Peripheral blood lymphocyte subsets in healthy Turkish children

Aydan İkincioğulları, Tanıl Kendirli, Figen Doğu, Yonca Eğin, İsmail Reisli
Şükrü Cin, Emel Babacan

Department of Pediatric Immunology and Allergy, Ankara University Faculty of Medicine, Ankara, Turkey

SUMMARY: İkincioğulları A, Kendirli T, Doğu F, Eğin Y, Reisli İ, Cin Ş, Babacan E. Peripheral blood lymphocyte subsets in healthy Turkish children. Turk J Pediatr 2004; 46: 125-130.

Immunophenotyping of peripheral blood lymphocyte subpopulations is essential for the diagnosis and follow-up of children with immunodeficiencies and other immune disorders. The relative size and absolute number distributions (median and 5-95%) of lymphocyte subsets, including cord blood (Coulter, EPICS-XL) were examined by flow cytometry in 190 healthy subjects from birth to 18 years of age with a view to obtaining normal reference values for Turkish children of the following age groups: cord blood (n:29), birth to 1 year (n:41), 1 to 2 years (n:30), 2 to 6 years 8n:30), 6 to 10 years (n:30), and 10 to 18 years (n:30). The relative size of CD2+, CD3+CD16-56-, CD3+CD8+ T lymphocytes increased while the relative size and absolute counts of those together with CD3+CD4+ and CD19+, CD20+ B lymphocytes decreased with age. The percentage of CD3-CD16+56+ NK cells increased from 0-1 year to 10-18 years; however, absolute count of CD3-CD16+56+ NK cells remained stable and unchanged in all age groups. The relative size and absolute count of activation markers (CD3+CD25+ and HLADR+) decreased from 0-1 year through 10-18 years age group.

This study has once more demonstrated that both the percentage and the absolute number of lymphocyte subsets in cord blood and peripheral blood of healthy infants and children changed with age. Therefore, comparison of results to those of age-matched healthy controls is of utmost importance in the reliable and accurate evaluation of lymphocyte subsets reflecting cellular immunity in children.

Key words: lymphocyte subsets, reference range, Turkish children.

During the past decade, immunophenotyping of blood lymphocytes has become an important tool in the diagnosis of immunologic and hematologic disorders such as immunodeficiencies, and lymphoproliferative and autoimmune diseases[1,2]. Recent application of flow cytometric immunophenotyping in childhood diseases has highlighted the need for reliable lymphocyte data ranges of normal infants and children[3]. Several reports about blood lymphocyte subsets in healthy children have been published[1,2,4-9]. However, only a small number of studies have systematically examined immunophenotypic changes from birth throughout childhood with the use of adequate lymphocyte markers which reliably cover the main lymphocyte subgroups[1]. Moreover, because of the higher blood lymphocte counts in neonates and infants compared to adults, differences in lymphocyte subpopulations are better reflected through the comparison of the absolute counts rather than their relative frequencies, a finding investigated by few studies[1,5].

The aim of the present study was to evaluate age-related changes in peripheral blood lymphocyte subpopulations in terms of both relative size and absolute counts and to obtain normal values for Turkish children.

Material and Methods

Between November 1998 and February 2000, 29 cord blood (CB) samples from healthy full term neonates and peripheral blood samples from 161 healthy children with no evidence of any infectious, hematologic or immunologic disorders were obtained. These children visited the hospital either for routine follow-up or for hepatitis B virus serology examination. Their

overall health conditions were assessed by questionnaires and physical examinations. Those suffering from conditions likely to affect the immune parameters were eliminated from the study. Informed consent was obtained from the parents of all subjects according to the guidelines of the Ankara University Medical School. Subjects (total of 190) were grouped into six age categories. The details of each group are shown in Table I.

Table I. Study Population

Group	1	2	3	4	5	6
Age	Cord blood	0-1 year	1-2 years	2-6 years	6-10 years	10-18 years
Median Age		6 months	16 months	3 years	7 years	12 years
Number of Children	29	41	30	30	30	30
Girls (n)	13	20	14	14	11	13
%	44.8	48.7	46.7	46.7	36.7	43.3
Boys (n)	16	21	16	16	19	17
%	55.2	51.3	53.3	53.3	63.3	56.7

Cord blood or peripheral blood samples (1.5-2.0 ml) were obtained in tubes containing ethylenediaminetetra-acetic acid (EDTA). Complete blood cell count (CBC), including an automated differential, was performed with Coulter JT. Lymphocyte counts were calculated according to the following formula: lymphocyte % x WBC/100[2].

Peripheral blood lymphocyte subgroups were examined using a panel of monoclonal antibodies (MoAb) (Table II) by lysed whole blood technique[6]. The following combination of mouse antihuman moAbs were used for two color staining: CD45-FITC/CD14-PE, CD3-FITC/CD4-PE, CD3-ITC/CD16+56-PE, CD3-FITC/CD8-PE, CD2-FITC/CD19-PE, CD45RA-FITC/CD4-PE, CD45RA-FITC/CD8-PE, CD3FITC/CD25-PE, HLA-DR-FITC/CD20-PE, CD4-FITC/CD45RO-PE, CD8-FITC/CD45RO-PE (Immunotecth, Marseille, France). Phycoerytrin (PE) or fluoroisothiocyanate (FITC)-labeled mouse IgG of the appropriate isotypes was used as negative control in all studies. Data were analyzed using Coulter System IITM software for the EPICS XL/XL MCL. Peripheral blood lymphocyte absolute numbers were calculated by the formula: lymphocyte subset % x TLC/100[2].

The median values of lymphocyte subsets and 5th and 95th percentiles (median and 5-95%) were determined for six age groups[1,3,6].

Results

The relative size and absolute number distributions (median and 5-95%) of lymphocyte subsets, whole blood counts, and absolute lymphocyte counts of 190 healthy children are given in Table III, Figure 1.

The absolute leukocyte and lymphocyte counts decreased from the 0-1 year group throughout adolescence (10-18 years). An age-related decline was observed in the absolute numbers of total T-cells (CD3+CD16-56-), B-cells (CD19+, CD20+), and NK cells (CD3-CD16+56+) as well (Table III).

Table II. MoAbs and Corresponding Cells

CD designation	Main cellular expression
CD45	Common leukocyte antigen
CD14	Monocyte
CD3	Total T lymphocyte
CD4	Helper T lymphocyte
CD8	Cytotoxic T lymphocyte
CD16+56+	Natural killer cells
CD19	Total B lymphocyte
CD20	Total B lymphocyte
CD45RA	Naive lymphocyte
CD45RO	Memory lymphocyte
CD25	IL-2 receptor p55 antigen, activated cells
HLA-DR	MHC Class II antigen, activated cells

MoAb: monoclonal antibodies.

The percentage of CD19+ and CD20+ B lymphocytes increased almost 1.5 fold during the first two years of life from the median value of 18% to 26% and 25%, respectively, followed by a gradual decrease to approximately 14% at 18 years. Absolute counts of CD19+ and CD20+ B lymphocytes remained around $1.5x10^9$/L at 0-1 year and 1-2 years then decreased to $0.4x10^9$/L at 10-18 years. The relative size of CD3+CD4+ T lymphocytes slightly decreased starting from 0-1 year

Table III. Relative Size and Absolute Numbers of Peripheral Blood Lymphocyte Subsets of Healthy Turkish Children

	Cord blood [n:29]	0-1 years [n:41]	1-2 years [n:30]	2-6 years [n:30]	6-10 years [n:30]	10-18 years [n:30]
	Median [5-95%]					
WBC [x10^9/L]	11.3 [7.0-17.6]	9.4 [5.6-13.11]	6.3 [4.4-12.9]	6.8 [4.0-10.4]	7.4 [3.7-11.1]	6.6 [4.3-11.4]
Lymphocyte [%]	40 [24-55]	61 [46-76]	62 [33-76]	51 51 51	44 [31-66]	42 [28-67]
[x10^9/L]	4.3 [3.3-7.1]	6.1 [3.2-10.8]	5.6 [2.2-8.1]	[27-69] 3.5 [1.5-5.2]	2.9 [1.5-7.6]	2.7 [1.7-5.7]
CD2+ [%]	64 [51-80]	70 [54-81]	70 [54-78]	74 [65-83]	76 [67-85]	80 [67-86]
[x10^9/L]	2.8 [1.0-4.9]	4.1 [2.5-8.4]	3.8 [1.4-6.9]	2.6 [1.1-3.9]	2.4 [1.1-5.4]	2.0 [0.2-2.4]
CD3+CD16-56-[%]	60 [46-77]	65 [51-79]	66 [51-77]	67 [55-79]	68 [57-81]	70 [58-82]
[x10^9/L]	2.6 [1.7-5.2]	3.8 [2.4-8.1]	3.7 [1.3-6.5]	2.4 [1.9-3.6]	2.0 [1.0-4.9]	1.8 [1.1-4.1]
CD3+CD4+[%]	42 [26-55]	44 [31-54]	41 [29-55]	38 [26-49]	36 [24-47]	39 [26-48]
[x10^9/L]	1.7 [1.1-3.7]	2.7 [1.4-5.2]	2.1 [0.7-4.5]	1.5 [0.6-2.0]	1.0 [0.5-2.7]	0.9 [0.6-2.4]
CD3+CD8+[%]	18 [7-28]	18 [10-31]	20 [15-33]	22 [9-35]	24 [17-37]	24 [16-32]
[x10^9/L]	0.7 [0.4-1.6]	1.1 [0.6-3.0]	1.0 [0.4-3.2]	0.7 [0.3-1.3]	0.8 [0.3-2.1]	0.6 [0.4-1.5]
CD3-CD16-56+[%]	8 [4-28]	11 [5-23]	9 [4-15]	10 [5-28]	14 [8-28]	15 [8-30]
[x10^9/L]	0.4 [0.2-1.7]	0.7 [0.2-1.8]	0.5 [0.2-1.3]	0.3 [0.2-1.2]	0.5 [0.2-0.9]	0.4 [0.2-1.0]
CD45RA+[%]	79 [35-89]	87 [72-93]	88 [82-94]	82 [72-97]	78 [61-87]	74 [66-95]
[x10^9/L]	2.9 [1.5-5.9]	5.0 [2.9-6.9]	4.9 [1.8-4.2]	3.0 [0.8-4.4]	2.2 [1.0-6.7]	1.9 [0.8-4.9]
CD4+CD45RA+[%]	37 [23-79]	35 [25-45]	33 [19-49]	29 [20-41]	26 [17-40]	27 [16-40]
[x10^9/L]	1.5 [1.0-4.4]	2.2 [1.2-5.6]	1.7 [0.5-4.2]	1.1 [0.5-6.6]	0.8 [0.3-2.4]	0.6 [0.4-2.0]
CD8+CD45RA+[%]	18 [11-28]	19 [12-28]	19 [14-32]	21 [13-31]	21 [15-32]	22 [16-33]
[x10^9/L]	0.8 [0.5-1.7]	1.2 [0.6-25]	1.0 [0.4-3.2]	1.0 [0.3-1.3]	0.7 [0.4-2.0]	0.6 [0.3-1.5]
CD45RO+[%]	13 [6-24]	17 [9-31]	17 [9-45]	26 [16-38]	32 [22-53]	33 [14-44]
[x10^9/L]	0.6 [0.2-1.0]	1.0 [0.5-2.2]	0.9 [0.4-3.3]	0.9 [0.4-1.6]	1.0 [0.5-2.0]	0.9 [0.4-2.0]
CD4+CD45RO+[%]	10 [4-18]	9 [6-21]	10 [5-18]	13 [8-42]	16 [9-23]	17 [8-26]
[x10^9/L]	0.4 [0.2-1.1]	0.6 [0.3-1.4]	0.5 [0.2-1.4]	0.5 [0.2-0.8]	0.5 [0.2-1.0]	0.5 [0.2-0.8]
CD8+CD45RO+[%]	2 [1-6]	4 [1-12]	4 [1-14]	6 [2-10]	8 [4-15]	8 [2-15]
[x10^9/L]	0.1 [0.04-0.7]	02 [0.07-1.4]	0.2 [0.07-1.5]	0.2 [0.06-0.5]	0.2 [%]0.09-0.8	0.2 [0.05-0.4]
CD25+[%]	5 [3-8]	4 [3-6]	4 [2-5]	4 [2-6]	4 [3-8]	4 [2-5]
[x10^9/L]	0.2 [0.1-1.5]	0.3 [0.2-0.5]	0.2 [0.1-0.4]	0.1 [0.08-0.2]	0.1 [0.05-0.2]	0.1 [0.05-0.2]
CD3+CD25+[%]	4 [2-6]	3 [2-5]	2 [1-4]	2 [1-4]	2 [1-5]	2 [1-4]
[x10^9/L]	0.2 [0.1-0.4]	0.2 [0.1-0.4]	0.1 [0.05-0.3]	0.1 [0.04-0.2]	0.1 [0.03-0.3]	0.1 [0.03-0.13]
HLADR+[%]	19 [11-34]	28 [15-48]	29 [19-43]	26 [18-38]	23 [17-31]	21 [16-35]
[x10^9/L]	0.9 [0.4-1.6]	1.8 [0.7-3.9]	1.5 [0.6-3.8]	0.9 [0.4-1.5]	0.7 [0.3-1.5]	0.5 [0.3-1.6]
CD19+[%]	18 [12-32]	24 [14-44]	26 [17-41]	20 [11-31]	17 [10-27]	15 [10-30]
[x10^9/L)]	0.9 [0.3-1.4]	1.5 [0.5-3.6]	1.4 [0.5-3.6]	0.7 [0.3-1.2]	0.5 [0.2-2.2]	0.4 [0.2-1.4]
CD20+[%]	18 [12-30]	24 [13-40]	25 [16-41]	20 [11-29]	16 [11-25]	14 [9-28]
[x10^9/L]	0.8 [0.5-1.6]	1.5 [0.5-3.1]	1.2 [0.5-3.3]	0.6 [0.3-1.1]	0.5 [0.2-2.0]	0.4 [0.2-1.3]
CD20+HLADR+[%]	16 [11-30]	23 [12-39]	23 [16-40]	19 [10-28]	15 [10-25]	13 [8-28]
[x10^9/L]	0.8 [0.4-1.6]	1.4 [0.2-3.1]	1.3 [0.5-3.3]	0.7 [0.3-1.1]	0.5 [0.2-2.2]	0.4 [0.2-1.3]

throughout 10-18 years of age. The absolute number of CD3+CD4+ T lymphocytes decreased three-fold from 0-1 year to 10-18 years age group. The percentage of CD3+CD8+ T-cells increased from 8% (0-1 year) to 24% (10-18 years). The absolute count of CD3+CD8+ T-cells decreased gradually from 0-1 year to 10-18 years. CD4+CD45RA lymphocytes were found to be 35% at 0-1 year group, followed by a slow decrease, to 27% at 10-18 years age group. CD4+CD45RO lymphocytes were measured to be 9% at 0-1 year group, followed by a gradual increase to 17% at 10-18 years age group. The relative size and absolute count of activation markers CD3+CD25+ and HLADR+ decreased from 0-1 year through 10-18 years age group.

The percentage of CD3-CD16-56+ NK cells increased from 0-1 year group to 10-18 years group, but absolute count of CD3-CD16+56+ NK cells remained stable and unchanged in all age groups.

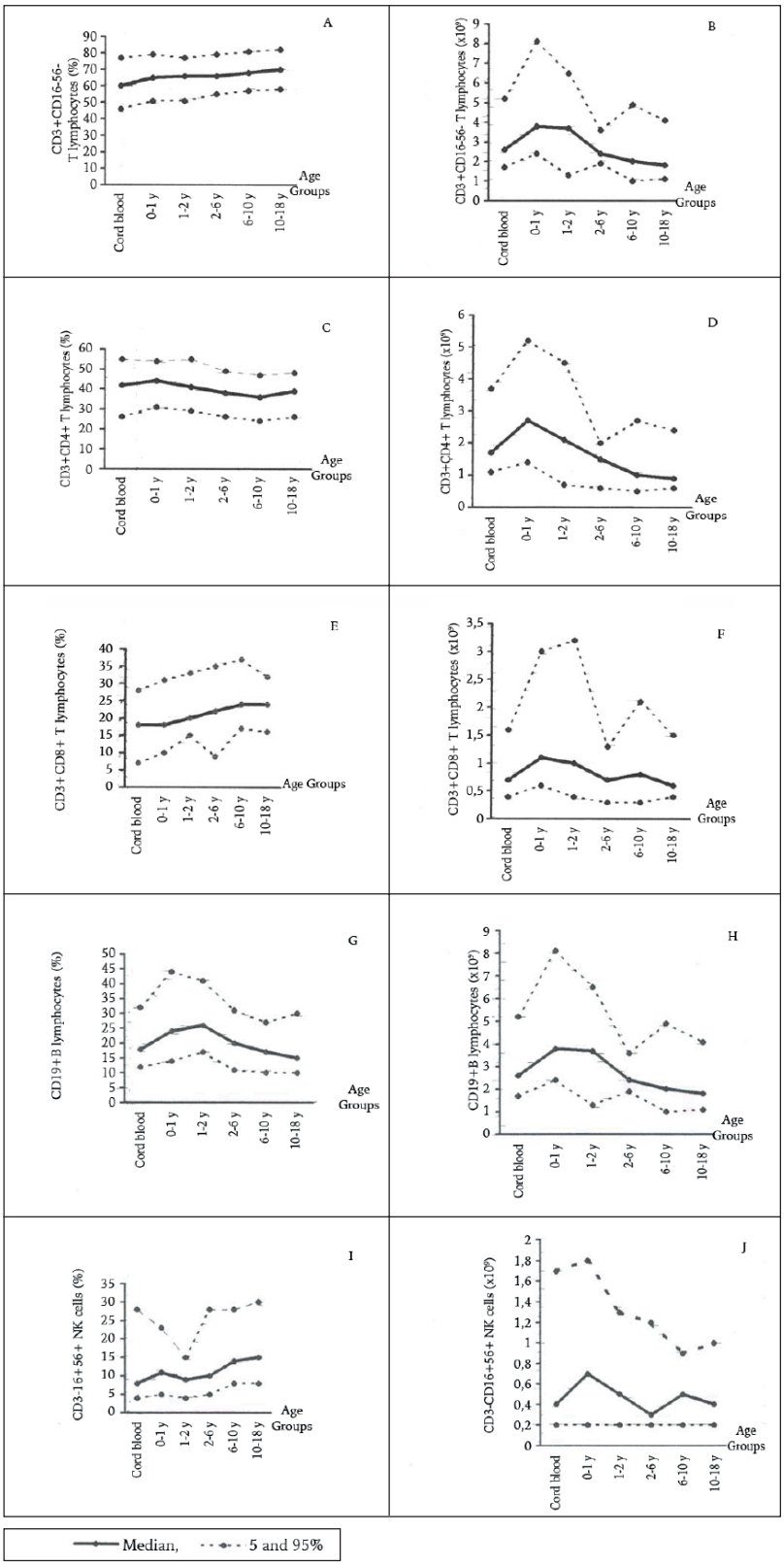

Fig. 1. The relative size and absolute number distributions (median and 5-95%) of CD3+CD16-56- total T lymphocytes (A, B), CD3+CD4+ helper T lymphocytes (C, D), CD3+CD8+ cytotoxic T lymphocytes (E, F), CD19+ B lymphocytes (G, H), and CD3-CD16+56+ NK cells (I, J).

The whole blood counts, relative size and absolute numbers of lymphocytes in 29 cord blood samples are also shown in Table III. Whole blood counts, absolute lymphocyte counts, absolute counts of CD3+CD4+ T lymphocytes, and relative sizes of CD4+CD45RA+ T lymphocyte levels were found to be higher than other age groups. However, relative sizes of CD2+ T lymphocytes, CD3+CD8+, CD4+CD45RO+ T lymphocytes and relative size of CD3-CD16+CD56+ NK cell levels were found to be lower than in other age groups.

Discussion

Peripheral blood T and B lymphocyte enumeration is frequently used for the diagnosis of immune and malignant discrepancies, especially in primary immune deficiencies such as SCID (severe combined immunodeficiency). It is known that leukocyte and lymphocyte counts are higher at birth and during the first years of life, then decline with age[5,11,12]. Thus, the understanding of T and B lymphocyte development and maturation as well as age-related changes of phenotype expression are critical for the recognition of diseases. Age-specific reference range of lymphocyte subsets should be used for appropriate clinical evaluation and treatment of pediatric patients[6,10]. CD4+ and CD8+ T lymphocyte counts follow the pattern of total T lymphocytes, with an increase at the first year of life as well as high numbers of CD4+ CD3+ T lymphocytes. These were mainly CD45RA+ naive T lymphocytes, probably new T cells freshly relased from the thymus[13,14,16]. The figures for CD19+ lymphocytes were more consistent, with a marked decrease in both percentage and actual numbers as the child ages. These findings are in accordance with the previously published data[1,5,8].

It has long been suggested that the human immune system is functionally less mature at birth and within the first year of life, then undergoes a process of sequential development that is programmed genetically and also stimulated by external antigenic exposure[5]. During the first years of life the immune system encounters many "new" antigens, which all together induce massive activation, proliferation and the maturation processes. These processes continue until sufficient levels of specific immune surveillance and memory function have been achieved. In fact, many report on blood lymphocyte subsets not higher values during childhood compared to adult hood.

In our study, the percentage of CD3-CD16+56+ NK cells was 8% in cord blood, as opposed to Comas-Bitter et al.[1] and Hannet et al.[3], who both found 20% in their studies. The percentage of CD3-CD16+56+ NK cells increased from 11% (0-1 year) to 15% (10-18 years), while absolute count of CD3-CD16+56+ NK cells remained stable and unchanged in all age groups. Our findings of absolute count of NK cells in cord blood differed from those of Comans-Bitter et al[1]. But there was no clear distinction in the absolute count of CD3-CD16+56+ NK cells between other age groups in our study nor in the studies of Comans-Bitter et al.[1], Hannet et al.[3], and Hulstaert et al[9]. The difference in the results may be attributed to racial and socio-economic factors.

Immunophenotypic changes of lymphocytes from birth throughout adulthood were systematically examined in a small number of studies[5,8]. The present study, which was based on extensive data obtained from 190 blood samples, provides reliable reference values for lymphocyte subsets from birth to 18 years.

Our findings strongly support that several differences can be observed in lymphocyte subsets from birth to 18 years of age. Despite a decline in the absolute count, the relative proportions of lymphocyte subsets varied and T cell percentages both in CD4+ and CD8+ subsets progressively increased with age. The multiplication of activated T-cells (CD3+HLADR+) with age suggests that activation and proliferation of immune effectors can be attributed to immune system maturation, such as thymic activity and exposure to novel antigens over time. An age-related decrease of B-cells was demonstrated. The percentage and absolute number of CD19+, CD20+ B lymphocytes increased from cord blood to 2-6 years, then gradually decreased through 10-18 years of age.

The relative size of lymphocytes can be determined more accurately by the lysed whole blood technique rather than by the analysis of density gradient separation[2]. This standardized technique is ideally suitable for pediatric patients as it processes small aliquots of whole blood and minimizes sample manipulation. The reason why our study population consisted of

children visiting the hospital was to reach a larger number of subjects from a similar socio-economic background.

During the interpretation of immunophenotyping data, one should be aware that changes in lymphocyte subsets can also be induced by other factors such as infections or medication[5]. We recommend that diagnostic blood lymphocyte phenotyping be performed on a clinically stable patient, if possible, free of infections and in the absence of any immunosuppressive treatment. The results should be interpreted on the basis of both relative size and absolute number of lymphocyte subsets, taking also into consideration the appropriate age-matched reference values.

In summary, this study has shown that both the percentage and the absolute number of various lymphocyte subsets in cord blood and peripheral blood of healthy infants and children changed with age. The definition of lymphocyte subset normal range is important in order to evaluate immune system diseases in infants and children. To do this correctly it is important that the percentages and absolute numbers of various lymphocyte subsets in cord blood, infants and children be evaluated and compared to individuals within the same age-related category rather than to adults[1,2,8,9].

REFERENCES

1. Comans-Bitter WM, Groot R, Beemd R, et al. Immunophenotyping of blood lymphocytes in childhood. J Pediatr 1997; 130: 388-393.
2. Lee BW, Yap HK, Chew FT, et al. Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: from birth to adulthood. Cytometry 1996; 26: 8-15.
3. Hannet I, Erkeller-Yuksel F, Lydyard P, Deneys V, DeBruyere M. Developmental and maturational changes in human blood lymphocyte subpopulations. Immunol Today 1992; 13: 215-218.
4. Vries E, Groot R, Bruin-Versteeg S, Comans-Bitter WM, Dongen JJ. Analysing the developing lymphocyte system of neonates and infants. Eur J Pediatr 1999; 158: 611-617.
5. Erkeller-Yuksel FM, Deneys V, Yuksel B, et al. Age-related changes in human blood lymphocyte subpopulations. J Pediatr 1992; 120: 216-222.
6. Kotylo PK, Fineberg NS, Freeman KS, Redmond NL. Reference ranges for lymphocyte subsets in pediatric patients. Am J Clin Pathol 1993; 100: 111-115.
7. Robinson M, O'Donohoe J, Dadain G, Wankowicz A, Barltrop D, Hobbs JR. An analysis of the normal ranges of lymphocyte subpopulations in children aged 5-13 years. Eur J Pediatr 1996; 155: 535-539.
8. Katevas P, Lembesopoulos C, Kamitaki C, Sakellariou D, Douna V. Establishment of normal ranges of lymphocyte subpopulations in cord blood and inperipheral blood in infants and children. Haema 1999; 2: 145-151.
9. Hulstaert F, Hannet I, Deneys V, et al. Age-related changes in human blood lymphocyte subpopulations. Clin Immunol Immunpathol 1994; 70: 152-158.
10. Deny T, Yogev R, Gelman R, et al. Lymphocyte subsets in healthy children during the first 5 years of life. JAMA 1992; 267: 1484-1488.
11. Yanase Y, Tango T, Okumura K, Tada T, Kawasaki T. Lymphocyte subsets identified by monoclonal antibodies in healthy children. Pediatr Res 1986; 20: 1147-1151.
12. Lisse IM, Aaby P, Whittle H, et al. T-lymphocyte subsets in west African children: impact of age, sex, and season. J Pediatr 1997; 130: 77-85.
13. Poppema S, Lai R, Visser L, Yan X. J. CD45 (Leucocyte Common Antigen) expression in T and B lymphocyte subsets. Leukemia Lymphoma 1996; 20: 217-222.
14. Hamann D, Baars PA, Hooibrink B, Lier RA. Heterogeneity of the human CD4 T-cell population: two distinct $CD4^+$ T-cell subsets characterized by coexpression of CD45RA and CD45RO isoforms. Blood 1996; 88: 3513-3521.
15. Gabriel H, Scmitt B, Kindermann W. Age-related increase of $CD45RO^+$ lymphocytes inphysically active adults. Eur J Immunol 1993; 23: 2704-2706.
16. Lai R, Visser L, Poppema S. Postnatal changes of CD45 expression in peripheral blood T and B cells. Br J Haematol 1994; 87: 251-257.