Age-related changes in blood lymphocyte subsets of south Indian children

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ABSTRACT

Background. Enumeration of lymphocyte subsets has been widely used for the diagnosis and monitoring of several haematological and immunological disorders. Various studies have demonstrated age, sex and racial differences in lymphocyte subset expression. Reference values are not available for Indian children and there is a need for this information to replace commonly used, but inappropriate, adult lymphocyte subset ranges.

Methods. One hundred thirty-eight healthy children between 3 and 15 years of age, attending a local government school in Chennai, South India were included in the study. Haemoglobin levels, and total and differential cell counts were determined using an automated counter and lymphocyte subsets were analysed by flowcytornetry.

Results. The mean (SD) absolute lymphocyte count declined with age from 4338 (1031) at 3 years to reach a plateau of 3096 (914) at 11-13 years ($p < 0.05$). A significant decline was also observed in the absolute numbers of CD3+, CD4+, CD8+ and CD19 + cells. However, the percentage values of CD3+, CD4+, CD8+, CD16/56+ cells and the CD4/CD8 ratio remained fairly stable across the age range.

Conclusion. Our data would prove useful in interpreting disease-related changes in lymphocyte subsets in Indian children of different age groups. Age-related decrease in the absolute lymphocyte count as well as numbers of CD4 and CD8 cells was found to occur between the ages of 3 and 11 years. A normogram relating age to CD4 count has been developed.

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INTRODUCTION

Children are born with immature immune systems that mature and expand during the initial years of life. As a result, the absolute sizes of lymphocyte subpopulations vary during childhood.[1,2] To evaluate the immune status of children, it is important to quantify the different lymphocyte subsets, keeping in mind age-related variations, which are known to occur.[3,4] Apart from age, race and gender are also known to have an impact on lymphocyte subset expresion.[5-14] To establish guidelines for the diagnosis and quantification of immunodeficiency, data from healthy children are required.

In children, immunophenotyping of blood lymphocytes, or lymphocyte subset analysis with monoclonal antibodies by flowcytometry is now routinely used in the diagnosis of congenital and acquired immune deficiencies as well as leukaemia and lymphoma. With the rising prevalence of paediatric HIV infection, assessment of immunodeficiency will be increasingly required. A literature survey did not yield any publication describing normal lymphocyte levels in Indian children. This study was carried out to address this gap in knowledge and generate data on the levels of all relevant lymphocyte subsets in healthy Indian children.

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METHODS

Subjects

This study was carried out in Chennai, South India and the subjects were 138 healthy children, between 3 and 15 years of age, attending a local government school. The study was approved by the Institutional Review Board of the Tuberculosis Research Centre and parental consent was also obtained. The subjects were categorized into 6 groups based on age-group A: 3–5years of age (*n* = 21), group B: >5–7years (*n* = 22), group C: >7–9years (*n* = 20), group D: >9–11years (*n* = 24), group E: >11–13years (*n* = 27) and group F: >13–15years (*n* = 24).

A complete physical examination was carried out to ensure that all children included in the study were free from infection. Children with known chronic illnesses as well as those with a history of fever, cold or cough in the previous 2 weeks were excluded. Children found to be clinically malnourished or anaemic were also excluded. The height and weight of the selected children were recorded. After ensuring lack of excessive physical activity in the previous 1hour, venous blood was collected in EDTA-coated tubes between 9.00 a.m. and 11.00 a.m. and prepared for analysis within 4 hours.

The total and differential cell counts and haemoglobin levels of all samples were determined using an automated blood cell analyser (Abx, France).

Monoclonal antibodies

The monoclonal antibodies used for immunophenotyping were anti-CD14, anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-CD19 and anti-CD16/56. Irrelevant isotype-matched antibodies (mouse IgGl and mouse IgG2a) were included as controls for non-specific immunofluorescence. All the monoclonal antibodies were directly conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and were purchased from Becton-Dickinson Immunocytometry (Becton-Dickinson, Mountain View, CA, USA).

Immunophenotyping

Cell surface phenotypes were analysed in whole blood by dual-colour flowcytometry using a standard protocol. Briefly, 50 µl of whole blood was incubated in the dark with 5 µl of appropriate antibodies for 30 minutes at 4°C. Unbound monoclonal antibod-

ies were washed off and red cells were lysed for 10 minutes with FACS-lysing solution (1 : 10) at room temperature, protected from light. Samples were centrifuged, washed in buffer, fixed in 1% paraformaldehyde and stored overnight at 4°C.

Cells were analysed on a FACSort Flow Cytometer (Becton-Dickinson) using the CELLQuest software. Data were collected per 10 000 cells for each tube and displayed as dot plots, T cells were defined as those cells expressing CD3 antigen and B cells as those cells expressing CD19 antigen. Natural killer (NK) cells were identified by the presence of CD16, CD56, or both, and by the absence of co-expression of CD3. CD4+ and CD8+ T cells were defined by the presence of CD4 and CD8 antigens, respectively, with co-expression of CD3.

Statistical analysis

The sample size was calculated by reviewing the past literature on the subject. Based on the expected standard deviation in cell counts of each age group, at a level of significance of 0.05 and power of 0.80, a sample size of approximately 20 in each age group (with an equal number of boys and girls) was considered adequate. Each child contributed only one sample to the dataset.

The results of all variables are expressed as age-specific means and standard deviations. Student's *t* test was used to test the difference between the genders. Simultaneous comparison of the mean values of lymphocyte counts and their subset variables was carried out using one-way analysis of variance (ANOVA). Multiple comparison test was performed to determine the pair-wise significant difference between the age groups.

RESULTS

The distribution of height, weight and haemoglobin level of the study population is shown in Table I. The height and weight were found to increase proportionally with age. Sixty-seven per cent of children were within the expected range (>80%) predicted) of weight for age, 21% were classified as grade I malnutrition and 12% as grade I1 (Indian Academy of Pediatrics classification of Protein–Energy Malnutrition). There were no severely malnourished children (grades I11 and IV). Some of the children were mildly anaemic, particularly in the younger age groups, but none had haemoglobin levels <8.5 g/dl.

TABLE I. Mean height, weight and haemoglobin level (SD) in different age groups

Age group (years)	*n*	Height (cm)	Weight (kg)	Haemoglobin (g/dl)
3–5	21	97.7 (3.4)	12.6(1.7)	9.4(1.0)
>5–7	22	108.0(4.0)	16.4(1.9)	9.5(2.3)
>7–9	20	117.6(7.5)	18.2(2.9)	9.9(1.1)
>9–11	24	127.6(7.1)	22.8(3.7)	11.2(1.2)
>11–13	27	137.1 (0.8)	27.5(4.8)	11.4(1.2)
>13–15	24	150.2 (7.2)	38.3(5.5)	12.4(1.2)

The mean percentage and absolute count of lymphocytes and their subset variables in the different age groups are listed in Table 11. Since there was no significant difference in the proportion of lymphocyte subsets between boys and girls, the values have been combined and the mean values listed. It was observed that the first 4 age groups (i.e.. 3–5, >5–7, >7–9 and >9–11 years) differed significantly in their mean values for absolute lymphocyte count and percentage, CD3+ cell count, CD4+ cell count, and CD8+ cell count ($p<0.05$). However, the percentages of CD3+, CD4+ and CD8+ cells did not show a consistent change. The >11–13 years age group did not differ significantly from the >13–15 years age group for any parameter.

The absolute counts of total lymphocytes and T cell subsets fell with increasing age, reaching a plateau by >11–13 years. The CD4+ cell count decreased from approximately 1300 cells/cmm at 3–5 years to about 850 cells/cmm at >13–15 years, which is similar to adult South Indian values- (Tuberculosis Research Centre, unpublished observations). There was significant correlation between CD4 counts and absolute lymphocyte numbers ($r=0.81$; $p<0.001$). Similarly, there was a statistically significant correlation between CD4 percentages and CD4 counts ($r=0.44$; $p<0.01$). The CD4/CD8 ratio remained >1.0 in all the age groups studied and did not show a significant variation with age.

Figure 1 is a normogram relating the absolute CD4 count to age. Data for boys and girls have been combined and the band around the mean, representing two standard deviations, reflects the wide variability of this parameter in healthy children.

The percentage and absolute counts of B lymphocytes (CD19+) and NK cells (CD16/56+) were similar in the first four age groups (i.e. 3–5,>5–7,>7–9and>9–11years), but declined significantly at >11–13 years of age, probably due to the falling absolute lymphocyte count. On the other hand, the percentage of NK cells showed a gradual increase from 10.5% at 3–5 years to 14.4% at >13–15 years. However, this increase was not found to be statistically significant.

TABLE 11, Mean percentage and absolute count of T lymphocyte subsets (SD) in different age groups

Variable	Age group (years)					
	3-5	**>5–7**	**>7–9**	**>9–11**	**>11–13**	**>13–15**
Lymphocytes (%)	38.5 (8.7))*	45.4 (12.3)*	41.7 (8.2)*	38.4 (6.5)*	33.2 (7.5)	32.6 (6.9)
Absolutecount	4338 (1031)*	4419 (1030)*	4095 (1357)*	3813 (966)*	3096 (914)	2978 (838)
CD3+ cells (%)	63.2 (6.8)	67.6 (5.2)	68.4 (5.2)	66.5 (5.7)	67.4 (7.6)	65.5 (6.7)
Absolute count	2705 (774)*	2978(744)*	2828 (1093)*	2563 (700)*	2116 (761)	1983 (7033)
CD4+ cells (%)	28.6 (4.7)	36.2 (4.4)*	34.2 (8.0)	33.4 (6.9)	33.7 (6.0)	31.7 (5.6)
Absolute count	1311 (458)*	1598 (430)*	1428 (768)*	1267 (379)*	1062 (428)	857 (278)
CD8+ cells (%)	27.7 (5.4)	29.8 (8.3)	28.3 (5.0)	27.3 (6.6)	28.2 (6.7)	27.6 (7.5)
Absolutecount	1198 (343)*	1321 (469)*	1170 (488)*	1050 (409)*	884 (355)	813 (474)
CD4/CD8 ratio	1.2 (0.5)	1.3 (0.3)	1.3 (0.5)	1.3 (0.5)	1.3 (0.4)	1.3 (0.4)
CD19+ cells (%)	15.7 (6.9)*	17.9 (4.2)*	17.2 (4.8)*	15.5 (6.4)*	13.9 (4.7)	11.8 (4.1)
Absolutecount	684 (314)*	790 (264)*	696 (267)*	590 (292)*	482 (306)	344 (I67)
CD16/56+ cells (%)	10.5 (3.9)	10.2 (4.4)*	13.2 (6.4)	12.0 (4.1)	13.1 (7.0)	14.4 (12.1)
Absolutecount	448 (191)	430 (247)	617 (191)*	467 (208)	361 (177)	366 (247)

*The mean difference is significant at the 0.05 level when compared to the > 1 3 - 1 5 years age group. Though a multiple comparison test was performed for all age groups, significant differences as compared to the >13–15years age group alone are highlighted **in** the table.

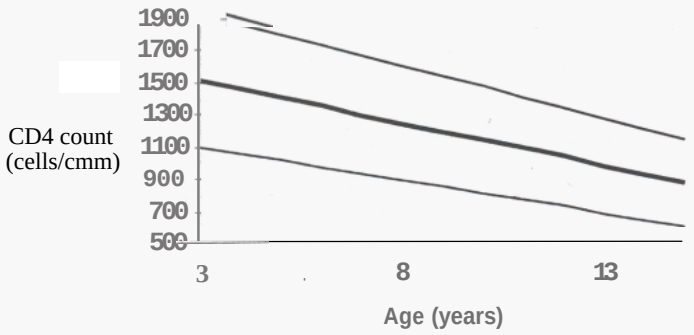

FIG 1. Normogram relating mean values (2 SD) of CD4 count to age.

DISCUSSION

The relative and absolute sizes of lymphocyte subpopulations vary during childhood due to maturation and expansion of the immune system in the first years of life. Previous studies have shown that the absolute and percentage values for most lymphocyte markers differ substantially not only between children and adults, but also between children of different age groups.[3,4,15-20] However, reference values for lymphocyte subsets of normal Indian children are lacking and it is important to generate these data for the correct interpretation of results obtained for Indian paediatric patients with possible immunological disorders.

Though the greatest changes in lymphocyte counts are known to occur during early infancy, that is, from birth to 3 years of age,[2,4,6,20] this group could not be included at the time of the study for practical reasons and will be taken up subsequently. The present data show that even beyond the age of 3 years there are age-related changes in the size of the lymphocyte subpopulations in healthy children. The most important change is the progressive decline in the absolute lymphocyte count that plateaus at >11-13 years of age. This results in a decrease in most of the lymphocyte subpopulations with age, notably the CD3+, CD4+, CD8+ and CD19+ cells. These findings are in line with observations from similar investigations carried out in other parts of the World.[15-20] The percentages of these subsets, however, remain fairly stable except for the CD19+ cells, which tend to decrease between 11 and 15 years of age. The CD4/CD8 ratio did not show any age-related change. The percentage of CD4 cells could therefore be used as an age-independent marker of immune competence in children.

Comparing our data with that of Kotylo *et al.* from the USA, the range of CD4+ cells in their series was 27%–57% (95% CI) for children 3 years of age and above, while in ours it was 29%-48% (2 SD) for 3–5-year-old children.[18] Similarly, the range for CD4+ cell count in their study was 560–2700 cells/cmm for children above 3 years, while in ours it was 740–2128 cells/mm for 3–5-year-olds. Our data therefore fall within the normal range for Caucasian children and are also similar to those of Shahabuddin et al. from Saudi Arabia.[12] Age-related changes appear to be more important than ethnic variation in children.

The reason for plotting CD4 count versus age as a normogram is that this is the most frequently used parameter for the evaluation of children with suspected immunodeficiency, particularly HIV infection. The plot depicting mean and two standard deviations around the mean shows a steady, gradual decline with age, as well as the normal wide variability of this biological parameter. Besides, CD4 counts are higher in children than in adults across all ages, and therefore, age-related cut-off values have to be specified for the diagnosis of mild, moderate and severe immunodeficiency. Studies need to be done in HIV-infected children at different stages of the disease to understand the correlation between CD4 count and opportunistic infections.

Our study confirms earlier reports on age-related changes in human blood lymphocyte subpopulations and provide some baseline data for use in India. However, this study was carried out in only one area of South India. Besides, a majority of the children included in this study belonged to the lower socioeconomic group. Similar data, therefore, need to be generated from other parts of the country, as well as from other socioeconomic strata before a reference chart on lymphocyte subsets in each age group of Indian children is made available for paediatricians, to be used for the diagnosis and monitoring of immune deficiency diseases. Our finding of a high correlation between absolute lymphocyte count and CD4 count suggests that the former could be used as a surrogate marker where facilities for CD4 testing are not available.

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