Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 1, 2012

Research Article

REFERENCE RANGES OF PERIPHERAL BLOOD LYMPHOCYTE SUBSETS IN SYRIAN HEALTHY ADULTS BY FLOW CYTOMETRY: INFLUENCE OF AGE AND SEX

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Received: 28 Sep 2011, Revised and Accepted: 9 Nov 2011

ABSTRACT

We established a normal reference range for peripheral blood lymphocyte subsets in a Syrian adult population. Lymphocyte subsets were examined in 100 healthy volunteers (31 males, 69 females) aged 17-78 years by Four-color flow cytometric analysis with a FACSCalibur. The percentage and absolute counts of CD3 (T cells), CD4 (T helper/inducer cells), CD8 (T suppressor/cytotoxic cells), CD16 and/or CD56 on CD3- cells (NK cells) and CD19 (B cells) lymphocytes were calculated. A significant correlation with age was seen in the absolute counts and percentage of CD3-CD16+ and/or CD56 +cells (NK cells) (p=0.003 and p=0.008 respectively). No significant correlation with age was observed for the absolute counts CD3 (T cells), CD8 (T suppressor/cytotoxic cells), and CD19 (B cells) (p=0.534, 0.908 and 0.453, respectively). No significant correlation with sex was observed.

Keywords: Reference range, B cells, T cells, Natural killer cells, Flow cytometry.

INTRODUCTION

With tremendous advances in the field of flow cytometry, the immunophenotyping of peripheral blood lymphocytes has evolved into the most important tool in the evaluation of immune status in patients with auto-immune diseases[1].

T lymphocytes are an important cell subset which is responsible for specific response against viral infections[2].

By using monoclonal antibodies directed against some immune markers present in the cells surface, we are able to evaluate the frequencies of the lymphocyte populations, as well as of other cellular groups. Using the "cluster differentiation" (CD) proteins, expressed by cells according to their specific functions in immune defense, we can measure the total lymphocytes population (CD3), and its subpopulations. The T-helper lymphocyte is CD4-positive while the cytotoxic Tcells are usually CD8-positive. Immunodeficiency associated with changes in T lymphocytes subsets can be diagnosed by measuring the level of these cells population in peripheral blood[3] . The T helper lymphocytes (CD3+CD4+ cells) are target cells for HIV, and this specific population decrease overtime during HIV infection, due to direct cytotoxicity, as well as by indirect mechanisms, like immune destruction of infected cells [4] .

The ageing process leads to marked changes in the composition, function and competence of the human immune system [5-6]. As a consequence of an altered immune system, older populations experience increased morbidity and mortality from respiratory tract pathogens[7], an increase in gastrointestinal infections [8]. Moreover antigen specific responses are diminished with orally administered vaccines [9]. Age-related phenotypic and functional changes to the T-cell component of adaptive immunity occur [10] while B-cell function and the innate immune system are less affected [11-12] .

However, little is known about whether males and females show differences on the immune system. We have been particularly interested in the percentages of cells in peripheral blood in older age groups, because of our studies in the peripheral immune response to stroke[13], which affects older age group.

Accumulating results suggest that the genetic and environmental differences among different ethnical populations lead to variable findings, which was also observed among the populations of neighborhood countries in Asia[14]

In this study, we managed to establish reference ranges for lymphocyte subsets in Syrian healthy adults with a wide range of age.

MATERIALS AND METHODS

Subjects

The subjects were adults above age 17 and were recruited between December 2010 and June 2011.The participants had to fill a questionnaire regarding loss/gain of weight, vaccination, infection in the past 4 weeks including viral, bacterial, fungal, and other pathogens, use of antibiotics in the past 4 weeks, hospitalization within the past 2 years, and history of medication, including analgesics, nonsteroidal anti-inflammatory agents, anti-ulcer drugs, anti-hypertensive drugs, and other cardiovascular drugs. Subjects who reported a positive history for any of these items were excluded from the study.

Blood specimens were collected (after an overnight fast of 12 hrs) in the anticoagulant EDTA. The samples were analyzed on the day of the collection.

Lymphocyte subsets were examined in 100 healthy volunteers (31 males, 69 females) aged 17-78. Samples were prepared by a whole blood lysis technique and analyzed by flow cytometry.

Flow cytometry analysis.

Lymphocyte subsets were analyzed on a FACScalibur Flow Cytometer (Becton Dickinson) with Cellquest software (Becton Dickinson). A single-platform, lyse-no-wash procedure was performed with Trucount tubes (Becton Dickinson) with the following two, four-color monoclonal antibody combinations supplied in the MultiTEST IMK kit (Becton Dickinson): CD3-fluorescein isothiocyanate-CD8-phycoerythrin-CD45-peridinin chlorophyll protein-CD4-allophycocyanin and CD3-fluorescein isothiocyanate-CD16 plus CD56-phycoerythrin-CD45-peridinin chlorophyll protein-CD19-allophycocyanin. The stained blood sample was lysed with a diluted lysing solution, and special care was taken not to expose the stained sample to light. CD3+ T cells, CD3+CD4+ T helper cells, and CD3+CD8+ T cytotoxic cells were identified according to published protocols. B cells were identified by CD19 expression, and NK cells were identified by the CD3−CD16+ and/or CD56+ phenotype. Statistical analyses were conducted using SPSS14

RESULTS

A total of 100 healthy blood donors ranging in age from 17 to 87 years were included in the study (mean age±SD:44.68±14.4 years old). Of these, 31 were male (mean age ± SD: 45.8±15.7 years old) and 69 were female (mean age ± SD: 44.1±13.9 years old). These

samples were selected on the basis of the exclusion criteria as mentioned earlier. The median age of males was 47 years and that for females was 50 years. Measurements were made of CD 19 (B cells), CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells), and CD3−CD16+ and/or CD56+ (NK cells).

Medians, means, ranges, standard deviation (SD) and standard errors (SE) were calculated for each parameter. Summary statistics for absolute counts and percentage of lymphocyte subsets in the total sample are given in table 1

After examining the effects of age on CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells), CD 19 (B cells) and CD3−CD16+ and/or CD56+ (NK cells).No significant correlation with age was observed for the absolute number of CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells) and CD 19 (B cells) (P=0.534 – 0.235 – 0. 908 and 0.453 , respectively). With aging, there was a significant increase in the absolute number of CD3–CD16+ and/or CD56+ (NK cells) (r=0.295 – p=0.003)

There was no significant change with age in the percentage for CD3+ (T cells), CD3+ CD8+ (suppressor T cells), CD4/CD8 and CD 19 (B cells) (P=0.083 – 0.141 – 0.145 and 0.77 , respectively).

The percentages of CD3−CD16+ and/or CD56+ (NK cells) increased with age (r=0.264 , P=0.008) and the percentage of CD3+CD4+ (helper T cells) decreased with age (r=-0.264 , p=0.008) as mentioned in table 2.

No significant correlation with sex was observed for the percentage of CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells), CD 19 (B cells) ,CD4/CD8 and CD3−CD16+ and/or CD56+ (NK cells) (P=0.86 – 0.79 – 0. 5 – 0.69 -0.39 and 0.11 , respectively).

The means, standard deviations (SD), medians, standard errors (SE) and ranges for males and females are tabulated in Table 3.

Table 1: Lymphocyte subset percentages and absolute-number reference ranges of study population

	Mean	Median	SD	SE	Range
T cells $CD3^+$ cells/µL	1225.5	850	1002.7	100.2	165-5174
helper T cells $CD3^+$ $CD4^+$cells/µL	525.1	353.5	504	50.4	4-2404
suppressor T cells $CD3^+$ CD8 $^+$cells/µL	427.1	308	344.3	34.43	44-1749
NK cells $CD3^-$ $CD16^+$cells/µL	243.2	174	200.3	20	15-1162
B cells $CD19^+$cells/µL	138.4	104.5	117.2	11.7	15-825
T cells $CD3^+$%	64.2	68	15.6	1.5	12-97
helper T cells $CD3^+$ $CD4^+$%	31.6	31	16.06	1.6	2-69
suppressor T cells $CD3^+$ CD8 $^+$%	23.9	23	9.3	0.9	4-57
NK cells $CD3^-$ $CD16^+$%	12.6	12	5.5	0.5	4-29
$CD19^+$% B cells	8	7	4.8	0.4	1-27
CD4/CD8 %	1.5	1.2	1.1	0.1	0.04-8.6

SD: standard deviation, SE: Standard Error of Mean , Range: Minimum – Maximum , CD: cluster of differentiation.

Table 2 : shows P values and correlation coefficient with age for the absolute number and percentage of CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells) , CD 19 (B cells) and CD4 CD3−CD16+ and/or CD56+ (NK cells)

	R	P
T cells $CD3^+$ cells/µL	-0.063	0.534
helper T cells $CD3^+$ $CD4^+$cells/µL	-0.120	0.235
suppressor T cells $CD3^+$ CD8 $^+$cells/µL	0.012	0.908
NK cells $CD3^-$ $CD16^+$cells/µL	0.295	0.003 *
B cells $CD19^+$cells/µL	-0.076	0.453
T cells $CD3^+$%	-0.174	0.083
helper T cells $CD3^+$ $CD4^+$%	-0.264	0.008*
suppressor T cells $CD3^+$ CD8 $^+$%	-0.148	0.141
NK cells $CD3^-$ $CD16^+$%	0.264	0.008*
$CD19^+$% B cells	0.178	0.77
CD4/CD8 %	0.147	0.145

When the analysis was done according to gender there were no significant differences among the gender in the following parameters: absolute counts of CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells), CD 19 (B cells) and CD3−CD16+ and/or CD56+ (NK cells) (P=0.7 – 0.26 – 0. 98 – 0.335 and 0.908 , respectively).

Table 3: Lymphocyte subset percentages and absolute-number reference ranges for males and females in the study population

	Mean	Median	SD	SE	Range	P value
T cells Male $CD3^+$ cells/µL	1179	757	905	162	232-3812	0.7
T cells Female $CD3^+$ cells/µL	1246	893	1049	126	165-5174	
helper T cells Male $CD3^+$ $CD4^+$ cells/µL	609	333	610	109	35-2404	0.26
helper T cells Female $CD3^+$ $CD4^+$ cells/µL	487	354	448	54	4-2079	
suppressor T cells Male $CD3^+$ $CD8^+$ cells/µL	426	275	347	62	44-1478	0.98
suppressor T cells Female $CD3^+$ $CD8^+$ cells/µL	427	338	346	42	47-1749	
NK cells Male $CD3^-$ $CD16^+$cells/µL	246	234	146	26	53-649	0.908
NK cells Female $CD3^-$ $CD16^+$ cells/µL	241	149	221	27	15-1162	
B cells Male $CD19^+$cells/µL	155	114	145	26	20-825	0.335
B cells Female $CD19^+$cells/µL	130	102	102	12	15-457	
T cells $CD3^+$ % Male	64	64	16	3	12-88	0.861
T cells $CD3^+$ % Female	64	70	15	2	15-97	
helper T cells Male $CD3^+$ $CD4^+$ %	32	35	15	3	3-62	0.791
helper T cells Female $CD3^+$ $CD4^+$ %	31	31	16	2	2-69	
suppressor T cells Male $CD3^+$ $CD8^+$ %	23	23	8	1.4	4-36	0.505
suppressor T cells Female $CD3^+$ $CD8^+$ %	24	22	10	1.1	5-57	
NK cells Male $CD3^-CD16^+$ %	14	13	6	1	6-29	0.117
NK cells Female $CD3^-CD16^+$ %	12	12	5	0.6	4-27	
B cells Male $CD19^+$ %	8	7	5	0.8	2-27	0.695
B cells Female $CD19^+$ %	8	7	5	0.5	1-21	
CD4/CD8 % Male	1.5	1.5	0.8	0.1	0.13-3.9	0.39
CD4/CD8 % Female	1.5	1.1	1.2	0.1	0.04-8.6	

SD: standard deviation , SE: Standard Error of Mean , Range: Minimum – Maximum , CD, cluster of differentiation .

DISCUSSION

In this study, we managed to obtain reference ranges for lymphocyte subsets in Syrian healthy adults with different age ranges. These reference ranges are applicable to adults between the ages of 17 and 78 years, which is the majority of clinically relevant adult population.

It seemed that Syrian population has lower lymphocyte subsets cell counts than Chinese population (Syrian vs Chinese: mean±SD : $CD3^+$ 1225±1002 vs 1428±424 cells/µL - $CD3^+CD4^+$ 525 ±504 vs 797±307 cells/µL - $CD3^+CD8^+$ 427±344 vs 543± 183 cells/µL - CD3-CD16+ 243± 200 vs 313±182 cells/µL and CD19+ 138 ±117 vs 242 ±111 cells/µL) [1].

Our results seemed different when compared with the Chinese subset in Singaporean population (Our study vs Singapore study : $CD3^+CD4^+$ 32 vs 35% - $CD3^+CD8^+$ 24 vs 27%) [14] , even when compared with Burkina Faso study (Our study vs Burkina Faso study : $CD3^+CD4^+$ 32 vs 41% but $CD3^+CD8^+$ 24 vs 24%) [15]

Many studies have been conducted to establish a reference range for peripheral blood lymphocyte subsets in different countries. These studies revealed variations in the normal range for lymphocyte subsets according to age [16-17-18-19-20], sex [21-22-23-24-25-26] .

Some studies have shown that the number of $CD4^+$ cells increased while the number of $CD8^+$ decreased with age,[19-27] while others state, that the numbers of both $CD4^+$ and $CD8^+$ increase, with age.[20]

Our study did not show any significant variation in lymphocyte subsets according to age for absolute counts of CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells) and CD 19 (B cells) (P=0.7 – 0.26 – 0. 98 – 0.335 and 0.908, respectively).

Our results do not show any significant differences in the percentages of CD3+ CD8+ (suppressor T cells), with respect to age unlike the study of Chng et al 2004[14] which reported that CD4+ CD8+ cell counts decreased with age (P= 0.0001).

However our results revealed a decreased in the percentage of CD3 +CD4+ (helper T cells) with age (r=-0.264, p=0.008) in reverse to Crooks et al 2010 [28]

Our results also show that the counts and percentage of CD3−CD16+ and/or CD56+ (NK cells), decreased with age (P=0.003 and P=0.008, respectively) similar to the findings of a previous Yan et al study 2010 [14].

When the analysis was done according to gender: we found no significant changes with gender in the absolute counts of the

following parameters: CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells), CD 19 (B cells) and CD3−CD16+ and/or CD56+ (NK cells) (p=0.7 - 0.26 – 0.98 – 0.33 and p=0.9, respectively).

Also there were no significant differences among the gender in the percentage of the following parameters: CD3+ (T cells), CD3+CD4+ (helper T cells), CD3+ CD8+ (suppressor T cells), CD 19 (B cells) , CD3−CD16+ and/or CD56+ (NK cells) and CD4/CD8 (p=0.86 - 0.79 – 0.5 – 0.69 - 0.11 and p=0.39, respectively) .which was different to the results shown by Das et al 29 where they found an increase in T lymphocytes CD3+ (p=0.008) and +CD4+ (helper T cells) (P<0,001) in females rather than males .

However, another study conducted by Chng et al 2004 14 showed only NK cell percentages and absolute counts were significantly different ($P < 0.0001$).

REFERENCES

1. Jiao Y, Qiu Z F, Xie J, et al. Reference ranges and age-related changes of peripheral blood lymphocyte subsets in Chinese healthy adults. Sci China Ser C-Life Sci, 2009, 52(7): 643-650, doi: 10.1007/s11427-009-0086-4
2. Bower J.E., Kemeny M.E., Taylor S.E. et al. Cognitive processing, discovery of meaning, CD4 decline, and AIDS-related mortality among bereaved HIV-seropositive men. J Consult Clin Psychol 1998 ;(6):979-86.
3. Lee B.W., Yap H.K., Chew F.T. et al. Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: from birth to adulthood. Cytometry 1996 ;(1):8-15.
4. Phillips A.N., Youle M., Lampe F. et al. CD4 cell count changes in individuals with counts above 500 cells/mm and viral loads below 50 copies/mL on antiretroviral therapy. AIDS 2002;(7):1073-5.
5. Aw D, Silva AB, Palmer DB: Immunosenescence: emerging challenges for an ageing population. Immunol 2007, 120:435-446
6. Kilpatrick RD, Rickabaugh T, Hultin LE, Hultin P, Hausner MA, Detels R, Phair J, Jamieson BD: Homeostasis of the naive CD4+ T cell compartment during aging. J Immunol 2008, 180:1499-1507
7. Meyer KC: Aging. Proc Am Thorac Soc 2005, 2:433-439.
8. Schmucker D, Owen R, Outenreath R, Thoreux K: Basis for age-related decline in intestinal mucosal immunity. Clin Dev Immunol 2003, 10:167-172.
9. 9.Hagiwara Y, McGhee JR, Fujihashi K, Kobayashi R, Yoshino N, Kataoka K, Etani Y, Kweon M, Tamura S, Kurata T, et al.: Protective Mucosal Immunity in Aging is Associated with Functional CD4+ T Cells in Nasopharyngeal - Associated Lymphoreticular Tissue.J Immunol 2003 , 170:1754-1762.
10. Ginaldi L, De Martinis M, Modesti M, Loreto F, Corsi MP, Quaglino D: Immunophenotypical changes of T lymphocytes in the elderly.Gerontol 2000, 46:242-248.
11. Castle S: Clinical relevance of age-related immune dysfunction. Clin Infect Dis 2000, 31:578-585.
12. Goronzy JJ, Lee WW, Weyand CM: Aging and T-cell diversity. Exp Gerontol 2007, 42:400-406.
13. Yan J, Greer JM, Etherington K, Cadigan GP, Cavanagh H, Henderson RD, O'sullivan JD, Pandian JD, Read SJ, McCombe PA: Immune activation in the peripheral blood of patients with acute ischemic stroke. J Neuroimmunol 2009, 206:112-117.
14. Chng W J, Tan G B, Kuperan P. Establishment of adult peripheral blood lymphocyte subset reference range for an asian population by single-platform flow cytometry: Influence of age, sex, and race and comparison with other published studies. Clin Diagn Lab Immunol, 2004, 11: 168—173
15. Klose N ., Coulibaly B., Tebit D. M, Nauwelaers F, Spengler H P, Kynast-Wolf G., Kouyaté B, Kräusslich H.-G., Böhler T. Immunohematological Reference Values for Healthy Adults in Burkina Faso .Clin Vaccine Immunol. 2007 June; 14(6): 782–784.
16. Denny, T., R. Yogev, R. Gelman, C. Skuza, J. Oleske, E. Chadwick, S. Cheng, and E. Connor. 1992. Lymphocyte subsets in healthy children during the first 5 years of life. JAMA 267:1481-1488.
17. Erkeller-Yuksel, F. M., V. Deneys, B. Yuksel, I. Hannet, F. Hulstaert, C. Hamilton, H. Mackinnon, L. T. Stokes, V. Munhyeshuli, F. Vanlangendonck, et al. 1992. Age-related changes in human blood lymphocyte subpopulation. J. Pediatr. 120:216-222.
18. Shahabuddin, S. 1995. Quantitative differences in CD8+ lymphocytes, CD4/CD8 ratio, NK cells, and HLA-DR+-activated T cells of racially different male populations. Clin. Immunol. Immunopathol. 75:168-170.
19. Tollerud, D. J., S. T. Ildstad, L. M. Brown, J. W. Clark, W. A. Blattner, D. L. Mann, C. Y. Neuland, L. Pankiw-Trost, and R. N. Hoover. 1990. T-cell subsets in healthy teenagers: transition to the adult phenotype. Clin. Immunol. Immunopathol. 56:88-96.
20. Wiener, D., S. Shah, J. Malone, N. Lowell, S. Lowitt, and D. T. Rowlands, Jr. 1990. Multiparametric analysis of peripheral blood in the normal paediatric population by flow cytometry. J. Clin. Lab. Anal. 4:175-179.
21. Bartlett, J. A., S. J. Schleifer, M. K. Demetrikopoulos, B. R. Delaney, S. C. Shiflett, and S. E. Keller. 1998. Immune function in healthy adolescents. Clin. Diagn. Lab. Immunol. 5:105-113.
22. Lee, B. W., H. K. Yap, F. T. Chew, T. C. Quah, K. Prabhakaran, G. S. Chan, S. C. Wong, and C. C. Seah. 1996. Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: from birth to adulthood. Cytometry 26:8-15.
23. Reichert, T., M. DeBruyere, V. Deney, T. Totterman, P. Lydyard, F. Yuksel, H. Chapel, D. Jewell, L. Van Hove, J. Linden, and L. Buchner. 1991. Lymphocyte subset reference ranges in adult Caucasians. Clin. Immunol. Immunopathol. 60:190-208.
24. Rudy, B. J., C. M. Wilson, S. Durako, A. Moscicki, L. Muenz, and S. D. Douglas. 2002. Peripheral blood lymphocyte subsets in adolescents: a longitudinal analysis from the REACH project. Clin. Diagn. Lab. Immunol. 9:959-965.
25. Santagostino, A., G. Gargaccio, A. Pistorio, V. Bolis, G. Camisasca, P. Pagliaro, and M. Girotto. 1999. An Italian national multicenter study for the definition of a reference ranges for normal values of peripheral blood lymphocyte subsets in healthy adults. Haematologica 84:499-504.
26. Tollerud, D. J., J. W. Clark, L. M. Brown, C. Y. Neuland, L. K. Pankiw-Trost, W. A. Blattner, and R. N. Hoover. 1989. The influence of age, race, and gender on peripheral blood mononuclear-cell subsets in healthy non-smokers. J. Clin. Immunol. 9:214-222.
27. Denny T, Yogev R, Gelman R, Skuza C, Oleske J, Chadwick E, et al. Lymphocyte subsets in healthy children during the first 5 years of life. JAMA1992;267:1481-8.
28. Crooks C V., Cross M L., Wall C R., 2010 - Age-related differences in integrin expression in peripheral blood lymphocytes. Immunity&Ageing, 7:5doi:10.1186/1742-4933-7-5.
29. Das BR, Bhanushali AA, Khadapkar R, Jeswani KD, Bhavsar M, Dasgupta A. Reference ranges for lymphocyte subsets in adults from western India: Influence of sex, age and method of enumeration. Indian J Med Sci 2008; 62:397-406