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Original Article

Immunophenotypic enumeration of $CD4^+$ T-lymphocyte values in human immunodeficiency virus-seronegative adults in Eastern India.

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ABSTRACT

Background: The enumeration of $CD4^+$ T-lymphocytes in Human Immunodeficiency Virus (HIV)-positive patients is an essential tool for HIV staging, initiation of anti-retroviral therapy (ART), monitoring response to ART and initiation chemoprophylaxis against opportunistic infections. Therefore, it is important to know the level of immunocompetence of a particular geographical region by enumerating the baseline $CD4^+$ T-lymphocytes in HIV-seronegative healthy adults. **Aim:** The aim was to enumerate $CD4^+$ T-lymphocytes counts of healthy HIV-seronegative adults in eastern India. **Materials and Methods:** Blood samples were obtained from hundred HIV-seronegative healthy adults (mean age 32.6±11.3 years) who attended integrated counselling and testing centre (ICTC-1) for HIV information. Immunophenotypic enumeration of $CD4^+$ T-lymphocytes was carried out using flow cytometer. **Results:** The mean absolute $CD4^+$ T-lymphocytes count was 823.9(±243.4)cells/µl. The established range of $CD4^+$ T-lymphocyte counts for men and women were 338 -1292 cells/µl (mean 793.4±243.5 cells/µl) and 402-1321 cells/µl (mean 885.9±234.8 cells/µl) respectively. Women had significantly higher absolute $CD4^+$ T-lymphocyte counts ($p<0.001$) when ompared to men. The distribution of mean absolute $CD4^+$ T-lymphocyte counts among different age groups showed that individuals within 18 to 27 years of age group had significantly higher CD4+ T-lymphocyte counts of 893.3±43.4) cells/µl. **Conclusions:** Our findings of $CD4^+$ T-lymphocyte counts among HIV-seronegative adults in east India corroborates emerging data that showed the presence of significant differences in reference to $CD4^+$ T-lymphocyte counts between different populations within and outside the country.

Key words: $CD4^+$ T-lymphocytes, HIV, ART, immunocompetence

INTRODUCTION

T-lymphocytes are defined by the expression of CD3$^+$ T cell subpopulations by the co-expression of CD4$^+$ or CD8$^+$ or HLA-DR.[1] CD4$^+$ T-helper lymphocytes play a central role in regulation of immune response.[2] These cells have capacity to help B cells for generating antibodies, to recruit and activate macrophages, to recruit neutrophils, eosinophils, and basophils to sites of infection and inflammation.[3] As the CD4$^+$ T-lymphocytes are main targets of human immunodeficiency virus (HIV), CD4$^+$-lymphocyte counts (LCs) are recognized as the most important measurement of overall HIV-induced immune impairment.[4] The enumeration of CD4$^+$ T-lymphocytes in HIV infected individuals is an essential tool for staging HIV disease, to make decisions for initiation of anti-retroviral therapy (ART), for monitoring response to ART and to initiate chemoprophylaxis against opportunistic infections.[5,6,7] Besides HIV disease, the clinical applications of CD4$^+$ LCs include diagnosis of primary and secondary immunodeficiency disorders, evaluation of immune-mediated diseases and the assessment of immune reconstitution following stem cell transplantation.[8,9,10]

Variability in CD4$^+$ LCs among healthy HIV-seronegative adults has been widely reported and has been attributed to biological, ethnic group influences as well as differences in the methodologies used for T-cell enumeration. Therefore, it is important to know the level of immunocompetence of a particular geographical region by enumerating the baseline CD4$^+$ LCs in HIV-seronegative healthy adults.

There have been studies of CD4$^+$ LCs among HIV-seronegative healthy adults reported from northeast, north, west, northwest, south region of India including a multi-centric study.[11-23] Also studies have been reported from different parts of the world.[24-36] To the best of our knowledge, no study has been conducted from eastern region of India. Hence, we undertook this present study to determine the baseline CD4$^+$ LCs among HIV-seronegative healthy adults who attended integrated counselling and testing centre 1 (ICTC-1) for HIV information in this part of the country with the aim of establishing a normal reference range among Indian population.

MATERIALS AND METHODS

Study area and population

The present study was carried out in the Department of Microbiology, integrated counselling and testing centre 1 (ICTC-1), which is a tertiary referral hospital of eastern India. Hundred HIV-seronegative healthy adult volunteers who attended ICTC-1 for HIV information aged between 18 to 55 years were included in the study. This group is sexually active, hence more vulnerable to contrast sexually transmitted diseases including HIV. The criteria for exclusion include (a) Any minor illness in the last one month (b) Any major illness, including surgery, trauma and accident in the last six months (c) Any chronic illness (d) Vaccination in the last six months (e) Pregnant women (f) Active drug administration (g) HIV seropositive volunteers. Interested subjects were included in this study after obtaining informed verbal consent. All the tests were done in accordance with the Medical College ethical committee guidelines. The findings were analyzed over a period of one year from July 2011 to June 2012.

Sample collection and processing

Five milliliters (ml) of unlysed whole-blood sample was collected at stipulated time interval between 10 am and noon. Two ml of blood was transferred to a sterile vial for HIV serology and 3 ml of blood transferred to K2 EDTA containing vacutainer tube for CD4$^+$ LCs.

HIV serology for screening

Samples were subjected to a rapid screening HIV 1 and 2 Immunodot Test (COMBAIDS® - RS Advantage- ST kit, Span Diagnostics Ltd., Surat, India). The test was done according to manufacturer's instructions. All hundred volunteers were HIV negative.

Immunophenotypic enumeration of CD4$^+$ T-lymphocytes by using flow cytometer

Immunophenotyping of lymphocytes was carried out by BD FACS™ Calibour system (Becton Dickinson, Fluorescent antibody cell sorter, Singapore) by using 50 µl of well mixed whole-

blood collected in K2 EDTA containing vacutainer tube. Three antibody panels were used i.e., BD Tri TEST™ CD3 fluorescein isothiocyanate (FITC)/CD4 phycoerythrin (PE)/CD45 peridinin chlorophyll protein (PerCP), a three-color direct immunofluorescence reagent to identify and determine the percentages and absolute counts of mature T-lymphocytes ($CD3^+$) and helper T-lymphocyte ($CD3^+CD4^+$) subsets in erythrocyte-lysed whole-blood, by using Tru Count™ tubes.[37,38] The absolute $CD4^+$ LCs in the present study were measured with the FACS Calibour system, using single platform technology which is regarded as a reliable and robust method for the enumeration of $CD4^+$ lymphocytes.[37,38]

Quality control

Tests were done in accordance to the manufacturer's guidelines.

Statistical analysis

The values of mean, median and standard deviation of $CD4^+$ T-lymphocytes were calculated using GraphPad® InStat statistical software. Statistical significance was defined when *P*-value < 0.05.

RESULTS

One hundred HIV-seronegative healthy adults who attended ICTC I for HIV information were included in the study: 67(67%) were male and 33(33%) were female. The age of the subjects included in the present study were ranged from 18 to 55 years, with a mean age of 32.6 ±11.3 years and median of 30.5 years. The mean age of male was 33.6 ±11.6 years (range, 18 to 55 years) and female was 30.7 ±10.7 years (range, 18 to 55 years).

The overall mean absolute $CD4^+$ LCs in the present study population was 823.9±243.4 cells/µl, median 847 cells/µl and reference range of 338 to 1321 cells/µl. The male showed mean absolute $CD4^+$ LCs of 793.4 ±243.5 (median=820), and female revealed mean $CD4^+$ LCs of 885.9 ±234.8 (median=896 cells/µl). The reference range of $CD4^+$ LCs was 338 to 1292 in male and 402 to 1321 cells/µl in female. The *P* value of mean absolute $CD4^+$ LCs equals to 0.001 (Table 1). In 11 subjects (one female and ten male), the absolute $CD4^+$ LCs were less than 500 cells/µl. The overall mean percentage of $CD4^+$ LCs in this present study was 40.5±8.9. The percentage of $CD4^+$ LCs in male and female was 40.1 (±9.2) and 41.3 (±8.8) respectively. The reference range in percentage was 22.6 to 61.2 and 26.1 to 60.4 in males and females respectively.

The subjects were grouped by age; 18-27, 28-37, 38-47 and 48-55 years. The distribution of mean $CD4^+$ LCs declined with age. Individuals between 18 to 27 years of age group had 893.3±43.4 cells/µl, followed by 832.4±43.6 in 28 to 37 years, 803.1±67 in 38 to 47 years and least 758.4±95.2 cells/µl in the age group of 48 to 55 years (Table 2).

Table 1: $CD4^+$ lymphocyte counts in HIV-seronegative healthy adults in Eastern India

P-value of mean absolute CD4 cell count equals to 0.0001, considered to be extremely statistically significant.

Study group	No. subjects	Absolute CD4 count (cells/µl)			% of CD4 cells		
		Mean (SD)	Median	Reference range	Mean (SD)	Median	Reference range
Male	67	793.4(±243.5)	820	338-1292	40.1(±9.2)	40.9	22.6-61.2
Female	33	885.9(±234.8)	896	402-1321	41.3(±8.8)	39.5	26.1-60.4
Total	100	823.9(±243.4)	847	338-1321	40.5(±8.9)	39.7	22.6-61.2

Table 2: Age distribution of CD4$^+$ lymphocyte counts in HIV-seronegative healthy adults in Eastern India

Age group (Years)	Gender				Overall Mean CD4 (SD)
	Male		Female		
	No. tested	Mean CD4 (SD)	No. tested	Mean CD4 (SD)	
18-27	21	862.6 (±225)	15	924 (±274.4)	893.3 (±43.4)
28-37	19	801.6 (±258.9)	11	863.3 (±140.8)	832.4 (±43.6)
38-47	18	755.8 (±235.7)	04	850.5 (±346.9)	803.1 (±67)
48-55	09	691.1 (±255.7)	03	825.7 (±231.5)	758.4 (±95.2)

Table 3: CD4 T-lymphocyte reference values reported by different Indian studies and its comparison with present study

Geographical location (India)	subjects	Absolute CD4 count (cells/µl)			% of CD4 cells			Ref. No.
		Mean	Median	Range	Mean	Median	Range	
North-east(NE)	44	Male: 711 Female: 766	Male:651 Female:745	Male:379-1128 Female: 547-1181	--	--	--	11
	14	848	--	--	36	--	--	12
West(W)	252	Male:727 Female: 845	Male:705 Female:839	Male:374-1398 Female: 380-1493	Male:36.9 Female:41.4	Male: 36.6 Female: 41.6	Male: 24.2-55.1 Female: 27.5-65	13
	65	Male:743.4 Female:790.4	Male:690 Female:741	Male:379-1800 Female: 321-1265				14
	94	865	--	430-1740	40.2	--	30.75-49.6	15
North (N)	84	Male:763.6 Female:797.9	--	Male:365-1328 Female: 415-1257	--	--	--	16
	125	Male: 687 Female:740	--	Male:640-734 Female:656-824				17
	40	818.4	--	--	--	--	--	18
South (S)	99	799	--	753.3-844.7	33	--	--	19
	213	Male: 865 Female:1021	Male:845 Female:954	Male:383-1347 Female: 448-1593	40.2	40.1	--	29
	44	1048	--	--	--	--	--	21
	30	834.6	--	--	--	--	--	22
Multi-centric study*	1027	--	--	--	E: -- W:39.46 N:37.38 S:32.43	E: -- W:38.75 N:37.26 S:33	E: -- W:15-65 N:15-60 S:14-51	23
Present study (East)	100	Male:793.4 Female:885.9	Male:820 Female:896	Male:338-1292 Female: 402-1321	Male:40.1 Female:41.3	Male:40.9 Female: 39.5	Male: 22.6-61.2 Female: 26.1-60.4	

Ref: Reference

*The Multicentric study was conducted by Indian Council of Medical Research in 1998. The mean and range of CD4 percent given in the table was the collective data obtained from 3 centers (north), 2 centers (west) and one center from south India.

DISCUSSION

This present study aimed to characterize CD4$^+$ LCs among HIV-seronegative healthy adults in eastern India, the first estimates of CD4$^+$ LCs in this part of the country. The CD4$^+$ LCs has been shown to be influenced by sex, age, race, time of specimen collection (diurnal rhythms), physical and psychological stress, pregnancy, drug administration (zidovudine, cephalosporin, cancer chemotherapy, nicotine and steroids), tuberculosis, viral infections, presence of anti-lymphocyte auto antibodies and procedures like spleenectomy.[39,40] Other factors that cause variations in the CD4$^+$ LCs were type of instrument used, processing and analyzing the whole-blood samples, integrity of the blood samples, staining reagents and fluorochromes, equipment calibration, preference and gating strategies used for the analysis of the results.[41,42]

Table 4: CD4 T-lymphocyte reference values reported by different countries worldwide and its comparison with present study

Geographical location	No. of subjects	Mean (SD) absolute CD4 cou (cells/µl)	Mean (SD) percentage CD count	Reference No.
Shanghai, China	614	727 (±255)	--	24
Thailand	150	910 (±300)		25
Saudi Arabia	209	869 (±310)	39.4(±7.9)	26
Asian population including China, Malaysia and India	232	838(±268)	35.6(±6.3)	27
Turkey	220	1095(±341)	47.37(±9.1)	28
Botswana	437	759 (±245)	--	29
Tanzania	147	980(±310)	--	30
Cameroon	203	980	--	31
Uganda	183	1256	--	32
Ethiopia	142	775(±225)	--	33
Central African Republic	150	933(±320)	--	34
Netherlands	1356	993(±319)		33
United Kingdom	676	830(±290)	43.6(±8.9)	35
Italy	965	940.5	45.1	1
United States (Caucasian population)	304	--	44(±7.6)	36
Present study (East India)	100	823.9(±243.4)	40.5(±8.9)	

The mean absolute CD4$^+$ LCs in the present study population was 823.9±243.4 cells/µl, median 847 cells/µl and reference range from 338 to 1321 cells/µl. Similar mean absolute CD4$^+$ LCs of 818.4 cells/µl were noted by Attili *et al*.[18] in north India, 834.6 cells/µl by Shahapur *et al.*[22] in south India and 848 cells/µl by Singh *et al.*[12] in northeast India. A wide variation in

mean absolute CD4$^+$ LCs has been reported from studies conducted in different parts of India. In south India, Kannangai *et al.*[21] and Murugavel *et al.*[20] had reported mean CD4$^+$ LCs of 1048 cells/µl and 926 cells/µl respectively. Uppal *et al.* in west India had revealed a mean of 865 cells/µl.[15] Kannangai *et al.* reported a mean of 1048 cells/µl in south India, Murugavel *et al.* 926 cells/µl in south India and Uppal *et al.* 865 cells/µl in west India. These results were higher than our study.[15,20,21] In comparison, lower mean absolute CD4$^+$ LCs values were reported by Das *et al.*[13] 771 cells/µl in west India, Ramalingam *et al.*[19] 799 cells/µl in south India and Ray *et al*.[17] 703 cells/µl in north India (Table 3). A huge variation in the mean absolute CD4$^+$ LCs has also been documented from various parts of the world. Similar mean absolute CD4$^+$ LCs of 838 cells/µl was observed by chng *et al*. among healthy Asian population comprising of individuals from China, India and Malaysia.[27] Jannossy *et al.* in Netherlands, Santagostino et al. in Italy, Yaman *et al.* in Turkey, Vithayasai *et al*. in Thailand, Al Quozi in Saudi Arabia, Jones *et al*. in Uganda, Jannossy *et al.* in Tanzania and Schnizlein-Bick *et al.* in Cameroon have reported higher absolute CD4$^+$ LCs values, while Jiang *et al*. in China, Bussmann *et al.* in Botswana and Jannossy *et al.* in Ethiopia have reported lower mean absolute CD4$^+$ LCs of 727, 759 and 775 cells/µl respectively (Table-4).[24-36] The mean percentage (%) of CD4$^+$ LCs in our study was 40.5±8.9 (median=39.7 and reference range from 22.6 to 61.2). A multicentric study was carried out by Indian Council of Medical Research (ICMR) in west, north and south parts of the country had revealed mean percentage of CD4$^+$ LCs of 39.46, 37.38, and 32.43% respectively.[23]

Categorization of data based on the gender of the subjects showed a significantly higher mean absolute CD4$^+$ LCs in females (885.9±234.4cells/µl) in comparison to males (793.4±243.5). Our findings are consistent with most of the studies conducted in India and other countries.[11,13,16,20,32,33] This may be due to the influence of sex hormones on lymphocyte subpopulations.

The distribution of mean absolute CD4$^+$ LCs among different age groups in our study revealed that the 18 to 27 age group had a significantly higher CD4$^+$ LCs of (893.3±43.4 cells/µl), followed by gradual decrease in CD4$^+$ LCs among subsequent higher age groups, while lowest count of 758.4±95.2 was recorded in the age group of 47 to 55 years. Similar age-related variations were observed by Oladepo *et al.* among healthy Nigerian adults.[43] They found out that the 18 to 25 year age group had a significantly higher mean CD4$^+$ LC of 861±288 cells/µl, while the lowest count of 774±433 cells/µl was observed among those older than 60 years of age.[43] This could account for the elderly falling ill more often than the younger ones who are more immunocompetent. However, Uppal *et al.*[15] in west India and Murugavel *et al*.[20] in South India revealed that none of the parameters differed significantly in any age groups, implying that in adulthood age had no significant influence on various parameters in their studies.

The mean absolute CD4$^+$ LCs in 11% (one female and ten males) of subjects in the present study were < 500 cells/µl. This implies 11% of healthy adult subjects had some amount of immunosupression.[44] Similar value of 10.6% reported by Ramalingam *et al.* in normal south Indian healthy individuals.[19] Rungta *et al.* in northwest India observed mean CD4$^+$ LCs in 20% of the controls were < 500 cells/µl.[14]

There were several limitations to this study. The sample size was small and a single geographical area was used. Also, rapid screening for HIV 1 and 2 would not detect recent seroconversion and this would have been included in the analysis.

CONCLUSIONS

Observations from our study corroborate emerging data that reported significant differences in reference CD4$^+$ LCs between different populations within and outside the country. The establishment of normal reference ranges within the local population is useful to clinicians for the management of HIV in India and other developing countries. It is essential for HIV staging, initiation of anti-retroviral therapy (ART), monitoring response to ART and initiation of chemoprophylaxis against opportunistic infections.

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