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Original Article

Reference values of T lymphocyte subsets among health adults in Inner Mongolia Region

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Abstract: Estimation of T-lymphocyte subsets continues to be an important aspect for monitoring HIV disease progression and response to antiretroviral therapy. Most of the diagnostic laboratories often rely on studies from western for CD4+T-lymphocyte reference values, which could, often be unreliable for usage in local settings. To establish the normal reference values of T lymphocyte subsets from healthy people of Inner Mongolia Autonomous Region, flow cytometry was performed to determine the reference ranges for lymphocyte subsets (CD3 and CD4 cells) in 400 healthy multiracial adult population from 12 League Cities in Inner Mongolia Region, China. The basic information including age, gender, nationality and history was collected. There were significant differences in the absolute counting, percentage of CD3+T lymphocytes, and CD4+T lymphocyte percentage counting among different age groups. There were significant differences in CD3+, CD4+T lymphocyte percentage in the groups with different genders. There were significant differences in CD3+T lymphocyte percentage count, absolute count of CD4+T lymphocytes and CD4+T lymphocyte percentage counting in the group with ages of 16-20. There were dramatic differences in CD3+T lymphocyte percentage count and CD4+T lymphocyte percentage counting in the group with ages of 31-40. There were significant differences in CD4+T lymphocyte percentage counting. By this study, age, gender and ethnic specific lymphocyte subset reference ranges have been locally established in Inner Mongolia Autonomous Region.

Keywords: T lymphocyte subsets, normal reference value, Inner Mongolia

Introduction

Under normal circumstances, the numbers and proportions of various lymphocyte subsets including T lymphocytes, B lymphocytes and NK cells are stable, maintaining the normal immune functions of bodies. When the quantities and functions of lymphocyte subsets are abnormal, a series of pathological changes and immune function disorders will take place. Therefore, measuring changes of human lymphocyte subsets is important for early detection and control of related diseases to guide the clinical treatment evaluation of the immune state [1].

T lymphocytes are a group of lymphocytes of the immune system with different functions. T lymphocyte subsets can not only reflect the immune state, but also can predict the disease progression and determining the therapeutic effects of drugs, so they have important clinical values [2]. In order to evaluate T lymphocyte subsets of patients with different diseases, the normal value of healthy people must be used as a reference. The normal values of T lymphocyte subsets currently used by the medical institutions in Inner Mongolia Autonomous Region are provided by instruments companies, which are detected from healthy people from abroad. However, there is no such data from our national people.

In the immune system, CD4+ regulatory T cells regulate as natural and acquired immune system functions by direct cell contact and secretion of cytokine, which controls the T cell overgrowth of self antigen reactions and plays an important role in maintaining immune tolerance and steady state. With the appearance and application of flow cytometry, it is possible and also necessary to establish healthy reference range for the distinguishing of healthy people and patients. Flow cytometry is a fast, flexible

and high precision technique for analyzing cells and particles. Flow cytometry has become the gold standard for the detection of CD4+T lymphocytes [3].

Lymphocytes were CD45-positive cells with a lower granularity, which include T lymphocytes (CD3+), B lymphocytes (CD19+) and NK cells (CD16+ 56+). T lymphocytes are also divided into T helper/T inducer cells (CD3+ CD4+) and T suppressor/T cytotoxic cells (CD3+ CD8+). CD3+ CD4+ cells are T Helper/Inducer lymphocytes, and CD3+ CD8+ cells are T Suppressor/Cytotoxic lymphocytes [4].

T lymphocytes have been widely used in developed countries, and its detection method has been standardized. The current detection methods are divided into two categories: single platform method and double platform method. Because the double platform method depends on two instruments at the same time, there is high error probability. At present, more than 70% of the international laboratories used the single platform determination method [5]. Thus, the present study also used the single platform method.

The reference range of the normal human lymphocytes has important significance for the diagnosis and prognosis of Acquired Immune Deficiency Syndrome (AIDS). CD4+T lymphocyte serves as an important representative mark of the HIV disease procession. The statistics of the CD4+T lymphocyte count information of healthy populations from all over the country is helpful for the establishment of the normal range of CD4+T lymphocytes, which will provide useful data for determining whether the Western antiretroviral treatment and management standard should be used in the HIV infected person [6].

It has been demonstrated that there were significant differences in races, ethnic groups, gender and ages of the India healthy adult T lymphocytes. Yaman A et al. established the reference ranges of lymphocyte subsets of Turkey healthy adults, which has been applied in clinics [7].

The normal reference value of T lymphocyte subsets of health adults in Inner Mongolia Region was established in the present investigation.

Materials and methods

Sample collection

Population structure of Inner Mongolia Autonomous Region was surveyed. Health examination time was set up to collect blood samples.

For experiments involving human subjects, approval was obtained from the institutional review board of local committee. Informed consent was provided according to the Declaration of Helsinki.

400 healthy adult (more than 16 years old) (in accordance with the requirements of the national population proportion sampling) from 12 League Cities in the whole autonomous region were selected as research subjects. 2-4 ml venous blood was drawn with evacuated blood tube with EDTA as the anticoagulant. At the same time, the basic personal information including name (or number), gender, age, nationality and history was investigated.

Flow cytometry

Flow cytometry was used to detect the blood samples of healthy adults from 12 League Cities. T lymphocyte subsets were analyzed to get the absolute counts and percentages of the T lymphocyte subsets to establish the normal reference range of T lymphocyte subsets of health adult in the Inner Mongolia Region. The details were as follows:

(1) Preparation of samples and reagents for detection;

(2) 20 μl anti-CD3/CD4/CD45 antibody was added in each tube;

(3) The samples and antibodies were mixed gently and evenly. 50 μl mixture was taken by using the reverse pipetting method and put in counting tube, which was added in quality control sample CD-CHEX;

(4) After mixed fully, the mixture was incubated for 15 min in the dark;

(5) 450 μl 1 × FACS hemolysin (America BD) was fully mixed and incubated in the dark at room temperature for 15 min;

(6) Flow cytometry analysis;

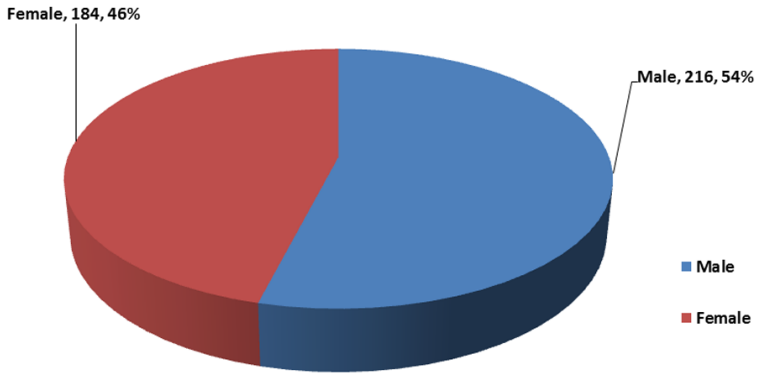

Figure 1. Gender distribution and composition of healthy population in Inner Mongolia.

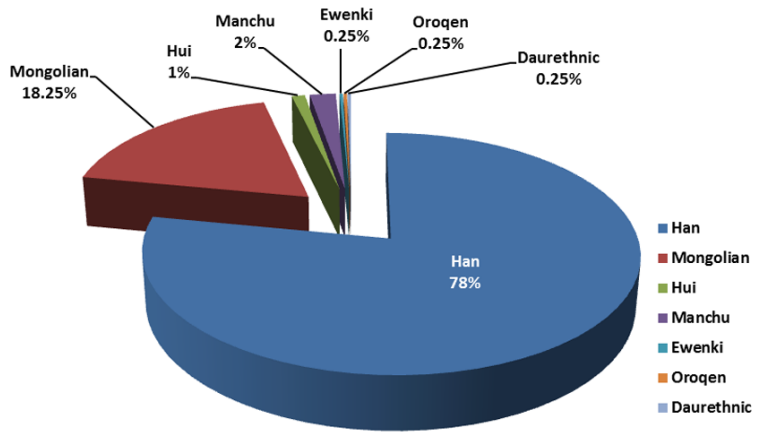

Figure 2. Ethnic distribution and composition of healthy population in Inner Mongolia.

(7) The CaliBRITE3 fluorescent microspheres and FACSComp automatic software was used to detect the inspection instrument. MultiSET automatic analysis software was used for the counting of lymphocyte subsets. Briefly, detection of quality control sample CD-CHEX was conducted. After the examination results of the quality control sample were in the range of quality control, each sample was examined one by one. Percentages of CD3, CD4, CD45 lymphocytes and the absolute values were reported.

(8) The data were collected by using the MultiSET software.

Statistical analysis

Data were analyzed by SPSS 13.0 statistical software (USA). Measurement data among groups were expressed as $\bar{\chi} \pm S$. Measurement data were analyzed by using t test, and enumeration data were analyzed by using the Wilcoxon method and the Kruskal-Wallis test. A $P < 0.05$ indicated that the difference was significant.

Results

Gender distribution

There were 400 research subjects in the present study, in which 216 cases were male and 184 cases were female with a gender ratio of 1.17:1 (**Figure 1**).

Age distribution

The ages of the subjects were between 16 and 65 years with a mean age of 36.81 ± 12.01 years, which were mainly young people (**Table 1**).

Ethnic composition and distribution

Of the 400 subjects, there were 312 cases of Han nationality (78%), 73 cases of Mongolia nationality (18.25%), 4 cases of Hui nationality (1%), 8 cases of Hui nationality (2%), 1 case of Daur Oroqen (0.25%) and 1 case of Ewenki (0.25%) (**Figure 2**).

Determination results of T lymphocyte subsets

Flow cytometry was used to detect CD3+ and CD4+T lymphocytes, their percentages and absolute counting. After examination, all the indicators were in line with the normal distribution.

As shown in **Table 2**, there were significant differences in the absolute counting, percentage of CD3+T lymphocytes, and CD4+T lymphocyte percentage counting among different age groups ($F = 3.994$, 9.421 and 5.957, $P < 0.05$). However, there was no dramatic difference in the absolute counting of CD4+T lymphocytes in the groups with different ages ($F = 2.071$, $P > 0.05$).

Table 1. Age distribution and constituent ratio of the Inner Mongolia health people

Ages (year)	People (cases)	Percentage (%)
16-20	37	9.25
21-30	95	23.75
31-40	116	29
41-50	79	19.75
> 50	73	18.25
Total	400	100.0

As demonstrated in **Table 3**, there were significant differences in CD3+, CD4+T lymphocyte percentage counting in the groups with different genders (t = 4.539 and 5.881, respectively. $P < 0.05$), while there was no great difference in the absolute counting of CD3+ and CD4+T lymphocytes (t = -0.783, 1.293, $P > 0.05$).

Table 4 illustrated that there were significant differences in CD3+T lymphocyte percentage count (t = -2.408), absolute count of CD4+T lymphocytes (t = -1.998) and CD4+T lymphocyte percentage counting (t = -2.816) ($P < 0.05$) in the group with ages of 16-20. There were dramatic differences in CD3+T lymphocyte percentage count (t = -2.434) and CD4+T lymphocyte percentage counting (t = -2.879, P < 0.05) in the group with ages of 31-40. There were significant differences in CD4+T lymphocyte percentage counting (t = -3.269, $P < 0.05$), but there was no statistically significant difference in the groups with ages of 41-50 but different genders (t = -3.269, $P < 0.05$).

The 95% reference value range of healthy adult T lymphocyte subsets of the Inner Mongolia Autonomous Region

According to the experimental design, of the 400 subjects, there were 312 cases of Han nationality, 73 cases of Mongolia, 4 cases of Hui, 8 cases of Man, 1 case of Daur Oroqen, 1 case of Oroqen and 1 case of Owenki. Because there was only limited samples and they did not have statistical significance, so Mongolia and Han nationality with large sizes were studies and compared (**Table 5**).

T lymphocyte subsets results of age and gender and other factors displayed that although they showed significant differences, but the difference was not large. Various indicators showed normal distribution, so the percentile method was used to establish the 95% reference value range of healthy adult T lymphocyte subsets of the Inner Mongolia Autonomous Region (**Table 6**).

Discussion

The healthy populations infected with HIV were selected from all the research subjects by HIV antibody screening to conduct detection of T lymphocyte subsets counts and the percentage of T lymphocytes. The normal reference range value of lymphocyte subsets of healthy adult of Inner Mongolia Region was established in the present study, which can be used for the determination of limit reference range value of lymphocyte subsets between healthy people and patients suitable for the local community of Inner Mongolia Region.

The correlations between T lymphocyte subset counts and percentage, and gender, age and nationality were revealed by analyzing various factors related to T lymphocyte subset examination values, which provided evidence for further analysis of specific progress status of people infected with HIV and AIDS patients.

T lymphocytes have been widely used in developed countries, and its detection method has been standardized [8]. The current detection methods are divided into two categories: single platform method and double platform method. Because the double platform method depends on two instruments at the same time, there is high error probability. At present, more than 70% of the international laboratories used the single platform determination method. Thus, the present study also used the single platform method.

In order to ensure the accuracy of the present study, the following aspects were used. First of all, 50 μl blood samples were detected by the reverse pipetting method using precision pipette. TruCOUNT tubes were stored in 2-25°C under closed drying conditions, which should be used within 1 hours. At the same time, FACS hemolysin and fresh sample were used in the experiment.

Secondly, FACSCALIBUR flow cytometry analyzer and automatic analysis software were employed in the present investigation to obtain samples automatically. Information of various subtypes of lymphocytes in peripheral blood

Table 2. T lymphocyte subsets of people in different groups ($\bar{X}$ ± S)

Ages (Year)	CD3+		CD4+	
	Percentage	Absolute number (/μl)	Percentage	Absolute number (/μl)
16-20	64.97 ± 7.45	1535.14 ± 492.91	31.59 ± 5.68	740.97 ± 212.40
21-30	68.45 ± 7.52	1602.98 ± 380.93	33.63 ± 6.27	781.61 ± 204.99
31-40	70.28 ± 7.40	1885.55 ± 590.92	34.29 ± 6.74	909.87 ± 287.18
41-50	69.33 ± 7.60	1705.62 ± 461.78	33.62 ± 6.70	819.24 ± 220.82
> 50	67.78 ± 8.39	1496.66 ± 357.33	34.90 ± 7.80	766.85 ± 212.87
F	9.421	3.994	5.957	2.071
P	< 0.01	< 0.01	< 0.01	> 0.05

Table 3. T lymphocyte subsets of people with different genders ($\bar{X}$ ± S)

Gender	CD3+		CD4+	
	Percentage	Absolute number (/μl)	Percentage	Absolute number (/μl)
Male	67.11 ± 7.95	1697.54 ± 502.98	32.09 ± 6.54	805.12 ± 253.68
Female	70.56 ± 7.14	1658.78 ± 482.56	35.91 ± 6.44	836.65 ± 230.54
t	4.539	-0.783	5.881	1.293
P	< 0.01	> 0.05	< 0.01	> 0.05

Table 4. T lymphocyte subsets in the groups with different gender but same ages ($\bar{X}$ ± S)

Ages	Gender	CD3+		CD4+	
		Percentage	Absolute number (/μl)	Percentage	Absolute number (/μl)
16-20	Male	64.43 ± 7.43	1545.38 ± 497.19	31.31 ± 5.96	746.00 ± 214.43
	Female	68.40 ± 7.33	1469.60 ± 514.39	33.40 ± 3.13	708.80 ± 219.50
21-30	Male	66.52 ± 6.88	1567.00 ± 403.04	31.96 ± 6.52	741.91 ± 206.25
	Female	70.12 ± 7.73	1632.74 ± 360.71	35.47 ± 5.77	824.82 ± 202.05
31-40	Male	68.72 ± 8.07	1966.17 ± 627.99	32.39 ± 6.52	922.02 ± 328.87
	Female	71.97 ± 6.55	1803.65 ± 547.11	35.78 ± 6.36	885.83 ± 244.57
41-50	Male	67.51 ± 7.95	1769.25 ± 409.52	31.12 ± 5.29	811.02 ± 190.27
	Female	69.88 ± 7.15	1624.25 ± 508.75	35.72 ± 7.21	827.64 ± 261.01
> 50	Male	67.18 ± 9.17	1524.42 ± 335.67	33.71 ± 8.17	764.92 ± 230.26
	Female	69.38 ± 7.13	1446.96 ± 381.13	37.31 ± 7.37	769.45 ± 190.56

was acquired to calculate the relative counting and absolute counting. Peripheral blood was examined to calculate the relative and absolute contents of lymphocytes and their subtypes by direct using of fluorescent antibody for one step and four-color fluorescence labeling. The designed disposable kit and simple operation reduced the cell loss and ensured quality control.

Thirdly, absolute cell counting of whole blood was conducted by using MultiTEST reagents. TruCOUNT tube was added into quantitative Beads in advance to ensure that each batch had the same content of TruCOUNT Beads. Furthermore, direct fluorescent antibody combinations were employed in the present study.

Quality control of data collection process and information recording and analyzing was conducted to ensure the reliability and objectivity. Before examination, fluorescent calibration microspheres and CD-CHEX Plus quality control reagents were employed in to instrument calibration and ensure that each detection was in range of quality control.

In the immune system, CD4+ regulatory T cells regulate natural and acquired immune system functions by direct cell contact and cytokine

Table 5. T lymphocyte subsets of different nationalities ($\bar{X} \pm S$)

Nationality	CD3+		CD4+	
	Percentage	Absolute number (/μl)	Percentage	Absolute number (/μl)
Han	68.75 ± 7.78	1685.61 ± 508.65	34.03 ± 6.66	828.29 ± 255.02
Mongolian	68.78 ± 8.26	1633.71 ± 431.66	32.98 ± 6.96	771.26 ± 196.20
t	-0.027	0.806	1.790	1.193
P	> 0.05	< 0.05	> 0.05	> 0.05

Table 6. 95% normal reference value range of T lymphocyte subsets of the healthy adult in the Inner Mongolia Autonomous Region

T lymphocyte subsets	Mean	Standard deviation	maximum	minimum	median	interquartile range	95% range
CD3+ number	1679.52	493.36	4458	713	1622.5	586.75	929-2912
CD3+ (Percentage)	68.71	7.77	89	45	69	10	53-83
CD4+ number	819.79	243.4	2226	366	776.5	326	427-1345
CD4+ (Percentage)	33.87	6.76	52	15	33	9	21-48

secretion mechanisms. Thus, it is important to maintain immune tolerance and homeostasis by controlling the self antigen reactive overgrowth of T cells targeting their self antigens. Flow cytometry has become the gold standard for the detection of absolute counting of CD4+T lymphocytes. Analyzing lymphocyte immunophenotyping by flow cytometry has gradually become one of the routine clinical examinations. Races, ecological environment and economic conditions and other factors of different countries or regions may affect the immune systems of different populations.

The reference range of normal human lymphocytes has important significance for the diagnosis and prognosis of AIDS. The most important CD4+T lymphocytes of the human immune system were regarded as the targets of HIV. After infected with HIV, lots of CD4+T lymphocytes were engulfed and destroyed, which mainly exhibited that the number of CD4+T lymphocytes in peripheral blood circulation was decreased, and thus the immune system was damaged, losing resistance to various diseases. With the development of the disease, acquired immune deficiency was induced, and secondary infection and malignant tumors were serious and difficult to be treated. Therefore, accurate and reliable detection of CD4+T lymphocytes is an important index to evaluate the immune status of HIV infections, predict disease progression ane evaluate the curative effect of anti-virus drugs and estimate prognosis.

CD4+T lymphocyte is an important representative mark of the HIV disease progression. The normal range of CD4+T lymphocyte count statistics from all over the country is helpful for the establishment of the normal reference range value of CD4+T lymphocyte count of national population, which in turn can help determine whether antiretroviral treatment on HIV infected people and management standards of the western world should be employed. In addition, the correlations between T lymphocyte subsets count and percentage and gender, ages and nationality were investigated.

In conclusion, the 95% reference value range of healthy adult T lymphocyte subsets of the Inner Mongolia Autonomous Region is established, which is important for both the theoretical research and clinical application.

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Disclosure of conflict of interest

None.

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