ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2003) 21: 95-103

Lymphocyte and NK Cell Subpopulations in HIV Seronegative Thais

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SUMMARY **Lymphocyte subpopulations, i.e. T, B and natural killer (NK) cells including NK cell subsets which express CD16 molecules (with or without co-expression of CD56 molecules) and NK cell subsets which express CD56 molecules (with or without co-expression of CD16 molecules) were enumerated by two color-flow cytometry in a total of 125 HIV seronegative Thai adults. The study demonstrated relatively low CD4 counts in the subjects, i.e. 26.3% of them had a CD4 count of less than 500 cells/µl. In contrast, their NK cell counts were relatively high. Statistical analyses of the percentage values showed that females had significantly higher CD3 (total T cells), but lower NK cell counts as compared to males ($p < 0.05$). Regarding age variation, an increase of 1.1% of CD4 cells per decade was seen. It was roughly estimated that about 86% of NK cells harbored both CD16 and CD56 molecules. Collective data from several studies including the present one suggest that high NK cell counts may be a compensation for low CD4 cell counts in Mongoloid people. Thus, the role of NK cells in the defense cascade against viral infections, especially human immunodeficiency virus infections deserves further investigation.**

Lymphocyte subpopulations comprising T, B and natural killer cells (NK) have been successfully enumerated by flow cytometry employing a panel of monoclonal antibodies to immunophenotypic surface markers. Determination of lymphocyte immunophenotypes may be useful in disease diagnosis, prognosis, assessing the efficacy of treatment and/or searching for clues to pathogenesis. However, normal values of certain lymphocyte subsets can vary according to age,[1-12] sex,[2,5-8,12] ethnic group,[2,5,6,8,9,11,12] circadian rhythm[13] and methodologies.[14-17] Changes of lymphocyte immunophenotypes from the reference ranges were reported in several diseases of infectious and noninfectious origins, e.g. acquired immunodeficiency syndrome (AIDS),[17,18] measles,[19] cancer[20] and diabetes mellitus.[21] In persons infected with human immunodeficiency virus (HIV), the level of CD4 cell counts is established as a guideline for the AIDS case definition or disease staging, starting anti-retroviral drugs and prophylaxis of opportunistic infections as well as a marker for treatment success.[22-24]

Collective data from Asian countries including Malaysia, Hong Kong and Thailand suggested that Mongoloid people have less CD4$^+$ T cells, but more NK cells compared to Caucasians.[5,6,8,9] A high NK cell number, therefore, seems to be a compensation for a low CD4 cell count. Thai clinicians often come across AIDS cases who have low

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CD4 cell counts, but are still capable to retain normal life activity. Whether NK cells play some role in the defense mechanism in those cases is not known. In supporting this observation, Ironson *et al.*[25] reported that NK cell cytotoxicity may be a factor which protected the health of HIV seropositive individuals who had very low CD4 cell counts.

Our study herein extended the work done by Webster *et al.*[8] who were the first to report in a multi-site study the reference ranges of lymphocyte immnophenotypes of healthy Thais with low CD4 counts and high NK cell numbers. In a more detailed approach, we determined T, B and NK cell numbers of every subject; thus, a summation of T, B and NK cells being equal to 100 ± 5% could be applied as an internal quality control by flow cytometry assay throughout the study. We collected blood samples during the same time of the day in order to minimize the effect of the circadian rhythm. Additionally we established the reference ranges for NK cell subpopulations.

MATERIALS AND METHODS

Subjects

A total of 125 HIV seronegative healthy subjects, consisting of 67 males and 58 females with an age range of 18-55 years (mean ± SD = 30.9 ± 10.0, median = 27.0 years) were enrolled in the study. All of them were native born Thais. They understood the purposes of the study and agreed to participate with informed consent.

Blood specimen collection

Five ml of venous blood were collected in EDTA vacutainer tubes during 9.00 to 11.00 a.m., and kept in an air-conditioned room before processing within two hours after collection. An aliquot of one ml of blood was sent for determination of the total white blood cell count as well as a differential cell count in an automated machine (Beckman-Coulter counter STKS, CA, USA). The remaining amount of blood was investigated by flow cytometry.

Anti-HIV testing

The subjects were screened for HIV infection by testing their plasma samples for anti-HIV antibody using both enzyme-linked immunosorbent assay (Vironostika Uni-Form II Ag/Ab, Organon Teknika, Boxtel, NL) and gel particle agglutination test (Serodia, Fujirebio, Japan). All subjects were nonreactive by these two assays.

Monoclonal antibodies to lymphocyte immunophenotypes

A panel of fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies used in this study were purchased from Becton-Dickinson Immunocytometry System (San Jose, CA, USA). It consisted of 1) CD45/CD14 (Simultest LeucoGATE) for verifying light scatter gating for lymphocytes; 2) IgG_1/IgG_1 (Simultest control) as an isotype control; 3) CD3/CD4 for staining total T lymphocytes and helper/inducer T lymphocytes; 4) CD3/CD8 for staining total T lymphocytes and suppressor/cytotoxic T lymphocytes; 5) CD3/CD19 for total T and total B lymphocytes; 6) CD3/CD16 and CD56 for staining total T and total NK lymphocytes; 7) CD3/CD16 for total T cells and NK cells which express the CD16 marker; 8) CD3/CD56 for total T cells and NK cells which express the CD56 marker.

Immunophenotypes for lymphocyte subsets are as follows: $CD3^+$ for total T cells, $CD3^+CD4^+$ for helper/inducer T cells, $CD3^+CD8^+$ for suppressor/cytotoxic T cells, $CD3^-CD19^+$ for B cells and $CD3^-CD16^+$and/or $CD56^+$ for total NK cells; and $CD3^-CD16^+$ or $CD3^-CD56^+$ for NK cell subsets.

Total NK cells were enumerated from lymphocytes expressing either $CD16^+$ or $CD56^+$ or both markers by double staining with anti-CD3-conjugated FITC and anti-CD16/CD56 conjugated PE. Some NK cells expressed both $CD16^+$ and $CD56^+$ markers ($CD3^-CD16^+ CD56^+$); and some NK cells expressed only $CD16^+$ ($CD3^- CD16^+ CD56^-$) or only $CD56^+$ ($CD3^- CD16^- CD56^+$).

Lymphocyte staining

The staining technique was modified from the US Centers for Diseases Control (CDC) revised guidelines 1997.[26] Anti-coagulated whole blood was thoroughly and gently mixed and distributed into eight 12 x 75 mm test tubes in amounts of 100 µl. Twenty microliters of each pair of monoclonal antibody were added into each tube, mixed and incubated at room temperature for 15 minutes in the dark. Then, two ml of 1x FACS lysing solution were added, followed by further incubation for another 10 minutes at room temperature. The tubes were spun at 200 x *g* for 5 minutes, and the supernatant of the lysing solution was discarded by aspiration. The pellets of stained cells were dispersed and washed

once with two ml of phosphate buffered saline (PBS) followed by suspending in 0.5 ml of 1% paraformaldehyde in PBS and analyzed by flow cytometry within 24 hours.

Flow cytometry analysis

The stained cells were analyzed on a FACScan flow cytometer (Becton-Dickinson) which was calibrated and adjusted by Cali-BRITE beads and AutoCOMP software. Two color-immunophenotype analyses were accomplished with the SimulSET software. The LeucoGATE antibody (CD45/CD14) identified lymphocytes, monocytes and granulocytes by light scatter gating; and the purity of at least 95% lymphocytes was obtained for immunophenotype analyses. The SimulSET software was programmed to enumerate 15,000 lymphocyte cells in a measurement.

Statistical analysis

The data of each lymphocyte subset obtained from all subjects were analysed for differences by gender and age variation. The Komogorov Smirnov test was used to verify the pattern of distribution of a dataset in order to determine the degree of fit. When a normal distribution of the dataset was obtained ($p \geq 0.05$), parametric analyses would be employed, i.e. the determination for sex differences of lymphocyte subsets would be performed by an independent t-test. On the other hand, if the distribution of the dataset was not normal ($p < 0.05$), non-parametric assays would be applied, i.e. the sex differences of the lymphocyte subsets would be determined by Mann Whitney-U test. The age variation was determined by simple linear regression.

RESULTS

The Komogorov Smirnov test showed a normal distribution of all the datasets in the present study, thus, an independent t-test was used to analyze sex differences in various parameters

The values of the total white blood cell counts and differential counts of the subjects are shown in Table 1. It was demonstrated that the number of WBC in males was significantly higher than in females ($p < 0.05$); but there was no statistical difference in lymphocyte counts or monocyte counts in both genders ($p > 0.05$).

The reference ranges of each lymphocyte subpopulation in term of percentages and absolute numbers are shown in Tables 2 and 3, respectively. Females had a higher total T cell count, but less total NK cells and less $CD3^-CD16^+$ NK cells than males (t-test; $p < 0.05$) (Table 2). There was no discrepancy by sex for the other parameters.

In addition to the determination of the total number of NK cells (cells which expressed either $CD3^-CD16^+CD56^+$ or $CD3^-CD16^+CD56^-$ or $CD3^-CD16^-CD56^+$), we also enumerated the NK cell subset which expressed CD16 molecules (either with or without co-expression of the CD56 marker), and the NK subset which expressed CD56 molecules (either with or without co-expression of CD16 marker). Based on the reference ranges for

Table 1 Differential leukocyte counts of the study population

Cell type	Sex	N	Mean ± SD	Median	95% Reference range
WBC x 10^3/μl	M	67	7.3 ± 2.0*	7.1	6.8-7.8
	F	58	6.4 ± 1.5*	6.2	6.0-6.8
	Both	125	6.9 ± 1.9	6.6	6.6-7.2
% Lymphocyte	M	67	30.7 ± 7.7	30.0	28.8-32.6
	F	58	31.1± 8.0	30.5	29.0-33.2
	Both	125	30.9 ± 7.8	30.0	29.5-32.3
% Monocyte	M	67	7.2 ± 2.5	7.0	6.6-7.8
	F	58	7.2± 2.3	7.0	6.6-7.8
	Both	125	7.2± 2.4	7.0	6.8-7.6

*There was a statistically significant difference when the numbers of cells of both sexes were compared ($p < 0.05$)

Table 2 Lymphocyte immunophenotype reference ranges with respect to sex and age by percentages

Lymphocyte immunophenotype	Sex	N	Mean	SD	Median	95% Reference range	Sex difference	Age variation
$CD3^+$	M	67	63.4	8.4	64.0	61.3-65.4	3.4%	
(total T cells)	F	58	66.8	7.4	66.0	64.9-68.7	F > M	NS
	Both	125	65.0	8.1	65.0	63.5-66.4		
$CD3^-CD19^+$	M	67	12.1	3.8	12.0	11.2-13.0		
(B cells)	F	58	13.0	4.9	13.0	11.7-14.3	NS	NS
	Both	125	12.5	4.3	12.0	11.8-13.3		
$CD3^+CD4^+$	M	67	31.7	6.0	32.0	30.2-33.2		1.1%
(T helper/inducer)	F	58	32.6	5.8	32.5	31.0-34.1	NS	per decade
	Both	125	32.1	5.9	32.0	31.1-33.1		
$CD3^+CD8^+$	M	67	26.9	7.1	26.0	25.2-28.6		
(T suppressor/cytotoxic)	F	58	28.7	7.5	29.0	26.8-30.7	NS	NS
	Both	125	27.7	7.3	28.0	26.5-29.0		
CD4/CD8 ratios	M	67	1.3	0.5	1.2	1.2-1.4		
	F	58	1.3	0.5	1.1	1.1-1.4	NS	NS
	Both	125	1.3	0.5	1.2	1.2-1.4		
$CD3^-CD16^+$ and/or	M	67	23.2	9.1	21.0	21.0-25.5	4.0%	
$CD56^+$	F	58	19.2	7.5	19.0	17.2-21.2	M > F	NS
(total NK cells)	Both	125	21.4	8.6	20.0	19.8-22.9		

NS, non-significant difference

both sexes of 19.8-22.9% for total NK cells, 18.1-20.9% for the CD16 NK cell subset and 17.0-19.8% for the CD56 NK cell subset, we can estimate by calculation that approximately 86% of NK cells carried both CD16 and CD56 molecules on the same cells (Table 4).

Regarding the variation in values of all lymphocyte subsets by age, only $CD3^+CD4^+$ cells showed an increase of 1.1% of cells per age decade (Table 2).

Dividing the absolute numbers of CD4 cells into three ranges, i.e. ≤ 399, 400-499 and ≥ 500 cells/μl, 26.4% of our subjects had a CD4 count below 500 cells/μl; and 13.6% had a CD4 count below 400 cells/μl (Table 5). The CD4 counts of our subjects ranged between 281 and 1,458 cells/μl (data not shown), with a 95% reference range of 624-703 cells/μl (Table 3).

DISCUSSION

Phenotyping for reference ranges of lymphocyte subsets can be influenced by the time of blood collection, duration and temperature of specimen storage before staining and flow cytometric analysis, and also by the methodologies.[13-16] A previous multi-center study reported the effects of the time lapsed from phlebotomy to staining and fixing, as well as the time lapsed from staining

Table 3 Lymphocyte immunophenotype reference ranges with respect to sex and age by absolute numbers

Lymphocyte immunophenotype	Sex	N	Mean	SD	Median	95% Reference range	Sex difference	Age variation
CD3$^+$	M	67	1,355.5	401.9	1,307.0	1,257.4-1,453.5		
(total T cells)	F	58	1,308.1	421.7	1,198.0	1,197.3-1,419.0	NS	NS
	Both	125	1,333.5	410.2	1,269.0	1,260.9-1,406.1		
CD3$^-$CD19$^+$	M	67	268.9	124.5	252.0	238.5-299.3		
(B cells)	F	58	257.4	128.2	234.5	223.7-291.1	NS	NS
	Both	125	263.6	125.9	245.0	241.3-285.8		
CD3$^+$CD4$^+$	M	67	689.8	240.8	663.0	631.1-784.5		53.8
(T helper/inducer)	F	58	633.8	198.7	584.5	581.6-686.1	NS	cells/μl
	Both	125	663.8	223.1	624.0	624.3-703.3		per decade
CD3$^+$CD8$^+$	M	67	587.5	229.6	542.0	531.5-643.6		
(T suppressor/cytotoxic)	F	58	570.3	277.4	527.5	497.4-643.3	NS	NS
	Both	125	579.6	252.0	531.0	534.9-624.2		
CD4/CD8 ratios	M	67	1.3	0.5	1.2	1.2-1.4		
	F	58	1.3	0.5	1.1	1.1-1.4	NS	NS
	Both	125	1.3	0.5	1.2	1.2-1.4		
CD3$^-$CD16$^+$ and/or	M	67	533.3	352.1	455.0	447.4-619.1	152.3	
CD56$^+$	F	58	381.0	202.3	366.5	327.8-434.1	cells/μl	NS
(total NK cells)	Both	125	462.6	301.0	403.0	409.3-515.9	M > F	

NS, non-significant difference

and fixing to analysis by flow cytometry.[15] In addition, a Japanese study suggested a circadian variation in circulating T cell subsets demonstrated in whole blood by flow cytometry technique. Repeatedly high numbers of lymphocytes were shown during night time and low values during daytime.[13]

In order to control variations induced by/from the above technical practices & issues, we performed phleblotomy during 9.00-11.00 a.m., followed by staining and fixing within 3 hours, and flow cytometry analyzed within 24 hours after staining and fixing. We also kept blood specimens in an air-conditioned room until stained.

Previous investigators demonstrated that changes in absolute numbers of lymphocyte subsets were not always consistent with changes in percentage values.[4] It should be kept in mind that the absolute number was derived from three variables: white blood cell count, total lymphocyte count and percentage of lymphocyte subsets. We also found that an analysis obtained from a dataset presenting by percentages may not give the same result as a dataset presenting absolute numbers (Tables 2 and 3). With respect to the sex difference in the values of WBC counts (Table 1), we suggest that statistical analysis of datasets presenting in terms of percentage values should be more accurate, because the result obtained is independent from other variable confounding factors.

Table 4 NK cell subpopulations by percentages and absolute numbers

Lymphocyte immunophenotype	Sex	N	Mean	SD	Median	95% Reference range	Sex difference	Age variation
Immunophenotypes by percentages								
$CD3^-CD16^+$ and/or	M	67	23.2	9.1	21.0	21.0-25.5	4.0 %	
$CD56^+$	F	58	19.2	7.5	19.0	17.2-21.2	M > F	NS
	Both	125	21.4	8.6	20.0	19.8-22.9		
$CD3^-CD16^+$	M	65	21.0	7.9	20.0	19.0-22.9	3.1 %	
	F	56	17.9	7.3	18.0	15.9-19.8	M > F	NS
	Both	121	19.5	7.8	19.0	18.1-20.9		
$CD3^-CD56^+$	M	65	19.6	8.1	17.0	17.6-21.6		
	F	56	17.0	7.3	17.5	15.0-18.9	NS	NS
	Both	121	18.4	7.8	17.0	17.0-19.8		
Immunophenotypes by absolute numbers								
$CD3^-CD16^+$ and/or	M	67	533.3	352.1	455.0	447.4-619.1	152.3	
$CD56^+$	F	58	381.0	202.3	366.5	327.8-434.1	cells/μl	NS
	Both	125	462.6	301.0	403.0	409.3-515.9	M > F	
$CD3^-CD16^+$	M	65	481.7	302.8	429.0	406.7-556.7	124.5	
	F	56	357.2	192.9	349.5	305.5-408.9	cells/μl	NS
	Both	121	424.1	264.2	377.0	376.5-471.6	M > F	
$CD3^-CD56^+$	M	65	447.7	289.7	351.0	375.9-519.5	106.9	
	F	56	340.8	200.5	328.0	287.1-394.5	cells/μl	NS
	Both	121	398.2	257.0	340.0	352.0-444.5	M > F	

NS, non-significant difference

The result of our study was compared to previous works done in blood donors as reported by 1) Webster *et al.*[8] in a multi-site study in Bangkok, i.e. Siriraj Hospital, Chulalongkorn Hospital and Armed Forces Research Institute of Medical Sciences (AFRIMS) in 1996, and 2) Vithayasai *et al.* in a Chiang Mai study.[9] The different studies presented their data in different ways and parameters. Collectively, some points of similarity and some points of discrepancy were found. Overall, all three studies demonstrated that Thai people had relatively low CD4 counts. The CD4 reference values of the present study and of that from Chulalongkorn Hospital were comparable *viz* mean = 32.1 *vs* 32.8% or 663.8 *vs* 730 cells/μl for both sexes, but both values were relatively lower than those pooled data generated by the Siriraj and AFRIMS laboratories *viz* 37.4% or 840 cells/μl, and lower than those reported by Chiang Mai Laboratory *viz* 36.1% or 910 cells/μl. The mean CD4/CD8 ratio in our study was about the same as that demonstrated by Webster *et al.*[8] *viz* 1.3 *vs* 1.35, while it was 1.5 by Vithayasai *et al.*[9] Both Webster *et al.*[8] and the present study revealed high NK cell counts especially in male subjects.

Gathering from various reports, the most important finding was the variation of lymphocyte reference ranges according to ethnic groups. Mongoloids had lower $CD4^+$ T cell counts, but a higher number of NK cells.[5,6,8] The values of $CD4^+$ T cells in Mongoloid people from different countries were similar, i.e. 32% in Thai people, 32% in Chinese living in Hong Kong,[6] 34.8% in Chinese living in Malaysia and 32.6% in native Malay people.[5] It was noteworthy that Indian people in Malaysia had higher CD4+ T cell

Table 5 Distribution of subjects with different ranges of CD4+ T cells

Sex	Number of subjects with CD4+ T cells of			Total
	≤ 399 cells/μl	400-499 cells/μl	≥ 500 cells/μl	
Male	9	9	49	67
Female	8	7	43	58
Both	17 (13.6%)	16 (12.8%)	92 (73.6%)	125 (100%)

counts; Indians are classified as Caucasian ethnic group. Reports for Caucasians from Western countries demonstrated higher CD4$^+$ T cell counts varying from 42 to 48%.[2-4,14] Our study found that 26.4% of Thai people had a CD4 count < 500 cells/μl, and 13.6% had a CD4 count < 400 cells/μl. The subject with the lowest amount of CD4 cells, 281 cells/μl, had normal WBC and CD4 counts; however, her lymphocyte count was only 15%. This subject was apparently healthy during the week of blood collection, and had no obvious medical history. Similarly, 2.9% of Chinese living in Hong Kong had CD4 counts < 300 cells/μl; and 15.9% had a CD4 count < 500 cells/μl.[6]

In the guidelines of the US and CDC, CD4 count levels are used as criteria for AIDS case definitions, for initiation of antiretroviral therapy and for pre-exposure prophylaxis of opportunistic infections.[22-24] Treatment of Mongoloid people infected with HIV based on CD4 count levels recommended for Caucasians should be reconsidered very carefully.

The compensation of Mongoloids for their low CD4 count is their high NK cell counts. The present study looked into those NK cells which expressed CD16 and/or CD56 molecules, since these two markers had been suggested to be responsible for key immunological functions of those NK cells. NK cells play a role in innate immunity; particularly, in the defense against tumor cells and viral infected cells.[27,28] CD16 molecules function as Fc receptors that bind IgG (Fc γ receptor III), thus, these receptors can link NK cells to the target cells coated with IgG and lead to target cell destruction via the mechanism so called antibody dependent cell cytotoxicity (ADCC).[27,28] CD56$^+$ NK cells play an important role in the production of several kinds of cytokines following an induction by monokines.[27,28] It was reported that activated NK cells may release RANTES, MIP-1α and MIP-1β, the cytokines capable of binding the HIV coreceptor, and thus blocking HIV replication.[29] NK cells also release tumor necrosis factor and gamma interferon which enhance immunological responses.[30] Our study demonstrated that 86% of NK cells expressed both CD16$^+$ and CD56$^+$ molecules on the same cell. NK cells also recognize and directly kill target cells through killer-activating receptors. Under normal circumstances this killing activity is superseded by killer-inhibitory receptors on recognition of MHC class I molecules. Unfortunately, the immunophenotypes of these molecules are not known.[28]

Activation of NK cells by immunological modulators or drugs may be a supplementary treatment for cancer and viral, especially HIV, infections. It has been suggested that NK cells may be a factor protecting the health of HIV seropositive individuals with very low CD4 cell counts.[25] A low level of NK cells in HIV infected cases was associated with rapid disease progression, compared to those who progressed more slowly.[31] NK cells are relatively resistant to HIV infection. Whether, the HIV can replicate in NK cells or not is still inconclusive.[32-34] NK cells can mediate killing activity which is not antigen specific. Therefore, NK cells can potentially overcome the problem of HIV antigenic diversity, which is an important factor limiting the roles of specific cytotoxic T lymphocytes and neutralizing antibody in HIV infection. A previous work done by de Souza *et al.*[35] reported that at comparable levels of NK cells, Thais had a higher cytotoxicity capability than North Americans. The role of NK cells in HIV infected Thais and other Mongoloid people deserves further investigation.

ACKNOWLEDGEMENTS

This investigation was supported by the Royal Golden Jubilee Scholarships for Ph.D. Program, the Thailand Research Fund; the Ministry of Public Health, Thailand; the Japanese Foundation on AIDS Prevention and Control; and the Japan Science and Technology Corporation.

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