

## Age-Related Changes in Blood Lymphocyte Subsets of Saudi Arabian Healthy Children

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**The age-related changes in absolute and percentage values of lymphocyte subsets in the peripheral blood of healthy children of different ages (1 month to 13 years) were studied by flow cytometry. The absolute and percentage values for most lymphocyte subpopulations differed substantially with age. Comparisons among age groups from infants through adults revealed progressive declines in the absolute numbers of leukocytes, total lymphocyte, and T, B, and natural killer (NK) cells. The percentages of T cells increased with age. Within the T-lymphocyte population, the CD8<sup>+</sup> subset increased but the CD4<sup>+</sup> subset decreased, resulting in a declining CD4<sup>+</sup>/CD8<sup>+</sup> ratio. The percentage of B cells declined, but that of NK cells remained unchanged. The percentage of HLA-DR<sup>+</sup> T cells increased over time, but their number changed inconsistently. Our findings confirm and extend earlier reports on age-related changes in lymphocyte subpopulations. These data should be useful in the interpretation of disease-related changes, as well as therapy-dependent alterations, in lymphocyte subsets in children of different age groups.**

Immunophenotyping of blood lymphocytes, or lymphocyte subset analysis, with monoclonal antibodies by flow cytometry is used routinely in the diagnosis of congenital and acquired immune deficiency syndromes, as well as leukemia and lymphoma, in children. In order to make a precise evaluation of affected individuals, reference values for lymphocyte subpopulations during childhood must be determined.

Age-related changes in blood lymphocyte subpopulations among healthy children have been reported, but their values are not yet well established for different age groups. Some studies on the reference ranges for T and B lymphocytes and their subsets in infants and children were done, but only a few lymphocyte markers were used (1, 4, 6, 15). Most of these earlier studies of age-related lymphocyte changes have been restricted to newborns (2, 7, 8, 13, 18, 24, 26, 31) or very young children (3) or have compared young adults to older adults (16, 21). Few reports have systemically documented immunophenotypic changes from birth through adulthood (5, 9-12, 17, 19, 30, 33, 34).

In the present study, age-related values for healthy infants and children of T, B, and natural killer (NK) cells and T-cell subsets in peripheral blood were determined and compared with corresponding values for healthy adults studied by the same technique.

### MATERIALS AND METHODS

**Subjects.** One hundred and two healthy children, 52 males and 50 females, with ages ranging from 1 month to 13 years and 30 healthy adult blood donors with ages ranging from 18 to 44 years were studied. All subjects were of Saudi Arabian origin. Informed consent from adult blood donors and from a parent or guardian of every child was obtained. The children had come to the healthy child clinic of King Khalid University Hospital, Riyadh, Saudi Arabia, for a routine health checkup or a vaccination. All children and adults were considered healthy if they had no past history of any disease; had normal blood pressure, pulse rate, and hemoglobin count; had no fever, cough, or infection; were not on any

medication; and (for the donors) had not donated blood in the past 3 months. In addition, all children and adult blood donors were screened for syphilis, human immunodeficiency virus, hepatitis B virus, and hepatitis C virus infection by routine serologic tests and were found negative. Blood samples were collected in EDTA tubes and used within 2 h of storage at room temperature. A complete blood count, including automated differential, was performed with a Coulter Counter.

Subjects were grouped into five age categories: group 1, ages 1 through 11 months; group 2, ages 1 through 2 years; group 3, ages 3 through 5 years; group 4, ages 6 through 13 years; group 5, ages 18 through 44 years.

**Flow cytometric analysis of lymphocyte subpopulations.** Whole-blood samples were stained with the Simultest immune monitoring kit having dual-color monoclonal antibodies (Becton Dickinson, Mountain View, Calif.) (Table 1). All samples were analyzed by a FACScan flow cytometer (Becton Dickinson) calibrated with CaliBRITE beads and AutoCOMP software; immunophenotyping results were obtained with SimulSET software (Becton Dickinson), as instructed by the manufacturer. Briefly, 100- $\mu$ l volumes of Simultest reagent were added to separate tubes and incubated for 15 to 20 min. Lysing solution (2 ml; Becton Dickinson) was added to each tube. Following 10 min of incubation, the tubes were centrifuged to remove lysed red cells and the cells were washed twice with the cell wash (Becton Dickinson). Washed cells were resuspended in 0.5 ml of the cell wash and analyzed immediately by the flow cytometer. Absolute lymphocyte subset counts were obtained as the product of the absolute lymphocyte count derived from a hematology analyzer and the percentages of the lymphocyte subset populations of interest, derived from the flow cytometer.

**Lymphocyte subset identification.** T cells were defined as those cells expressing the CD3 antigen, and B cells were defined as those cells expressing CD19. NK cells were identified by the presence of CD16, CD56, or both and by the absence of the coexpression of CD3. In most cases, the sum of lymphocyte lineage percentages (percent T cells + percent B cells + percent NK cells) was 100%  $\pm$  4% in each age group. This calculation works as a quality control verification for all lymphocyte subset determinations by flow cytometry and rules out the presence of any preparation artifacts in the samples. CD4<sup>+</sup> and CD8<sup>+</sup> cells were defined by the presence of CD4 and CD8, respectively, with coexpression of CD3. Activated T cells were those cells expressing HLA-DR on CD3<sup>+</sup> T cells.

**Statistical analysis.** Analysis of variance was conducted by using the Statpac Gold statistical analysis package, version 3.2 (Walonick Associated, Inc., Minneapolis, Minn.). The results are presented as the means  $\pm$  1 standard deviation of the percentage and absolute number for each lymphocyte population. The statistical significance of the observed differences in the percentages and numbers of lymphocyte subsets for any two groups was evaluated by Student's *t* test. A *P* value of <0.05 (two tailed) was considered statistically significant.

### RESULTS

**Absolute counts of WBC and lymphocyte subpopulations.** The mean of the absolute leukocyte (WBC) count declines sharply across age groups by a factor of approximately 1.4, from 10,330 cells/mm<sup>3</sup> in infants to 7,221 cells/mm<sup>3</sup> in older

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TABLE 1. Monoclonal antibody combinations<sup>a</sup>

Reagents (FITC/PE)	CD no. (FITC/PE)	Functional identity of relevant subsets
LeucoGATE	CD45/CD14	Immunophenotype 3 cell differential (lymphocytes, monocytes, and neutrophils)
Isotype control	IgG1/IgG2	Direct conjugate control for nonspecific staining
Leu-4/Leu-3	CD3/CD4	CD4 <sup>+</sup> T cells
Leu-4/Leu-2	CD3/CD8	CD8 <sup>+</sup> T cells
Leu-4/HLA-DR	CD3/HLA-DR	Activated (MHC class II expression) T-cells
Leu-4/Leu-11+ Leu-19	CD3/CD16 + CD56	NK cells (CD3 <sup>-</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> )
Leu-4/Leu-12	CD3/CD19	T (CD3 <sup>+</sup> CD19 <sup>-</sup> ) and B (CD3 <sup>-</sup> CD19 <sup>+</sup> ) cells

<sup>a</sup> FITC, fluorescein isothiocyanate; PE, phycoerythrin; MHC, major histocompatibility complex; IgG1, immunoglobulin G1.

children (Table 2). The absolute count for other cell types declines even more steeply: more than twofold (from 6,918 to 2,782 cells/mm<sup>3</sup>) for total lymphocytes, twofold (from 4,479 to 1,997 cells/mm<sup>3</sup>) for T cells, fourfold (from 1,652 to 427 cells/mm<sup>3</sup>) for B cells, and twofold (from 552 to 289 cells/mm<sup>3</sup>) for NK cells. In all the cases, the decline is statistically significant ( $P < 0.05$ ). In comparison with those for the older children, the absolute counts of the total lymphocytes and T and B cells for adults are even lower, while the absolute counts of WBC and NK cells are higher; however, these differences are not statistically significant ( $P > 0.05$ ). Although, the decline is progressive over the five age groups studied, the decrease in lymphocyte subset counts from group 3 to group 4 is not statistically significant; but that from group 1 to group 2 is significant. The mean values for lymphocytes and most of their subsets for group 2 and group 3 show significant decreases from those for group 1 and groups 1 and 2, respectively ( $P < 0.05$ ).

**Percentages of lymphocyte subpopulations.** The percentage of lymphocytes in the total WBC population declines with age from 67.4% of the WBC in infants (group 1) to 47.5% in young children (group 3) to 38.2% in older children (group 4) and to 29.9% in adults (group 5) (Table 3) ( $P < 0.05$ ). The absolute number of B cells drops more than twofold between group 1 and group 3, whereas the percentage of B cells declines less than twofold over a longer time. This is because the rate of decline in B-cell absolute numbers is steeper than the change in total lymphocytes. The percentage value drops from 23.8% in infants (groups 1 and 2) to 19.6% in group 3 children to 13.4% in group 4 children and remains at 13.9% in adults (group 5) ( $P < 0.05$  for the transition between infants and groups 3, 4, and 5 and between group 3 and groups 4 and 5). T cells decline in number less rapidly than total lymphocytes. Sixty-five percent of lymphocytes in infants are T cells, but this increases to 73.3% in older children ( $P < 0.05$ ) and to 74.3% in adults ( $P < 0.05$ ).

There is no significant change in the percentage of NK cells from group 1 to group 4, because the decline of the absolute values of NK cells is similar to that of the total lymphocyte count. However, the percentage of NK cells for adults (14.2%) is significantly different from the percentages of NK cells for groups 1 through 4 ( $P < 0.05$ ). The percentage of NK cells varied from 8.1% for infants to 10.5% for older children.

TABLE 2. Absolute values of WBC and lymphocyte subsets for healthy children

Cell type	Mean cell count (cells/mm <sup>3</sup> ) ± 1 SD (range) <sup>a</sup> for group:				
	1 (n = 40)	2 (n = 28)	3 (n = 24)	4 (n = 10)	5 (n = 30)
WBC	10,330 ± 3,360 (6,500-19,660)	9,820 ± 2,742 (5,000-16,300)	6,669 ± 2,215 <sup>1,2</sup> (2,100-11,300)	7,221 ± 1,883 <sup>1,2</sup> (5,500-10,710)	7,842 ± 2,192 <sup>1,2</sup> (4,129-10,788)
Lymphocytes	6,918 ± 2,260 (4,290-13,130)	5,185 ± 2,008 <sup>1</sup> (2,024-10,600)	3,070 ± 1,066 <sup>1,2</sup> (1,134-5,220)	2,782 ± 1,305 <sup>1,2</sup> (1,940-6,426)	2,244 ± 540 <sup>1,2</sup> (1,148-3,507)
CD3 <sup>+</sup> T cells	4,479 ± 1,456 (2,900-9,191)	3,317 ± 1,446 <sup>1</sup> (1,052-7,100)	2,312 ± 1,246 <sup>1,2</sup> (1,030-7,260)	1,997 ± 793 <sup>1,2</sup> (1,360-4,200)	1,666 ± 429 <sup>1,2</sup> (700-2,700)
CD19 <sup>+</sup> B cells	1,652 ± 715 (683-3,750)	1,251 ± 670 <sup>1</sup> (370-2,914)	716 ± 509 <sup>1,2</sup> (220-2,720)	427 ± 472 <sup>1,2</sup> (80-1,744)	298 ± 121 <sup>1,2,3</sup> (110-620)
CD3 <sup>-</sup> CD16 <sup>+</sup> /CD56 <sup>+</sup> NK cells	552 ± 291 (130-1,537)	423 ± 279 <sup>1</sup> (92-1,308)	307 ± 181 <sup>1</sup> (127-810)	289 ± 141 <sup>1,2</sup> (110-517)	328 ± 184 <sup>1</sup> (100-730)
HLA-DR <sup>+</sup> T cells	276 ± 190 (60-919)	338 ± 194 (101-830)	203 ± 153 (60-790)	271 ± 111 (140-440)	235 ± 147 (100-720)
CD4 <sup>+</sup> T cells	3,050 ± 1,138 (1,390-6,696)	2,062 ± 846 <sup>1</sup> (607-3,854)	1,491 ± 930 <sup>1,2</sup> (510-5,220)	1,085 ± 504 <sup>1,2</sup> (600-2,455)	895 ± 227 <sup>1,2,3</sup> (100-1,300)
CD8 <sup>+</sup> T cells	1,548 ± 612 (630-3,283)	1,311 ± 703 (296-3,390)	898 ± 406 <sup>1,2</sup> (400-2,270)	888 ± 203 <sup>1,2</sup> (590-1,227)	905 ± 364 <sup>1,2</sup> (400-1,900)

<sup>a</sup> The group or groups whose mean values are significantly ( $P < 0.05$ ) different from the listed value are indicated by superscript numbers.

TABLE 3. Percentages of lymphocyte subsets for healthy children

Cell type or ratio	Mean % total lymphocytes (or % WBC <sup>a</sup> ) ± 1 SD (range) <sup>b</sup> for group:				
	1 (n = 40)	2 (n = 28)	3 (n = 24)	4 (n = 10)	5 (n = 30)
Lymphocytes	67.4 ± 7.5 (51–86)	54.3 ± 17.4 <sup>1</sup> (19–78)	47.5 ± 11.7 <sup>1,2</sup> (20–71)	38.2 ± 9.6 <sup>1,2,3</sup> (28–60)	29.9 ± 7.4 <sup>1,2,3,4</sup> (19–49)
CD3 <sup>+</sup> T cells	65.4 ± 5.8 (47–79)	64.4 ± 7.0 (47–77)	65.5 ± 6.1 (50–76)	73.3 ± 4.4 <sup>1,2,3</sup> (65–80)	74.3 ± 6.9 <sup>1,2,3</sup> (61–85)
CD19 <sup>+</sup> B cells	23.8 ± 5.8 (14–44)	23.8 ± 5.9 (14–38)	19.6 ± 5.4 <sup>1,2</sup> (10–28)	13.4 ± 6.1 <sup>1,2,3</sup> (4–27)	13.9 ± 5.2 <sup>1,2,3</sup> (5–27)
CD3 <sup>-</sup> CD16 <sup>+</sup> /CD56 <sup>+</sup> NK cells	8.1 ± 3.3 (3–15)	8.5 ± 4.7 (4–21)	9.1 ± 4.0 (4–19)	10.5 ± 4.1 (5–17)	14.2 ± 7.0 <sup>1,2,3,4</sup> (5–29)
HLA-DR <sup>+</sup> T cells	3.9 ± 1.6 (1–7)	6.3 ± 1.9 <sup>1</sup> (3–11)	5.8 ± 2.3 <sup>1</sup> (2–12)	10.9 ± 4.3 <sup>1,2,3</sup> (7–17)	10.4 ± 3.9 <sup>1,2,3</sup> (5–21)
CD4 <sup>+</sup> T cells	45.6 ± 7.4 (28–62)	40.5 ± 5.8 <sup>1</sup> (29–50)	41.4 ± 6.0 <sup>1</sup> (25–51)	39.1 ± 4.4 <sup>1</sup> (31–45)	42.8 ± 6.8 (29–55)
CD8 <sup>+</sup> T cells	22.9 ± 5.2 (14–38)	25.1 ± 5.4 (16–34)	26.5 ± 5.9 <sup>1</sup> (17–35)	34.4 ± 8.6 <sup>1,2,3</sup> (19–47)	39.7 ± 7.8 <sup>1,2,3,4</sup> (27–56)
CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio	2.1 ± 0.7 (0.7–4.4)	1.7 ± 0.5 <sup>1</sup> (0.9–2.8)	1.7 ± 0.6 <sup>1</sup> (0.8–2.8)	1.2 ± 0.4 <sup>1,2,3</sup> (0.7–2.0)	1.1 ± 0.3 <sup>1,2,3</sup> (0.5–1.8)

<sup>a</sup> For total lymphocyte values.

<sup>b</sup> The group or groups whose mean values are significantly ( $P < 0.05$ ) different from the listed value are indicated by superscript numbers.

**T-cell subsets and activated T cells.** The analysis of the age-related changes in the percentages and numbers of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes per cubic millimeter and in the ratio between these two major T-cell subsets (Table 3) indicates that in the early stages of life, T lymphocytes with the helper/inducer phenotype prevail over the T cells with the suppressor/cytotoxic phenotype. A progressive reduction in the percentages and numbers of CD4<sup>+</sup> lymphocytes develops in the second year of life ( $P < 0.05$  for the transition between group 1 and group 2). In contrast, an increase in the percentages of CD8<sup>+</sup> lymphocytes has been observed ( $P < 0.05$ ), while the numbers of these cells per cubic millimeter decline from infancy up to the age of 5 years, but later on do not vary greatly from the values for the group of adult donors. On the basis of the variations in the values of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes, the CD4<sup>+</sup>/CD8<sup>+</sup> ratio decreases from 2.1 for 1- to 11-month-old infants to 1.2 for 6- to 13-year-old children ( $P < 0.05$ ), while no further significant decrease was observed in the values for the adult group.

The percentage of T cells expressing the activation marker HLA-DR increases from 3.9% in group 1 to 6.3% in group 2 to 10.9% in group 4 to 10.4% in group 5. The transition between group 1 and other groups is statistically significant ( $P < 0.05$ ). The absolute values of these cell populations vary from 276 to 235 cells/mm<sup>3</sup>, and the difference is not statistically significant ( $P > 0.05$ ).

## DISCUSSION

The present findings show that the absolute and percentage values for most lymphocyte markers differ substantially not only between children and adults but also between children from different age groups. Therefore, the reference values for lymphocyte subsets of neither adults nor children of mixed-age groups can be used for infants and children.

There is a significant decline from infants to adults in absolute counts of WBC, total lymphocytes, T, B, and NK cells, and CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets. These observations confirm the findings of other investigations (9, 12, 30, 33, 34).

It has been known for nearly 60 years (14) that total WBC and absolute lymphocyte counts are highest at birth and decline with age. Although the absolute counts decline, the relative proportions of lymphocyte subsets vary, and total T-cell and CD8<sup>+</sup> T-cell percentages increase with age (4). Two more-recent studies have reported that the percentages of T cells, including both CD4<sup>+</sup> and CD8<sup>+</sup> subsets, increase with age and that the CD4<sup>+</sup>/CD8<sup>+</sup> ratio is unchanged across age groups (30, 34). Our findings tend to differ with this observation, as we found decreases in CD4<sup>+</sup> T-cell percentages and the CD4<sup>+</sup>/CD8<sup>+</sup> ratio. Other workers have also shown an elevated

CD4<sup>+</sup>/CD8<sup>+</sup> ratio in cord blood (7, 25) or in the first 5 years of life (5, 33). This is further supported by the results of studies of Japanese children by Yanase et al. (33) and Yachie et al. (32), who found a decreased CD4<sup>+</sup>/CD8<sup>+</sup> ratio as a result of an increased percentage of CD8<sup>+</sup> T-cell subsets and a decreased percentage of CD4<sup>+</sup> T-cell subsets over time. It is reasonable to believe that this discrepancy between immunophenotype patterns seen in Caucasian, Japanese, and Saudi Arabian populations may be a reflection of significant differences in the relative representation of CD8<sup>+</sup> cells in T-cell subsets (20), which could be due to the influence of the racial and ethnic background. We have demonstrated in another study that healthy Saudi Arabian male blood donors have a significantly higher percentage and number of CD8<sup>+</sup> T cells and a lower CD4<sup>+</sup>/CD8<sup>+</sup> ratio than healthy Caucasian males (23). Several studies have demonstrated the different influences of racial and environmental factors on the human lymphocyte immunophenotype (20, 22, 27–29).

B cells, which were found to decline with age in our study, were reported to show no age-related changes by Osugi et al. (17). The same workers reported that the NK cell percentage increased significantly with age, whereas it remained unchanged in our study. In two other studies, the NK cell percentage was reported to decline with time (9, 10). These different findings may also be attributed to the influence of racial and environmental factors.

HLA-DR is an activation marker on T cells. There is a significant increase in the percentage of HLA-DR<sup>+</sup> T cells, whereas there is a little change in their number, over time in this study. Most of the earlier reports support our findings (9, 12, 34).

In conclusion, the data presented in this report confirm and extend the findings on age-related changes in human blood lymphocyte subsets reported earlier. Absolute and percentage values for most lymphocyte subsets in healthy adults differ significantly from those for children. Most importantly, among children, lymphocyte subsets vary significantly with age. In addition, racial and environmental factors may have some influence on the lymphocyte immunophenotype of children.

Therefore, when evaluating the immune status of children, consideration of age-related changes along with racial and environmental factors should be taken into account. The findings of this study are important for interpreting changes in various diseases, including infections which occur in children of different age groups.

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