Lymphocyte Subsets Identified by Monoclonal Antibodies in Healthy Children

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**ABSTRACT. The distributions of lymphocyte subsets and
monocytes in the peripheral blood mononuclear leukocytes
of 72 normal children from 2 months to $13^5/_{12}$ yr were
examined using quantitative immunofluorescence analysis
with monoclonal antibodies. Distinct decreases with age
were found in the total leukocyte counts, the percentages
and the absolute numbers of peripheral blood mononuclear
leukocytes. The percentages of Leu-2a$^+$ cells, Leu-7$^+$ cells,
and Leu-M3$^+$ cells significantly increased with age,
whereas the percentages of Leu-3a$^+$ cells, Leu-4$^+$ cells,
and 2H7$^+$ cells significantly decreased with age. As a
result, ratios of Leu-3a$^+$/Leu-2a$^+$ decreased with age. No
prominent differences with age were found in the propor-
tions of Leu-10$^+$ cells and HLA-DR$^+$ cells. (*Pediatr Res*
20: 1147–1151, 1986)**

Abbreviations

PBL, peripheral blood mononuclear leukocyte
MEM, minimal essential medium

Since a number of monoclonal antibodies directed against cell
surface antigens of human lymphocyte subsets have recently
become available, quantitative immunofluorescence analysis
with these monoclonal antibodies and flow cytometry techniques
has become technically possible (1, 2). The application of these
monoclonal antibodies to clinical medicine has been providing
not only new dimensions to the diagnosis of human diseases but
also a new clinical parameter to monitoring therapeutic progress
(3).

In proceeding to carry out the study on lymphocyte subsets in
various kinds of diseases commonly encountered in childhood,
we have noticed that ranges of values of lymphocyte subsets in
healthy infants and young children that were examined as normal
controls are apparently different from those in young adults and
elderly humans previously reported (Yanase Y, Tango T, Oku-
mura K, Tada T, Kawasaki T, unpublished data). Therefore, a
more detailed examination was performed to determine the
normal percentage of each lymphocyte subset in healthy children.
Data from children with diseases can be compared with data
from healthy controls.

PATIENTS, MATERIALS, AND METHODS

Donors. Seventy-two heparinized venous blood samples were
obtained from apparently healthy children attending the Depart-
ment of Pediatrics, Japanese Red Cross Medical Center at Tokyo
from May 1983 to April 1984. The mean age of healthy children
was 3.95 ± 3.18 (mean ± SD) yr old, ranging from 2 months to
$13^5/_{12}$ yr of age with a male/female ratio of $^{42}/_{30}$.

Monoclonal antibodies. Fluorescein-conjugated monoclonal
antibodies, termed Leu-2a, Leu-3a, Leu-4, Leu-7, Leu-10, Leu-
M3, and HLA-DR were purchased from Becton Dickinson
Monoclonal Center (Mountain View, CA). Leu-2a is directed at
the suppressor/cytotoxic T cell subset (2, 5–7); Leu-3a, at the
helper/inducer T cell subset (2, 6, 7); Leu-4, at total T cells (7);
Leu-7, at natural killer/killer cells (8, 9); Leu-10, at B cells and
activated T cells; Leu-M3, at mature monocytes/macrophages
(10); HLA-DR, at B cells, monocytes/macrophages and activated
T cells (11, 12); and 2H7, at B cells (13).

Analysis of stained cells with flow cytometry. Human PBL were
prepared by centrifugation of heparinized venous blood on Fi-
coll-Isopaque (Ficoll-Paque; Pharmacia, Uppsala, Sweden) at
room temperature and washed three times in ice cold MEM
(eagle MEM; Nissui, Tokyo, Japan). These cells were then stained
for 40 min in microtiter plates on ice by reacting 5 × 10^5 target
cells/well with saturation levels of directly fluorescein-conjugated
monoclonal antibodies. Cells were then washed with MEM three
times and analyzed immediately using a fluorescence activated
cell sorter (FACS-II; Becton-Dickinson, Mountain View, CA).
After setting forward light scatter gate on the lymphocyte cluster
and monocyte cluster, 1 × 10^4 cells were analyzed. Immunoflu-
orescence staining with fluorescein-conjugated mouse monoclo-
nal non-specific IgG1, IgG2 and IgM (Becton-Dickinson Mono-
clonal Center) was used as a negative control. The numbers of
cell populations were estimated from fluorescence-positive cells
with background cells subtracted, and their proportions were
expressed as percentages of the analyzed cells.

Statistical analysis. Studied individuals were divided into four
groups: group A (less than 1 yr, $n = 15$); group B (from 1 to $2^{11}/_{12}$
yr, $n = 20$); group C (from 3 to $4^{11}/_{12}$ yr, $n = 14$); and group D
(from 5 to $13^5/_{12}$ yr, $n = 23$).

All the individuals were examined for Leu-2a$^+$ cells, Leu-3a$^+$
cells, Leu-4$^+$ cells, Leu-7$^+$ cells, and Leu-10$^+$ cells. On the other
hand, 65 individuals (group A nine, group B 19, group C 14,
and group D 23) for 2H7$^+$ cells, 70 individuals (group A 13,
group B 20, group C 14, and group D 23) for Leu-M3$^+$ cells, and
69 individuals (group A 12, group B 20, group C 14, and group
D 23) for HLA-DR$^+$ cells were examined.

All the values of lymphocyte subsets, monocytes, and the Leu-
3a$^+$/Leu-2a$^+$ ratios were expressed as arithmetic mean ± 2 SD.

Age-related differences among 4 groups were tested for significance by multiple comparison test using Scheffe's procedure (14). A probability of <0.05 was considered significant.

RESULTS

Age effect. Total Leukocyte and Differential Counts. As shown in Figure 1, remarkable decreases with age were found in the total leukocyte counts, the percentages of PBL and the absolute numbers of PBL among the four age groups.

According to the results of Scheffe's multiple comparison test (Table 1), an almost identical changing pattern was observed in the three items. That is, the youngest age group A had significantly high values as compared with the other three age groups, plus a significant difference in values between group B and group D was observed (pattern a). In the total leukocyte counts, however, there was no significant difference between group A and group B (pattern a').

Percentages of Lymphocyte Subsets and Monocytes and Leu-3a⁺/Leu-2a⁺ Ratios. As shown in Figures 2 to 4, the percentages of Leu-2a⁺ cells, Leu-7⁺ cells, and Leu-M3⁺ cells closely increased in parallel with age, whereas the percentages of Leu-3a⁺ cells, Leu-4⁺ cells, and 2H7⁺ cells clearly decreased with age. As a result, the Leu-3a⁺/Leu-2a⁺ ratio markedly decreased with age, while no prominent changes with age were shown in the percentages of Leu-10⁺ cells and HLA-DR⁺ cells.

According to the results of Scheffe's multiple comparison test (Table 2), three kinds of decreasing patterns with age were observed. In the percentage of Leu-4⁺ cells, a significant decrease
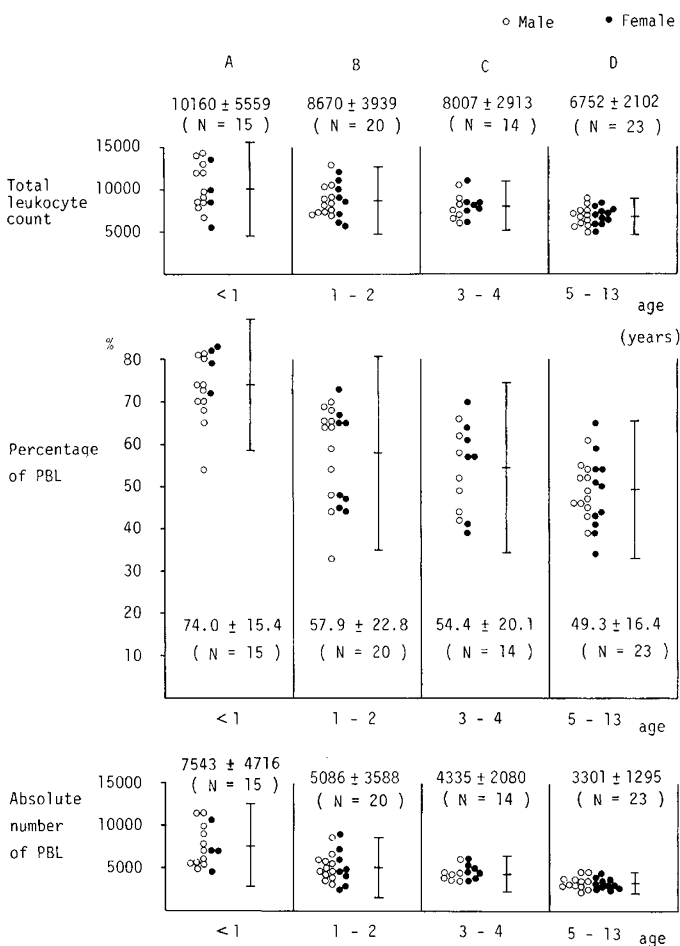
Table 1. *Results of Scheffe's multiple comparison test in total leukocyte and differential counts among four age groups*

Comparison of age group	A-B	A-C	A-D	B-C	B-D	C-D	Changing pattern
Total leukocyte count		*	†		*		a' ↘
Percentage of PBL	†	†	†		*		a ↘
Absolute number of PBL	†	†	†		‡		a ↘

* Significant difference between two age groups, $p < 0.05$.
† Significant difference between two age groups, $p < 0.001$.
‡ Significant difference between two age groups, $p < 0.01$.
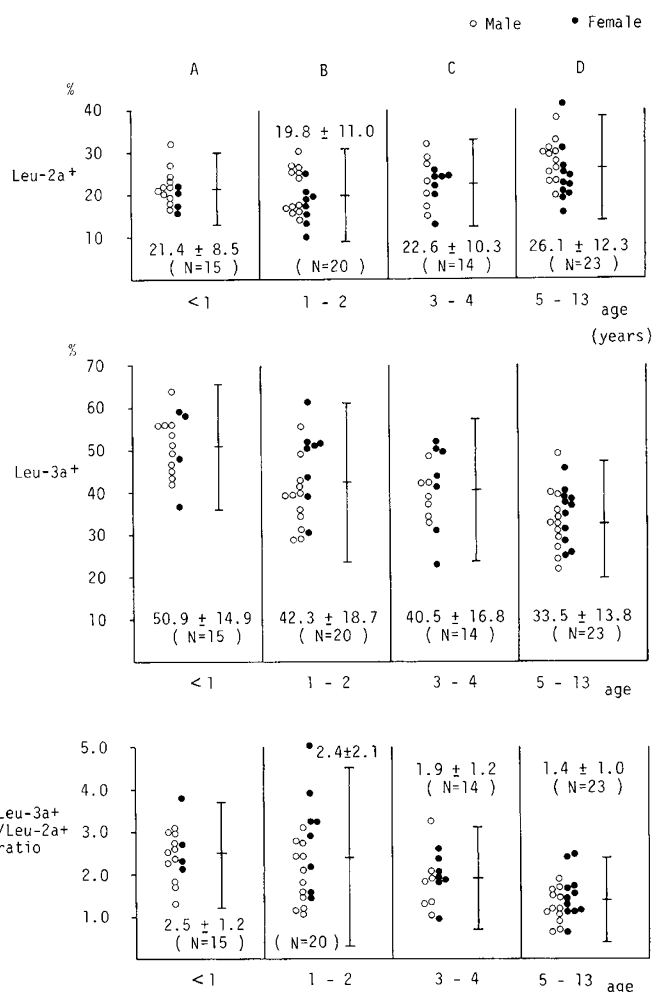

Fig. 2. Percentages of Leu-2a⁺ cells, Leu-3a⁺ cells, and Leu-3a⁺/Leu-2a⁺ ratios in healthy children. Data are presented as the mean ± 2 SD.

Fig. 1. Total leukocyte counts, percentages of PBL, and absolute numbers of PBL in healthy children. Data are presented as the mean ± 2 SD.

occurred after the age of 1 yr, but no significant difference among the three age groups older than 1 yr was shown (pattern b). The change in the percentage of Leu-3a⁺ cells belonged to pattern a. In the ratio of Leu-3a⁺/Leu-2a⁺ and the percentage of 2H7⁺ cells, significant decreases were observed between children less than 3 yr old (group A or B) and older than 5 yr (group D), though there were no significant differences between group A and group B (pattern c). On the other hand, three different kinds of increasing trend with age were seen in the percentages of Leu-2a⁺ cells, Leu-7⁺ cells, and Leu-M3⁺ cells.

Absolute Numbers of Lymphocyte Subsets and Monocytes. As shown in Table 3, distinct decreases with age were observed in the absolute numbers of Leu-2a⁺ cells, Leu-3a⁺ cells, Leu-4⁺ cells, Leu-10⁺ cells, 2H7⁺ cells, and HLA-DR⁺ cells, whereas no major change with age was indicated in the absolute numbers of Leu-7⁺ cells and Leu-M3⁺ cells.
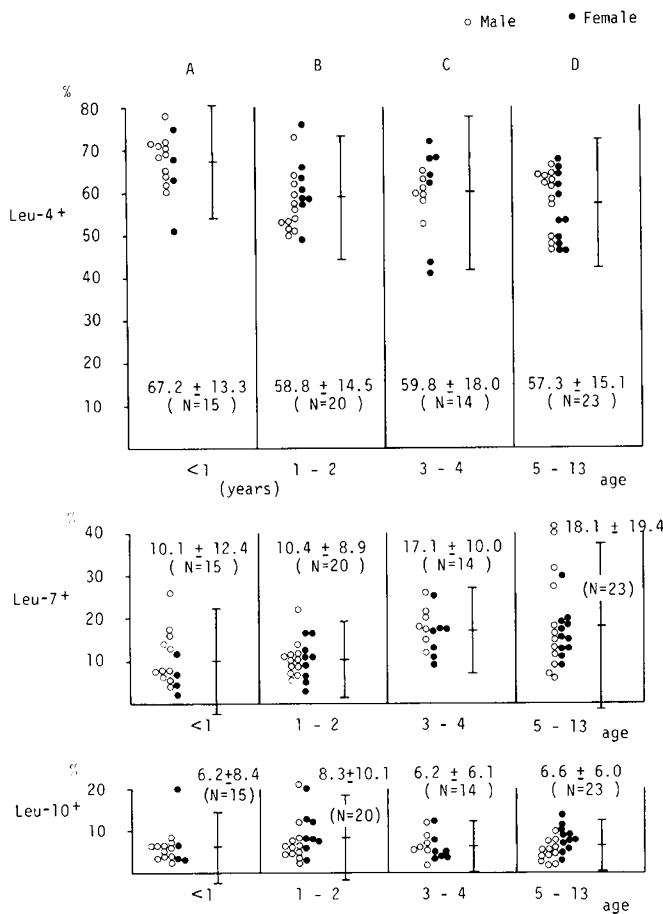
o Male • Female

Fig. 3. Percentages of Leu-4⁺ cells, Leu-7⁺ cells, and Leu-10⁺ cells in healthy children. Data are presented as the mean ± 2 SD.
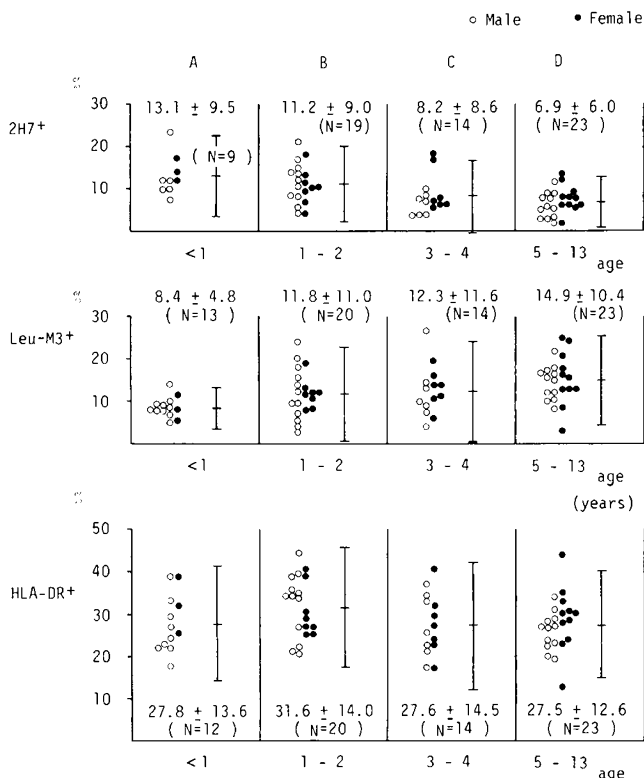
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Fig. 4. Percentages of 2H7⁺ cells, Leu-M3⁺ cells, and HLA-DR⁺ cells in healthy children. Data are presented as the mean + 2 SD.

Table 2. *Results of Scheffé's multiple comparison test in percentages of lymphocyte subsets and monocytes, and Leu-3a⁺/Leu-2a⁺ ratios among four age groups*

Comparison of age group	A-B	A-C	A-D	B-C	B-D	C-D	Changing pattern
Percentage of Leu-2a⁺ cells				*			↗
Percentage of Leu-3a⁺ cells	†	†	‡	*			a ↘
Leu-3a⁺/Leu-2a⁺ ratio			‡		‡		c ↘
Percentage of Leu-4⁺ cells	*	†	‡				b ↘
Percentage of Leu-7⁺ cells			†	*			↗
Percentage of Leu-10⁺ cells							
Percentage of 2H7⁺ cells			*		†		c ↘
Percentage of Leu-M3⁺ cells			*				↗
Percentage of HLA-DR⁺ cells							

* Significant difference between two age groups, $p < 0.01$.
† Significant difference between two age groups, $p < 0.05$.
‡ Significant difference between two age groups, $p < 0.001$.

The results of Scheffé's multiple comparison test were expressed in Table 4. The changing pattern in the absolute number of Leu-2a⁺ cells belonged to pattern b. Changing patterns in the absolute numbers of Leu-3a⁺ cells, Leu-4⁺ cells, 2H7⁺ cells, and HLA-DR⁺ cells were consistent with pattern a. The absolute number of Leu-10⁺ cells changed as pattern c.

Sex effects. No significant differences which could be attributed to sex were shown in the total leukocyte counts, the percentages, and the absolute numbers of PBL as well as of lymphocyte subsets and monocytes, or the Leu-3a⁺/Leu-2a⁺ ratios.

DISCUSSION

In the present study we demonstrated that the total leukocyte counts, the percentages, and the absolute numbers of PBL prominently decreased with age. In childhood the alterations of the normal total and differential leukocyte counts with age have already been established (15, 16), and our results are in agreement.

Age-related changes in cell-mediated and humoral immunity have progressively been studied during the normal aging of experimental animals and humans (17), but there are few studies on the effects of age on the lymphocyte subsets in childhood. No consistent observations of the aging of human lymphocyte subpopulations, *i.e* the T cell subpopulation identified by the sheep red blood cell rosette forming method and the B cell subpopulation identified by the cell surface immunoglobulin analysis method have been reported (18-24). The reasons for the discrepancies in the reported proportional and absolute number alterations of T and B cell subpopulations with age reported are suggested to be due to technical differences, gender differences, and age classification (25).

With regard to exclusion of the technical differences, recent development of the application of monoclonal antibodies and flow cytometry methods allows us to obtain more precisely analyzed data. Consistent with our results, Yachie *et al.* (26) reported that the proportion of OKT4⁺ (helper/inducer T) cells was high and the proportion of OKT8⁺ (suppressor/cytotoxic T) cells was low in cord blood T cells as compared with in normal adults, and OKT4⁺/OKT8⁺ ratios decreased with age and reached near adult levels around 3 to 5 yr of life. The results of this study were also consistent with a study by Abo *et al.* (9) in which it was shown that there was a progressive increase with age in the proportion of HNK-1⁺ (Leu-7⁺) cells.

The prominent decrease of B (2H7⁺) cells and the increase of monocytes (Leu-M3⁺ cells) with age was demonstrated, although we could not find the relationship between the functional alteration with age and these changes in this study. However, in adulthood there are various reports regarding the effect of age on

Table 3. *Age-dependent differences in absolute numbers of lymphocyte subsets and monocytes among four age groups**

Age group Age (yr) (*n*)	A <1 (15)	B 1–2 (20)	C 3–4 (14)	D 5–13 (23)
Leu-2a$^+$ cells	1614 ± 1131	1019 ± 990	1003 ± 779	865 ± 530
Leu-3a$^+$ cells	3817 ± 2570	2094 ± 1527	1798 ± 1372	1104 ± 616
Leu-4$^+$ cells	5026 ± 2996	2971 ± 2188	2641 ± 1739	1890 ± 872
Leu-7$^+$ cells	852 ± 1517	531 ± 704	717 ± 408	599 ± 649
Leu-10$^+$ cells	447 ± 478	443 ± 707	256 ± 226	219 ± 235
2H7$^+$ cells†	1149 ± 1278	591 ± 811	362 ± 476	233 ± 242
Leu-M3$^+$ cells‡	672 ± 667	590 ± 645	532 ± 530	485 ± 369
HLA-DR$^+$ cells§	2337 ± 2266	1625 ± 1363	1189 ± 784	910 ± 556

* Mean ± 2 SD.
† The number of subjects was 65 (A, nine; B, 19; C, 14; and D, 23).
‡ The number of subjects was 70 (A, 13; B, 20; C, 14; and D, 23).
§ The number of subjects was 69 (A, 12; B, 20; C, 14; and D, 23).

Table 4. *Results of Scheffé's multiple comparison test in absolute numbers of lymphocyte subsets and monocytes among four age groups*

Comparison of age group	A-B	A-C	A-D	B-C	B-D	C-D	Changing pattern
Absolute no. of Leu-2a$^+$ cells	*	*	†				b ↘
Absolute no. of Leu-3a$^+$ cells	†	†	†		*		a ↘
Absolute no. of Leu-4$^+$ cells	†	†	†		*		a ↘
Absolute no. of Leu-7$^+$ cells							
Absolute no. of Leu-10$^+$ cells			‡		‡		c ↘
Absolute no. of 2H7$^+$ cells	*	†	†		‡		a ↘
Absolute no. of Leu-M3$^+$ cells							
Absolute no. of HLA-DR$^+$ cells	‡	†	†		*		a ↘

* Significant difference between two age groups, $p < 0.01$.
† Significant difference between two age groups, $p < 0.001$.
‡ Significant difference between two age groups, $p < 0.05$.

T cell subpopulations identified by monoclonal antibodies (27–30), although the reason for the differences shown of in these results is unknown.

Regarding values of normal adults in our laboratory, no significant differences in the values of any of the lymphocyte subsets and monocytes examined between young adults (from 18 to 29 yr) and group D (from 5 to 13$^5/_{12}$ yr) were observed (data not shown). In addition, the levels of the lymphocyte subsets and monocytes of children more than 3 yr of age were considered to be consistent with those of young adults, since no significant differences in the values of any of the lymphocyte subsets and monocytes examined between group B (from 1 to 2$^{11}/_{12}$ yr) and group C (from 3 to 4$^{11}/_{12}$ yr) or between group C and D were shown, and the differences between group B and D were seen to be significant for most items in our results.

In order to evaluate diseases in children which may be associated with immunoregulatory changes, it is extremely important to determine the normal ranges of lymphocyte and monocyte subpopulations. PBL from individuals classified according to age must be analyzed using well-established and more specific methods. Therefore, it is essentially important for each individual laboratory to establish its own individual age-related normal reference ranges.

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Announcements

Annual Meeting

The American Pediatric Society and The Society for Pediatric Research announce the abstract deadline for the 1987 Annual Meeting (April 27–May 1, Disneyland Hotel, Anaheim, CA) has been set as *December 3, 1986.*

For further information contact: SPT—Debbie L. Wogenrich, The Society for Pediatric Research, Department of Pediatrics, UNM School of Medicine, Albuquerque, NM 87131, (505) 277-6629 **or** APS—Dr. Audrey K. Brown, Secreatry-Treasurer, Department of Pediatrics, Box 49, SUNY, Health Sciences Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, (718) 270-1692.

Pediatric Symposium

The IIIrd International Symposium on Pediatric Surgical and Neonatal Intensive Care will be held September 15–18, 1987 at the Hotel Sofitel, Paris.

Congress President: G. Huault. President Organizing Committee: F. Beaufils. President Scientific Committee: M. Dehan. *Information:* SOCFI-14, Rue Mandar–75002, Paris, France.

9th Summer Program in Methods of Immunologic Research and Diagnosis

This program is offered by the Ernest Witebsky Center for Immunology, University at Buffalo, State University of New York, June 7–20, 1987. It is a 2-wk program that consists of combinations of core and elective lectures, demonstrations, discussions, and practical laboratory exercises; participants personally carry out techniques at the bench. Open to postdoctoral level, supervisory senior technologists, and graduate students beyond the master's level.

For detailed program description contact James F. Mohn, M.D., Director, The Ernest Witebsky Center for Immunology, 210 Sherman Hall, Buffalo, New York 14214. (716) 831-2848. Inquiries should be received by 20 February 1987, to insure consideration for acceptance.

Pediatric Respiratory Disease

The National Heart, Lung, and Blood Institute, National Institutes of Health, has recently published an evaluation report on Pediatric Respiratory Disorders. This report relates to major advancements made in six categories of pediatric respiratory disorders and includes assessment of opportunities and needs in the epidemiology, diagnosis, management, and prevention of pediatric lung diseases. Copies of this report are available on request. Send reprint requests to Pushpa V. Thadani, Ph.D., Structure and Function Branch, DLD, NHLBI, Westwood Building, Room 6A07, Bethesda, MD 20892.