Reference Range for T Lymphocytes Populations in Blood Donors from Two Different Regions in Brazil

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This study defined the normal variation range for different subsets of T-lymphocyte cells count in two different Brazilian regions. We analysed the T-lymphocytes subpopulations (CD3+, CD4+, CD8+) in blood donors of two Brazilian cities, located in North (Belem, capital state of Para, indian background) and Northeast (Salvador, capital state od Bahia, African background) regions of Brazil. Results were compared according to gender, stress level (sleep time lower than 8 hours/day), smoking, and alcohol intake. Lymphocytes subpopulations were measured by flow cytometry. Five hundred twenty-six blood donors from two Brazilians cities participated in the study: 450 samples from Bahia and 76 samples from Pará. Most (60%) were men, 59% reported alcohol intake, 12% were smokers, and 80% slept at least 8 h/day. Donors from Bahia presented with significantly higher counts for all parameters, compared with Para. Women had higher lymphocytes levels, in both states, but only CD4+ cells count was significantly higher than men's values. Smokers had higher CD4+ counts, but sleep time had effect on lymphocytes levels only for Para's donors (higher CD3+ and CD4+ counts). That state had also, a higher proportion of donors reporting sleep time <8 h/day. The values for CD3, CD4 and CD8+ cells count were significantly higher in blood donors from Bahia than among those from Pará. Female gender, alcohol intake, stress level, and smoking were associated with higher lymphocyte counts. The use of a single reference range for normal lymphocytes count is not appropriate for a country with such diversity, like Brazil is.

Key-Words: CD4, CD8, blood donors, reference values, Brazil.

T lymphocytes are an important cell subset which is responsible for specific response against viral infections [1]. By using monoclonal antibodies directed against some immune markers presented in 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 T-cells 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 [2].

The T helper lymphocytes (CD3+CD4+ cells) are target cells for HIV, and this specific population is decreased overtime during HIV infection, due to direct cytotoxicity, as well as by indirect mechanisms, like immune destruction of infected cells [3]. Once the continuous drop in CD4 count is a characteristic of HIV infection, and there is a direct relationship between the CD4+ cells count in peripheral blood and the risk of opportunistic infections (OIs) and neoplasms, this evaluation has been used as the main surrogate marker for HIV infection, serving to define the moment of starting antiretroviral therapy, and prophylaxis against OIs. It is also important to evaluate the frequency of CD8+ cells, since this population is responsible by cytotoxic activity, and also reflects the ability of immune system in controlling viral infections [3]. Such evaluation can be performed through the measurement of absolute CD4+ and CD8+ lymphocyte count, or by using the percentage values, as well as the CD4+/CD8+ cells ratio. To evaluate HIV disease stage we can measure for absolute counts and percentage of T lymphocytes, coupled with HIV RNA viral load measurement [4]. Reference range values are used for clinical monitoring of patients and are defined by normal parameters values from healthy population [5].

The use of flow cytometry to detect peripheral blood lymphocyte subpopulations needs a careful definition of reference ranges in terms of subset percentage and absolute numbers in order to permit comparison of results obtained from different populations. It is well known that the reference values for many immune parameters used in the clinical care of patients, is affected by their genetic background, as well as by other factors like nutrition, geographical situation, use of some drugs, and time of resting previously to the evaluation [6-9].

The T lymphocytes populations can be characterized by the presence of the CD3, CD4 and CD8 molecules in cell's surface. It is usually defined by flow cytometry, using dye labeled monoclonal antibodies against these molecules. Some different reference range values were published from other countries and these results could be associated with ethnic, nutrition and behaviors factors according to the population studied [10-14]. Therefore, it is needed a evaluation of these parameters in different populations, to provide a reliable reference range for the normal values, which can be used for different regions of the country, regardless the genetic background of their populations.

In Brazil, we have no reported evaluation of reference values for these parameters. The values in use for that purpose were obtained from studies conducted in North hemisphere, from

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populations with a main Caucasian genetic background [15]. The Brazilian populations have a wide ethnic diversity, with a mix of europeans, blacks (brought to the country during slave trade, in colonial period), orientals, and the native indian population [16]. Thus, the existing reference ranges for evaluation of these cells population may not be proper for use in the country. In addition, the different life-styles of these populations can add some other problems for the use of foreign reference ranges.

The goal for this study was to define reference ranges of relative and absolute numbers of lymphocytes subsets, by evaluating a cohort of healthy adults from two different Brazilian regions, with different ethnics background, by using a standard protocol to reduce the variability in both, sample preparation methodology, and flow cytometer operations, so that the data might reflect inherent biological variability in the population and not simply a variation in methods.

Material and Methods

Salvador, is the capital of Bahia state, and has the third largest city in the country. Salvador is considered a city with African characteristics, and an estimated 80% of its population is black or racially mixed, with a predominant black-and-white miscegenation. On the other hand, Belém, (the Pará's state capital) is predominantly composed by a population with a Caucasian origin, and presents less miscegenation. The remaining areas of Pará, are characterized, by a predominance of Indian-white miscegenation, which generated the "caboclo", a typical inhabitant of that region [17]. Thus we decided to use samples obtained from bone marrow donors, who were originated from small cities in the inland, where the population is predominantly "caboclo". The volunteers signed inform consents and answer a standard epidemiological questionnaire used in both blood banks. Were analyzed gender, use of alcohol or tobacco, and stress level (characterized by daily sleep time stratified in groups with less or more than 8 sleep hours per day).

The protocol for preparation, lyse and panel definition for monoclonal antibodies for T lymphocytes was derived from the guidelines of the International Federation of Clinical Chemistry (IFCC) [18].

The samples were collected using K^3EDTA at 26°C and analyzed by flow cytometry in a FACSCalibur equipment (Becton Dickinson). In this protocol we used the monoclonal antibodies CD45 (PE-Cy5), CD3 (PerCP), CD4 (FITC) and CD8 (PE) to determinate absolute and percentage values for T lymphocytes. For each sample 10,000 events were acquired.

We pooled data and analyzed the frequency distribution of each variable which were described by mean and standard deviation. The reference range was defined by interquartile values (from 2.5% to 97.5%) The influence of gender, smoking, alcohol intake, and stress level were evaluated by the t-test. All statistics were performed by using the software SPSS 11.0.

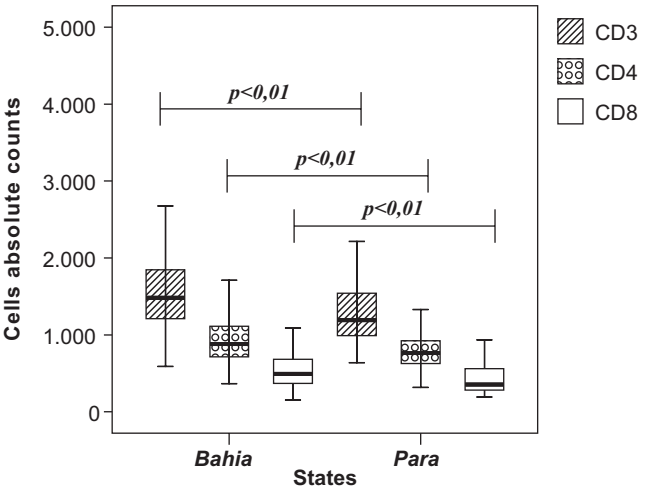
Results

Five hundred twenty-six blood donors from two Brazilians cities participated in the study: 450 samples were collected from the Centro de Hematologia da Bahia (HEMOBA) and 76 samples came from the Centro de Hematologia do Pará (HEMOPA).

Most of donors (59,5%) were men and 59,2% reported alcohol intake at least twice weekly. Among them, 10,9% were smokers and 77,9% had a minimum daily sleep time of eight hours.

Figure 1 shows the difference between mean count and median values for T lymphocytes CD3+, CD4+ and CD8+ in Bahia and Pará states. In Bahia the mean for absolute and percentage lymphocyte values was significantly higher for T lymphocytes CD3+ (1581; 77,9% cells), CD4+ (943; 43,2% cells) and CD8+ (549; 25,4% cells) cells, when compared to values found in Pará State´s blood donors (1298; 62,4% cells, 792; 37,7% cells and 417; 18,6% cells, for CD3+, CD4+ and CD8+, respectively, $p<0.01$ in use for T-test).

Figure 1. Comparison between the absolute values for the CD3+, CD4+ and CD8+ T lymphocytes populations from Bahia and Pará states.



In Bahia 66,3% were men, 11,7% were smokers, 62,8% reported to drink alcohol at least two times per week, and 81,6% used to sleep 8 or more hours per day. In Pará, 31,6% were men, 7,9% smokers, 44,7% drink alcohol at least twice weekly, and 63,2% used to sleep 8 or more hours per day.

Median values found for T cells subpopulations according demographic data from Bahia are shown in Table 1. Were adjusted results for gender, smoking, alcohol intake and stress level in blood donors. We determinate blood donors stress, by inquiring their sleep time (more or less than 8 hours per day). Alcohol intake was measured considering the a limit of drinking equal or greater than two alcohol doses (destilated drinks), two or more days a week. An intake of less than that limit was considered as a negative alcohol intake.

In Bahia, we detected a significant difference in T CD4+ cells count comparing men (898 cells/mm^3) and women (1032

Table 1. Absolute results for the CD3+, CD4+ and CD8+ T lymphocytes according to demographics characteristics of blood donors from Bahia state.

	Bahia Cells/ μL		
	CD3+	CD4+	CD8+
Men	1,521	898*	537
Women	1,698	1,032*	570
Smokers	1,622	1,013*	456
No smokers	1,470	864*	499
Stress	1,537	809	513
No stress	1,483	891	495
Alcohol	1,505	881	500
No alcohol	1,466	864	486

*p<0.05 for comparison between the specific variables.

Table 2. Absolute results for the CD3+, CD4+ and CD8+ T lymphocytes according to demographics characteristics of blood donors from Pará state.

	Pará Cells/μL		
	CD3+	**CD4+**	**CD8+**
Men	1,243	768	393
Women	1,323	802	428
Smokers	1,543	960*	509
No smokers	1,276	773*	412
Stress	1,372*	842*	435
No stress	1,171*	706*	387
Alcohol	1,357	828	436
No alcohol	1,250	762	402

*p<0.05 for comparison between specific variables.

Table 3. Absolute results for lymphocytes subsets of blood donors form Bahia and Pará states, according to gender, smoking, alcohol intake, and stress level.

	Cells/μL		
	CD3+ Bahia/Pará	**CD4+ Bahia/Pará**	**CD8+ Bahia/Pará**
Men	1,521/1,243*	897/768*	537/393*
Women	1,698/1,323**	1,032/802**	571/428**
Smokers	1,694/1,543	1,094/960	534/509
No smokers	1,568/1,276**	924/773**	552/412**
Stress	1,595/1,372	958/842	550/435
No stress	1,517/1,171**	877/706**	546/387**
Alcohol	1,579/1,357*	941/828*	549/436*
No alcohol	1,583/1,250**	946/762**	549/402**

*p<0.05; **p<0,01.

cells/mm³, p=0.03 value for T-test) and smokers (1,013 cells/mm³) *versus* no smokers (864 cells/mm³, p=0.02 value for T-test). The stress level showed no impact on these parameters.

On the other hand, in Pará, no difference was found between women and men, but it was detected for smokers, and individuals with less than 8 h of daily sleep time (Table 2). Both regions presented with similar results for the T CD3+ and T CD8+ cells count. For the stress level we found significant difference in T CD3+ and T CD4+ lymphocytes count (p= 0.01 and p= 0.03, respectively).No relationship was observed between alcohol intake and levels of T-cells count neither for donors in Para or in Bahia states, but the comparison between Pará and Bahia showed a difference between donors from the two states, when compared according to the alcohol intake.

The comparison between the two states provided significant differences, for all but smokers groups, with

individuals from Bahia presenting with significantly higher counts of different lymphocytes subpopulations. Table 3 summarizes the results for the two states.

Discussion

The present study detected significant differences for the reference ranges results in T lymphocytes from donors from Bahia and Pará states. The levels of absolute lymphocyte count, CD cells, CD8+ cells were higher in Bahia than Para. It was also higher when adjusted for gender, stress level, and alcohol intake, and smoking. The only group with comparable results was the non-smokers.

Brazilian population have very ethnic diversity which varies according to the region, as a result of the destination of the different groups of immigrants received during the colonial period. In general, the ethnic composition of Brazilian population includes Europeans (mainly in South/Southeast regions), Africans (brought to the country during slave traffic, and predominantly located in Northeast region, especially in Bahia), Asian immigrants (Southeast region) and Indians (native population, currently predominant in North region). Were evaluated two states from Brazil: Bahia and Pará situated in Northeast and North region, respectively.

The Indigenous populations exert a profound cultural influence in Brazilian North region, with a direct impact on gastronomy, music, and other cultural manifestations. The remaining populations from this group in Brazil, live in North and Central-West regions. In Pará, according with data from Brazilian Institute of Statistics and Geographyl (IBGE) exist 20.185 Indians and 34 different tribes [19].

In Bahia, the African influence is also very present: in the state 77,5% populations are from African descendant, and in Salvador the black, or racially mixed (mainly black and white miscegenation) inhabitants represents 80% (2,277,591 estimated individuals) of the general population, according to IBGE data.

The values we obtained from Para state for T lymphocytes are similar to those reported for Asian countries, confirming the likely influence of the Amerindian ethnicity, since they are believed to originate from Asian continent, during migration occurred thousands of years ago. We didn't found any published specific reference values of T-cells for Indians populations.

Similarly, the values obtained for T lymphocytes ranges, in Bahia, are comparable to those described for general population, in Italy, and some African countries, which can also be a reflex of the already described miscegenation between European African ancestors, widely expressed in Bahia´s population. When we compare other published reference ranges for blood donors from Cameroon, and Central Africa the results are quite similar, with only a 5% difference in CD4+ T-lymphocytes count [20,21].

Likewise, some studies performed in Italy, Turkey and Switzerland, in comparable population groups, showed a consistently similar range for normal values of T lymphocytes between them. The difference for T CD3+ cells was only 3.7% higher in Italy in comparison with Turkey and 1% higher of that from Switzerland. In T CD4+ cells, the absolute values were 6% greater in Italy, compared with Turkey and 2,9% greater in comparison with Switzerland, a pattern also seen for T CD8+ cells (donors from Italy presented values 2,6% greater than those from Turkey, and 3.2% higher than donors from Switzerland [22-24].

Comparing populations with an Asian genetic background, provides a similar picture: the difference for reference range was very narrow for blood donors from China and India. For T lymphocytes CD3+ cells, in China, the absolute mean values was 0,4% higher than that from India, and for T-cells CD4+ lymphocytes the difference was only 0,7%. For T-CD8+ cells it was only 4,3% [25-26].

Our study demonstrated that absolute values T CD3+, T CD4+ and T CD8+ cells were higher in women in comparison with men in both states, but the only significant difference was found for comparison of CD4+ count. Similar results were reported in other countries, raising the hypothesis that sexual hormones may influence the frequency of T lymphocytes subpopulations leading to this difference [26].

Some behavioral issues, like smoking, stress level, and alcohol intake have been already considered as having an impact on the immune system. In our study, T lymphocytes values were higher for smokers than those found for non-smokers. Smoking is considered to be capable to decrease the immune response. Smoking is considered to be a factor that can impair the innate and acquired immune response (27). It can lead to a persistent immune activation which could increase CD4+ cells count, as observed in our results from Salvador blood donors.The detection of such difference only in Bahia suggest that other factors may be responsible for this finding, including the genetic background that could differentially modulate such response. On the other hand, stress level (measured by less hours of sleep time, in our study) was significantly different for the two groups, and could also contribute for the observed difference, since 36,8% of donors in Pará reported less than eight daily hours of rest against only 19.4% in Bahia.

For the variables studied, only results for smokers did not present significant differences between states for T lymphocytes values. T lymphocytes values were higher in Bahia in comparison with Pará state, when compared gender (men higher than women), stress level and alcohol intake.

One of limitations in our study regards to the smaller sample size of blood donors in Para state. However, the significant difference obtained in the comparison between the populations of the two states indicates it had enough power to detect variations in the studied variables. In addition, some of the already published works have used similar number of patients, and the results were also comparable [20].

Therefore, the observed differences for reference ranges in T lymphocytes populations between Bahia and Pará demonstrate the need of defining specific reference values

for such parameters in settings characterized as having populations with a great genetic diversity. Brazil is a large country, with potentially important differences in population composition, depending on the target region. These findings demonstrate that the T lymphocytes reference range currently used in Brazil may not be appropriate for routine use in all regions of the country.

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