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Flow Cytometric Analysis of T-Lymphocytes Subsets in Adult Thais

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SUMMARY Flow cytometer (FACScan) was used to determine the range of T lymphocyte subpopulations in normal Thai blood donors at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai. Reference population consisted of 150 healthy HIV seronegative blood donors. T lymphocyte subsets were analysed using two-color immunophenotyping of peripheral blood lymphocytes with a lysed whole blood technique and enumerated. The study showed that the normal values for $CD3^+$ lymphocytes (percent), $CD4^+$ lymphocytes (percent), $CD8^+$ lymphocytes (percent), CD4/CD8 ratio, absolute $CD3^+$ lymphocyte count, absolute $CD4^+$ lymphocyte count and absolute $CD8^+$ lymphocyte count were 64 ± 8.8, 36.1 ± 6.4, 25.7 ± 7.3, 1.5 ± 0.6, $1,630 \pm 600$ cells/µl, 910 ± 300 cells/µl and 670 ± 350 cells/µl, respectively. We found that the values of CD3, CD4 and CD4/CD8 ratio were significantly lower than those in the Caucasians but those of CD8 was not significantly different. This observations have important clinical implication for the use of T lymphocyte subsets measurement, especially in the management of HIV infection in Thais. These normal ranges can be used as a reference for the decisions in clinical practice.

In the last decade, the use of flow cytometry has made possible the precise identification of peripheral blood lymphocytes, particularly lymphocyte subpopulations, which have been linked to specific immune functions.[1-2] The clinical relevance of lymphocyte population measurements (immunophenotyping) is already established for a number of disorders and is now being investigated for a variety of other diseases. For example, the relative proportion of lymphocyte subsets may be altered in certain disease states, such as depletion of the CD4 positive subset of T-lymphocytes during the course of HIV infection.[3-4] While flow cytometry promises a wide range of applications, the most common routine clinical use today, is immunophenotyping, a procedure which counts the number of cells in specific lymphocyte subsets. Alterations in the number of cells of a specific lineage, for example B cells, may suggest a diagnosis of leukemia, lymphoma, or immunodeficiency. Additionally, the system can be used to identify unusual cells with aberrant or disease-specific phenotypes which may be presented in a small number.[5] Although immunophenotyping does not "make" a clinical diagnosis, its results enter into the fund of information used to make the diagnosis.[3-4]

For the determination of the relative and absolute numbers of specific lymphocyte subset populations, flow cytometry has inherent advantages over other techniques such as microscopy. This is because a flow cytometer can analyze large number of lymphocytes quickly (> 1,000 cells/sec), objectively, quantitatively and reproducibly, which

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greatly increases the statistical confidence and accuracy of the data. The development of two-color flow cytometric analysis also permits identification of several lymphocyte subsets in a single measurement. Lymphocyte subsets can be identified on the basis of multiple parameters: two light scatter qualities (reflecting size and granularity of the cells) and the level of expression of two or more specific cell surface antigens.

In the era of AIDS epidemic, enumeration of $CD4^+$ T lymphocyte by flow cytometry has become one of the important surrogate markers for diagnostic and therapeutic use. Despite the relentless progression of the epidemic in Thailand especially Northern part, there is a few reference ranges for Thais. The reference ranges are necessary because of biological variation within a cell population combined with the variation inherent in the measurement system. Therefore there is a need to establish reference ranges of T lymphocyte subsets in normal adult Thai population. We conducted the study at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University, Chiang Mai, Thailand. With the purpose of clinical implication in mind, we studied only subpopulation of T lymphocytes because the clinical utility of enumeration of other lymphocyte subsets had not been established.

MATERIALS AND METHODS

Study population

Subjects for the study were recruited from the blood donor program at Maharaj Nakorn Chiang Mai Hospital. Selected individuals were native born Thai, 20-49 years of age, in overall good health, currently not taking any medications, non-smoker, HIV antigen and antibody seronegative, hepatitis B seronegative and Venereal Disease Research Laboratory non-reactive. The 20-49 year-old group was selected because this age group was the most HIV-affected group. Study subjects consisted of 150 donors, separated into 3 groups of 50 individuals by age: 20-29, 30-39 and 40-49 years old. Blood samples (5 ml) were collected by venipuncture between 9 a.m. and 11 a.m. An aliquot (1 ml) of each sample was prepared and sent for hematological analysis within 6 hours.

Reagents

Table 1 shows the combination of fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated reagents used to determine the expression of each antigen reported in this study.

The lysing solution used in this study was 1x lysing solution freshly prepared from 10x stock solution (89.9 g NH_4Cl, 10.0 g $KHCO_3$, 370.0 mg tetrasodium EDTA, adjust to pH 7.3 in 1 l of glass distilled water).

Cell preparation and staining

Blood was collected by venipuncture using EDTA as anticoagulant. Ten microliters (μl) of each monoclonal reagent pair was added to 50 μl of whole blood in 12 x 75-mm test tubes. The whole blood and monoclonal mixture was gently vortexed and incubated at room temperature for 20 minutes in the dark. Then 2 ml of 1x lysing solution was added, and the mixture was vortexed and incubated for 10 minutes at room temperature in the dark. The sample was subsequently centrifuged for 5 minutes at room temperature at 500 x *g*. After the supernate was decanted, the pellet was resuspended in 2 ml PBS by vortexing. A second centrifugation

Table 1. Lymphocyte antigen studied and the antibodies used for their identification

Antigen (cellular distribution)	Antibody used, including fluorochrome (Becton Dickinson Immunocytometry Systems, San Jose, CA)
Leu4/CD3 (All T cells)	Anti-Leu-4 FITC in the Leu4/Leu3 and Leu4/Leu2 Simultest reagent
Leu3/CD4 (T helper cells)	Anti-Leu-3a PE in the Leu4/Leu3 Simultest reagent
Leu2/CD8 (cytotoxic and some NK cells	Anti-Leu-2a PE in the Leu4/Leu-2 Simultest reagent

was performed as above and the pellet was resuspended in 1% paraformaldehyde for fixation. The cells were then analyzed with flow cytometer.

Flow cytometry analysis

Data were acquired using FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The analysis gate that included at least 95% lymphocytes and no more than 5% monocytes was established from a minimum of 10,000 total events acquired by staining the whole blood with LeucoGATE reagent (CD45/CD14). A minimum of 2,000 lymphocyte events was then acquired from each tube.

Markers for determining cells positive and negative for any reagent were set by the SimulSET software using directly conjugated antibodies of irrelevant specificity (Simultest control) as negative controls. Each two-color immunophenotype analysis was run and analyzed under the supervision of the SimulSET software which determined lymphocyte gate and marker settings and calculated the percentage of events singly or doubly positive for each reagent. Dot plots of each two-color analysis accompany these results.

Table 2. SimulSET software output quality control criteria[2]

1. Adequate separation between cellular populations (SimulSET software was able to define a lymphocyte gate)
2. Nonspecific staining (negative control) < 5%
3. Percentage of total lymphocytes included in the analysis gate > 95%
4. Number of lymphocyte events > 2,000
5. Percentage of gated lymphocytes > 90%
6. Percentage of granulocytes in the analysis gate < 10%
7. Percentage of monocytes in the analysis gate < 5%
8. Percentage of debris in the analysis gate < 10%

Quality control

The output of SimulSET software (dot plots plus quadrant statistics) was reviewed using a set of written criteria (Table 2). All CD4 quadrant statistics were corrected for nonspecific staining by nonlymphocytes. Samples with the following criteria were classified as not evaluable and were eliminated from analysis (i) missing demographic data (age, sex, race); (ii) missing CBC or differential; (iii) sample preparation deficiencies leading to failure of the gate setting algorithm used by the SimulSET software; or (iv) percentage of $CD3^+$ T cells of the third and fourth tubes differed by ≥ 5% and/or failure to satisfy the logical requirement that the sum of % $CD4^+$ T cells + % $CD8^+$ T cells approached the $CD3^+$ cells within ± 10% of the CD3 value.

Statistical analysis

Mean and standard deviation of each parameter were calculated. Histogram of WBC count, % T cells, % $CD4^+$ T cells, % $CD8^+$ T cells, CD4/CD8 ratio, lymphocyte count, T cell count, $CD4^+$ T cell count and $CD8^+$ T cell count were generated to evaluate the distribution. The parameters which did not distribute normally were recalculated to produce logarithmic data and reevaluated. All normally distributed data in each age group were compared using ANOVA. Least significant difference (LSD) was used to determine difference between each age group.

RESULTS

The parameters which were not normally distributed included WBC count, lymphocyte count, T cell count, CD4 count and CD8 count. All logarithmic parameters were normally distributed as shown by the histogram.

The mean, standard deviation, and reference ranges for WBC count, lymphocyte percent, T cell percent, CD4 percent, CD8 percent, CD4/CD8 ratio, lymphocyte count, T cell count, CD4 count, and CD8 count were shown in Table 3. The study showed that in normal Thai adults, the normal ranges of T lymphocyte percent (mean ± SD) was 64.0 ± 8.8%, of $CD4^+$ T lymphocytes was 36.1 ± 6.4%, of $CD8^+$ T lymphocytes was 25.7 ± 7.3%, and

Table 3. Reference ranges for WBC count and T lymphocyte subsets (mean ± SD)

Parameters	Total	Age group (year-old)		
		20-29	30-39	40-49
WBC (x1,000 cells/μl)	7.8 ± 2.3	7.1 ± 1.6	7.5 ± 2.0	8.7 ± 2.9
Lymphocyte percent	33.8 ± 8.1	34.6 ± 8.0	34.3 ± 7.3	32.4 ± 8.9
CD3 percent	64.0 ± 8.8	65.8 ± 8.8	63.7 ± 8.3	62.4 ± 9.3
CD4 percent	36.1 ± 6.4	35.3 ± 6.0	35.7 ± 6.4	37.3 ± 6.7
CD8 percent	25.7 ± 7.3	27.7 ± 7.1	26.1 ± 7.2	23.5 ± 7.2
CD4/CD8 ratio	1.5 ± 0.6	1.4 ± 0.5	1.5 ± 0.6	1.7 ± 0.6
Lymphocyte count (cells/μl)	2,560 ± 850	2,410 ± 670	2,540 ± 780	2,720 ± 1,040
CD3 count (cells/μl)	1,630 ± 600	1,570 ± 440	1,610 ± 540	1,710 ± 780
CD4 count(cells/μl)	910 ± 300	840 ± 230	890 ± 260	1,000 ± 370
CD8 count (cells/μl)	670 ± 350	670 ± 260	670 ± 340	670 ± 440

Table 4. Lymphocyte immunophenotyping data from published studies (only selected parameters are shown)

Parameters	Reichert *et al.*[8] n = 271	Erkeller-Yuksel *et al.*[7] n = 101	Hulstaert *et al.*[8] n = 85
Lymphocyte percent	30-33	32 (28-39)	31 (27-34)
CD3 percent	73 ± 6.2	72 (67-76)	75 (71-79)
CD4 percent	43 ± 7	42 (38-46)	48 (43-54)
CD8 percent	33 ± 7	35 (31-40)	25 (22-31)
CD4/CD8 ratio	1.4 ± 0.6	1.2 (1.0-1.5)	1.8 (1.4-2.4)

Values shown as mean ± SD or (range)

of CD4/CD8 ratio was 1.5 ± 0.6. The absolute T lymphocyte count, $CD4^+$ T lymphocyte count, $CD8^+$ T lymphocyte count were 1,630 ± 600, 910 ± 300 and 670 ± 350 cells/μl, respectively.

If we compared each parameter among age groups, we found that WBC count of the 40-49 year-old group was significantly higher than the 30-39 year-old group ($p < 0.05$) and the 20-29 year-old group ($p < 0.01$). However, lymphocyte percent, T cell percent and CD4 percent were not significantly different among these groups. Percentages of $CD8^+$ T cells in 40-49 year-old group was significantly lower than other 2 groups ($p < 0.01$). Forty to forty-nine year-old group

had CD4/CD8 ratio higher than the other 2 groups. When we compared lymphocyte count, T cell count, CD4 count, and CD8 count, there were no difference among these groups except CD4 count between 20-29 year-old group and 40-49 year-old group in term of statistical significance.

DISCUSSION

This report presents the results of a study which defines the reference ranges for T lymphocyte subsets in healthy Thai adults by using flow cytometric immunophenotyping. Key elements that control the quality of the process of a sample preparation and flow cytometric analysis are:

- a uniformly lysed whole blood staining technique
- the use of LeucoGATE to establish reproducible light scatter acquisition gates, which quantitates the recovery and purity of lymphocytes analyzed by correlating specific antigen expression with light scatter signal.
- definition and enumeration of percent positive cells using automated SimulSET software.
- the use of T cell percentage as an internal control for the process or analysis, this is performed by verifying that the summations of T cell between CD3/CD4 and CD3/CD8 samples were not different more than 5%, and the sum of CD4+CD8 approached the $CD3^+$ cells within ± 10% of the CD3 value.

Several published normal value studies that reported data based on defined Caucasian populations using immunophenotyping by flow cytometry are summarized in Table 4.[6-8] Comparison of the published values in Table 4 for each lymphocyte subset shows good agreement except for T lymphocyte as reported by Hulstaert *et al.*[8] where CD4 and CD8 lymphocytes were measured as $CD3^+CD4^+$ and $CD3^+CD8^+$ cells which were the same reagent combinations we used in our study. When our results were compared with the studies in Table 4, mean of $CD3^+$ lymphocyte percent in Thais showed approximately 8-11% lower ($p<0.01$). The mean percentage of $CD4^+$ T lymphocyte has been consistently reported in Causasians as greater than 40% which is higher than the value in Thais ($p<0.01$). Percentage of $CD8^+$ T lymphocytes in this study was comparable to those observed in Caucasians when measured as $CD3^+CD8^+$. Otherwise the reported CD8 lymphocyte percents are higher due to the inclusion of $CD3^-$ $CD8^+$ cells. The CD4/CD8 ratio for Thais was lower than that reported by Hulstaert *et al.*[8] for Caucasians which was not unexpected because CD4 percent in Thais was lower.

Our study established normal ranges of T lymphocyte subsets using a clinical applicable methods and confirmed that these parameters could be used in practice only after the normal ranges in the specific group of interest studied. We demonstrated clearly that CD4 percent in Thais was significantly lower than Caucasians. All decisions in clinical practice using CD4 percent and CD4 count especially in HIV infection should use this normal ranges for reference. Although we did not use lymphosum (%T+%B+%NK = 100±5%) as quality control criteria, we used tube-to-tube consistency check of CD3 percent and summation of CD4 and CD8 percent compare with CD3 percent which was thought to be enough and could be applied for clinical sample services. This assumption was confirmed when we compare our results with a recent published reference ranges in healthy Thai adults[9] in which standard recommended protocols were used, CD4 percentage was not different although our CD4 count was higher ($p < 0.05$) due to a higher absolute lymphocyte count.

In conclusion, lymphocyte immunophenotyping reference ranges were determined for a reference population of adult Thais at Maharaj Nakorn Chiang Mai Hospital. The study showed that Thais had lower CD3 percent, CD4 percent, and CD4/CD8 ratio than Caucasians. These observations have important clinical implication for the use of lymphocyte subset measurement especially in the management of HIV infection in Thais.

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