Blood lymphocyte T subsets reference values in blood donors by flow cytometry.

Etablissement des valeurs de référence des lymphocytes T chez les donneurs de sang parcytométrie en flux

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RÉSUMÉ

Objectifs : Déterminer les valeurs de référence des sous-populations lymphocytaires T par cytométrie de flux dans une population de donneurs de sang de la région de Sfax et évaluer l'influence de l'âge et du sexe sur leurs numérations.

Méthodes : L'étude a porté sur 143 donneurs de sang du centre régional de transfusion sanguine de Sfax. Les valeurs de référence ont été déterminées par technique double plate-forme (compteur de globules pour la numération en valeur absolue des lymphocytes circulants et cytomètre EPICS XL pour l'évaluation en pourcentages des lymphocytes T CD3+, CD3+/CD4+ et CD3+/CD8+). L'analyse statistique a été faite par les tests de Student et d'ANOVA.

Résultats : Les valeurs de référence des sous populations lymphocytaires T ont été exprimées en moyenne et en intervalles de confiance à 95%: CD3+ : 1415 ± 348 cellules/µL [1357–1473], CD3+/CD4+ : 786 ± 220 cellules/µL [732,31–811,7], CD3+/CD8+ : 639 ± 258 cellules/µL [596-862] avec un ratio CD4+/CD8+ de 1,46 ± 0,77 [1,36–1,62]. Elles sont influencées par l'âge et le sexe.

Conclusion : Notre étude a établi les valeurs de référence des lymphocytes T chez les donneurs de sang dans la région de Sfax. D'autres études plus large et portant sur d'autres régions de la Tunisie et sur les autres populations lymphocytaires B et NK sont fortement recommandées.

Mots-clés

CD3+/CD4+; CD3+/CD8+; cytométrie de flux; sous populations lymphocytaires T; valeurs de référence.

SUMMARY

Aims: To determine region-specific reference ranges of lymphocyte T subsets in blood donors and to assess the influence of gender and age on lymphocyte T susbsets.

Methods: Blood samples from 143 blood donors were collected in the Blood Transfusion Center of Sfax. Lymphocyte T subsets were analyzed using a dual-platform method with a flow cytometer (percentages) and an automated hematology analyzer (white blood cells and lymphocytes). ANOVA and Student's tests were used for data analysis.

Results: Reference values were expressed as mean and 95% confidence intervals for T cells: CD3+: 1415 ± 348 cells/µL [1357–1473], CD3+/CD4+: 786 ± 220 cells/µL [732.31–811.7], CD3+/CD8+: 639 ± 258 cells/µL [596-862] and CD4+/CD8+ ratio was 1.46 ± 0.77 [1.36–1.62]. Gender and age influenced blood lymphocyte T subsets.

Conclusion: Our study leads to the establishment of peripheral blood lymphocyte T subset reference ranges in blood donors in the region of Sfax. A study on a more diversified population, including more important number of individuals from various regions of Tunisia and including enumeration of other lymphocyte subsets (B cells and NK cells) is required.

Key-words

CD3+/CD4+; CD3+/CD8+; flow cytometry; lymphocyte T subsets; reference ranges.

INTRODUCTION

Analysis of peripheral blood lymphocyte populations (T, B, and natural killer (NK) cells) and subpopulations (CD4 and CD8) by flow cytometry (FC) has become an essential tool in the evaluation of immunological and pathological disorders. For instance, T lymphocyte subsets enumeration has a great importance in assessment of both hereditary and acquired immunodeficiency disorders. Furthermore, CD4+ T cells enumeration is imperative for the follow-up and the treatment of human immunodeficiency virus (HIV) infection (1, 2). It is also useful in solid organ transplantation and immunosuppressive therapy monitoring (3).

Therefore, the establishment of accurate reference values of lymphocyte subsets in normal subjects is essential and each population should have their own defined reference values as recommended (4). However, such data shows variations due to circadian fluctuations and influence of gender, age, ethnicity, and lifestyle differences (5, 6).

This study was conducted to establish the normal absolute and percentage values of CD3+/CD4+ and CD3+/CD8+ lymphocyte subsets and CD4+/CD8+ ratio in blood donors (BD) of Sfax region and assess the influence of gender and age on lymphocyte T subsets.

METHODS

Reference population

A total of 185 healthy, adult, BD coming to the Blood Transfusion Center of Sfax were initially included in the study. All donors were screened for HIV (Antigen (Ag) and antibody (Ab)), hepatitis B (Ag) and C (Ag and Ab) and syphilis by serological methods. Cases with positive results in any of these tests were excluded from the study. Besides, BD who had rate of leukocytes or lymphocytes out of our references ranges were also excluded (7).

A total of 143 BD were finally included in the study. In order to analyze the lymphocyte T subtypes variation according to age, BD were classified into 4 groups: group 1 (18-25 years), group 2 (26-35 years), group 3 (36-45 years) and group 4 (> 45 years).

Blood collection

Blood specimens were collected by venipuncture and anticoagulated with ethylene diamine tetraacetic acid (EDTA). Anticoagulated blood samples were used for complete blood counts and absolute lymphocyte counts (ALCs), using an automated hematology analyzer (ABX MICROS 60-OT, France) and for immunophenotyping by FC.

Flow cytometry analysis

Flow cytometric study was done within 2 hours after collection of specimens. A dual-platform, lyse-no-wash procedure was performed for each subject with the following three color monoclonal antibody combinations supplied by Beckman Coulter (Cyto-Stat ® Trichrome™): CD45-fluorescein isothiocyanate (FITC)/CD4-phycoerythrin (PE)/CD3 phycoerythrin-cyanine (PC)-5 and CD45 FITC/ CD8 PE/CD3 PC5 and isotypic control. Fifty microliters (µL) of anticoagulated blood was mixed with 10 µL of each of the antibodies and incubated at room temperature for 15 minutes in the dark. Then, hemolysis was performed by an appropriate lysing reagent (Opti-Lyse©, Beckman Coulter, France) according to manufacturer's instructions. Then, processed samples were introduced to the FC (Epics XL, Beckman Coulter), equipped with a 488 nm laser argon, forthwith by using fluorescence gating strategy based on CD45/SSC. For each sample analyzed, a minimum of 10 000 events was acquired. Absolute values of CD3+/CD4+ and CD3+/CD8+ cells were calculated by multiplying ALCs by the percentage of the particular corresponding T-cell subset obtained by FC.

Quality control

Daily calibration of both, the hematological analyser and the FC was performed using respectively a sample of blood check (Control normal ABX Minotrol 16) and the Flowcheck - Flowset fluorospheres (Beckman Coulter).

Statistical analysis

Data were analyzed using the SPSS statistical package program version 11.5 software. The mean and standard deviation (SD) values were calculated for all parameters. The Gaussian distribution of each variable was checked by the Kolmogorov–Smirnov (KS) test. For determination of reference intervals, both parametric (mean ±2 SD) and non parametric (2.5 and 97.5 percentiles for upper and lower limits) methods were used. The influence of gender and age on the distribution of blood lymphocytes T subsets were evaluated by ANOVA and Student's tests respectively. The Pearson's correlation coefficient was used to check for significant differences between age groups. P values of <0.05 were considered statistically significant.

RESULTS

A total of 143 healthy donors met our criteria and had blood samples processed for the study. Absolute counts and/or percentages of the indicated cell populations are presented as mean values with SD, medians and 95% confidence intervals. Reference ranges were extracted from the 2.5 th to 97.5 th percentiles (Table 1).

Influence of gender

The study population consisted of 98 men and 45 women. A comparison of the parameters between these 2 groups is presented in Table 2. Overall, no statistically significant differences were observed between the 2 groups.

Absolute counts and percentages of CD3+cells and CD3+/CD4+ cells were higher in women than in men as well as the CD4 +/CD8 + ratio ($p > 0.05$). However, absolute count and percentage of CD3+/CD8+ cells were higher in men than in women ($p = 0.7$ in both cases).

Influence of age

Statistical significant difference in CD3+ cells absolute counts was observed between the groups ($p < 0.05$). Likewise, CD3+ cells percentages were different between the groups ($p=0.8$) but without statistically significance (Table 3).

A trend of decreasing absolute count and percentage of CD3+/CD8+cells with age ($r=-0.115$, $p=0.209$ and $r=-0.072$, $p < 0.438$) was observed but without significant difference. Absolute count and percentage of CD3+/CD4+ cells ($r = 0.7$, $p = 0.45$ and $r = 0.16$, $p<0.05$) showed a tendency to increase in the group older than 45 years. Consequently, CD4+/CD8+ ratio ($r=0.22$, $p=0.16$) showed an increase with age.

DISCUSSION

The reference ranges for CD3+/CD4+ cells, CD3+/CD8+ cells and CD4+/CD8+ ratio of BD in the region of Sfax are established. This study is the first published study for our local population that gives the reference interval of lymphocyte T subsets in a relatively large sample size.

Similar to previously reported findings in the literature, the results of this study showed differences from data obtained in other countries. Such differences may originate from several factors such as ethnical and/or genetic differences, environmental factors (diet, smoking, exercise, stress levels…) and methodological and/or instrumental variations (choice of monoclonal antibodies used, analyzes on whole blood or on separate cells...) (8,9).

Table 1. Means, medians, extremes, confidence intervals and reference ranges of lymphocyte T subsets.

Parameters	Mean ± SD	Median	Min – Max	Confidence intervals [a]	Reference ranges [b]
WB					
Cells/µL	6610 ±1640	6200	4000 -9500	6304 - 6884	4290 - 9530
Lymphocytes					
Cells/µL	2087 ± 486	1995	1116 - 3402	2006 - 2167	1291 - 3099
%	32.12 ± 7.16	51.3	10 - 51.9	37.21 - 40.46	19.7 - 48
CD3+					
Cells/µL	1415 ± 348	1346	810 -2395	1357 - 1473	870 - 2236
%	69.17 ± 9.32	69.31	43 - 95	67.51 -71.11	50.6 - 90
CD3+CD4+					
Cells/µL	786 ± 220	761	162 -1308	732.31- 811.7	383.8 -1235
%	38.83 ± 9.18	37	17 - 67	36.6 - 40	21.6 - 60.8
CD3+CD8+					
Cells/µL	639 ± 258	628	183 -1742	596 - 862	273 -1256
%	30.36 ± 9.38	30	15 -59	28.81- 31.91	15.6 -53.8
CD4+/CD8+					
Ratio	1.46 ± 0.77	0.5	0.5 – 4.84	1.36 -1.62	0.54 -3.26

[SD]: Standard deviation, [a] : 95% confidence intervals , [b]:2.5 th and 97.5 th percentiles.

Table 2. Lymphocyte T subsets percentages and absolute-number reference ranges of study population by gender.

Parameters	Mean ± SD		Reference ranges [a]		p-value[b]
	Male	Female	Male	Female	
Age (years)	37.10 ± 9.87	35,24 ±10,02			0.6 [c]
WB					
Cells/µL	6760 ± 1950	6610 ± 1640	4170 - 9532	4260 - 9300	0.490
Lymphocytes					
Cells/µL	2085 ± 518	2091 ± 413	1212 -3113	1419 -3175	0.938
%	31.89 ± 7.51	32 .6 ± 6.37	17.63 - 47.83	19.63 -50.86	0.577
CD3+					
Cells/µL	1408 ± 380	1430 ± 270	840 - 2365	877 -1988	0.725
%	69.3 ± 7.51	68 .89 ± 7.06	50 - 92.1	53.48 -84 .55	0.977
CD3+CD4+					
Cells/µL	764 ± 219	832 ± 218	314 -1238	443 -1264	0.091
%	38.07 ± 9.42	40.49 ± 10.54	20.48 -61	25 -62	0.172
CD3+CD8+					
Cells/µL	658 ± 265	598 ± 239	272 -1360	197 - 1014	0.783
%	31.26 ± 9.15	28.4 ± 9.68	15.48 -55	13.45 - 44.85	0.703
CD4+/CD8+					
Ratio	1.39 ± 0.61	1.7 ± 1.01	0.52 -2.85	0.6 - 4.84	0.63

[SD]: standard deviation, [a]: 2.5 th and 97.5 th percentiles [b]: analysis by ANOVA test, [c]: Student's test

Table 3. Lymphocyte T subsets means and absolute-number reference ranges of study population by age group.

Parameters	Mean ± SD				p-value[a]
	19 -25 (n=24)	26 -35 (n=37)	36 -45 (n=60)	>45 (n=22)	
WB					
Cells/µL	6290 ± 1010	6711 ± 1490	6270 ± 1660	7040 ± 2130	0 .032
Lymphocyte					
Cells/µL	2068 ± 375	2167 ± 572	2020 ± 444	2152 ± 558	0.528
%	33.45± 7.83	30.9 ± 6.68	33.34± 7.62	29.4 ± 8 .39	0.577
CD3+					
Cells/µL	1379 ± 323	1425 ± 415	1403 ± 308	1470 ± 372	0.049
%	66.71± 10.66	66.71 ± 9.53	69.41± 9.31	70 ± 9.27	0.810
CD3+CD4+					
Cells/µL	760 ± 173	748 ± 178	753 ± 234	904 ± 264	0.091
%	37.25 ± 7.94	37.64 ± 8.26	38.95± 11.23	42.55 ± 9.50	0.172
CD3+CD8+					
Cells/µL	618± 260	717 ± 314	627 ±230	576 ± 204	0.891
%	29.47 ± 9.15	31.97 ± 9.71	31.03± 9.34	26.64 ± 8.65	0.202
CD4+/CD8+					
Ratio	1.41 ± 0.6	1.33± 0.615	1.5± 0.84	1.82 ± 0.92	0.025

[SD]: Standard deviation, [a]: Analysis by ANOVA test

In this study, we followed the same procedure of specimen collection to reduce at the most the inter-individual variability. To avoid circadian lymphocyte fluctuation and that associated with physical effort and stress, we chose a morning time for phlebotomy (between 8 am and 11 am) after a rest of at least 15 minutes.

For analytical procedure, we followed the recommendations of the guideline of the Centers for Disease Control (CDC) established for the follow-up of HIV infected patients (10) with a dual-platform, lyses-no-wash method. Although it isn't any more recommended since 2003, dual-platform method remains very widely used for studying lymphocyte T subsets (11-13). Reference values established with this method should not be used for interpretation of results obtained with single platform. Indeed, in spite of essays of standardization, quality controls still showed great differences between laboratories coefficients of variation (approximately 25 % for the enumeration of absolute count for any method used and 8 % for the same technique). This variation of results could be explained by measurement error's addition of both automats (hematology cell counter and FC) needed in the dual-platform method (14).

Furthermore, we have used a triple staining associating CD45 antibody. In fact, CD45 based lymphocyte gating strategy is the most appropriate approach for more accurately and reliably lymphocyte identification. It allowed to select lymphocytes and to eliminate background fluorescence noise. The dual combination CD3+/CD4+ and CD3+/CD8+ represent the cornerstone of analysis; the first one allowed not to count monocytes (CD3-/CD4+), as for the second, it eliminates the false count of NK lymphocyte (CD3-/CD8+).

Results of many studies establishing the lymphocyte T subset reference values are summarized in the table 4. Results were expressed, either in the form of values situated in more or less 2 SD with regard to the observed average value, or in the form of the values between the 2.5th and 97.5th percentile of a set of observed values. Results of these studies are very variable and difficult to be compared. Factors which can contribute to the variation of those results include, essentially, differences of the characteristics of the studied populations, the inclusion and exclusion criteria, the techniques used and the statistical methods of establishing of reference values. According to the table 4, inter-population variation of lymphocyte T subset enumeration seems very clear.

Inter-population variation of lymphocyte T subset enumeration

Our results were different from those of many other reported populations indicating the need of addressing geographical variations while interpreting the lymphocyte reference ranges (table 4). These observations suggest that each population should have its own lymphocyte reference ranges which should be regularly updated as socio-demographic factors change.

In a study carried out in Oman, in 2013, including 50 healthy subjects, authors found that reference values of CD3+ and CD3+/CD4+ cells are similar furthermore to those of Asian studies (21,22).

The most important ethnic variations are the ones observed for African (13, 18). Indeed, Lugada and al., established in 2004 reference values of Ugandan population from 3000 HIV seronegative subjects of any age. Enumeration was performed with the same technical procedure as our study (13). No homogeneity was found between values reported in this study and those of our study. Indeed, Lugada and al. reported higher values of CD3+/CD4+ cells and CD4+/CD8+ ratio, suggesting the influence of ethnical and/or environmental factors.

The study of Chng WJ and al. carried out in 2004, with the aim of comparing lymphocyte reference values according to gender, age and race, concerned 232 BD (184 Chinese, 22 Malay, 19 Indians and 9 of different race among Caucasian and Eurasian) (6). Indians seemed to have higher values of CD3+ cells and CD3+/CD4+ cells than Chinese or Malay, whereas other lymphocyte subpopulations were comparable between Malay and Chinese.

Influence of gender on lymphocyte T subset enumeration

We observed significant gender differences in some lymphocyte T subsets. These differences are thought to be mainly caused by the effect of sex hormones (23, 24). The involved mechanisms could be correlated with an accelerated thymocytes apoptosis by the male androgens, or with an effect of the fixation of estrogens on their specific receptors on lymphocytes T (18). However, the socio-demographic and lifestyle factors characterizing each population also seem to contribute and influence such gender differences in lymphocyte subsets because the pattern of differences between men and women are quite distinct, depending on the population studied.

For instance, in our study, absolute counts and percentages of CD3+, CD3+/CD4+ cells and CD4+/CD8+ ratio were higher among women than men and a similar observation has been addressed in some reports (8,25). In the study of Jentsch-Ullrich and al, absolute count of CD3+/CD4+ cells and CD4+/CD8+ ratio were higher for women older than 50 years (15). The significant reduction of CD3+/CD8+ cells in the group aged over 50 years was independent from the gender.

Influence of age on lymphocyte T subset enumeration

Our study includes only adult subjects. It was not possible to include children, teenagers and old subjects, because the age of our BD was between 18 and 65 years, which is the regulatory age for blood donation in Tunisia.

We found age-associated variation in some lymphocyte T subsets. In particular, with advancing age, we observed tendencies towards increasing absolute count of CD3+ and CD3+/CD4+ cells and towards decreasing absolute count and percentage of CD3+/CD8+ cells. These findings are consistent with those of other reports (15,18). In general, aging affects the potential activity of hematopoietic stem cells, the involution of the thymus, and the decline in T lymphocytes (26). Thus, the immune system may be postulated to have evolved in such a way as to be programmed to balance declining numbers of cytotoxic T-cells CD3+/CD8+ (involved in adaptive immunity) with increasing numbers of NK-cells (innate immunity).

However, some reports found contradictory results. Some observed tendencies towards increasing percentage of CD3+/CD8+ cells and consequently CD4+/CD8+ ratio decreasing (25,27-28) whereas others observed tendencies towards increasing of both CD3+/CD4+ and CD3+/CD8+ cells (21).

This disparity of results could be explained by the variability of age group included in every study as well as the number of subjects in each age group. According to the literature's data, children, in particular under 4 years, had the most marked variation of lymphocyte enumeration compared to adult (28).

In fact, in a study including children, newborns, absolute count of CD3+/CD4+ cells decreased with age, but percentage of CD3+/CD4+ cells and CD3+/CD8+ cells increased with a constant CD4+/CD8+ ratio (29).

Besides, a more recent multicenter study conducted in the United States in 2003 and including the widest pediatric population (807 children, from birth to the age of 18), showed that absolute count of CD3+cells , CD3+/CD4+ cells and CD3+/CD8+ cells decreased with age and this from age group 6 to 12 months (30). In the literature, there are no arguments to refuse using, to the old subjects, the reference values established for the youngest. Indeed, many studies reported quite variations of lymphocytes population for old subjects compared to other age groups (25).

CONCLUSION

Our study leads to the establishment of peripheral blood lymphocyte T subset reference ranges of BD in the region

Table 4. Reference ranges of lymphocyte T subsets reported in literature. NA :not available

Parameters/ Country	Allemagne (15)	Suisse (16)	Netherlands (17)	Singapore (6)	Italy (9)	Ethiopie (18)	Turkey (12)	Saudi Arabia (19)	Florida (20)
N (Male/Female)	100 (50/50)	70 (44/26)	51 NA	232 (104/128)	968 (532/436)	142 (92/50)	220 (105/115)	209 NA	100 NA
Age intervals (years)	19 - 85	24 - 70	>16	16 – 65	16 - 65	15 - 45	18-80	<1 -13	21-67
Techniques	Dual platform	Single platform	Single platform	Single platform	NA	Dual platform	Dual platform	NA	Dual platform
CD3+ (cells/µL)	780 - 2240	540 -1740	700 - 2100	800 -2680	600- 2460	850- 2560	725-296	870-2236	983-3572
CD4+ (cells/µL)	490 - 1640	310- 1140	300 - 1400	400 -1450	490- 1670	370- 1240	437-2072	383.8-1235	491-2000
CD8+ (cells/µL)	170 -880	140 - 820	200 - 900	240 - 1210	220- 1110	310- 1620	307-1184	273-1256	314-2087
CD4+/CD8+ ratio	0.9 - 5	1 - 5	1 – 3.6	0.7 - 2.8	NA	0.4 - 2.4	1.06 -2.76	0.5-3.26	0.6- 4.4

of Sfax. A study on a more diversified population, including more important number of individuals from various regions of Tunisia and including enumeration of other lymphocyte subsets (B cells and NK cells) is required.

Disclosure of interest

No conflicts of interest concerning this article.

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