Reference Ranges of Lymphocyte Subsets of Healthy Adults in Turkey

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Key Words

Lymphocyte subsets · Healthy adults · Flow cytometry

Abstract

Abstract: The aim of this study was to determine the reference ranges of lymphocyte subsets in serologically HIV-negative healthy adults in Turkey. **Materials and Methods:** Blood samples from 220 healthy adults, 105 female and 115 male, collected into tubes containing EDTA were investigated for lymphocyte subsets using flow cytometry. The age range was 18–80 years (44.80 ± 16.69). **Results:** The mean percentage and absolute values of the lymphocyte subsets were as follows: CD3: $72.70 \pm 8.44\%$, $1,680 \pm 528$ cells/ μ l; CD4: $47.37 \pm 9.10\%$, $1,095 \pm 391$ cells/ μ l; CD8: $28.99 \pm 5.99\%$, 669 ± 239 cells/ μ l; CD19: $10.96 \pm 4.44\%$, 254 ± 122 cells/ μ l and CD56: $7.03 \pm 3.26\%$, 161 ± 92 cells/ μ l, respectively. The ratio of CD4/CD8 was 1.68 ± 0.43 . There was no statistically significant difference in the percentages and absolute values of lymphocyte subsets between the genders ($p > 0.05$). **Conclusion:** Immunophenotyping has been used to establish reference values of lymphocyte subsets in normal healthy adults in Turkey.

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Introduction

T cells are defined by the expression of CD3; T cell subpopulations by the coexpression of CD4 or CD8 or HLA-DR; B lymphocytes by the expression of CD19, while natural killer lymphocytes are identified by positivity of CD16 and/or CD56 without CD3 [1–4]. Enumeration of various lymphocyte subsets is widely used in the diagnosis and therapeutic monitoring of various disease states [5]. With the development of flow cytometry and whole blood analysis, enumerating various lymphocyte subsets has become more sensitive. It is therefore important to establish reference values in normal healthy adults using these techniques [6–9]. Although a lot work has been done using flow cytometric immunophenotyping, there is little agreement as to normal reference ranges as these values have not been standardized yet [10].

Therefore, the aim of this study was to determine the reference values of certain specific lymphocyte surface antigens in healthy adults using flow cytometric immunophenotyping in Turkey.

Subjects and Methods

Subjects

Two hundred and thirty-four adults were initially enrolled in the study. They were examined by an infectious disease specialist. They were asked if they had an infection during the previous 4 weeks, taken any antibiotics or other medications during this pe-

Table 1. Percent distribution of age groups for both the study group and general population in Adana, Turkey

Age group	Years	Study population, %	General population, %
Group 1	18–29	36.4	39.4
Group 2	30–44	28.2	33.4
Group 3	45–59	26.4	16.8
Group 4	60–80	9.1	9.5

Table 2. The mean $\pm$ SD, median and 95% reference range of percentages and absolute values of lymphocyte subsets in 220 healthy adults

Lymphocyte subsets		Mean $\pm$ SD	Median	95% reference range
CD3	%	72.70 $\pm$ 8.44	73.20	52.30–84.64
	cells/ μ l	1,680 $\pm$ 528	1,637	725–2,960
CD4	%	47.37 $\pm$ 9.10	49.35	30.00–60.34
	cells/ μ l	1,095 $\pm$ 391	1,055	437–2,072
CD8	%	28.99 $\pm$ 5.99	29.65	17.76–39.94
	cells/ μ l	669 $\pm$ 239	630	307–1,184
CD19	%	10.96 $\pm$ 4.44	10.85	3.90–20.79
	cells/ μ l	254 $\pm$ 122	240	74–586
CD56	%	7.03 $\pm$ 3.26	7.15	0.10–13.20
	cells/ μ l	161 $\pm$ 92	155	3.08–367
CD4/CD8		1.68 $\pm$ 0.43	1.65	1.06–2.76

riod and if they had any vaccination within the past 6 months. Fourteen adults were excluded from the study because of infection and/or medication. The participants were then grouped into four age categories: group 1, 18–29 years; group 2, 30–44 years; group 3, 45–59 years, and group 4, 60–80 years. One hundred and five female (42.5 $\pm$ 17.4 years) and 115 male (47.2 $\pm$ 15.7 years) subjects participated in the study. Statistically, there was no significant difference in age and gender between the study groups and those subjects who were excluded.

According to the Governmental Statistics Institute, the population of the city of Adana was 916,000 according to the 2000 census. The distribution of the age groups in the study is similar to that of the general population except for group 3 as shown in table 1.

This study was approved by the Institutional Ethic Committee and informed consent was obtained from each participant.

Processing of Blood Samples

The ranges of CD3, CD4, CD8, CD19, CD56 and the CD4/CD8 ratios in the 220 healthy adults were determined using a flow cytometry instrument (EPICS XL-MCL, Beckman Coulter, USA) at the Central Laboratory of the Faculty of Medicine, University of Çukurova, Turkey. Blood samples were drawn simultaneously for

white cell blood count and flow cytometric analysis during a period of 5 months. The flowing fluorochrome-conjugated monoclonal antibodies T4-RD1/T8-FITC, CD3-FITC, B4 (lytic)-RD1/J5-FITC (Beckman Coulter, USA) and NKH-1 RD1 were used in this study.

Two milliliters of peripheral venous blood was withdrawn from each person and placed into a tube containing ethylenediaminetetraacetic acid. All the blood samples were processed within 2 h of collection. For each parameter, 100 μ l of blood was put into a 12 $\times$ 75 mm test tube and 10 μ l of the appropriate fluorochrome-conjugated monoclonal antibody solution was added. The samples were incubated in the dark at room temperature for 20 min. Later, 600 μ l Immunoprep A (erythrocyte lysing agent), 265 μ l Immunoprep B (leukocyte stabilizer) and 100 μ l Immunoprep C (cell membrane fixative) solutions were added to the samples in the TQ-Prep Workstation (Beckman Coulter, USA).

Analysis of each lymphocyte subset was made using an EPICS XL-MCL flow cytometer (Beckman Coulter) and the values were determined as a percentage of all the parameters. Absolute values of lymphocyte subsets (numbers of cells/ μ l) were calculated by the formula: WBC $\times$ lymphocyte percentage $\times$ subset percentage [1, 11]. An initial fluorescence gating strategy was performed based on CD45+/CD14– back-gating and/or CD45+/side scatter gating. For each analysis, 50,000 cells were counted.

A range of internal quality assurance procedures was employed, including daily calibration of flow cytometer optical alignment and fluidic stability using Flow-Check (Beckman Coulter) fluorospheres and daily monitoring of whole blood preparation procedures and monoclonal antibody reactivity using Immuno-Trol (Beckman Coulter) control cells.

Statistical Analysis

Superior Performance Statistical Software (SPSS, version 10.0) program for Windows statistical package was used to analyze all the data. The mean, median and standard deviation were calculated for each immunological parameter. The 95th percentile reference ranges were determined by using 2.5 and 97.5 percentiles. The normal distribution of variables was tested using the Shapiro-Wilk and Kolmogorov-Smirnov tests and histograms. Since the data were not normally distributed, the Mann-Whitney U was used to compare the distribution of immunohematological parameters between genders and the Kruskal-Wallis test between the age groups. A p value <0.05 or <0.016 was considered significant. When multiple comparisons were used to determine significant age groups, Bonferroni correction was applied (p/n = 0.10/6 = 0.016).

Results

The overall leukocyte count was 7,558 $\pm$ 1,820 per μ l and those of female and male subjects were 7,367 $\pm$ 1,747 and 7,732 $\pm$ 1,874 per μ l, respectively. The corresponding counts for lymphocytes were 2,196 $\pm$ 668, 2,140 $\pm$ 663 and 2,252 $\pm$ 673 per μ l. All the subjects were serologically HIV negative.

The mean and median percentages and absolute values of the lymphocyte subsets with reference ranges are given in table 2. The percentages and absolute values of

Table 3. The mean $\pm$ SD, 95% reference range and p value of the percentages and absolute values of the lymphocyte subsets in 115 male and 105 female subjects

Lymphocyte subsets		Male (n = 115)		Female (n = 105)		p value
		mean $\pm$ SD	95% reference, range	mean $\pm$ SD	95% reference, range	
CD3	%	71.89 $\pm$ 8.56	51.29–86.46	73.58 $\pm$ 8.27	54.59–83.98	NS
	cells/ μ l	1,668 $\pm$ 470	704–2,691	1,693 $\pm$ 588	766–3,125	NS
CD4	%	47.70 $\pm$ 8.88	29.91–60.12	46.99 $\pm$ 9.37	29.74–61.41	NS
	cells/ μ l	1,112 $\pm$ 373	438–1,890	1,076 $\pm$ 410	394–2,234	NS
CD8	%	29.46 $\pm$ 5.82	18.13–39.91	28.48 $\pm$ 6.15	16.86–40.27	NS
	cells/ μ l	687 $\pm$ 235	310–1,136	648 $\pm$ 242	239–1,354	NS
CD19	%	10.85 $\pm$ 4.91	3.98–22.18	11.09 $\pm$ 3.86	3.70–20.11	NS
	cells/ μ l	253 $\pm$ 130	79–617	256 $\pm$ 114	62–551	NS
CD56	%	6.49 $\pm$ 3.24	0.10–12.48	7.26 $\pm$ 3.19	0.40–13.33	NS
	cells/ μ l	151 $\pm$ 89	3–325	173 $\pm$ 94	8–449	NS
CD4/CD8		1.66 $\pm$ 0.40	1.04–2.57	1.71 $\pm$ 0.46	1.08–3.00	NS

NS = Not significant.

Table 4. The mean $\pm$ SD and 95% reference range of the percentages and absolute values of lymphocyte subsets in the four groups of healthy adults

Lymphocyte subsets	Group 1, 18–29 years (n = 80)		Group 2, 30–44 years (n = 62)		Group 3, 45–59 years (n = 58)		Group 4, 60+ years (n = 20)		p value
	mean $\pm$ SD	95% reference range	mean $\pm$ SD	95% reference range	mean $\pm$ SD	95% reference range	mean $\pm$ SD	95% reference range	
CD3									
%	73.23 $\pm$ 7.79	55.40–83.08	73.32 $\pm$ 8.90	49.62–87.46	72.31 $\pm$ 8.72	52.24–85.86	69.73 $\pm$ 8.63	52.20–86.40	NS
cells/ μ l	1,697 $\pm$ 521	690–2,867	1,727 $\pm$ 530	777–3,101	1,628 $\pm$ 542	704–3,107	1,618 $\pm$ 526	708–2,685	NS
CD4									
%	45.54 $\pm$ 7.94	30.20–59.87	48.47 $\pm$ 10.08	27.48–61.50	47.58 $\pm$ 9.33	29.42–61.08	50.64 $\pm$ 8.70 ^a	30.00–60.10	0.027
cells/ μ l	1,053 $\pm$ 343	426–1,837	1,150 $\pm$ 446	478–2,305	1,077 $\pm$ 417	345–2,108	1,145 $\pm$ 298	594–1,727	NS
CD8									
%	26.43 $\pm$ 5.67	16.11–39.32	28.80 $\pm$ 5.19 ^a	18.31–39.91	31.27 $\pm$ 6.28 ^{a,b}	17.23–40.92	33.26 $\pm$ 3.46 ^{a,b}	26.00–39.00	0.000
cells/ μ l	609 $\pm$ 215	250–1,102	681 $\pm$ 244	309–1,278	701 $\pm$ 252	240–1,397	772 $\pm$ 232 ^a	356–1,122	0.016
CD19									
%	11.63 $\pm$ 3.33	4.62–19.70	10.60 $\pm$ 3.45	4.00–19.46	10.88 $\pm$ 6.07 ^a	3.70–24.90	9.67 $\pm$ 5.29 ^a	3.00–22.00	0.006
cells/ μ l	269 $\pm$ 106	89–610	251 $\pm$ 120	91–617	245 $\pm$ 138	44–617	229 $\pm$ 146	68–574	NS
CD56									
%	8.60 $\pm$ 3.13	0.40–13.69	6.70 $\pm$ 2.79 ^a	3.60–12.97	5.47 $\pm$ 3.19 ^a	0.10–11.31	6.29 $\pm$ 2.70 ^a	3.20–9.60	0.000
cells/ μ l	199 $\pm$ 94	8–467	154 $\pm$ 75 ^a	62–320	122 $\pm$ 90 ^{a, b}	3–362	146 $\pm$ 81	46–286	0.000
CD4/CD8	1.78 $\pm$ 0.42	1.08–2.81	1.71 $\pm$ 0.40	1.06–2.67	1.58 $\pm$ 0.48 ^{a, b}	1.05–3.07	1.52 $\pm$ 0.25 ^a	0.96–2.01	0.002

* NS = Not significant.

^a p < 0.016 versus group 1; ^b p < 0.016 versus group 2; ^c p < 0.016 versus group 3.

the lymphocyte subsets in male and female subjects are shown in table 3 while those of age groups are shown in table 4. For the gender comparison, the difference was not significant. For age group comparison, the percentage and absolute values for CD3 were not significant but all other values except absolute values for CD4 and CD19 were significant; p value ranged from 0 to 0.027.

Discussion

Investigators using flow cytometric immunophenotyping have not been able to establish generally acceptable reference values [12] probably due to variation in techniques, and biological factors such as age, gender and race in populations may explain the differences [13–16].

In this study, the percentage and absolute values were higher only for CD4 and lower for CD56 than the values reported previously [1, 4, 11, 17–21]. The mean values of CD3, CD8 and CD19 are similar to the values reported for Italian, Kuwaiti and Romanian populations [1, 17, 19]. The CD4 values are similar to those of Italians [1] and CD56 values were lower than those of Kuwaiti [17], Saudi Arabia [4], and Romanian populations [19]. However the values of CD4 and CD56 were more similar to those of Kuwaiti and Saudi Arabia, who are close neighbors of Turkey. Ethnic and racial differences are factors that may influence the levels of lymphocyte subsets [17, 18, 22]. Environmental factors, including various infectious agents [4, 7, 23, 24], may also influence the number and subsets of lymphocytes. Of equal importance may be the variation introduced by use of different instruments and procedures [17].

However, biological characteristics such as age, gender and race have been shown to affect the results of the flow cytometric immunophenotyping [13–16]. Of these characteristics, the race factor has not been investigated as much as the others [12, 22]. On the other hand, the age factor has been shown to affect immunophenotyping greatly. It has been reported that CD4 and CD19 lymphocytes are low in small children, decreasing with age [13, 15, 23]. The reference ranges of the lymphocyte subsets change little in persons under 65 years of age, but there are important changes in immunophenotyping after 65 years of age [12, 14, 23].

In this study, there was no statistically significant difference between the percentages and absolute values of CD3 and CD4 according to age groups ($p > 0.016$) except that the percentage of CD4 was significantly lower in group 1 than in group 4 ($p \leq 0.016$), a finding not consis-

tent with previous reports [13, 15, 23]. The percentage of CD8 showed a significant increase with age in group 4 ($p > 0.016$) similar to other reports [13, 15, 23]. In addition the percentage of CD19 decreased with age (group 1 was higher than groups 3 and 4, this finding is also similar to previous reports) [13, 15, 23]. The percentage of CD56 was higher in group 1 than the other groups ($p \leq 0.016$) a finding not consistent with previous reports [13, 15, 23].

In our study gender did not affect the percentages and absolute values of lymphocyte subsets. However, Kam et al. [11] and Uppal et al. [25] reported that only CD4 was significantly higher in female than in male subjects. In addition, Uppal et al. [25] found higher CD8 values for male than female subjects. Santagostino et al. [1] reported significant differences in CD3, CD4 and NK according to gender, but not in the ratio of CD4/CD8. Kaaba et al. [17] found no difference in CD2, CD3, CD4, CD8, CD19 and CD56 according to gender similar to our present finding.

The CD4/CD8 ratio of 1.68 ± 0.43 obtained in this study is within the reported ratio of 1.5 ± 0.6 – 2.0 ± 0.02 [17–19]. This ratio was 1.71 ± 0.46 in female and 1.66 ± 0.40 in male subjects. The difference between the genders was not found to be statistically significant ($p > 0.05$).

The results obtained by flow cytometric immunophenotyping of healthy persons will aid in the evaluation of data obtained from patients, especially with leukemias, immunodeficiency and autoimmune diseases. However, it will be necessary to perform population-based studies in many countries to obtain reference values that are universally acceptable. In such studies, it will be necessary to standardize the techniques. Consequently, it is important to determine reference ranges of lymphocyte subsets according to age and gender in healthy populations in order to successfully evaluate the results from patients.

Conclusion

Immunophenotyping has been used to establish reference values of lymphocyte subsets in normal healthy adults in Turkey.

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