REFERENCE RANGES FOR LYMPHOCYTE SUBSETS IN A DEFINED MALAYSIAN POPULATION

J S Dhaliwal, T Balasubramaniam, C K Quek, H K Gill, B A Nasuruddin

ABSTRACT

The aim of this study was to establish the lymphocyte subset reference ranges in a defined Malaysian population as well as to determine inter-racial differences for these values. Normal blood obtained from 152 subjects (55.9% Malay, 26.3% Chinese and 17.7% Indian) was immunophenotyped. Results obtained (expressed as mean ± SD %), absolute count (x 10^6 cells/mm^3) were as follows : CD3:66.5 ±8.6%, 2,066; CD4:33.2 ±8.5%, 1,028; CD8:31.6 ±8.9%, 982; CD19:12.0 ±0%, 5,374, and CD56 +CD16:20.9 ± 9%, 1,638. There were no significant differences between the percent lymphocyte subsets of the three racial groups. However, the absolute number of CD4 cells and CD19 cells in Chinese was significantly lower ($p<0.05$) compared to the Indian and the Indian and Malay groups respectively. Comparison of our results with other reports showed that the percentage of Natural Killer cells in this population is higher than that reported for Caucasian population.

Keywords: reference ranges, lymphocyte subsets, NK cells, HLA-DR cells, absolute counts.

SINGAPORE MED J 1995; Vol 36: 288-291

INTRODUCTION

The need to estimate lymphocyte subsets has increased markedly especially in cases of Human Immunodeficiency Virus infection. The availability of reference values allows the assessment of an individual's immune status. The absolute number of CD4+ T cells is an important measure of the progression of HIV infection in adults[1,2]. It is also used as an indicator to begin prophylactic therapy against opportunistic infections[3] as well as to monitor efficacy of treatment. The demand for such assays is likely to increase in future. Such data however, is only useful in relation to the appropriate reference ranges. In this respect it has been reported that lymphocyte subsets have been reported to vary according to race[4]. It is, therefore, necessary to establish reference ranges for the population of interest in order to ensure the correct interpretation of the results. In this paper, we describe the establishment of lymphocyte subset reference ranges for a defined Malaysian population and compare the means between three racial groups found in Malaysia.

MATERIALS AND METHODS

Blood

Control blood was obtained from various sources. This included staff of the Institute and age-matched control blood sent with patient specimens. Control blood was accompanied by a form to determine if the donor had any immunological or haematological abnormalities or was on any medication (except birth control pills). The age of the subjects included in this study ranged from 13 to 68 years with a mean of 33.1 years and a median of 30.5 years. Approximately 2 mls of blood was placed in vials containing EDTA for transport to the laboratory at ambient temperature. On receipt of the blood, the Total White Blood Cells (TWBC), percentage lymphocytes and absolute number of lymphocytes were determined using a haematological cell counter (Model T540, Coulter Co). Blood was stored at room temperature and processed within six hours of venisection.

Division of Immunology
Institute for Medical Research
Jalan Pahang
50588 Kuala Lumpur
Malaysia

J S Dhaliwal, PhD
Scientist

T Balasubramaniam, AIMHLT
Senior Technician

C K Quek
Technician

H K Gill, PhD
Scientist

B A Nasuruddin, MD, MSc (Lond)
Head

Correspondence to : Dr J S Dhaliwal

Monoclonal antibodies

Monoclonal antibodies used for the enumeration of T and B cell subsets were selected according to the recommendations of the U. S. National Committee for Clinical Laboratory Standards[5]. Selection of monoclonal antibodies was based on the need to correctly identify all lymphocyte subsets, as CD antigens may be found on more than one population of cells. The following monoclonal antibodies were used in each panel: Anti-CD45 FITC/Anti-CD 14 PE (to identify the lymphocyte population), IgG1 FITC/IgG1 PE (negative isotype control), Anti-CD3 FITC/Anti-CD 19 PE (T and B cells), Anti-CD3 FITC/Anti-CD4 PE (T helper cells), Anti-CD3 FITC/Anti-CD8 PE (T suppressor/cytotoxic cells), Anti-CD3 FITC/Anti-CD16 + Anti-CD56 PE (NK cells). All monoclonal antibodies were purchased from Becton Dickinson, Mountain View, CA.

Staining of cells

0.1 ml of blood is carefully placed in a 12 x 75 mm round bottomed polystyrene tube (Falcon 2052, Becton Dickinson, Lincoln Park, New Jersey, USA). 20 microlitres of the appropriate antibody conjugate is then added to the blood and mixed thoroughly followed by a 15 minute incubation in the dark at 4°C. Red blood cells are then lysed by adding 2 mls of a 1:10 dilution of FACSLYSE (Becton Dickinson, Mountain View, CA) in distilled water. The tubes are incubated for not more than 10 minutes at room temperature followed by centrifugation at 300g for 10 minutes. The cells are then washed once with Phosphate Buffered Saline (PBS) (pH 7.2) before resuspension in PBS pH 7.2 containing 1% formaldehyde. Tubes are kept overnight before analysis in a FACSCAN (Becton Dickinson, Mountain View, CA).

Analysis of data

Cells were analysed in a FACSCAN using Simulset software (Becton Dickinson, Mountain View, CA). Absolute lymphocyte numbers obtained from the haematological cell counter were entered into the software for each patient. Approximately 2,000 lymphocytes were analysed in each tube. Results obtained were in percentages and absolute numbers of the various subsets tested. In order to ensure validity of the analysis, data was only included if (a) %T cells + %B cells + %NK cells = 90 - 100%, (b) 90% or more of the total lymphocyte population was analysed, and (c) the forms accompanying the specimens were completed legibly and correctly.

Statistical Analysis

Statistical analysis was carried out using SPSS version 5.0 for Windows. Comparison of the means was carried out using the Student-t test.

RESULTS

Lymphocyte subset data from a total of 185 normal specimens was analysed to determine the reference ranges of T cells, CD4+ T cells, CD8+ T cells, CD19+ (B cells) and Natural Killer Cells. Only 152 controls met the criteria described in Materials and Methods (Analysis of data). The breakdown of the normal population studied in terms of race and sex is shown in Table I. The controls consist of 85 (55.9%) Malays, 40(26.3%) Chinese and 27 (17.7%) Indians. Table II shows the mean percentages and absolute values (±SD) as well as their ranges for each of the three races. Since it has been established that most lymphocyte subset data is not normally distributed, the range was derived using the 5 and 95 percentile values. It can be seen that there is very little difference in lymphocyte subset values when the figures from each race is compared. Comparison of means using the t-test show no significant differences between all three races when comparing all the five lymphocyte subsets shown. However,

Table I – Breakdown of study population by race and sex

	Malay	Chinese	Indian
Male	54	26	19
Female	31	14	8
Total	85	40	27

Table II - Reference ranges for lymphocyte subset percentages and absolute numbers by race

		CD3Cells (T Cells)	CD4Cells	CD8Cells	CD19Cells (B Cells)	NKCells
Race: Malay	**(n=85)**					
Mean ± SD	(%)	65.6 + 8.4	33.2 ± 8.5	30.2 ± 7.8	12.7 ± 5.2	21.2 ± 9.0
Median	(%)	65.5	34	30	12	20
Range	(%)	53.0 - 79.7	19.0 - 46.0	17.3 - 42.8	6.0 - 25.4	8.3 - 40.5
Mean ± SD	(x 10^6/L)	2,092 ± 905	1,052 ± 526	965 ± 470	414 ± 283*	649 ± 349
Median	(x 10^6/L)	1,970	964	917	347	577
Range	(x 10^6/L)	1,059 - 4,026	452 - 2,079	421 - 1,821	152 - 849	286 - 1,378
Race : Chinese	**(n=40)**					
Mean ± SD	(%)	67.7 ± 9.1	31.5 ± 9.2	33.7 ± 10.6	10.2 ± 4.6	21.8 ± 9.7
Median	(%)	67.5	30.5	35	10	20
Range	(%)	54.1 - 82.8	16.3 - 46.9	17.0 - 53.8	2.0 - 20.0	8.0 - 37.8
Mean ± SD	(x 10^6/L)	1,921 ± 786	884 ± 421*	967 ± 485	270 ± 159*	624 ± 355
Median	(x 10^6/L)	1,827	826	930	274	551
Range	(x 10^6/L)	711 - 3,646	268 - 1,709	283 - 1,939	33 - 559	149 - 1,333
Race : Indian	**(n=27)**					
Mean ± SD	(%)	67.8 ± 8.3	35.8 ± 7.0	32.9 ± 9.1	12.4 ± 4.6	19.0 ± 8.2
Median	(%)	69	37	31	12	17
Range	(%)	50.4 - 80.0	21.8 - 46.0	20.8 - 52.6	6.0 - 23.0	7.4 - 37.8
Mean ± SD	(x 10^6/L)	2,201 ± 655	1,168 ± 402*	1,054 ± 379	406 ± 183*	627 ± 327
Median	(x 10^6/L)	2,166	1,258	930	378	572
Range	(x 10^6/L)	1,268 - 3,709	581 - 2,028	524 - 2,010	152 - 715	173 - 1,392

*. $p<0.05$

Table III – Reference ranges for lymphocyte subset percentages and absolute numbers for the whole study population

		CD3Cells (T Cells)	CD4Cells	CD8Cells	CD19Cells (B Cells)	NKCells
Race : All races (n=152)						
Mean ± SD	(%)	66.5 ± 8.6	33.2 ± 8.5	31.6 ± 8.9	12.0 ± 5.0	20.9 ± 9.1
Median	(%)	66	33.5	31	11	19
Range	(%)	53.0 - 80.0	19.0 - 46.0	17.6 - 49.0	5.0 - 22.4	8.0 - 37.4
Mean ± SD	(x 10^6/L)	2,066 ± 836	1,028 ± 487	982 ± 458	374 ± 247	638 ± 345
Median	(x 10^6/L)	1,969	950	919	309	568
Range	(x 10^6/L)	988 - 3,912	431 - 1,976	385 - 1,808	130 - 716	227 - 1,354

Table IV - Comparison of lymphocyte subset percentages from different studies (% Mean ± SD)

Ref	Country	Race	Number	Blood Prep	CD3	CD4	CD8	CD19	NK
10	US	Caucasian	304	LWB	73±6.5	44±7.6	33±7.4	14±4.2	14±6
11	US	ns	16	LWB	78.5±4.5	45.4±5.3	30.5±4.4	8.9±3.2	13.2±2.1
12	US	ns	150	LWB	73	47	25	11	nd
13	UK	ns	600	LWB	76.3±9.2	43.6±8.9	29.5±8.2	nd	nd
7	Japan	ns	50	Ficoll	69.5±6.1	38.1±5.8	28.8±6.5	nd	16.1[a]±6.7
14	Japan	Japanese	23	LWB	66±11	36±8.8	34±6	16±6	19±8
Present Study	Malaysia	Malay Chinese Indian	152	LWB	66.5±8.6	33.2±8.5	31.6±8.9	12.0±5.0	20.9±9.1

[a] : Only Leu 11 was used to determine the population of NK cells
ns : not stated
nd : not done
LWB : Lysed Whole Blood method
Ficoll : White blood cells were separated using Ficoll

Chinese subjects had significantly lower CD4 absolute counts compared to Indians and significantly lower CD19 absolute counts compared to both Malays and Indians. Table III shows the data after combining the whole study population as a single group. Table IV shows a comparison of T subset values recently reported in the literature. Comparison of the means between this study and the US study with 304 Caucasians using the t-test showed a significant difference ($p<0.001$) whilst comparison with the Japanese study with 23 subjects showed no significant difference. Comparisons were not made with the other studies because the race was not stated or the methodology differed considerably.

DISCUSSION AND CONCLUSIONS

Analysis of comparative data in Table IV shows that there is little intra-race variation but significant inter-race variation with respect to CD4, CD8 and NK cells. There can be a number of reasons why there would be differences when comparing lymphocyte subset percentage data from different racial groups. These include assay methodology, as well as genetic and environmental factors.

Assay variations may be attributed largely to processing methods, monoclonal antibodies, analysis methods and the lag period between drawing blood and processing of the specimen. This has been reviewed extensively by Landay and Muirhead[5]. It has been demonstrated that certain subsets may be lost if lymphocytes are separated using Ficoll instead of the whole blood lysis method[6]. Although each of these factors may not significantly alter the percentage values, their effects taken together may result in a shift of the mean values. Therefore the data used for comparison was selected to ensure that such differences were kept to the minimum. Kitajima et al[7] (see Table IV) for example used CD 16 alone instead of CD16 + CD56 for the enumeration of NK cells. It has been demonstrated that CD16 is found in all resting NK cells. This introduces a variation in NK cell percentages. Although it may be more relevant to compare the absolute numbers of lymphocyte subsets, it is quite clear that there are variations when absolute numbers are taken into account. This is due to diurnal variation in lymphocyte numbers as well as intrinsic differences in values obtained from haematological cell counters. The difference in lymphocyte subset percentages may be due to genetic variation. It is possible that there are racial differences in subset values. This possibility may be examined by studying ethnic populations in different settings to reduce the influence of environmental factors. The lymphocyte subset percentage reflects the individual's response to external factors eg infection. Thus differences in lymphocyte subset percentages may be due to varying environmental factors. For example, Natural Killer cells are capable of killing a broad range of human solid tumours, leukaemic and virus infected target cells. Decreased NK

activity and/or numbers of circulating NK cells is associated with development and progression of cancer as well as acute and chronic viral infections[8]. It is not inconceivable that NK levels may be higher in populations where exposure to viruses may be higher.

ACKNOWLEDGEMENTS

We thank Dato Dr M. Jegathesan, Director, Institute for Medical Research, for permission to publish this paper.

REFERENCES

1. Fahey JL, Taylor JMG, Detels R. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. N Engl J Med 1990; 322:166-72.
2. Gardner LI Jr, Brundage JF, McNeil JG, Milazzo MJ, Redfield RR, Aronson NE, et al and the Military Medical Consortium for Applied Retrovirology. Predictors of HIV-1 disease progression in early and late stage patients : The US Army Natural History Cohort. J Acquir Immune Defic Syndr 1992; 5:782-93.
3. Center for Disease Control. Guidelines for prophylaxis against *Pneumocystis carinii* pneumonia for persons infected with immunodeficiency virus. MMWR 1989; 38 (suppl 5) : 1-19.
4. Prince HK, Hirji S, Waldbeser S, Plaeger-Marshall S, Kleinman S, Lanier L. Influence of racial background on the distribution of T cell subsets and Leu 11 +ve lymphocytes in healthy blood donors. Diagnostic Immunology 1985; 3:33-9.
5. Landay AL, Muirhead KA. Procedural guidelines for performing immunophenotyping by flow cytometry. Clin Immunol Immunopathol 1989; 52:48-60.
6. Brown G, Greaves MF. Enumeration of absolute numbers of T and B cells in human blood. Scand J Immunol 1974; 3:161-72.
7. Kitajima I, Mariyama I, Matsubara H, Osame M, Igata A. Immune dysfunction in hypophosphatemic Vitamin D resistant rickets : immunoregulatory reaction of 1α(OH) vitamin D3. Clin Immunol Immunopathol 1989; 53:24-31.
8. Jondal M. The human NK cell: A short overview and a hypothesis on NK recognition. Clin Exp Immunol 1987; 70:255-62.
9. Ortaldo JR, Heberman RB. Heterogeneity of natural killer cells. Ann Rev Immunol 1984; 2:359 -94.
10. Reicheret T, De Bruyere M, Deneys V, Totterman V, Lydard, P, Yuksel, et al Lymphocyte subset reference ranges in adult Caucasians. Immunol & Immunopath 1989; 60:190-208.
11. Lal RB, Rudolph DL, Schmid DS, Lairmore MD. Concomitant augmentation of CD4+CD29+ helper inducer and dimunition of CD4+CD45RA+ suppressor inducer subset in patients infected with human T cell lymphotropic virus types I and II. Clin Exp Immunol 1992; 87:293-7.
12. Kotylo PK, Sample RB, Redmond NL, Hibner GC. Reference ranges for lymphocyte subsets. A comparison of standard vs rapid whole blood lysis techniques. Arch Pathol Lab Med 1991; 115:181-4.
13. Bofil M, Janossy G, Lee CA, MacDonald-Burns D, Phillips AN, Sabin C, et al. Laboratory control values for CD4 and CD8 T lymphocytes. Implications for HIV-1 diagnosis. Clin Exp Immunol 1992; 88:243-52.
14. Senju M, Makiyama K, Hara K, Hulstaert F, Lowder JN, Jewell DP. Two colour immunofluorescence and flow cytometric analysis of peripheral blood lymphocyte subsets in Caucasian and Japanese healthy subjects. Jpn J Med 1991; 30:509-15.