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Normal CD4 & CD3 Lymphocyte Counts in Healthy HIV Seronegative Adults in Tertiary Care Hospital Vadodara, Gujarat, India.

KEYWORDS: CD4/CD8 normal ranges, healthy Indians - reference ranges

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ABSTRACT *INTRODUCTION : The enumeration of CD4 and CD3 positive cells, surrogate markers for HIV disease progression, is helpful in management and follow up of immunocompromised HIV positive patients. In assessing the degree of immune deficiency in HIV-positive patients of a particular region, knowledge of reference range of T-cell subset counts of healthy individuals of that particular region is essential.*
OBJECTIVES : To determine the reference ranges of CD4 and CD3 counts in normal healthy adults and its variation in relation to age and sex.
METHODOLOGY : This is a cross sectional study carried out in SSGH, at Microbiology Department, includes 185 healthy HIV seronegative Residents, Doctors and Health care workers of both sexes between the age 20-70 years over a period 3 years from August 2009 to August 2012.
RESULTS : In our study Mean CD4 count 929.32 cells/µl which ranges between 887.76 - 970.89, cells/µl and Mean CD3 count is 1719.42 cells/µl which ranges from 1647.68 - 1791.16, cells/µl.
INTERPRETATION : As there is heterogenicity in baseline CD4 T lymphocyte count of normal healthy adults , Our findings on T-cell subset reference ranges of normal healthy individuals validate the utility of determination of CD4 cell

Introduction:
CD4+ T helper lymphocytes play a central role in regulation of immune response. The CD4+ T lymphocytes are the crucial cells in the cascade of events in forming immune response to the foreign antigen and hence monitoring the CD4+ T cell counts to understand the extent of immune deficiency is a common practice. CD4+ T cells are also the primary target cells for human immunodeficiency virus (HIV). Hence CD4+ T lymphocyte count is the most important marker of immune dysfunction in HIV disease progression. The estimation of CD4+ T cell counts is used to decide the initiation of anti retroviral therapy (ART), to monitor the efficacy of ART and to start treatment for opportunistic infections (OIs). To develop the threshold levels of CD4+ T cell counts, data from western countries are being used in India. (Ashwini Shete, December 2010) CD 4 counts normally differ from one locality to other and vary among different ethnic groups. The mean CD4 count among normal Indians is significantly lower than that in the western population. It is important to know the baseline CD4 count to draw a criterion for consideration of therapy and we cannot use western standards for treatment of Indian patients.(P.R.Shahpur, 2008) The CD4+ T cell counts are known to be influenced by race and environmental factors. Hence it is important to establish the reference ranges for the CD4+ T cell counts in the target population to understand the immune dysfunction.(Ashwini Shete, December 2010)

Flow cytometry is an accepted standard method for determination of absolute count of CD4+, CD3+ and CD8+ T- lymphocytes. Absolute T-lymphocyte subset counts are preferred over percentages by both clinicians and laboratory personnel, as the percentages are relative values and involve the use of multiplatform methods, which are prone to errors and analytical bias, while single platform methods have the potential to yield a less variable analysis. The correct interpretation of the results of these tests depends on the precision and reproducibility of the method used and the availability of reference ranges of these counts in healthy individuals in a community . (Krishna Ray, September 2006) As the absolute Tlymphocyte counts are expected to differ from one study to the other depending upon the region of study, number of subjects, method of enumeration, etc., more studies in different parts of the country are required to be conducted, in order to discern the regional diversity in reference ranges. (Krishna Ray, September 2006).

To evaluate for HIV, it is necessary to determine normal values and variations thereof in the reference Indian population after careful screening for the absence of diseases that could alter these values. It is also of great interest to know how our target population differs from other populations studied across the world, and whether such differences need to be taken into account while interpreting data with regard to the immune status of such individuals in the Indian setting(Uppal, 2003)

Aim : The present cross-sectional study was therefore carried out to determine the reference ranges of absolute counts of T-cell subsets and their ratios in normal HIV seronegative healthy Indians. Also to see its distribution according to age and sex.

Materials and Methods :
The cross sectional study was carried out from August 2009 to August 2012 in the HIV and CD4 testing Laboratory, Microbiology Department, Medical College Baroda.The centre for estimating CD4/CD3 counts under National AIDS Control Organization (NACO) exist in this Department. The study includes total 185 healthy volunteers.

Selection of Subjects : Healthy volunteers, seronegatives for both HIV and HBs Ag and non-reactive for RPR, were selected for the study. Subjects included in the study were enrolled after obtaining basic personal information. Informed verbal consent was obtained from each subject. The selected individuals were doctors, residents, laboratory staff and staff members working in the ART center, in the age group of 21-60 yr.

Exclusion Criteria :
1. Pregnancy and lactation with in past 6 months.

Volume : 2 | Issue : 1 | October 2012 | ISSN - 2249-555X

2. Individuals who had vaccination during the past six months.
3. Consumed alcohol during the past one week or were regular smokers.
4. H/o infections or communicable diseases including viral infections, accidents , trauma etc.. within past 6 months.
5. Any major surgery with in past 6 months.
6. Any chronic infections.

Collection of Blood: The blood samples of the healthy controls were collected between 9.00 to 12.00 h with all biosafety precautions, initially 3 ml, in plain vaccutainers for HIV, HBsAg and RPR testing and later 2 ml, in K-3 liquid EDTA vacutainer (Becton Dickinson, Mountain View CA) for CD4/ CD3 testing.

HIV Testing: Sera were separated on the same day and tested for HIV antibodies on the same day of collection, by WHO approved ELISA/Rapid kits supplied by NACO, New Delhi, following its testing guidelines (National AIDS Control Organisation, 2007). The kits however, differed from time to time.

CD4 and CD3 Testing :After collection samples are processed within 2 hours. CD4 counts were determined by flow cytometry which is considered to be a gold standard.(Rungata, 2008) CD4/CD3 counts were measured using a FACSCountTM (Becton Dickinson) system according to the manufacturer's instructions.

Reagents: Ready to use paired reagent tubes contained known number of fluorochrome labeled reference beads and fluorochrome conjugated monoclonal antibodies in a buffered solution (Becton Dickinson). These beads functioned as fluorescence and quantitation standard for determining absolute counts of CD4+ and CD3+ cells. Reference bead concentration differed from lot to lot.

Controls: Ready to use control kit consisted of paired control bead sets containing fluorochrome integrated (2 µm) polysterene beads at four levels of concentration (zero, low , medium and high) (Becton Dickinson). The bead concentration differed slightly from lot-to-lot, except the size which was constant.

Quality Control: Quality control of the test was strictly monitored following CDC guidelines 1997(guidelines, 1997) and the instructions from the manufacturers of kits and reagents.

Internal quality Control: In order to keep a check on lot-to-lot variation of the CD4/CD8 monoclonal antibodies, two different samples (one normal and the other HIV-positive) were run in parallel with the reagents from both the lots and reproducibility was verified. The coefficient of variation (CV) ranged between ± 20%. Daily entries were made in the control as well as reagent logs and were monitored continually for changes in the parameters.

Statistical Analysis : Data were entered and analyzed by using Microsoft Excel 2007. The means and standard deviations (SD) were calculated for each marker. The frequency of distribution of each variable was analyzed. If Gaussian distribution was described by the mean and standard deviation, then the reference range was defined by the mean ± 2 SD.

Results :
Total 185 healthy volunteers were included. Which includes 125 females and 60 males.

Sex wise distribution is presented in Table -1 , which also includes CD4 & CD3 mean and its range. Women had comparatively higher CD4 mean count than their male counterparts. The difference was statistically insignificant. CD3 mean count also shows same pattern of distribution.Table – 2 and Table – 3 shows age wise distribution of mean CD4 / CD3counts in females and males respectively. Changes in age influenced the subset values marginally in both the sexes. However, the age dependant changes did not achieve any statistical significance.

Table – 1 Sex wise distribution

Sex	Lowest value	Highest value	Subsets	Mean (Cells/µl)	95% CI (Cells/ µl)
Female	493	2000	CD4	975.38	921.23 - 1029.53
	745	3499	CD3	1729.28	1636.32 - 1822.24
Male	310	1319	CD4	833.38	778.24 - 888.52
	748	2686	CD3	1698.86	1588.45 - 1809.27
Total	310	2000	CD4	929.32	887.76 - 970.89
	745	3499	CD3	1719.42	1647.68 - 1791.16

Table – 2 Age wise distribution in Females.

Age group	Sex	Total samples	Subsets	Lowest	Highest	Mean (Cells/µl)	95% CI (Cells/µl)
21-30	F	98	CD4	538	1966	1009	948.29 to 1070.35
			CD3	812	3260	1783.75	1685.09 to 1882.41
31-40	F	16	CD4	493	1514	861.75	700.36 to 1023.13
			CD3	745	2506	1525.93	1208.41 to 1843.46
41-50	F	9	CD4	726	1523	1089.77	890.13 to 1289.42
			CD3	1378	2332	1829.66	1577.91 to 2081.41
51-60	F	2	CD4	1004	1328	1166	892.40 to 3224.40
			CD3	2314	2413	2363.5	1734.54 to 2992.45

Table – 3 Age wise distribution in Males

Age group	Sex	Total samples	Subsets	Lowest	Highest	Mean (Cells/ µl)	95% CI (Cells/ µl)
21-30	M	23	CD4	520	2000	855.73	727.83 to 983.64
			CD3	748	3499	1702.17	1469.22 to 1935.12
31-40	M	28	CD4	434	1305	873.10	785.73 to 960.48
			CD3	876	2686	1813.10	1646.13 to 1980.07
41-50	M	3	CD4	789	923	841.66	664.17 to 1019.16
			CD3	1228	2323	1605	566.72 to 2335.94
51-60	M	6	CD4	625	1115	795.8333	615.05 to 976.61
			CD3	1050	2316	1586.833	1091.14 to 2082.52

Discussion :
This study provides the first estimates of CD4 & CD3 lymphocytes count among healthy individuals in the Vadodara District. Wide variations in mean CD4 counts has been reported from studies conducted in different parts of India. Reports from South India document CD4 counts of 1048 cells / µl (n = 46) and 799 cells/ µl (n = 99) in different studies.(P.R.Shahpur, 2008; Ramalingam S, 2001; Rungata, 2008) In North East the count observed was 848 cells/µl (n = 14)(Singh YG, 2000) and in North West it was 763.81 cells/ µl (n = 65)(Rungata, 2008) . The mean CD4 reference count of the present study is 929 cells / µl close to that of the studies from South India and North East. Higher CD4 counts observed in females than in males of the present study are also similar to the findings of North West study.

Wide variations are also observed all over the world in the different studies. This may be inherent to the population , due to the selection processes for the different studies or an artifact of the different laboratory methods for estimation of

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CD4 count (A.C. Crampin, 2011).

As the results obtained from our population are different from the results of other populations studied. This difference suggests that each population should have its own reference ranges for lymphocyte subsets. As a result, populations where HIV and AIDS are a major problem should study their own HIV-AIDS cohorts to see if traditional thresholds for CD4 used for the determination of treatment and prophylaxis and for AIDS definition are applicable to their populations as these ranges were defined in terms of local populations. Immunological progression markers for HIV-AIDS may have to be reestablished for different populations, and this process will require long-term prospective cohort studies aimed at describing the progression of HIV in each population.

In addition to the regional changes, the variations could also be due to use of different equipment and techniques, as multiplatform conventional flow cytometry and ELISA based immuno-capture Kit were used in different studies and could have given rise to procedural and instrumental errors.(Krishna Ray, September 2006)

Though, the base line CD4 cell count in healthy females in the present study was higher than that of males, the difference was statistically insignificant. However, significantly higher mean CD4/CD8 ratios found in females compared to males confirmed findings of previous studies .(Krishna Ray, September 2006; Saxena RK, 2004; Uppal, 2003)Bofill et al(Bofill M, 1992) found that seronegative women had 28.0 per cent higher CD4 counts than that of men and suggested that the gender difference could be due to diurnal variation. Diurnal variation, however, cannot explain the significant gender difference observed in this study, as all the samples were collected between 9:00 to 12:00 h. While smoking was also reported to be associated with higher CD4 count, this could not be the reason for the difference as all female subjects in the present study were nonsmokers. Sex hormone effect could be the possible explanation for the observed gender difference in CD4 counts, as the circulating lymphocytes have receptors for androgens and oestrogens (Krishna Ray, September 2006).

Conclusion : T-lymphocyte subset reference range for Healthy individuals determined in the present study can be used routinely as normal ranges for guiding clinical decisions. However, wide ranges in the observed values imply that there is a considerable variation in these counts in the Indian population. A regional diversity in values was also noticed indicating the need to establish a national reference range for absolute counts in healthy Indian men and women. The data available on reference ranges for CD4+ T cell counts in Indian population are not comparable and there is a need to generate reference ranges in normal population in a well designed multicentric study. The availability of standardized procedures, pretested and calibrated controls and external quality assurance programmes can help generate robust reference values. The establishment of such reference ranges for CD4+ T cells count would provide region-wise differences, if any, in CD4 counts in India and would serve as baseline data for comparison of studies done for analysis of CD4 counts in HIV infected patients.

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