LYMPHOCYTE IMMUNOPHENOTYPE REFERENCE RANGES IN HEALTHY THAI ADULTS: IMPLICATIONS FOR MANAGEMENT OF HIV/AIDS IN THAILAND

H Kyle Webster[1], Kovit Pattanapanyasat[2], Praphan Phanupak[3], Chantapong Wasi[2], Choedchai Chuenchitra[4], Lupe Ybarra[1] and Leonard Buchner[1]

[1]Becton Dickinson Immunocytometry Systems, San Jose, CA, USA; [2]Siriraj Hospital, Mahidol University, Bangkok, Thailand; [3]Chulalongkorn Hospital, Chulalongkorn University, Bangkok, Thailand; [4]Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand

Abstract. Lymphocyte immunophenotype reference ranges for T, B, and NK subsets were determined for healthy adult Thais in a multi-center study in Bangkok. Immunophenotyping was by flow cytometry using lysed whole blood. A standard protocol for flow cytometry instrumentation, reagents and quality control was used to minimize site differences and to facilitate comparison of the Thai reference values to those found for Caucasians in previous studies. Major differences were determined for CD3(T), CD4 (T helper/inducer) and CD16+56 (NK) lymphocyte percentages and CD4 lymphocyte absolute counts. Age trends and sex differences were also observed. Compared to Caucasians, Thais, particularly Thai males, had lower CD3 and CD4 T lymphocyte percentages and absolute numbers whereas the percentage of NK lymphocytes was higher. Heterogeneity attributed to biological variation of CD4 T lymphocyte but not other immunophenotype subset distributions was also observed in a well defined geographic population. This study demonstrates the importance of ethnicity, age, sex and possibly environment as factors that influence distribution characteristics of normal lymphocyte immunophenotype reference values. These observations have important implications for the use of lymphocyte subsets - particularly CD3+CD4+ T lymphocyte measurements as applied to HIV disease staging, AIDS definition and the overall clinical management of HIV/AIDS in Thailand.

INTRODUCTION

The use and misuse of CD4+ T lymphocyte measurements have critical implications for the effective management of individuals at risk or infected with Human Immunodeficiency Virus (HIV) or progressing into Acquired Immunodeficiency Disease Syndrome (AIDS). Utility of CD4+ T lymphocyte measurements involves clinical considerations for HIV infection classification, AIDS definition, assessment of prognosis, therapeutic decision making and the design of clinical trials (Stein *et al*, 1992; Laurence, 1993). Misuses of CD4+ T lymphocyte measurements arise primarily from failures to recognize biological variability and the variability inherent in laboratory methodologies and the implication for application and interpretation of normal reference values - particularly lower range values that indicate thresholds for clinical decision making.

Correspondence: Dr H Kyle Webster, Director, Medical Affairs, Becton Dickinson Asia-Pacific, 30 Tuas Avenue 2, Singapore 2263.
Tel: (65) 860-1459; Fax: (65) 860-1511

Immunophenotyping using lysed whole blood stained with monoclonal antibodies and analyzed by flow cytometry is the current standard method for measurement of CD4 + T lymphocytes and other lymphocyte phenotypes (CDC, 1994). The clinical utility of enumerating lymphocyte subsets other than CD4+ T lymphocytes has not been established although an elevation of CD8+ T lymphocytes is clearly associated with early HIV-1 infection (Nicholson *et al*, 1985; Giorgi *et al*, 1987). Other potentially useful observations involve changes in markers of lymphocyte activation (CD38, HLA-DR) on CD8+ T lymphocytes (Giorgi *et al*, 1993; Landay *et al*, 1993; Levacher *et al*, 1992).

Enumeration of the major T, B and NK lymphocyte subsets is recognized as having value for quality control in flow cytometry (CDC, 1994; Reichert *et al*, 1991). Specific guidelines for the performance of CD4+ T lymphocyte determinations in persons with HIV infection have been published by the United States Centers for Disease Control (CDC) (CDC, 1994). The CDC guidelines recommend a lymphocyte panel that includes CD3+, CD4+, CD8+, CD19+, and CD16+56+ lymphocyte

immunophenotype subsets. Reference range studies using standardized methodology are designed to estimate the distribution of target values that normally occur in a large well defined study population. Factors of age, sex, ethnicity and circadian rhythm are known to influence normal lymphocyte subset values (Laurence, 1993). There are numerous studies that have used flow cytometry to describe demographic and disease associated variations in lymphocyte immunophenotypes (Reichert *et al*, 1991; Erkeller-Yuksel *et al*, 1992; Hulstaert *et al*, 1994; Prince *et al*, 1985; Tollerud *et al*, 1989; Bofill *et al*, 1992; Goto and Nishioka, 1989). However, few studies actually report estimates of reference distributions and precisely determined range limits that are essential to confidence in clinical usage (Reichert *et al*, 1991).

Flow cytometry is a relatively new clinical laboratory resource in developing countries and there are no published reference ranges for Thai or other Southeast Asian populations despite the relentless progression of regional HIV/AIDS epidemics. There is therefore, a need to established standards for clinical flow cytometry, quality assurance and normal reference values for lymphocyte immunophenotypes in developing countries. As an approach to this objective, we conducted a multi-center study in Thailand at three laboratory sites in Bangkok. Reference ranges were developed from distributions of lymphocyte subset populations characterized by monoclonal antibodies to the following cluster differentiation (CD) antigens: CD3+ (T-lymphocytes), CD3+ CD4+ (T helper/inducer lymphocytes), CD3+ CD8+ (T suppressor/cytotoxic lymphocytes), CD3-/CD19+ (B lymphocytes) and CD3-CD16+ and/or CD56+ (NK lymphocytes).

MATERIALS AND METHODS

Study population

Each of the three institutions participating in the multi-center study were located in Bangkok: Chulalongkorn Hospital (Chulalongkorn University), Siriraj Hospital (Mahidol University), and the Armed Forces Research Institute of Medical Sciences (AFRIMS) (a collaboration between the Royal Thai Army Medical Department and the Walter Reed Army Institute of Research, Washington DC, USA). Subjects for the study were recruited from the blood donor programs at each institution. Informed consent was obtained for each subject and the protocol was approved by institutional review boards at each site. Selected individuals were native born Thai, 18-50 years of age, in overall good health, not on medications, and HIV and hepatitis B seronegative. Blood samples (5 ml) were collected by venipuncture between 8 am and 12 pm. An aliquot (1 ml) of each sample was prepared and transported within 6 hours to Siriraj Hospital for hematological analysis. To reduce methodological variability and to focus on individual biological variation, a standard protocol was used at all three sites to define sample preparation, reagents, flow cytometry operation, as well as data acquisition, processing, and analysis.

Panel of monoclonal antibody reagents

The combinations of fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugated monoclonal antibody reagents as well as the antigen(s) expressed were as follows: CD45/CD14 (Simultest LeucoGATE) to establish and verify light scatter gating of lymphocytes; IgG_1/IgG_{2a} (Simultest Control) as an isotype control for marker positioning; CD3/CD4 to quantitate T lymphocytes and T helper/inducer lymphocytes; CD3/CD8 for T lymphocytes and T suppressor/cytotoxic lymphocytes; CD3/CD19 for T and B lymphocytes; and CD3/CD16+56 to measure T lymphocytes and NK lymphocytes (Becton Dickinson Immunocytometry Systems, San Jose, CA). This six tube panel is consistent with the guidelines recommended by the Centers For Disease Control (CDC, 1994).

Cell preparation and staining

Peripheral blood (5ml) samples were collected into K_3EDTA tubes and processed for flow cytometry within six hours of venipuncture. Twenty microliters of each monoclonal antibody reagent pair was added to 100 μl of whole blood in 12 × 75 mm test tubes. The whole blood and monoclonal antibody mixture was gently vortexed and incubated for 15 minutes at room temperature in the dark. Following the incubation period, 2 ml of (1×) FACS Lysing Solution was added to each tube. The mixture was gently vortexed and incubated for 10 minutes at room temperature in the dark. Samples were then centrifuged at 200*g* for 5 minutes at room

temperature. Subsequently, the supernate was aspirated and the pellet resuspended in 2ml PBS with 0.1% azide. The samples were gently vortexed and centrifuged at 200*g* for 5 minutes at room temperature. The supernatant was aspirated as before and the pellet was resuspended in 0.5 ml of 1.0% paraformaldehyde in either sheath fluid or PBS with 0.1% azide. All samples were analyzed within 12 hours of collection.

Flow cytometry analysis

Data at each site were acquired and analyzed on identically configured FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometers. FACScan setup and instrument calibration including PMT and compensation adjustments were performed using AutoCOMP software and CaliBRITE beads in accordance with manufacturer's recommendations.

SimulSET software which provides automatic acquisition, gating, counting, and computation was used for this study. A minimum of 15,000 events were acquired using the LeucoGATE (CD45/CD14) tube. The lymphocytes were identified based on the intensity of the CD45/CD14 immunofluorescence and a corresponding light scatter gate (FSC *vs* SSC) that included 98% or greater of the lymphocytes. The SimulSET software then calculated the purity of the light scatter gate by counting monocytes, granulocytes, and debris. Debris included platelets, nucleated erythrocytes, and unlysed erythrocytes that were also included within the gate. If the contaminating monocytes were fewer than 3% of the cells within the gate, the program proceeded to the IgG_1/IgG_{2a} (isotype control) tube. This process of establishing a proper light scatter gate included > 95% of all lymphocytes. Daily optimization of instrument settings enhanced the ability to gate on lymphocytes without including monocytes. Another feature of the software was its ability to calculate results as a percentage of lymphocytes within the analysis gate rather than as a percentage of the total events in the gate.

A minimum of 2,000 lymphocyte events were acquired from each tube after LeucoGATE (CD45/CD14). The IgG_1/IgG_{2a} (Simultest Control) tube determined the limits of background fluorescence. With few exceptions, the marker settings determined with the isotype control tube were used to analyze the remainder of the tubes in the panel. marker repositioning was only performed in cases to correct for undercompensation. Flow cytometry results were reported as the lymphocyte subset percentage of total lymphocytes.

Quality control

For all subjects for whom complete flow cytometry, laboratory, and demographic data were available, the SimulSET software printouts were evaluated using the following quality control criteria for acceptance (Reichert *et al*, 1991). Adequate separation between cellular populations as defined by SimulSET software's ability to set a lymphocyte gate; Nonspecific staining as defined by the negative control ≤ 5%. Lymphocyte gating was verified as follows: percentage of total lymphocytes included in the analysis gate ≥ 95%; percentage of granulocytes included in the analysis gate ≤ 6%; percentage of monocytes included in the analysis gate ≤ 3%; and percentage of debris included in the analysis gate ≤ 10%. Tube-to-tube consistency checks were performed to include agreement of all four CD3 values within 8% and a gated event difference that did not exceed 500 events. The lymphosum (%T + %B + %NK = 100% ± 5% was used as an internal process control (purity) with %T calculated as the average CD3 measurement, %B calculated as the %CD3- CD19+ and %NK calculated as the %CD3-CD16+ 56+.

Hematology

A 1 ml aliquot of the peripheral blood sample was separated at the Chulalongkorn and AFRIMS collection sites and transported to Siriraj Hospital within three hours. All samples were analyzed within six hours on a Coulter J3 hematology instrument to obtain the WBC and lymphocyte differential percentage. Absolute lymphocyte subset counts were determined as the product of the WBC, lymphocyte differential percentage and the flow cytometry generated lymphocyte subset percentage.

Statistical analysis

Statistical procedures were essentially those as described in a previous paper (Reichert *et al*, 1991). Combinability of data for each lymphocyte subset was tested using the Kolmogorov-Smirnov and

Krushkal-Wallis tests (Conover, 1980). Since there were 3 sites being tested, the alpha level used to determine significance was adjusted to 0.0167 for the Kolmogorov-Smirnov test. Combined data for each lymphocyte immunophenotype were used to generate histograms and sample statistics for estimation of parameters for the best fits to each of seven selected statistical distributions (truncated gaussian, inverse gaussian, log normal, gamma, weibel, burnbaum-saunders and gompertz) (Reichert *et al*, 1991; Christensen, 1990). The Kolmogorov-Smirnov statistic was used to verify that the best fit distribution determined for the combined data was appropriate for the data at each site. The mathematical form of the best-fitting distribution was then used to estimate the reference range for each lymphocyte subset. The central 95% of the area under the fitted distribution curve is then the reference range. Regresion statistics were used with each lymphocyte subset analyzed covariantly for detection of age trends or sex differences (Reichert *et al*, 1991).

RESULTS

There were 313 subjects enroled in the study from the three clinical sites. Following evaluation of clinical report forms and flow cytometry files, 217 subject datasets were found acceptable. Incomplete clinical information accounted for elimination of 26 subjects while the remaining 70 subjects were unacceptable based on flow cytometry quality control (QC) criteria. The causes of QC failure were in order of descending frequency: gated events check failure, Lymphosum (T+B+NK) check failure, excess gate contamination or failure of software to set a gate, and CD3 check failure. Rigorous application of these measures of internal consistency to the flow cytometry data removed a large fraction of otherwise defective samples from the database used for statistical analysis.

Histograms were generated from analysis of each lymphocyte immunophenotype and the best fit for mathematically defined distribution determined

Table 1

Statistical characteristics of the distribution for each lymphocyte immunophenotype (percentages for all study sites).

Lymphocyte immunophenotype	Sex	N	Mean	Median	95% Lower range		Reference upper range	SD
CD3+* (Total T lymphocytes)	Male	131	65.3	66.25	46.2 (42.9-49.3)	-	78.9 (77.6-80.1)†	8.4
	Female	86	70.2	70.25	57.8 (55.9-59.7)	-	82.7 (80.9-84.7)	6.4
CD3+CD8+ (T suppressor/cytotoxic lymphocytes)	Male	131	27.6	27.0	17.1 (16.1-18.1)	-	42.7 (40.3-45.3)	6.6
	Female	86	30.2	29.5	19.5 (18.3-20.8)	-	44.6 (41.8-47.6)	6.4
T helper/suppressor lymphocyte ratio	Both	216	1.35	1.27	0.65 (0.61-0.69)	-	2.49 (2.33-2.66)	0.48
CD3-CD19+ (B lymphocytes)	Both	217	14.7	14.0	7.7 (7.4-8.4)	-	25.4 (24.4-27.4)	4.5
CD3-CD16+ and/or CD56+ (NK lymphocytes)	Male	131	20.6	20.0	3.9 (1.9-6.0)	-	38.5 (36.4-40.5)	8.5
	Female	85	15.3	14.0	6.4 (5.7-7.3)	-	31.1 (27.5-35.2)	6.4

* Total CD3+ value calculated as the average of the CD3 value taken from the CD3/CD4, CD3/CD8, CD3/CD19, and CD3/CD16+56 tubes.

† 90% confidence interval on reference range limits

(Reichert *et al*, 1991; Christensen, 1990). Characteristics of the distribution of each lymphocyte subset including mean, median, standard deviation, reference range and variations for sex and age are summarized in Tables 1-3. Data from all three sites were observed to be combinable by Kolmogorov-Smirnov and Krushkal-Wallis test (Conover, 1980) for all lymphocyte immunophenotypes except the CD3+CD4+ at Chulalongkorn Hospital. An effort was made to account for the source of variation at this hospital. A cross-over study (data not shown) involving Chulalongkorn Hospital and AFRIMS was conducted with 40 subjects accessioned at Chulalongkorn Hospital in which samples were split and aliquots run at both sites. The results from both sites showed good agreement and the CD3+ CD4+ lymphocyte distributions resembled the original reference range data collected at Chulalongkorn Hospital. This finding ruled against technical factors and suggested an undefined source of biological variation associated only with the CD3+CD4+ T lymphocyte subset in the Chulalongkorn subject population. Volunteer selection criteria, individual case report forms, factors of age and sex distributions and social risk factors for the Chulalongkorn population were carefully reviewed. There was no obvious site specific explanation for the heterogeneity in CD3+CD4+ lymphocyte distribution.

Lymphocyte immunophenotypes as percentages

B Lymphocytes (CD3-CD19+): CD19+ B lymphocytes were characterized by an underlying log normal distribution slightly skewed to higher values (mean 14.7%, 7.7-25.4 range, Table 1). There were no age or sex differences observed.

Natural killer cells (CD3-, CD16+ and/or CD56+): The distribution for males (truncated normal) was symmetric whereas the female distribution (log normal) was skewed to higher values. The lower reference range limits overlapped between the male and female distributions whereas the upper reference range limits differed by 7.4% and the confidence intervals did not overlap. Analysis of sex showed a large mean difference, males having a NK cell percentage greater than females (20.6% versus 15.3% $p = 0.0001$, Table 1). An analysis of age showed no significant trends.

T lymphocytes (CD3+): The percentage of CD3+ lymphocytes (total T lymphocytes) was determined by gating on the total lymphocyte population. Confidence bounds on the male and female lower reference range limits did not overlap with the male limit being lower by 11.6% (Table 2). Analysis by sex showed females had a higher mean value than males (70.2 versus 65.3%, $p = 0.001$) (Table 3). The underlying distribution for females was Gaussian (truncated normal) whereas the underlying male distribution was an inverse log normal and skewed to lower values. Analysis of age showed an average decrease of - 1.2% per decade, $p = 0.0195$. Most of the decrease appeared at ages greater than 38. There was no female/male difference observed with age.

T suppressor/cytotoxic lymphocytes (CD3+ CD8+): The fitted distribution for males and females was log normal and slightly skewed to higher values. The mean values by sex for percent CD3+CD8+ lymphocytes were 27.6% (17.1-42.7, range, males) and 30.2% (19.5-44.6, range, females) - a 2.6% mean difference ($p = 0.0134$) (Table 1). Analysis by age showed an average decrease for both sexes of - 1.3% per decade, $p = 0.004$ (Fig 2b).

T helper/inducer lymphocytes (CD3+CD4+): The distribution of CD3+CD4+ T lymphocyte percentage for Chulalongkorn Hospital unlike the other subsets was not combinable with the distributions for both the Siriraj and AFRIMS sites. Separate CD3+ CD4+ T lymphocyte reference ranges are therefore, presented for Chulalongkorn and Siriraj/AFRIMS. The best fitting distribution at all sites was log normal. At Chulalongkorn the mean (unadjusted for sex and age) percentage for CD3+ CD4+ T lymphocytes was 32.8% (18.4-47.2%, range) compared to 37.4% (24.1-50.7, range) at Siriraj/AFRIMS, $p \leq 0.0001$ (Table 2). Sex and age difference were observed for all sites (Table 3). Males at Chulalongkorn showed a remarkable mean CD3+ CD4+ T lymphocyte value of 29.9% (18.6-41.2%, range) which was lower than that observed for both males and females at AFRIMS/Siriraj (36.1% (22.9-49.3) and 40.4% (27.8-53.1) range, $p \leq 0.0001$) (Table 2). Analysis by age showed an average increase for both sexes of +1.0% per decade, $p = 0.029$).

T helper/suppressor ratio: The underlying distribution for the CD3+CD4+/CD3+CD8+ ratio (all sites) was log normal. There was no significant difference in ratio (mean 1.35, range 0.65-2.49) between males and females (Table 1). An age trend

Table 2

Statistical characteristics of the distribution for CD3+CD4+ T helper/inducer lymphocytes. (Percentages and absolute counts for each study site)

Lymphocyte immunophenotype	Sites	Sex	N	Mean	Median	95% Lower range	Reference upper range	SD
CD3+CD4+ (T helper/inducer lymphocytes)	A&S*	Both	154	37.4	37.0	24.1 - (22.5-25.6)	50.7 (49.1-52.2)†	6.8
,,		Male	90	36.1	36.0	22.9 - (20.9-24.9)	49.3 (47.3-51.3)	6.7
,,		Female	64	40.4	40.5	27.8 - (25.5-30.1)	53.1 (50.1-55.4)	6.5
,,	CH°	Both	62	32.8	33.0	18.4 - (16.1-20.8)	47.2 (44.8-49.6)	7.3
,,		Male	40	29.9	30.0	18.6 - (16.0-21.2)	41.2 (38.6-43.8)	5.8
,,		Female	22	35.9	36.0	22.6 - (18.5-26.7)	49.2 (45.1-53.3)	6.8
Absolute lymphocyte count		Both	212	2.4	2.29	1.5 - (1.4-1.6)	3.5 (3.4-3.6)	0.5
Absolute CD3+CD4+ (Absolute T helper/inducer lymphocytes)	A&S*	Both	152	840	836	470 - (441-501)	1,404 (1,317-1,496)	240
		Male	89	815.5	775.7	430.1 - (393-471)	1,410.7 (1,289-1,544)	252.9
,,		Female	63	933.2	907.9	573.4 - (528-623)	1,437.5 (1,323-1,562)	221.8
,,	CH°	Both	59	730	709	428 - (390-470)	1,161 (1,058-1,275)	190
,,		Male	37	697.4	693.8	430.1 - (386-479)	1,070.7 (961-1,192)	167.2
,,		Female	22	750.1	740.2	409.8 - (345-487)	1,264.3 (1,064-1,502)	220.1

* AFRIMS and Siriraj Hospital
° Chulalongkorn Hospital
† 90% confidence interval on reference range limits

was observed that showed an increase of +0.12% per decade ($p \leq 0.0004$).

T helper/inducer (CD3+CD4+) lymphocyte immunophenotype as absolute count

Absolute lymphocyte count: The absolute number of lymphocytes was calculated as the product of the WBC count and percentage total lymphocytes in the differential count as determined by a hematology analyzer. All samples were analyzed using the same instrument (Siriraj Hospital). The best fitting distribution was log normal which was skewed to higher values. The median absolute lymphocyte count was 2,290 cells/μl (Table 2). There were no significant differences for age or sex.

Absolute T helper/inducer lymphocytes (CD3+

Table 3

Sex and age variation for the major lymphocyte immunophenotype subsets.

Lymphocyte immunophenotype	Variation with age	Sex diference
CD3+* (Total T lymphocytes)	- 1.2% per decade	5.0% Female > Male
CD3+CD4+ (T helper/inducer lymphocytes)	+ 1.0% per decade	3.8% Female > Male
CD3+CD8+ (T suppressor/cytotoxic lymphocytes)	-1.3% per decade	2.5% Female > Male
T helper/suppressor lymphocyte ratio	+ 0.12 per decade	NS†
CD19+ (B lymphocytes)	NS	NS
CD3-CD16+ and/or CD56+ (NK lymphocytes)	NS	6.3% Male > Female
Absolute lymphocyte count	NS	NS
Absolute CD3+CD4+ (Absolute T helper/inducer)	60.8cells/μl†† per decade	79.4 cells/μl Female > Male

* Total CD3+ value calculated as the average of the CD3 values taken from the CD3/CD4, CD3/CD8, CD3/CD19 and CD3/CD16+56 tubes.

† NS, Not significant

†† 18-40 years of age

CD4+): The absolute number of CD3+CD4+ lymphocytes was determined from the product of the absolute lymphocyte count (hematology analyzer) and the percentage of the CD3+CD4+ subset (flow cytometer). As with percentages, age, sex, and site differences were observed for CD3+ CD4+ T lymphocyte absolute counts. The distribution for the CD4+ absolute count for all sites both male and female was log normal and skewed to higher values. At Chulalongkorn Thai males had a mean absolute CD4 count of 697 cells/μl (430-1,071, range) whereas females were higher at 750 cells/μl (410-1,264 cells/μl, range) (Table 2). CD4 absolute count values were lower for both males and females at Chulalongkorn compared to AFRIMS/Siriraj (815 cells/μl (430-1,411) and 933 cells/μl (573-1,437), range). Whereas males had the same lower reference range limit at all sites (430 cells/μl) females at Chulalongkorn showed a mean shift with a lower limit of 410 cells/μl. Although Thai males had the same lower reference limit the male upper reference limit at Chulalongkorn was 340 cells/ml lower than that for AFRIMS/Siriraj. An age trend of +60.8 cells/μl per decade was observed for both males and females, age 18-40, p = 0.033 (Table 3).

Critical value tables

A set of critical values (Table 4) for the mean and standard deviation of each lymphocyte immunophenotype subset in the Thai study was established using statistical procedures previously published (Reichert *et al*, 1991). An independent laboratory can determine whether their reference population based on a smaller sample size (n = 50) is sufficiently similar (with 95% confidence) to the current multi-center study reference ranges by comparing their mean and standard deviation to the value ranges in Table 4. If the laboratories mean

Table 4

Critical values for the mean and standard deviation for each lymphocyte immunophenotype.

Lymphotype immunophenotype	Sites	Sex	N	Mean (min, max)	SD (min, max)
CD3+ (Total T lymphocytes)	All	Male	131	65.3* (62.94, 67.70)	8.4 (6.57, 10.73)
,,	All	Female	86	70.2 (68.47, 72.00)	6.4 (5.11, 7.62)
CD3+CD4+ (T helper/inducer lymphocytes)	A & S*	Both	154	37.4 (35.48, 39.24)	6.8 (5.44, 8.12)
,,		Male	90	36.1 (34.26, 38.00)	6.7 (5.4, 8.06)
,,		Female	64	40.4 (38.63, 42.21)	6.5 (5.18, 7.73)
,,	CH°	Both	62	32.8 (30.78, 34.85)	7.3 (5.89, 8.79)
,,		Male	40	29.9 (28.29, 31.48)	5.8 (4.62, 6.89)
,,		Female	22	35.9 (33.98, 37.75)	6.8 (5.45, 8.13)
CD3+CD8+ (T suppressor/cytotoxic lymphocytes)	All	Male	131	27.6 (25.98, 29.47)	6.6 (5.01, 7.97)
,,	All	Female	86	30.2 (28.47, 32.02)	6.4 (4.98, 8.15)
T helper/suppressor lymphocyte ratio	All	Both	216	1.35 (1.22, 1.48)	0.48 (0.34, 0.62)
CD3-CD19+ (B lymphocytes)	All	Both	217	14.7 (13.68, 16.26)	4.5 (3.45, 5.89)
CD3-CD16+ and/or CD56+ (NK lymphocytes)	All	Male	131	20.6 (17.71, 22.84)	8.5 (7.42, 11.07)
,,	All	Female	85	15.3 (13.62, 17.03)	6.4 (4.49, 8.51)
Absolute lymphocyte count	All	Both	212	2.4 (2.21, 2.49)	0.5 (0.41, 0.65)
Absolute CD3+CD4+ (Absolute T helper/inducer lymphocytes)	A & S	Both	152	840† (780,920)	240 (180,310)
,,		Male	89	815.5 (745,886)	252.9 (203,303)
,,		Female	63	933.2 (872,995)	221.8 (178,266)
,,	CH	Both	59	730 (680,780)	190 (140,230)
,,		Male	37	697.4 (652,743)	167.2 (132,197)
,,		Female	22	750.1 (689,811)	220.1 (177,263)

Note: Total CD3+ value calculated as the average of the CD3 value taken from the CD3/CD4, CD3/CD8, CD3/CD19, and CD3/CD16+56 tubes.

* Percentages

† Cells/μl

and standard deviation for a specific lymphocyte subset fall within the relevant critical values then there is concordance between populations and the published reference ranges can be used. It is essential that the independent laboratory reference population be based on the same selection criteria as used for the published study and that the same flow cytometry methodology be used. For CD4 T lymphocytes there are two sets of critical values reflecting the heterogeneity observed for the AFRIMS/Siriraj and Chulalongkorn reference populations. Where another laboratory reference sampling is non concordant then that laboratory must develop independent reference ranges.

DISCUSSION

We observed in the Thai study that the proportions of T (CD3+), T-helper/inducer (CD3+ CD4+) and NK (CD3-/CD16+ and/or CD56+) and the absolute number of CD3+ CD4+ T lymphocytes were different in healthy adult Thais compared to published data on adult Caucasians. These differences in lymphocyte subset values were most evident in Thai males. To place the Thai data in perspective, we have drawn upon published normal value studies (Reichert *et al*, 1991; Erkeller-Yuksel *et al*, 1992; Hul-staert *et al*, 1994) that reported data based on defined Caucasian populations and comparable approaches to immunophenotyping by flow cyto-metry. Differences among studies involved primarily monoclonal antibody reagents. Data from these studies are summarized in Table 5. Comparison of the published values in Table 5 for each lymphocyte subset shows good agreement except for T lympho-cytes as reported by Hulstaert *et al* (1994) where CD4 and CD8 lymphocytes were measured as CD3+ CD4+ and CD3+ CD8+ cells. This reagent combination was the same as that used in the Thai study.

The percentage of B (CD19+) lymphocytes in

Table 5*

Lymphocyte immunophenotype data from published studies.

Study	Reichert *et al* (1991) n = 261	Erkeller-Yuksel *et al* (1992) n = 101	Hulstaert *et al* (1994) n = 85
CD3+	73 (6.2*) (61-85)	72 (67-76)	75 (71-79)
CD4+	43 (7) (28-58)	42 (38-46)	48 (43-54)
CD8+	33 (7) (19-48)	35 (31-40)	25 (22-31)
CD4/CD8	1.4 (0.6) (0.6-2.8)	1.2 (1.0-1.5)	1.8 (1.4-2.4)
CD3-, CD16+, CD56+	14 (6) (6-29)	14 (10-19)	11 (8-15)
CD19	14 (7) (7-23)	13 (11-16)	13 (11-16)
Absolute lymphocyte	(1,100-1,400)	2,100† (1,600-2,400)	1,600 (1,300-1,900)
Percentage lymphocyte	(30-33)	32 (28-39)	31 (27-34)
WBC	(5,700-5,900)	5,900 (4,600-7,100)	5,300 (4,400-6,300)
Absolute CD4	ND	800 (700-1,000)	700 (600-980)
Absolute CD8	ND	700 (500-900)	418 (309-531)

* Values are percentages as mean (SD) and/or (range)

† Values are absolute number (cells/ml) as mean and/or (range)

Thais (14.7) (7.7-25.4 mean, 95% range) was comparable to that observed in the Caucasian studies. There were no observed differences for age or sex with B lymphocytes. NK lymphocyte (CD 3-/ CD16+ and/or CD56+) percentage was notably increased in Thai males (20.6% (3.9-38.5) mean, 95% range) compared to Thai females and Caucasians. The mean difference was about 6%. Reichert *et al* (1991) noted a difference between Caucasian men and women that was about 1.7%. In a previous study by Prince *et al* (1985) CD16 + NK cells were reported as higher in Asians - Chinese and Japanese - than Caucasians although no distinction was made by sex. Tollerud *et al* (1989) found no differences between Caucasians and Black Americans or difference by sex for CD16 + NK cells. Elevated NK lymphocyte levels in Thais, Chinese, and Japanese groups compared to Caucasian reflect a possible genetic basis. However, in the Thai population female NK lymphocyte values were comparable to Caucasians suggesting the influence of environmental or sociological factors.

CD3+ lymphocytes in Thai males showed an approximate 7-10% mean decrease compared to Caucasians. A higher CD3 percentage (5%) was observed in Thai females compared to Thai males. In Caucasians, the male to female difference was 2.5%, half that noted for Thais. A decrease in CD3+ lymphocytes with age of - 1.2%/decade was observed in Thais. There was no age trend reported for Caucasians.

Percentage of CD8+ T suppressor/cytotoxic lymphocytes in adult Thais was comparable to those observed in Caucasians when measured as CD3+ CD8+ [Hulstaert *et al* (1994) Table 5]. Otherwise the reported CD8 lymphocyte values are higher due to the inclusion of CD3- CD8+ cells. Thai CD3+ CD8+ lymphocytes showed a 12-15% higher upper reference range than that observed in the Hulstaert *et al* (1994) study. Unlike previous studies CD3+ CD8+ T lymphocyte values in Thais showed differences for both age (-1.3%/decade decrease) and sex (females were 2.5% greater than males).

The mean percentage of CD4+ T lymphocytes has been consistently reported in Caucasians as greater than 40% with a spread on the 95% range from 28-58% (Table 5). Interestingly Giorgi *et al* (1990) observed a mean CD4+ lymphocyte value of 45% (7% SD) in a large (n = 2,787) group of asymptomatic, seropositive males who were predominantly Caucasian. Compared to healthy and asymptomatic HIV+ Caucasians, Thai CD4+ T lymphocyte percentages showed lower mean values with 33% at Chulalongkorn and 37% at AFRIMS/Siriraj (unadjusted for sex and age, Table 2). At Chulalongkorn the estimate for the 2.5% quantile dropped to 18.4%. This 18.4% lower range was bounded by a 16.1-20.8% confidence interval (90%) placing the lower limit of normal for Thai males within approximately 2% of the US CDC surveillance definition of AIDS (≤ 14%, category 3) (CDC, 1992). Thai females compared to males had higher mean CD4+ lymphocyte percentages (Table 3). At AFRIMS/Siriraj Thai females had a mean CD4+ T lymphocyte percentage of 40.4% (27.8-53.1%, range) which was comparable to the Caucasian data (Table 5). The relative difference (Approx 3.5%) between mean CD4 + T lymphocyte percentage for males and females was similar in both Thais and Caucasians (Table 3, Reichert *et al,* 1994). CD4+ T lymphocytes increased with age at a rate of about + 1.0% per decade in both Thais and Caucasians. Absolute numbers of CD4+ T lym-phocytes were similar for Thais and Caucasians (comparison of means unadjusted for sex and age, Tables 2, 4). However Thai lower reference range values differed from the Caucasian values in that both Thai groups had values below 500 cells/μl (470 cells/μl at AFRIMS/Siriraj and 428 cells/μl at Chulalongkorn, means unadjusted for age and sex, Tables 2, 5). This distinction is important in view of published CDC recommendations that target CD4+ lymphocyte counts of 500 cells/μl in HIV seropositive individuals as a clinical threshold for inclusion in the overall consideration to start or withhold AZT therapy (CDC, 1992). The two studies in Table 5 that reported CD4+ T lymphocyte absolute counts did not note sex difference although Reichert *et al* (1994) reported one for percentages. In the Thai study males had consistently lower CD4+ T lymphocyte absolute counts compared to females (Table 2) and particularly notable were lower range boundaries less than 400 cells/μl.

As a final point of comparison the CD4/CD8 ratio for Thais was notably lower than that reported by Hulstaert *et al* (1994) for Caucasians [(1.35 (0.65-2.49) versus 1.8 (1.4-2.4) mean, range)] (Tables 2, 5).

The comparative reference range data suggest two distinctive features of lymphcytes in the Thai population. First, Thai males compared to Caucasians have higher NK lymphocyte (percentage) and

lower CD3+ CD4+ T lymphocyte percentage and absolute number (based on lower range boundaries). Second, compared to Thai males, Thai females have significantly lower NK levels and higher CD3+ CD4+ T lymphocyte levels making Thai females more comparable to the Caucasian population. These observations have both immunological and clinical implications. Elevated levels of NK cells in normal individuals at risk to HIV infection or as candidates for HIV vaccines may be important immunologically. Recent work suggest that NK cells may play a role in polarizing the immune response towards TH1 type immunity (Garside and Mowt, 1995). However once HIV infection occurs there is evidence for progressive depletion of NK cells (CD3- CD8+ CD16+) (Mansour *et al*, 1990) and early depressed cytokine functional capacity (Rosenberg and Fauci, 1991). NK cells can also be infected with HIV-1 *in vitro* (Chehimi *et al*, 1991). Thus there may be some clinical value to measuring NK cells levels and function in healthy at risk populations.

Lymphocyte immunophenotype, multi-site reference ranges developed in this study are the first to systematically characterize an adult Thai reference population. Careful selection of the reference individuals and rigorous standardization of pre-analytical and analytical procedures across study sites provided a good estimate of biological and methodological variation. Consequently unique ethnic, age, sex and possibly environmental characteristics have been identified for thai lymphocyte subset distributions. Lymphocyte subset distributions were similar at all sites except for CD4+ T lymphocytes at Chulalongkorn. Methodologic variation was ruled against using a cross-over study. The lower CD4 + T lymphocyte values observed at Chulalongkorn appear, therefore to represent biological heterogeneity in the Thai reference population. Additional studies particularly in the rural populations of Thailand will be needed to further characterize the distributions of CD4 + T lymphocytes in the Thai population.

When methodological variables are not controlled - despite the use of a well developed reference population - reference ranges and the clinical decisions based on them become unreliable. A recent international study by Vulgelers *et al* (1993) emphasized this point. This tricontinential study examined decline in CD4+ T lymphocytes following seroconversion in homosexual men characterized as homogenous for AIDS incidence, index diagnoses and ethnicity over 8 years at five study sites employing different flow cytometry instruments and procedures. Major site differences were observed for CD4+ T lymphocyte depletion that were attributed to inter-laboratory variation. For example, Kaplan-Meier survival curves showed that the time at which 50% of the cohort in Vancouver dropped below 500 CD4+ cells/µl was 4.4 years whereas in Sydney it was 1.2 years, an approximate 3 fold difference. Similar differences between sites were observed for CD4+ T lymphocyte counts dropping below 200 CD4+ cells/µl. Therapeutic guideline or policy being applied across clinical sites in such a situation are compromized in that some individuals are being treated too soon and others too late.

The impact of such methodologic variation on the individual and the health care system may be substantial. Such lessons do not, however, need to be repeated. In Thailand efforts are already underway to standardize clinical flow cytometry practices throughout Ministry of Public Health medical centers and hospitals. As a result the development of national clinical guidelines involving CD4+ T lymphocyte measurements for HIV/AIDS management should be uniformly applicable throughout Thailand.

In conclusion, lymphocyte immunophenotype reference ranges were determined for a reference population of adult Thais living in the Bangkok metropolitan area. Ethnic, age, sex and other biologic variation were observed for the Thai population - particularly Thai males, when compared to published reference ranges on Caucasians. These observations have important implications for the use of lymphocyte subset measurements in the management of HIV/AIDS or other clinical applications.

ACKNOWLEDGEMENTS

We thank Sunee Sirivichayakul, Sasiwimol Ubonyan (Chulalongkorn University Hospital), Charin Thepthai (Siriraj Hospital, Mahidol University) Pangsri Tongtawe, Prapasson Kongcharoen and Suchitra Sukwit (AFRIMS) for their dedicated efforts as flow cytometer operators.

REFERENCES

Brofill M, Janossy GJ, Lee CA, *et al.* Laboratory control values for CD4 and CD8 T lymphocytes. Implications for HIV - 1 diagnosis. *Clin Exp Immunol* 1992; 88 : 243-52.

Centers for Disease Control. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR* 1992; 41 : 1-19.

Centers for Disease Control. 1994 Revised guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus (HIV) infection. *MMWR* 1994; 43 : 1-21.

Chehimi J, Bandyodedhyay S, Prakash K, *et al.* *In vitro* infection of natural killer cells with differen human immunodeficiency virus type 1 isolates. *J Virol* 1991; 65 : 1812-22.

Christensen R. Enropy minimax sourcebook, volume XI. Fitting distributions to data. Lincoln: Enropy limited, 1990; pp 81-146.

Conover WJ. Practical nonparametric statistics, 2nd edi New York: Wiley, 1980.

Erkeller-Yuksel FM, Deneys V, Uysel B, *et al.* Age related changes in human blood lymphocyte subpopulations. *J Pediatrics* 1992; 120 : 216-22.

Garside P, Mowt AM. Polarization of T-cell responses: A phylogenetic consequence of nonspecific immune defense. *Immunol Today* 1995; 16 : 220-3.

Giorgi JV, Nisharian PG, Schmid I, Hultin LE, Chong H, Detels R. Selective alterations in immunoregulatory lymphocyte subsets in early HIV lymphadenopathy associated virus infection. *J Clin Immunol* 1987; 7 : 140-50.

Giorgi JV, Cheng H, Margolick JB, *et al.* Quality control in the flow cytometric measurements of T-lymphocyte subsets: The multi-center AIDS cohort study experience. *Clin Immunol Immunopathol* 1990; 55 : 173-86.

Giorgi JV, Liu Z, Hultin LE, Cumberlan WG, Hennessey K, Detels R. Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: Results of 6 years of follow-up. *J Acq Imm Def Syn* 1993; 6 : 904-12.

Goto M, Nishioka K. Age and sex related changes of the lymphocyte subsets in healthy (Japanese) individuals: An analysis by two-dimensional flow cytometry. *J Gerontol Med Sci* 1989; 44 : 51-6.

Hulstaert F, Hannet I, Deneys V, *et al.* Age related changes in human blood lymphocyte subpopulations. *Clin Immunol Immunopathol* 1994; 70 : 152-8.

Landay AL, Mackewicz CE, Levy JA. An activated CD8+ T cell phenotype correlates with anti-HIV activity and asymptomatic clinical status. *Clin Immunol Immunopathol* 1993; 69 : 106-16.

Laurence J. T-cell subsets in health, infectious disease and idiopathic CD4+ T lymphocytopenia. *Ann Int Med* 1993; 119 : 55-62.

Levacher M, Hulstaert F, Tallet S, Ullery S, Poadalo JJ, Bach BA. The significance of activation markers on CD8 lymphocytes in Human Immunodeficiency Syndrome: Staging and prognostic value. *Clin Exp Immunol* 1992; 90 : 376-82.

Nicholson JKA, McDougal JS, Spira TJ. Alterations of functional subsets of T helper and T suppressor cell populations in acquired immunodeficiency syndrome (AIDS) and chronic unexplained lymphadenopathy. *J Clin Immunol* 1985; 5 : 269-74.

Mansour I, Doenel C, Rouger P. CD16+ NK cells decrease in all stages of HIV infection through a selection depletion of the CD16+ CD8+ CD3- subset. *AIDS Res Hum Retrovir* 1990; 6 : 1451-9.

Prince HE, Hirji K, Waldbeser LS, Plaeger-Marshall S, Kleinman S, Lanier LL. Influence of racial background on the distribution of T-cell subsets and Leu II positive lymphocytes in healthy blood donors. *Diag Immunol* 1985; 3 : 33-7.

Reichert TA, De Bruyere M, Deneys V, *et al.* Lymphocyte subset reference ranges in adult Caucasians. *Clin Immunol Immunolopathol* 1991; 60 : 190-208.

Rosenberg F, Fauci AS. Immunopathogenesis of HIV infection. *FASEB J.* 1991; 5 : 2382-90.

Stein DS, Korvick JA, Vermund SH. CD4+lymphocyte cell enumeration for predication of clinical course of human immunodeficiency virus disease. A Review. *J Infect Dis* 1992; 165 : 352-63.

Tollerud DJ, Clark JW, Brown LM, *et al.* The influence of age, race, and gender on peripheral blood mononuclear-cell subsets in healthy nonsmokers. *J Clin Immunol* 1989; 9 : 214-22.

Vulgelers PJ, Schechter MT, Tindall B, *et al.* Differences in time from HIV seroconversion to CD4+ lymphocyte end-points and AIDS in cohorts of homosexual men. *AIDS* 1993; 7 : 1325-9.