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Developmental changes in lymphocyte surface markers

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The aim of the study was to establish normal values for T_G and T_M cells in neonates and small babies who are more susceptible to infections than children and adults. For the same reason, the T- and B-lymphocyte ratios were also determined. The percentage of B- and T-cells in neonates was significantly lower, while their absolute number higher, than the adult value. The percentage of T_G cells was significanlty higher in neonates than in the other age groups. There was a significant decrease in the ratio of T_M cells as compared to adult values. Spontaneous thymidine uptake showed an inverse correlation with age, but the difference was not significant. There was a nearly identical response to PHA in the observed age groups, at least to the single dose of PHA applied.

The cell surface markers of lymphocyte populations and the relevance of their functional changes were extensively studied during the past decade. Recently, T-cell subsets became the prime targets of these investigations, revealing further heterogeneity of surface membrane components and functional characteristics [8, 35, 36, 39]. One of the most accessible approaches for the counting of T-lymphocyte subpopulations employs their capacity to form EA-rosettes. Moretta et al. [24, 26] described the T_G and T_M cells that possess specific receptors for the Fc-fragment of either the IgG (T_G cells) or the IgM molecules (T_M cells). These two lymphocyte populations differ markedly in their morphological, cytochemical and functional characteristics [16, 28]. The IgM-Fc "positive" subpopulation provides help for B-lymphocytes in their PWM-induced proliferative response and differentiation process. The T_G (IgG-Fc positive) cells, on the other hand, cannot provide help. Furthermore, upon contact with IgG, T_G cells inhibit the PWM-induced maturation of B cells into plasma cells [28, 29]. Although some authors ascribe this inhibitory effect in cord blood to cells other than T_G lymphocytes [18, 30], these two cell types seem likely candidates for the suppressor and inducer subpopulations described originally for mouse lymphocytes.

The ratio of T_G and T_M cells was found to be significantly altered in a number of primary and secondary immunodeficiencies [11, 17, 27, 40]. A close study of this question seems particularly relevant in neonates since most of the primary immunodeficiencies appear in the first few weeks of life. Further, newborn babies and especially prematures are "naturally"

more susceptible to viral, bacterial and fungal infections [10, 43]. In the present study we describe our findings in normal babies and children. Apart from T- and B-lymphocyte counts, the number and ratio of T_G and T_M cells was determined along with the PHA-induced proliferative response of their cells.

Materials and Methods

Subjects. Group 1 consisted of 0 to 6-day old mature, healthy newborns, Group 2 of 1 to 20-month old babies admitted to the hospital for minor surgical interventions. Two to 18-year old children hospitalized for similar reasons formed Group 3, while Group 4 consisted of 20 to 40-year old healthy blood donors. The number of subjects for each test is indicated in the legends to the figures.

Sera. Antibodies used in the EA-rosettes were obtained by repeated immunization of rabbits with ox red blood cells (haemolytic titre in the IgM-rich serum was 1 : 4000, in the IgG-rich serum 1 : : 16 000). IgM was obtained by precipitation with 33% ammonium-sulfate followed by Sephadex G-200 gel filtration. An IgG fraction was prepared by DEAE-cellulose (REANAL) chromatography following the salt-fractionation procedure [11, 33].

Purity of the IgG- and IgM-fractions was established by immunoelectrophoresis.

All sera were inactivated at 56°C for 1 hour.

Preparation of erythrocytes for EA-rosettes. Less than a week old ox RBCs were washed three times inPBS. A 2%RBC suspension was incubated at 37°C for 60 min with a subagglutinating concentration of anti ox-RBC IgG or anti ox-RBC IgM in buffer. Sensitized cells were washed in PBS [11, 24, 33].

Lymphocyte separation. Lymphocytes were separated from heparinized venous blood by Ficoll-Uromiro gradient centrifugation [4]. Monocytes were removed from the aliquots used for surface marker analysis by adherence to a glass surface followed by carbonyl iron treatment. Cell viability was determined using the trypan blue dye-exclusion method. T-cells used in Fc-receptor analysis were separated by the E-rosette technique [15].

B-lymphocytes were counted by direct immunofluorescent technique using FITC-labelled anti-human pig IgG (SEVAC) and also by a heterologous anti-B-cell serum used in an indirect fluorescence system.

T-lymphocyte numbers were assayed by the E-rosette technique [20] and by indirect immunofluorescence using a heterologous anti-T-cell serum.

EA-rosettes. 5×10^6/ml lymphocytes or separated T-cells were mixed with an equal volume of 2% sensitized ox-RBCs and centrifuged at 100 *g* for 5 min. Rosettes were counted after 1 hour incubation at 4°C. Prior to assaying the EA–IgM rosettes, the lymphocytes were incubated overnight in serum-free medium [11, 22, 24, 33].

Lymphocyte cultures. 1) 72 hour cultures were set-up from separated lymphocytes in TC-199 medium containing Hepes buffer and 20% inactivated normal human AB-positive serum. Cell concentration was 2×10^6/ml; 200 μl aliquots were cultured in Microtest II tissue culture plates with or without 5 μg/ml PHA (Wellcome) at 37°C for 56 hours. A further 16 hour incubation period followed the addition of 1 μCi ^{3}H-TdR (spec. activity: 150 mCi/mM, Amersham). Thymidine uptake was measured by liquid-scintillation counting of the TCA-precipitable material in an Intertechnique spectrometer. 2) In some instances whole-blood cultures were also set up. 0.2 ml of heparinized peripheral blood was diluted to 4 ml with the above medium and cells were cultured in the same way as in the case of separated lymphocytes.

Statistical analysis. Kruskal-Wallis H-test was used [46]. A p value of less than 0.01 was considered significant.

Results

Results are summarized in Figs 1 and 2. Since the absolute number of lymphocytes is high in neonates and small babies, the absolute number of lymphocyte subpopulations is also higher than later in life. The percentage of B-lymphocytes in neonates is significantly lower than in the other 3 age groups. After a few months the ratio of B-cells to total lymphocytes reaches adult values and it is at this time that the highest B-cell numbers are detected. T-cell ratios show an increase parallel to development, with low values in the first few weeks and a subsequent rise to adult values. T_M lymphocyte percentages show a similar tendency. Neonatal T_M percentage values are significantly lower than those of adults.

There was a revers tendency in the percentage of T_G-cells: it was highest

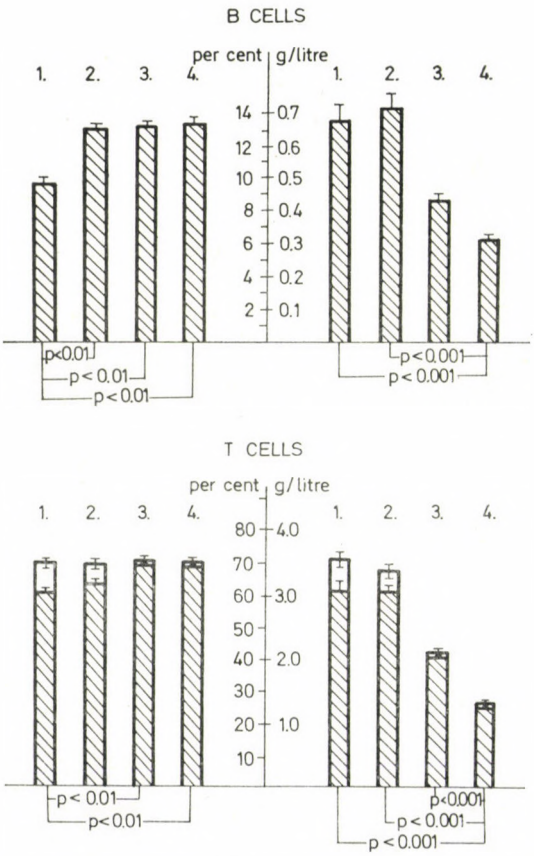

Fig. 1 Distribution of B and T lymphocytes according to age. Columns represent the mean percentage values or the mean absolute numbers with the standard error of the mean. Open columns represent the T-cell ratios as determined by a hetero-antiserum to T-cells. Number of subjects tested: 40

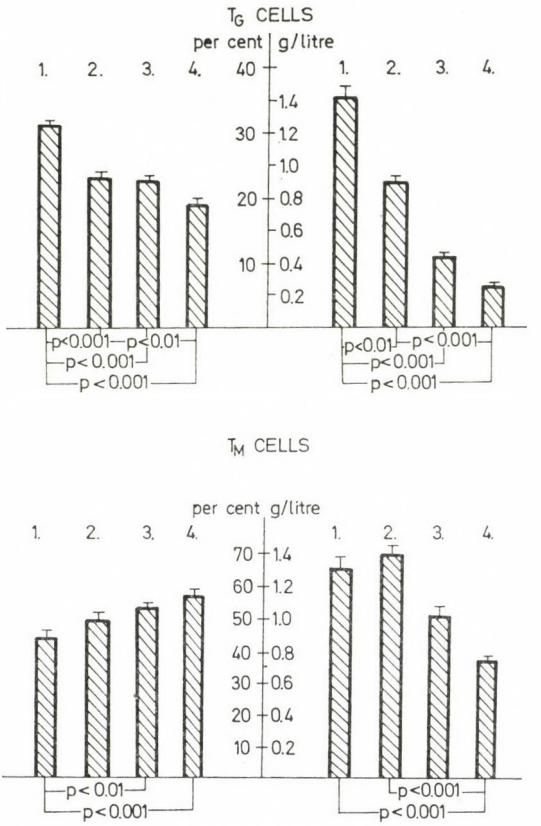

Fig. 2 Distribution of EA-IgG and EA-IgM rosette-forming cells in isolated T-lymphocytes Symbols as in Fig. 1. Number of subjects tested: 30

in the group of neonates and showed a gradual decline to adult values in small babies.

When we analysed the ratio of IgG–Fc and IgM–Fc receptor positive cells from unfractionated lymphocytes, we found results similar to those obtained from isolated T-cells. Significant alterations were found only in the youngest age-group.

Comparing the values from fractionated and unfractionated cells, it became apparent that while IgM–EA rosettes were only formed by T-cells, there were other cell types (B- and L-lymphocytes) involved in the formation of IgG–EA rosettes.

PHA-stimulated lymphocyte transformation showed no significant differences in the investigated age groups (Table I). Spontaneous metabolic activity as measured in unstimulated cultures, was also the same in the neonates as in older children or adults. Their PHA reactivity was nearly the same as that of the others. Whole blood cultures gave similar results. Comparing the stimulation values in

TABLE I

PHA*-induced lymphocyte transformation

	1.			2.		
	U	S	SI	U	S	SI
Separated lymphocytes 5×10^5 cells/well	1850 (±1090) n = 12			1710 (±1260) n = 20		
		40 040 (±28200) n = 12			50 300 (±31 700) n = 20	
			24 (±14) n = 12			42 (±39) n = 20
Whole blood microcultures 5×10^4 cells/well	640 (±370) n = 23			490 (±290) n = 14		
		45 830 (-32 450) n = 23			42 870 (±20 370) n = 14	
			96 (±90) n = 23			112 (±83) n = 14

	3.			4.		
	U	S	SI	U	S	SI
Separated lymphocytes 5×10^5 cells/well	1680 (±990) n = 26			1230 (±680) n = 21		
		71 920 (±58 060) n = 26			58 950 (±29 480) n = 21	
			57 (±53) n = 26			62 (±37) n = 21
Whole blood microcultures 5×10^4 cells/well	440 (±230) n = 6			320 (±170) n = 6		
		45 850 (±33 680) n = 6			36 090 (±25 670) n = 6	
			115 (±99) n = 6			118 (±54) n = 6

* PHA dose, 5 μg/ml
U: unstimulated, cpm
S: stimulated, cpm
SI: stimulation index

separated and whole blood cultures it appeared that stimulation indices rose considerably in the presence of autologous erythrocytes.

Discussion

Primary and secondary immunodeficiencies are characterized by varying degrees of T-cell dysfunction. These functional changes, however, are often not reflected in the number of peripheral T-cells or in the usual ***in vitro*** functional tests [41]. At the same time a shift in the ratio of T-cell subsets is demonstrable in the majority of cases [5, 11, 25, 27, 42]. One of the more widely used and accepted methods for the identification and isolation of T-cell subsets is the EA-rosette technique, outlined by Moretta et al. [16, 26, 28, 29].

The aim of this study was to establish the normal values of T_G and T_M cells in neonates and small babies who are more susceptible to infections than children and adults. For the same reason the T- and B-lymphocyte ratios were also determined. In contrast to the findings of other authors [2, 3, 6, 13, 14] we found that the percentage of B-cells in neonates is significantly lower than in adults. At the same time, however, the absolute number of B-cells is higher than the adult value. This is explained by the high initial WBC count and relative lymphocytosis in neonates. The percentage of B-cells reaches the adult values at a few months of age, while the absolute number attains the peak in this age group.

As far as T-cells are concerned the results in the literature are somewhat contradictory. Some authors found in neonates normal "adult" values [2, 10], while others reported reduced T-cell ratios [1, 3, 13, 14, 37, 43]. According to our findings the percentage of T-cells in neonates and young babies as determined by E-rosetting is significantly lower than in adults. It is around the age of 2 years when this value reaches that of the adults. When the number of T-cells was estimated with a heterologous anti-T-cell serum we found nearly identical percentages in all the examined groups. It seems therefore that noenates have a larger proportion of immature, E-rosette negative, T-cell pool, that may be demonstrated by other means, such as anti-T sera [1, 2, 38].

As regards the T_G—T_M cells of newborn babies, in most of the studies cord blood lymphocytes were tested [14, 18, 19, 23, 30, 31]. T_G cells were found to be increased in cord blood, showing a rapid decline to adult values by about 3 months. A reverse tendency was shown for T_M cells. We also found the percentage of T_G cells significantly higher in neonates than in the other 3 groups. Thus, the absolute number of T_G cells was highest in newborn babies and lowest in adults. These tests were carried out on peripheral blood lymphocytes.

There was a significant reduction in the ratio of T_M cells of neonates as compared to adult values. In young babies both the percentage and absolute number of T_M cells showed an

increase. In older children the percentage of these cells rose further, while their absolute number gradually declined. Similar results were obtained when the tests were carried out on unfractionated lymphocytes rather than separated T-cells. Naturally, these suspensions also contained some B-cells and other non-T lymphocytes as well as a few monocytes. In comparison to adult values both the relative and absolute number of EA-IgG positive cells was significantly higher in neonates in this test-system too. The ratio of IgM-Fc receptor positive cells, on the other hand, showed no significant difference from the adult value.

Looking at the results obtained with unseparated lymphocytes and isolated T-cells it appears that cells other than T-lymphocytes also take part in the formation of IgG-EA rosettes. In contrast, the IgM-EA rosettes in this test system are formed entirely from T-cells.

According to some of the earlier papers, B-cells and other non-T lymphocytes take no, or very little part in the formation of IgG- and IgM-EA-rosettes [44, 45]. Others, however, claim that under optimal conditions these receptors can be found on a significant proportion of the above-mentioned cells [12, 33]. Because of all this uncertainty, we feel that the enumeration of T_G and T_M cells should be carried out from isolated T-cells.

There are also somewhat conflicting reports concerning the lymphocyte responses to PHA in cord blood. Most authors found a higher spontaneous ^{3}H-thymidine uptake by neonatal lymphocytes [9, 24, 32, 34]. Maximum PHA responses were higher than those of the adult controls and were found at lower PHA concentrations (1—10 μg/ml). With higher PHA doses the responses were comparable in the two age groups [3, 6, 7]. In our experiments spontaneous thymidine uptake showed an inverse correlation with age, but the differences were not significant.

There was a nearly identical response to PHA in the observed age groups, at least to the single dose of PHA used in the experiments. Separated lymphocytes and whole blood cultures gave essentially identical results. Since this latter test requires only minute amounts of blood, ongoing studies are designed to establish the responses to various doses of mitogens as well as the kinetics of these responses.

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