sup>+</sup> cells which were low at D<sub>0</sub> and T8<sup>+</sup> cells which were low at D<sub>0</sub> and D<sub>3</sub>.

Table 3. Densities of white blood cells and lymphocytes and percentages of lymphocytes at D<sub>0</sub>, D<sub>3</sub> and D<sub>7</sub> of patients and normal controls

Donors	WBC/mm <sup>3</sup>	% lymphocytes	lymphocytes/mm <sup>3</sup>
Controls (n = 17)	6456 ± 255*	38 ± 1.3	2453 ± 137
Patients at D <sub>0</sub> (n = 18)	6119 ± 433	22 ± 2.3**	1426 ± 207**
Patients at D <sub>3</sub> (n = 16)	6313 ± 508	35 ± 2.5	2215 ± 247
Patients at D <sub>7</sub> (n = 14)	7157 ± 432	39 ± 2.5	2787 ± 264

\*mean ± S.E.

\*\*significantly different from normal control value

Table 4. Means and SE of absolute numbers of T cell and null cell subpopulations in 1 mm<sup>3</sup> of blood of normal donors and malaria patients

Cells	Normal (n = 17)	Patients at D <sub>0</sub> (n = 18)	Patients at D <sub>3</sub> (n = 16)	Patients at D <sub>7</sub> (n = 14)
T3 <sup>+</sup>	1467 ± 90	872 ± 151*	1386 ± 159	1731 ± 176
T4 <sup>+</sup>	992 ± 17	588 ± 94*	916 ± 100	1111 ± 112
T8 <sup>+</sup>	798 ± 51	355 ± 67*	532 ± 68	730 ± 120
Leu-11 <sup>+</sup>	335 ± 35	221 ± 32*	349 ± 40	420 ± 41
Leu-7 <sup>+</sup>	304 ± 37	122 ± 18*	288 ± 38	356 ± 47
Leu-7 <sup>+</sup> , Leu-1 <sup>+</sup>	66 ± 11	19 ± 4*	48 ± 8	59 ± 14

\*significant difference as compared with normal control value

**Table 5.** Means and SE of percentages of T cell and null cell subpopulations and T4 : T8 ratios in normal controls and malaria patients

Cells	Normal (n = 17)	Patients at D <sub>0</sub> (n = 18)	Patients at D <sub>3</sub> (n = 16)	Patients at D <sub>7</sub> (n = 14)
T3 <sup>+</sup>	59.9 ± 1.9	58.4 ± 2.8	62.5 ± 1.6	62.2 ± 2.1
T4 <sup>+</sup>	40.7 ± 1.8	41.1 ± 2.7	42.5 ± 2.0	40.6 ± 2.0
T8 <sup>+</sup>	32.5 ± 1.0	23.8 ± 2.2*	23.8 ± 1.3*	26.9 ± 2.8
T4 : T8	1.3 : 1	1.7 : 1*	1.8 : 1*	1.5 : 1
Leu-11 <sup>+</sup>	13.5 ± 1.1	15.9 ± 0.7	16.2 ± 1.2	15.9 ± 1.3
Leu-7 <sup>+</sup>	12.8 ± 1.5	9.7 ± 1.3*	10.1 ± 1.1	13.3 ± 1.2
Leu-7 <sup>+</sup> , Leu-1 <sup>+</sup>	2.8 ± 0.5	1.7 ± 0.4*	2.2 ± 0.3	2.0 ± 0.4

\*significant difference as compared with normal control value

The T4:T8 ratios at D<sub>0</sub> and D<sub>3</sub> of the patients were (1.7:1 and 1.8:1, respectively), thus, higher than that of the normal controls (1.3:1).

The total number of null cells (the sum of absolute numbers of Leu-11<sup>+</sup> and Leu-7<sup>+</sup> cells) of the patients was significantly lower than normal at D<sub>0</sub> (Table 4). However, the mean percentage of Leu-11<sup>+</sup> cells in malaria patients was not different from the normal value (Table 5). In contrast, during the acute phase, the percentages of Leu-7<sup>+</sup> and Leu-7<sup>+</sup>, Leu-1<sup>+</sup> cells of the patients were lower than those of the normal subjects. The percentages of these cells became normal at D<sub>3</sub> and D<sub>7</sub>.

## DISCUSSION

In the preliminary part of the present study, subpopulations of lymphocytes in peripheral blood of 17 healthy individuals were stained with dye-labeled monoclonal antibodies specific to various surface markers of the lymphocytes. Cells with different markers were then enumerated using either the FACS or FM. The results revealed that the percentages of lymphocyte subpopulations stained with one kind of dye-labeled antibody enumerated by both methods were more or less similar. However, the percentage of lymphocytes with double markers

(Leu-7<sup>+</sup>, Leu-1<sup>+</sup>) which showed both green (fluorescein) and red (phycoerythrin) colors on the same cell was higher when counted by FACS than by FM. The FACS is advantageous over the conventional FM since it can detect two colors, green and red, at the same time at a much faster rate than FM. Counting of the 10,000 cells in any sample by FACS could be finished before the red color, which had a short fluorescent half-life, faded away. The FM required a longer period of time to count a total of 300 cells. Thus, the percentage of Leu-7<sup>+</sup>, Leu-1<sup>+</sup> cells counted by the FACS appeared higher than that by FM. It was also found that the preparation of cells which had been labeled with dye-labeled antibody could be kept at 4° C for up to 1 week for FACS analysis. However, the same preparation could be stored for only 24 hours for the FM.

Numbers in subpopulations of null cells in the PBL and null cell purified fractions of the same individuals were compared. It was found that the percentages of the Leu-11<sup>+</sup> and Leu-7<sup>+</sup> cells in the null cell purified fractions (22.2% and 20.7%, respectively) were higher than the percentages of their counterparts in the PBL (12.3% and 13.0%, respectively). However, the percentages of these cells in the null cell pools

were not as high as one would expect to obtain after the purification process in which T and B cells were eliminated. Besides, the percentage of Leu-7<sup>+</sup>, Leu-1<sup>+</sup> cells in the null cell fraction (2.0%) was arbitrarily, though not statistically, lower than that of the PBL (3.1%). It is known that detection of surface markers of cells requires fresh cells with high viability.<sup>10</sup> The null cell purification process in the present study involved many complicated steps. The process might cause a loss of, or damage to, certain surface markers. Thus, some of the cells might have lost their properties to be stained by the dye-labeled antibodies. The percentages of cells positive for the monoclonal antibodies used were, therefore, not as high as expected. For this reason, fresh PBL were used throughout the later part of the study.

Lymphocytopenia was observed in malaria patients during the acute phase. The finding agrees with those reported previously.<sup>1,3,11</sup> Attempts have been made to explain this finding. Playfair and Desouza<sup>12</sup> found evidence for homing of lymphocytes to the reticuloendothelial system in murine malaria. Normal lymphocytes were found to be killed when incubated with *P. berghei* infected mouse serum but not with normal mouse serum. This finding indicates that there might be some lymphocytotoxic factor(s) in sera of the infected mice. Wells and his colleagues<sup>13</sup> demonstrated that there was an autoantibody against lymphocytes in sera of patients infected with *P. falciparum* or *P. vivax*.

The imbalance of T4:T8 in this study occurred because of significant depletion of T8<sup>+</sup> cells (suppressor or cytotoxic T cells). The finding supports the hypothesis of Greenwood *et al.*,<sup>14</sup> who proposed that there might be a selective depletion of a T lymphocyte subpopulation, perhaps suppressor T cells, during acute malaria. Selective depletion of this subpopulation could contribute to

the enhancement of immunoglobulin production which is a characteristic of acute malaria.

The reduction of T8<sup>+</sup> cells was not associated with cell damage due to interaction with the parasites in the blood of the patients. Cytotoxic T cells (T8<sup>+</sup> cells) had no destructive effect on malaria parasite infected red blood cells.<sup>15</sup> It is not yet known why the number of T8<sup>+</sup> cells was reduced during the acute phase of malaria.

Alteration in the null cell subpopulations is one of the immunological changes found in patients with malaria. Wyler<sup>1</sup> and Greenwood *et al.*<sup>2</sup> found an increase in the proportion and absolute numbers of null cells in peripheral blood of children during acute malaria. The relationship between numbers of null cells and NK cell activity in malaria patients has been studied.<sup>8,16</sup> This was done by investigating the activity of NK cells against radioactively labeled target tumour cells (K562) *in vitro*. The results showed an increase in null cell numbers during convalescence phase as compared with numbers of the cells during acute infection. In contrast to the number, the NK cell activity was lower during the convalescence period.

Abo and his colleagues<sup>17</sup> reported that NK cells which were HNK-1<sup>+</sup>, T3<sup>-</sup> exhibited a higher level of activity than the cells with HNK-1<sup>+</sup>, T3<sup>+</sup>. It is possible that the NK cells during the convalescence phase in the experiments of Chaicumpa *et al.*<sup>8</sup> were HNK-1<sup>+</sup>, T3<sup>+</sup> cells which had low NK activity. The finding in the present study supports this hypothesis, as during the convalescence phase (D7) the absolute number and percentage of Leu-7<sup>+</sup>, Leu-1<sup>+</sup> cells (Leu-7 was used instead of HNK-1 and Leu-1 was used instead of OKT3) were significantly higher than the number and percentage of their counterparts during the acute phase.

NK cell activity in relation to surface markers was studied by Lanier and co-workers.<sup>18,19</sup> They found that lymphocytes which had the highest NK activity were a Leu-11<sup>+</sup>, Leu-7<sup>-</sup> subset. Unfortunately in the present study, the cells with Leu-11<sup>+</sup>, Leu-7<sup>-</sup> combination were not determined. However, during acute phase of malaria in which Chaicumpa *et al.*<sup>8</sup> found high NK cell activity when compared with the convalescence period and equal to the activity in normal individuals, the percentage of Leu-11<sup>+</sup> cells were normal while that of Leu-7<sup>+</sup> cells were low (Table 5). The Leu-11<sup>+</sup> cells at the acute malaria might be Leu-11<sup>+</sup>, Leu-7<sup>-</sup> cells which had highest NK activity.<sup>18,19</sup> It is also possible that the Leu-7<sup>+</sup> cells which were reduced during the same period were Leu-11<sup>-</sup>, Leu-7<sup>+</sup> cells which had low NK cell activity.<sup>18,19</sup> If these explanations were true then the NK cell activity would be expected to be normal or increased during acute phase of malaria. In fact, Ojo-Amaize and his colleagues<sup>7</sup> found that the NK cell activity of children with acute malaria was higher than during the convalescence. After treatment, the percentage of Leu-7<sup>+</sup> cells returned to the normal level while the percentage of the Leu-11<sup>+</sup> cells was not changed. Again the Leu-7<sup>+</sup> cells which increased during the convalescence might, in fact, be Leu-11<sup>-</sup>, Leu-7<sup>+</sup> cells with low NK activity. Therefore, the NK activity would be expected to be low during the convalescence period of malaria.

It was also found that the absolute numbers and percentages of Leu-7<sup>+</sup> and Leu-11<sup>+</sup> cells were normal at D7. Thus, the NK cell activity should be normal during the convalescence phase. However, the activity was found to be low at this period.<sup>7,8</sup> The possibility exists that the null cells during the convalescence period were immature NK cells which possess similar Leu-11 and Leu-7 surface markers to the mature one,

No antibodies specific to surface markers which differentiate between mature and immature NK cells are yet available. It is also possible that the Leu-11<sup>+</sup> and Leu-7<sup>+</sup> cells during convalescence were K cells. These K cells may have an important role in ADCC during the convalescence phase of infection when there are high levels of specific antibodies.

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