Immunophenotyping of blood lymphocytes in childhood

Reference values for lymphocyte subpopulations

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Objective: **Immunophenotyping of blood lymphocytes is an important tool in the diagnosis of hematologic and immunologic disorders. Because of maturation and expansion of the immune system in the first years of life, the relative and the absolute size of lymphocyte subpopulations vary during childhood. Therefore we wished to obtain reference values for the relative and the absolute size of all relevant blood lymphocyte subpopulations in childhood.**

Study design: **We used the lysed whole blood method for analysis of lymphocyte subpopulations in 429 blood samples from neonates (n = 20), healthy children (n = 358), and adults (n = 51). The following age groups were used: 1 week to 2 months (n = 13), 2 to 5 months (n = 46), 5 to 9 months (n = 105), 9 to 15 months (n = 70), 15 to 24 months (n = 33), 2 to 5 years (n = 33), 5 to 10 years (n = 35), and 10 to 16 years (n = 23).**

Results: **Our results show that the absolute number of $CD19^+$ B lymphocytes increases twofold immediately after birth, remains stable until 2 years of age, and subsequently gradually decreases 6.5-fold from 2 years to adult age. The $CD3^+$ T lymphocytes increase 1.5-fold immediately after birth and decrease threefold from 2 years to adult age. The absolute size of the $CD3^+/CD4^+$ T-lymphocyte subpopulation follows the same pattern as the total $CD3^+$ population, but the $CD3^+/CD8^+$ T lymphocytes remain stable from birth up to 2 years of age, followed by a gradual threefold decrease toward adult levels. In contrast to B and T lymphocytes, the absolute number of natural killer cells decreases almost threefold in the first 2 months of life and remains stable thereafter. Our study also showed that changes in the absolute size of lymphocyte subpopulations are not always consistent with changes in their relative size. This demonstrates that the relative counts of lymphocyte subsets do not reflect their actual size and are therefore of limited value.**

Conclusion: **On the basis of this study we strongly recommend that immunophenotyping of blood lymphocytes for the diagnosis of hematologic and immunologic disorders be based on the absolute rather than on the relative size of lymphocyte subpopulations. Our data can be used as age-matched reference values for blood lymphocyte immunophenotyping. (J Pediatr 1997;130:388-93)**

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Full-color agenda-size foldouts for Table II and the Figure can be obtained from Prof. J. J. M. van Dongen, MD, PhD, at the address given in column 2 of these footnotes.

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During the past decade immunophenotyping of blood lymphocytes has become an important tool in the diagnosis of immunologic and hematologic disorders such as immunodeficiencies, lymphoproliferative diseases, and autoimmune diseases. Accurate interpretation of the immunophenotyping data of individuals with these disorders requires reliable reference values.[1, 2] Because it is known that the relative and absolute size of lymphocyte subpopulations vary with age,[1-6] it is important to use age-matched reference values.

Several reports about blood lymphocyte subpopulations in children have been published.[2-10] However, the results of most studies cannot be used as general reference values for diagnostic purposes, because of various shortcomings, such as (1) limited numbers of blood samples were analyzed in the first years of life, resulting in age groups with broad age ranges[3, 4, 6-8]; (2) either relative or absolute counts were reported instead of both[8, 9]; (3) no 5th to 95th percentile values were provided[3, 4, 6]; (4) no whole blood methods were used,[3, 9] although they are known to be the most reliable methods for obtaining absolute counts for lymphocyte subsets[8, 10, 11]; and (5) limited numbers of lymphocyte markers were used, which did not cover the main lymphocyte subsets.[2, 5, 8]

See commentary, p. 347.

NK Natural killer [cells]

In this study we present reference values for immunophenotyping of blood lymphocytes obtained by analysis of more than 375 blood samples from healthy children (16 years of age or younger), including approximately 200 blood samples taken during the first year of life. All blood samples were analyzed with an appropriate antibody panel using the lysed whole blood method to determine the relative and absolute size of the main lymphocyte subsets, as they are studied in immunologic and hematologic disorders.

METHODS

Blood samples. Heparinized blood samples from 20 healthy term neonates were obtained from the umbilical cord immediately after delivery. Heparinized blood samples from 358 healthy children and 51 healthy adults (without infectious diseases or immunologic and hematologic disorders) were obtained by venipuncture. The blood samples were collected according to the informed consent guidelines of the medical ethics committee of the Erasmus University Rotterdam/University Hospital Rotterdam. The 254 blood samples in the age range from 2 months to 2 years were obtained from 254 healthy children participating in the Dutch vaccination program; the vaccination time points determined the clustering of the blood samples and thereby the age divisions in this group of children. The children were divided into the following age groups: 1 week to 2 months (n = 13), 2 to 5 months (n = 46), 5 to 9 months (n = 105), 9 to 15 months (n = 70), 15 to 24 months (n = 33), 2 to 5 years (n = 33), 5 to 10 years (n = 35) and 10 to 16 years (n = 23).

Immunophenotyping. Two-color flow cytometric immunophenotyping using the lysed whole blood method (Becton Dickinson, San Jose, Calif.) was performed to determine the following lymphocyte subsets: $CD19^+$ B lymphocytes, $CD3^+$ T lymphocytes, $CD3^+/CD4^+$ T lymphocytes, $CD3^+/CD8^+$ T lymphocytes, $CD3^+/HLA\text{-}DR^+$ activated T lymphocytes, and $CD3^-/CD16\text{-}56^+$ natural killer cells. For this purpose 25 μl aliquots of whole blood were incubated for 10 minutes at room temperature with combinations of the following optimally titrated fluorescein isothiocyanate and phycoerythrin conjugated CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), CD16 (Leu-11c), CD19 (Leu-12), CD56 (Leu-19), and HLA-DR (L243) antibodies (Becton Dickinson). After incubation the cells were washed, followed by lysis of the erythrocytes using FACS Lysing Solution (Becton Dickinson), and subsequently analyzed by flow cytometry.

All analyses were performed with a FACScan flow cytometer (Becton Dickinson) that had been calibrated with CaliBRITE beads and AutoCOMP software (Becton Dickinson).

Calculation of relative and absolute lymphocyte counts. The relative size of each lymphocyte subset was expressed as the percentage within the total lymphocyte population (i.e., the "lymphocyte gate" as determined manually on the basis of forward scatter and side scatter characteristics). The lymphocyte gate was checked for its purity by use of CD14 (Leu-M3)/CD45 (HLe-1) (Becton Dickinson) double staining and was regarded to be correct, if the gate included at least 95% of all lymphocytes and contained less than 5% contamination with monocytes, granulocytes, or cell debris (SimulSET software, Becton Dickinson). In this way the lymphocyte gate can be set appropriately in all blood samples, except for some neonatal cord blood samples, which contain erythroid cells (normoblasts, unlysed erythrocytes, or both) in their lymphocyte gate. In such cases the relative size of the lymphocyte population was corrected for this contamination.

The relative size of the total lymphocyte population was determined by flow cytometric blood cell differentiation (relative frequency of lymphocytes, monocytes, and granulocytes), based on the above described scatter characteristics and CD14/CD45 double staining (SimulSET software).

The absolute leukocyte counts were determined in quadruple with a Coulter Counter model Z1, certified to ISO9002 quality assurance (Coulter Electronics, Luton, England). The reliability of the quadruple leukocyte countings

Table I. Relative size of lymphocyte subpopulations in blood

Lymphocyte subpopulations	Age groups									
	Neonatal (n = 20)	1 wk-2 mo (n = 13)	2-5 mo (n = 46)	5-9 mo (n = 105)	9-15 mo (n = 70)	15-24 mo (n = 33)	2-5 yr (n = 33)	5-10 yr (n = 35)	10-16 yr (n = 23)	Adults (n = 51)
$CD19^+$ B	12%	15%	24%	21%	25%	28%	24%	18%	16%	12%
lymphocytes	(5-22)	(4-26)	(14-39)	(13-35)	(15-39)	(17-41)	(14-44)	(10-31)	(8-24)	(6-19)
$CD3^+$ T	62%	72%	63%	66%	65%	64%	64%	69%	67%	72%
lymphocytes	(28-76)	(60-85)	(48-75)	(50-77)	(54-76)	(39-73)	(43-76)	(55-78)	(52-78)	(55-83)
$CD3^+/CD4^+$ T	41%	55%	45%	45%	44%	41%	37%	35%	39%	44%
lymphocytes	(17-52)	(41-68)	(33-58)	(33-58)	(31-54)	(25-50)	(23-48)	(27-53)	(25-48)	(28-57)
$CD3^+/CD8^+$ T	24%	16%	17%	18%	18%	20%	24%	28%	23%	24%
lymphocytes	(10-41)	(9-23)	(11-25)	(13-26)	(12-28)	(11-32)	(14-33)	(19-34)	(9-35)	(10-39)
CD4/CD8	1.8	3.8	2.7	2.5	2.4	1.9	1.6	1.2	1.7	1.9
ratio per $CD3^+$	(1.0-2.6)	(1.3-6.3)	(1.7-3.9)	(1.6-3.8)	(1.3-3.9)	(0.9-3.7)	(0.9-2.9)	(0.9-2.6)	(0.9-3.4)	(1.0-3.6)
$CD3^+$/HLA-DR^+	2%	5%	3%	3%	4%	6%	6%	7%	4%	5%
T lymphocytes	(1-6)	(1-38)	(1-9)	(1-7)	(2-8)	(3-12)	(3-13)	(3-14)	(1-8)	(2-12)
$CD3^-/CD16\text{-}56^+$	20%	8%	6%	5%	7%	8%	10%	12%	15%	13%
NK cells	(6-58)	(3-23)	(2-14)	(2-13)	(3-17)	(3-16)	(4-23)	(4-26)	(6-27)	(7-31)

The relative frequencies are expressed within the lymphocyte population: median and percentiles (5th to 95th percentiles).

Table II. Absolute size of lymphocyte subpopulations in blood

Lymphocyte subpopulations	Age groups									
	Neonatal (n = 20)	1 wk-2 mo (n = 13)	2-5 mo (n = 46)	5-9 mo (n = 105)	9-15 mo (n = 70)	15-24 mo (n = 33)	2-5 yr (n = 33)	5-10 yr (n = 35)	10-16 yr (n = 23)	Adults (n = 51)
Lymphocytes	4.8	6.7	5.9	6.0	5.5	5.6	3.3	2.8	2.2	1.8
	(0.7-7.3)	(3.5-13.1)	(3.7-9.6)	(3.8-9.9)	(2.6-10.4)	(2.7-11.9)	(1.7-6.9)	(1.1-5.9)	(1.0-5.3)	(1.0-2.8)
$CD19^+$ B	0.6	1.0	1.3	1.3	1.4	1.3	0.8	0.5	0.3	0.2
lymphocytes	(0.04-1.1)	(0.6-1.9)	(0.6-3.0)	(0.7-2.5)	(0.6-2.7)	(0.6-3.1)	(0.2-2.1)	(0.2-1.6)	(0.2-0.6)	(0.1-0.5)
$CD3^+$ T	2.8	4.6	3.6	3.8	3.4	3.5	2.3	1.9	1.5	1.2
lymphocytes	(0.6-5.0)	(2.3-7.0)	(2.3-6.5)	(2.4-6.9)	(1.6-6.7)	(1.4-8.0)	(0.9-4.5)	(0.7-4.2)	(0.8-3.5)	(0.7-2.1)
$CD3^+/CD4^+$ T	1.9	3.5	2.5	2.8	2.3	2.2	1.3	1.0	0.8	0.7
lymphocytes	(0.4-3.5)	(1.7-5.3)	(1.5-5.0)	(1.4-5.1)	(1.0-4.6)	(0.9-5.5)	(0.5-2.4)	(0.3-2.0)	(0.4-2.1)	(0.3-1.4)
$CD3^+/CD8^+$ T	1.1	1.0	1.0	1.1	1.1	1.2	0.8	0.8	0.4	0.4
lymphocytes	(0.2-1.9)	(0.4-1.7)	(0.5-1.6)	(0.6-2.2)	(0.4-2.1)	(0.4-2.3)	(0.3-1.6)	(0.3-1.8)	(0.2-1.2)	(0.2-0.9)
$CD3^+$/HLA-DR^+ T	0.09	0.3	0.2	0.2	0.2	0.3	0.2	0.2	0.06	0.09
lymphocytes	(0.03-0.4)	(0.03-3.4)	(0.07-0.5)	(0.07-0.5)	(0.1-0.6)	(0.1-0.7)	(0.08-0.4)	(0.05-0.7)	(0.02-0.2)	(0.03-0.2)
$CD3^-/CD16\text{-}56^+$	1.0	0.5	0.3	0.3	0.4	0.4	0.4	0.3	0.3	0.3
NK cells	(0.1-1.9)	(0.2-1.4)	(0.1-1.3)	(0.1-1.0)	(0.2-1.2)	(0.1-1.4)	(0.1-1.0)	(0.09-0.9)	(0.07-1.2)	(0.09-0.6)

Absolute counts ($\times 10^9$/L): median and percentiles (5th to 95th percentiles).

was high, because the mean coefficient of variation appeared to be 4% ± 3%.

The absolute size of each lymphocyte subset was calculated from the relative size of the lymphocyte subset, the relative size of the total lymphocyte population, and the absolute leukocyte count.

Statistical analysis. For nine age groups the median values of lymphocyte subsets and the 5th and 95th percentiles were determined without distributional assumptions, because the distribution of lymphocyte subset parameters in most age groups were too skewed for calculations according to normal distributions. The age group 1 week to 2 months was too small (n = 13) to use a distribution-free method. Therefore the 5th to 95th percentile range in this age group was determined according to the mean ± 1.645 SD (B- and T-lymphocyte subsets) or by determining the antilog of the outcomes of the logarithmically transformed data (total lymphocytes, activated T lymphocytes, and NK cells).

RESULTS

The relative sizes of the lymphocyte subpopulations during childhood are shown in Table I. The percentage of $CD19^+$ B lymphocytes increases twofold during the first 5

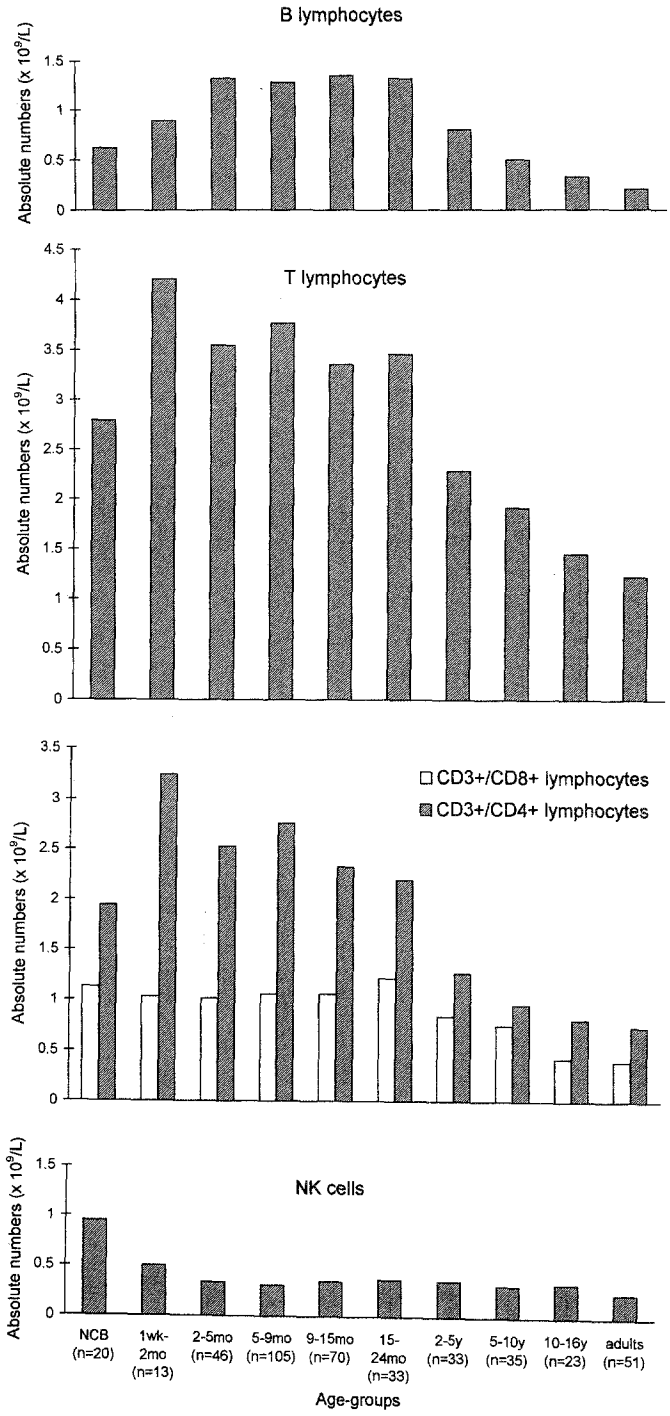

Figure. Histograms of median absolute size of the main lymphocyte subsets in neonates, children, and adults. The age range of the childhood age groups and the number of individuals per age group are indicated. *NCB*, Neonatal cord blood.

months of life from a median value of 12% to 24%, and remains stable until approximately 5 years, followed by a gradual decrease to approximately 13% at adult age. The relative frequency of $CD3^+$ T lymphocytes remains within narrow median limits of 60% to 75%. Also the fluctuations in $CD4^+$ and $CD8^+$ T-cell subsets are limited, but their relative distribution, the so-called CD4/CD8 ratio, shows age-related changes with higher median values (2.0 to 3.0)

during the first 2 years of life and lower values (1.0 to 2.0) at birth, in older children, and in adults. The relative frequency of activated T lymphocytes ($CD3^+/HLA\text{-}DR^+$) increases slowly during childhood from 2% at birth to a value of 5% at adult age. The percentage of NK cells ($CD3^-/CD16^+\text{-}CD56^+$ lymphocytes) shows a dramatic threefold decline immediately after birth from 20% to 7%, followed by a slow twofold increase to a median value of 13% at adult age.

The absolute sizes of the main lymphocyte subpopulations are shown in Table II and the Figure. The absolute number of lymphocytes increases 1.3-fold immediately after birth, remains relatively stable until 2 years of age, and subsequently gradually decreases threefold to adult levels. A comparable pattern is seen in the $CD19^+$ B lymphocytes with a twofold increase during the first 5 months of life and a 6.5-fold decrease from 2 years to adult age. The number of $CD3^+$ T lymphocytes increases 1.5-fold immediately after birth and decreases threefold from 2 years to adult age. The absolute number of $CD3^+/CD4^+$ T lymphocytes follows the same pattern as the total $CD3^+$ population, but the number of $CD3^+/CD8^+$ T lymphocytes remain stable from birth up to 2 years of age, followed by a gradual threefold decrease toward adult levels. The number of activated T lymphocytes increases immediately after birth, remains stable until the age of 10 years, and subsequently decreases to low levels. In contrast to B and T lymphocytes, the absolute number of NK cells decreases almost threefold during the first 2 months of life and thereafter remains stable.

DISCUSSION

Our study shows that changes in the absolute size of lymphocyte subpopulations are not always consistent with changes in their relative size. For instance, the relative median size of $CD3^+$ T lymphocytes remains stable at 64% to 72% from the age of 2 years to adult age, but in the same age period the median absolute number decreases threefold. Furthermore, the twofold relative increase of NK cells during childhood is not caused by an increase of NK cell numbers, but is related to the decrease of B- and T-lymphocyte numbers. This demonstrates that the relative distribution of lymphocyte subsets does not reflect their actual size and is therefore of limited value.

During the first year of life the immune system encounters many "new" antigens, which together induce massive activation, proliferation, and maturation processes. These processes continue until sufficient levels of specific immune surveillance and memory function have been reached. Many reports on blood lymphocyte subsets indeed noted higher values during childhood as compared with adult values.[2-10] However, because of the small sample size or limited subset analysis during the first years of life, these studies were unable to identify the changes we describe here in relative and absolute size of lymphocyte subsets from birth to adolescence. These changes in B- and T-lymphocyte counts are in agreement with changes in the size of the bone marrow precursor–B-cell compartment and the thymic cortex during the same age period.[12, 13]

Our extensive study on more than 375 childhood blood samples provides reliable reference values for lymphocyte subsets from birth to adult age. Such age-matched reference values have proven their value in the diagnosis and monitoring of immune disorders, such as pediatric acquired immunodeficiency syndrome.[1, 2] However, when interpreting immunophenotyping data, one should be aware that changes in lymphocyte subsets can also be induced by other causes—infections or medication.[14]

We recommend that diagnostic blood lymphocyte phenotyping be performed when the patient is in a clinically stable phase without infections or immunosuppressive treatment. The results should be interpreted on the basis of the absolute rather than the relative size of lymphocyte subsets, according to appropriate age-matched reference values.

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