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Distinct Subpopulations of Thymus-Dependent Lymphocytes

TRACING OF THE DIFFERENTIATION PATHWAY OF T CELLS BY USE OF PREPARATIVELY ELECTROPHORETICALLY SEPARATED MOUSE LYMPHOCYTES

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Summary. Thymus-dependent cells from thymus and peripheral lymphoid organs were preparatively separated by means of free flow electrophoresis into various subpopulations which were defined in terms of θ (theta) antigen content, negative surface charge, graft-versus-host (GvH) reactivity, hydrocortisone sensitivity, cell volume and morphological details. Most thymocytes in the cortex have a low negative surface charge, high θ antigen content, are hydrocortisone-sensitive and immuno-incompetent. On the basis of electronic cell sizing this group consists of a large population of 90 μm^3 cells (T_1) and a small population of 175 μm^3 cells (T_2), the latter being less hydrocortisone-sensitive than the former.

A minority of thymocytes resides in and around the medulla and has high negative surface charge, a medium θ antigen content, is hydrocortisone-resistant and reveals low GvH reactivity. These cells are medium sized (125 μm^3), electrophoretically bimodal (T_3 had a medium and T_4 a high negative surface charge) and on the basis of morphological criteria are metabolically more active than the thymocytes of low negative surface charge.

In the peripheral lymphoid organs, all thymus-dependent cells show high negative surface charge and have the lowest observed θ antigen content and the highest observed GvH reactivity. These cells fall into two populations of which one is 125 μm^3 with lower negative surface charge and the other is 90 μm^3 with a somewhat higher negative surface charge. These 125 μm^3 cells (T_4), which morphologically resemble the 125 μm^3 thymocytes, are less GvH-reactive than the 90 μm^3 (T_5) cells, which seem to be resting cells.

On the basis of these data, a possible sequence of steps in the maturation of T cells was constructed as follows: in the cortex of the thymus T_1 thymocytes are transformed into T_2 and these develop into T_3 and T_4 thymocytes which have higher negative surface charge, lower θ antigen content and are in an advanced stage of maturity. After further loss of θ antigen these cells, which are in the medulla,

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emigrate into the periphery and are finally transformed into highly immunocompetent T_5 cells possessing the highest observed negative surface charge.

INTRODUCTION

It is now firmly established that there are two classes of lymphocytes, the thymus-dependent (T cells) which mediate cell-bound immunity and thymus-independent (B cells) which mediate humoral immunity (Miller, Basten, Sprent and Cheers, 1971). Both types of cells originate in the bone marrow but they subsequently follow different differentiation pathways. T cells acquire immunocompetence by passage through the thymus, whereas B cells acquire immunocompetence without this thymic influence (Davies, 1969; Miller and Mitchell, 1969). The vital role of the microenvironment of the thymus in T-cell development is demonstrated by the fact that the deficiency in cell-bound immunity following neonatal thymectomy can be corrected by a thymus graft but not by the injection of thymocytes (Osoba and Miller, 1963). Even short exposure of such a thymus-deprived animal to a thymus graft results in a temporary restoration of GvH reactivity, which is lost again after removal of the graft (Stutman, Yunis and Good, 1972).

The major prerequisite for the investigation of distinct steps in T-cell development in the thymus is the isolation of cells at distinct stages of differentiation. The most frequently applied methods are the irradiation of animals with X-rays and treatment with hydrocortisone, both of which cause an involution of the cortex, which contains the majority of thymocytes, but leave the medulla almost unaffected (Murray, 1948; Warner, 1964). As a result of these treatments, it could be observed that, in contrast to the hydrocorticosteroid-sensitive cells, resistant cells in the medulla are phytohaemagglutinin (PHA) reactive (Elliott, Wallis and Davies, 1971; Blomgren and Svedmyr, 1971), have a larger cell volume similar to that of lymph node cells (Schlesinger and Golakai, 1967; Blomgren and Andersson, 1969, 1971), cooperate with B cells (Andersson and Blomgren, 1970), show GvH reactivity (Blomgren and Andersson, 1969), react in mixed lymphocyte culture (MLC) and cell-mediated lymphotoxicity (CML) (Blomgren and Svedmyr, 1971) and have less thymocyte-specific alloantigens but more H-2 antigens on the cell surface (Schlesinger and Golakai, 1967; Lance, Cooper and Boyse, 1970; Leckband and Boyse, 1971; Raff, 1971). Similar results were obtained by physical separation of thymocytes by means of density gradient techniques. Cells with a higher density revealed properties like those of the corticosteroid-sensitive cells, whereas cells with lower density behaved like the resistant population (Takiguchi, Adler and Smith, 1971; Konda, Nakao and Smith, 1972). By use of radioisotope labelling techniques it could be shown that the medullary thymocytes are continuously produced by proliferation of cortical thymocytes (Hinrichsen, 1965; Joel, Hess and Cottier, 1971). Taken together, these data indicate that the maturation of T cells in the thymus is characterized by profound functional and structural changes.

Evidence has appeared that these changes also affect the net negative surface charge. The majority of murine thymocytes possess a low negative surface charge density and a minority has high surface charge density, as could be shown by means of preparative electrophoresis with the free flow electrophoretic cell separator (Zeiller and Hannig, 1971; Zeiller, Holzberg, Pascher and Hannig, 1972). This majority is immunoincompetent and possesses a high amount of thymus-specific antigen. The minority is immunocompetent and shows a low thymus-specific antigen content (Zeiller *et al.*, 1972; Zeiller and Dolan,

1972). In contrast to the thymus, no T cells of low electrophoretic mobility (EPM) were found in the peripheral lymphoid organs (Zeiller, Hannig and Pascher, 1971a; Zeiller *et al.*, 1972; Nordling, Andersson and Häyry, 1972). These observations suggested that the maturation of T cells in the thymus is accompanied by an increase in negative surface charge. It was the main aim of the present experiments to investigate the functional and structural properties of T-cell subpopulations which can be physically separated on the basis of differing net negative surface charge density.

MATERIALS AND METHODS

Animals and cell suspensions

Adult spf DBA/2J/Bom and C3H/Tif/Bom were obtained from inbred colonies of Bomholtgaard, Denmark. C3D2F1 hybrids were produced by mating DBA females with C3H males. In all experiments, 14–18-week-old male C3H mice of F1 hybrids were used as cell donors. Spleens (Spl), thymus (Thy) and lymph nodes (LN) were pooled from fifty animals per experiment and cell suspensions prepared in TC Medium Puck G (Difco), supplemented by 1 per cent (w/v) bovine albumin (Armour Pharmaceutical Company, Chicago). Blood (Bl) leucocytes were obtained by Ficoll gradient centrifugation (Böyum, 1968). Before electrophoretic separation the cell suspensions were washed twice with TC Medium Puck G and twice with Zeiller–Hannig's electrophoretic separation medium (Hannig and Zeiller, 1969). The final cell suspensions were filtered through a gauze layer and the required cell concentrations were adjusted.

Cell electrophoresis

The cells were separated in an improved model of a free flow electrophoretic cell separator based on the commercially available FF IV (DESAGA, Heidelberg; Bender and Hobein, Munich, Germany). The procedure of this method has been described in detail elsewhere (Hannig and Zeiller, 1969; Zeiller, Liebich and Hannig, 1971b). The present separations were performed at 210 mA with an electric field of 80–90 V/cm, at a temperature of 5–7° and a buffer flow rate of 550 ml/hour. Five-millilitre cells suspension ($4–6 \times 10^7$ cells/ml) were separated per hour. The mean exposure time of individual cells to the electric field was approximately 200 seconds. Equal volumes of each fraction of separated material were collected in 1 ml TC Medium Puck G containing 1 per cent bovine albumin. The total cell frequencies in the fraction tubes were determined as described recently (Zeiller *et al.*, 1971b). The viability of the cells in the fraction tubes was determined by the Trypan Blue exclusion test and was in the range of 90 per cent viable cells in the individual fractions.

Electronic cell volume determination

The volume of separated lymphocytes was measured by use of a particle volume analyser developed by AEG–Telefunken, Ulm, according to the design of Thom, Hampe and Saňerbrey (1969). The equipment used consisted of a particle detector MS DK 1-1105/1, a preamplifier MS KV 1-1105/1, a power supply MS HS 4-1105/1, and a multichannel analyser MS VAS 1105 (Telefunken, Ulm, Germany). The detector capillary had a diameter of 70 ± 10 μm^3. The cell size corresponding to a given threshold was obtained by calibrating the instrument with rat erythrocytes (effective volume = 54 μm^3, spherical volume = 31·6 μm^3) and Latex particles (Dow Chemical Latex number 41955, 2·02 μm diameter).

The cell size histograms were plotted by means of a Hewlett Packard X-Y-Recorder Model number 7034A. The advantage of this electronic particle size analyser is its ability to measure cell volumes in a homogeneous electric field thus eliminating coincidence error (Thom *et al.*, 1969; Thom and Kachel, 1970), which in normal Coulter instruments is the cause of artificial positive skewness of volume distribution curves. Thus

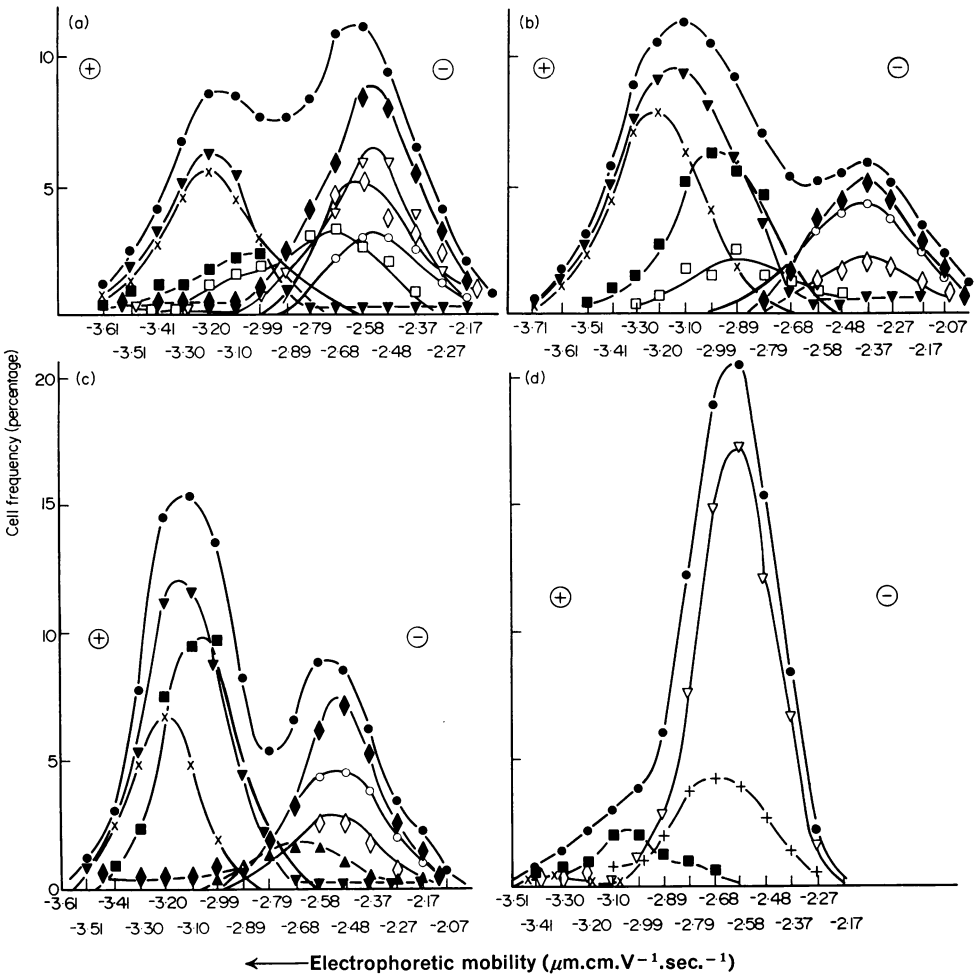

FIG. 1. Electrophoretic separation profiles of C3H mouse leucocytes from (a) spleen, (b) lymph node, (c) blood and (d) thymus. (●) Total nucleated cells; (◆) MBLA positive cells; (▼) θ C3H positive cells; (▽) surface immunoglobulin-positive cells. The other symbols correspond to those used in Table 2 to indicate cells with defined volumes. The dashed parts of the curve could not be experimentally established due to overlapping.

Thom's equipment produces Gaussian size distribution curves with homogeneous populations such as erythrocytes or latex particles. When asymmetric curves nevertheless appear they indicate the presence of subpopulations overlapping in size. They were analysed by construction of Gaussian curves as described elsewhere (Bhattachraya, 1967). The validity of this method is supported by the fact that the subpopulations so obtained were

relatively homogeneous in certain defined electrophoretic fractions, as indicated in Fig. 1a–d. The curves obtained were transposed onto Gaussian sum frequency paper. The relative frequency of volumetrically defined cells in the individual fractions of the electropherograms was determined by planimetric evaluation of the Gaussian curves in the size histograms.

Electron-microscopic preparations

Electron-microscopic preparations of electrophoretically separated cells were prepared as described (Zeiller *et al.*, 1971b) and the sections were viewed in a Siemens Elmiscop 101.

Spleen weight assay

The GvH reactivity of lymphocytes was qualitatively and quantitatively determined by the Simonsen spleen weight assay according to recommendations in the literature (Michie, 1969). Distinct numbers (geometrically increasing) of unseparated and electrophoretically separated C3H (H-2k/2k) lymphocytes obtained from Thy, LN and Spl were adjusted to a constant total number of cells by addition of C3D2F1 (H-2k/2d) hybrid mice Spl cells and the resulting cell suspensions were injected intraperitoneally (0·1 ml/mouse) into newborn C3D2F1 hybrid mice. Ten days later the recipients were killed and the Spl indices calculated (Michie, 1969). The GvH reactivity of the donor cells was analysed in terms of linear regression functions of the log spleen indices. The difference in GvH reactivity between the two distinct cell samples was evaluated according to the equation:

$$Px = \bar{x}_1 - \bar{x}_2 - \frac{\bar{y}_1 - \bar{y}_2}{b_{yx}}.$$

($\bar{x}_{1,2}$ = total means of parental cells injected, $\bar{y}_{1,2}$ = total means of log spleen indices, b_{xy} = mean regression coefficient.)

Antisera

Anti-θ C3H antiserum (titre 64) kindly provided by Dr Mitchison was diluted 1/2 with PBS before use. Anti-MBLA (mouse B cell-specific antigen) antiglobulin was prepared as described recently (Zeiller and Pascher, 1973). Antisera and normal mouse sera were previously heat-inactivated and properly absorbed with C3H erythrocytes. Rabbit anti-mouse immunoglobulin was a gift from Dr Baudner, Behringwerke. FITC Fluorescein isothiocyanate (FITC) labelled goat anti-rabbit γ-globulin lyophilisate (Lot 3907) was obtained from Cappel Laboratories, Downington. In order to obtain optimal staining conditions, the concentrations of the anti-immunoglobulins were adjusted using the method described by Wick and Beutner (1970).

Testing of surface antigens

The θ C3H and MBLA antigen-positive cells in the electrophoretically separated fractions were determined by cytotoxicity testing and the cytotoxic indices were calculated as described recently in detail (Zeiller and Pascher, 1973). The θ antigen content of distinct separated T cells was compared in the following way. Constant volumes of packed cells were incubated with increasing volumes of inactivated anti-θ antiserum (1/20, 1/40, 1/80, 1/160, 1/320). After incubation for 30 minutes at room temperature, the cells were removed by centrifugation and then cytotoxicity of the supernatant was tested on a

constant number of thymocyte target cells (15×10^6 cells/ml) as described by Zeiller and Dolan (1972).

Indirect immunofluorescence staining using viable cells was performed according to Möller (1961). After staining and washing, the cells were immediately examined by transmitted light in suspension under cover glass sealed with paraffin. A Zeiss fluorescence microscope was used, equipped with an Osram (Germany) HBO 200 W/4 mercury lamp, an exciter filter BG/12 and a Barrie filter 53/44. The cells in each field were counted after examination under the phase-contrast.

RESULTS

ELECTROPHEROGRAMS OF LEUCOCYTES FROM SPL, LN, BL AND THY

The electropherograms of the leucocytes from C3H and C3D2F1 mouse Spl, LN, Bl and Thy can be divided into approximately sixteen fractions within an electrophoretic mobility range of $-2{\cdot}07$ to $-2{\cdot}79$ μm.cm.V^{-1}.sec.$^{-1}$ (low mobility range (LMR)) and $-2{\cdot}89$ to $-3{\cdot}61$ μm.cm.V^{-1}.sec.$^{-1}$ (high mobility range (HMR)). In Bl and LN most of the leucocytes were in the HMR, Spl possessed approximately equal numbers of cells in both EPM ranges and the majority of the thymocytes were found in the LMR. No difference was observed between C3H and C3D2F1 mouse leucocyte electropherograms.

The total cell loss during electrophoretic separation did not exceed 10 per cent and the viability of the cells in the individual separated fractions was >90 per cent. The electropherograms obtained were highly reproducible as described (Zeiller *et al.*, 1972).

DISTRIBUTION OF T- AND B-CELL-SPECIFIC SURFACE MARKERS ON SEPARATED LEUCOCYTES FROM SPL, LN, BL AND THY

The cytotoxic indices obtained with anti-θ C3H antiserum and anti-MBLA antiglobulin for the individual fractions of electrophoretically separated C3H cells were determined as described (Zeiller and Pascher, 1973). The θ antigen was present on all thymocytes and on 80, 70 and 60 per cent of the leucocytes of high EPM in LN, Bl and Spl respectively, but was not present on leucocytes of low EPM in the peripheral organs (Fig. 1a–d). MBLA was shown to exist on approximately 70 per cent of the cells in the LMR of the three peripheral lymphoid organs but on not more than 5–10 per cent of cells in the HMR fractions. The investigation of surface immunoglobulin by use of the indirect immunofluorescence technique revealed surface immunoglobulin on <0·5 per cent of thymocytes and on <2 per cent of Spl and LN cells in the HMR. A considerable number of Spl and LN cells in the LMR carried immunoglobulin receptors but more cells carried MBLA. In the medium EPM range considerable numbers of θ antigen, MBLA and immunoglobulin-negative cells were detected in Spl (10–120 per cent), LN (5–10 per cent) and Bl (10–15 per cent).

COMPARISON OF THE θ ANTIGEN CONTENT OF SEPARATED SPL AND THY LEUCOCYTES

Lymphocytes from C3H Spl and Thy were electrophoretically separated and the fractions pooled within the EPM ranges of $-3{\cdot}30$ to $-3{\cdot}61$ (H_I), $-2{\cdot}89$ to $-3{\cdot}20$ (H_{II}), $-2{\cdot}17$ to $-2{\cdot}79$ (L_{III+IV}) μm.cm.V^{-1}.sec.$^{-1}$. Constant numbers of separated cells were

absorbed with increasing amounts of anti-θ antiserum and the cytotoxic activity of the supernatant was tested on constant numbers of thymocyte target cells. Fig. 2 shows that thymocytes from the L_{III+IV} fractions possess the greatest anti-θ antibody binding capacity; this capacity decreases in H_{II} and is still less in H_I. The binding capacity of unseparated thymocytes was similar to that of H_{II} thymocytes. The smallest binding capacity was observed in the Spl H_I and H_{II} fractions. Since the Spl H_{II} fractions in contrast to the Spl H_I fractions were contaminated by a considerable amount of non-thymus dependent cells (Fig. 1a) their θ antigen binding capacity observed was considered invalid. Spl L_{III+IV} fractions showed no binding of anti-θ antiserum.

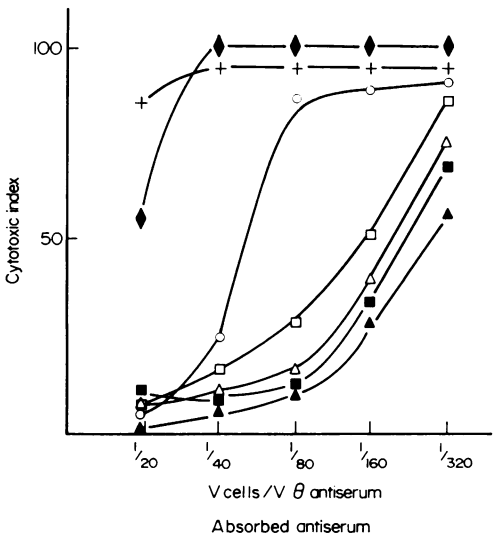

FIG. 2. Cytotoxicity of anti-θ antiserum which has previously been absorbed with increasing amounts of electrophoretically separated Spl cells: (○) H_I cells; (◆) H_{II} cells; (+) L_{III+IV} cells; and thymocytes: (□) H_I cells; (■) H_{II} cells; (▲) L_{III+IV} cells; (△) unseparated thymocytes.

GVH REACTIVITY OF SEPARATED LEUCOCYTES FROM THY, LN AND SPL

In order to test qualitatively and quantitatively the GvH reactivity of separated Thy, LN and Spl C3H mouse leucocytes, the cells were pooled into four separate groups within the EPM fractions $-3{\cdot}30$ to $-3{\cdot}61$ (H_I), $-2{\cdot}89$ to $-3{\cdot}20$ (H_{II}), $-2{\cdot}58$ to $-2{\cdot}79$ (L_{III}) and $-2{\cdot}17$ to $-2{\cdot}48$ (L_{IV}) $\mu m.cm.V^{-1}.sec.^{-1}$ Linearly rising regression lines of the spleen indices could be obtained with unseparated leucocytes as well as with electrophoretically separated leucocytes in the fractions H_I and H_{II} of all three organs. The L_{III} fraction of Spl and LN (but not of Thy) also showed some reactivity. None of the L_{IV} fractions showed any reactivity. These results agree closely with the distribution of θ-positive cells. Fig. 3a–c clearly demonstrate that the GvH reactivity decreases continuously from the H_I to the L_{III} fractions. Table 1 compares quantitatively the GvH reactivity of the different samples with that of normal unseparated thymocytes, which was given the base evaluation of 1. Before separation, Spl leucocytes were nearly one third less active than LN but considerably more active than Thy leucocytes. After separation, the Spl and LN leucocytes in the H_I fraction showed identical activity and this was nearly three times that of unseparated Spl. In the H_{II} fraction of Spl and LN the

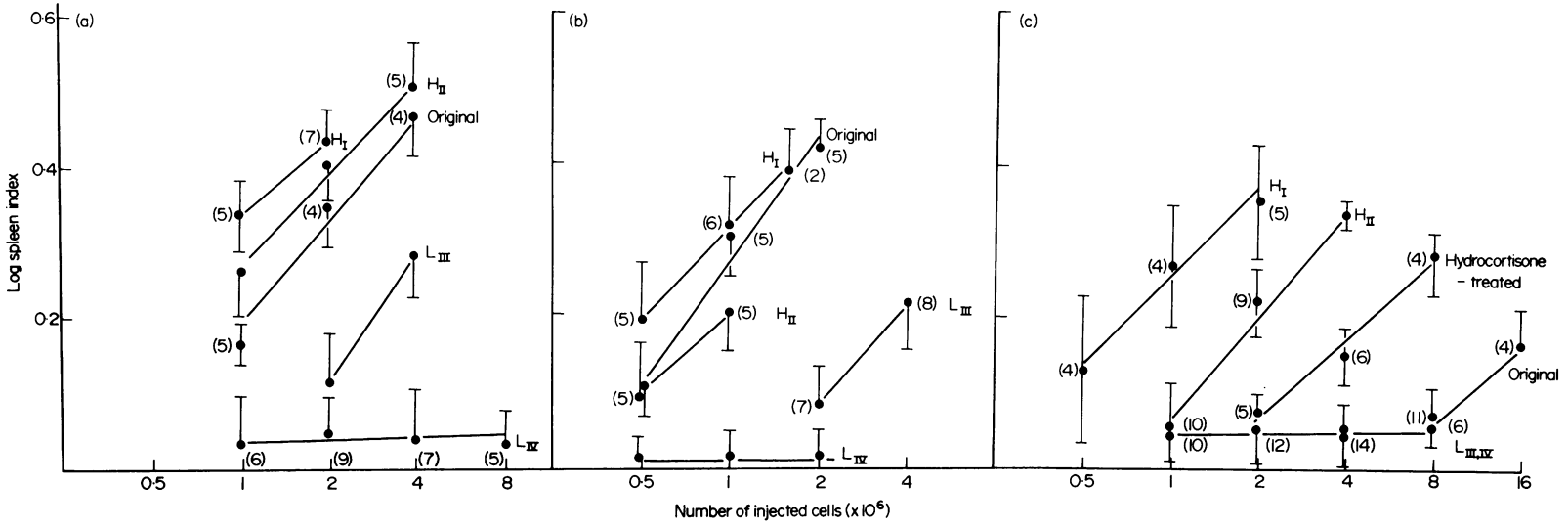

FIG. 3. Regression lines of GvH reactivity of electrophoretically separated lymphocytes from C3H mice: (a) spleen (b) lymph node and (c) thymus. The numerical values given in parentheses correspond to 90 per cent confidence limits. The labels on the curves H_I, H_{II}, L_{III} and L_{IV} are explained in detail in the text.

TABLE 1

COMPARISON OF THE GvH REACTIVITY OF ELECTROPHORETICALLY SEPARATED AND UNSEPARATED LYMPHOID CELLS FROM MOUSE SPLEEN, LYMPH NODE, THYMUS AND CORTISONE-TREATED THYMUS. THE ACTIVITY OF THE UNSEPARATED THYMUS WAS ARBITRARILY GIVEN A VALUE OF 1 FOR PURPOSES OF COMPARISON WITH THE OTHER CELLS

Lymphoid cells	Electrophoretic mobility (μm.cm.V^{-1}.sec.$^{-1}$)	Relative GvH reactivity			
		Spleen	Lymph node	Thymus	Hydrocortisone-treated thymus
Unseparated		21	28	1	4
H_I	−3·30 to −3·61	38	40	28	—
H_{II}	−2·89 to −3·20	27	21	10	—
H_{III}	−2·58 to −2·79	7	5	None	—
H_{IV}	−2·17 to −2·48	None	None	None	—

GvH reactivity was lower. In the Thy, neither electrophoretic separation nor hydrocortisone treatment could produce a high enrichment of GvH reactive leucocytes to compare with that in the peripheral organs. Hydrocortisone-treated thymocytes produced an increase in GvH reactivity four times that of untreated thymocytes, but the number of cells was too small for GvH testing after electrophoretic separation. In untreated animals, separated thymocytes in the H_I and H_{II} fractions were much less active than those in the periphery but twenty-eight and ten times higher respectively than unseparated thymocytes.

CELL SIZE OF SEPARATED LEUCOCYTES FROM SPL, LN AND BL

In the fractions of separated leucocytes from LN, Spl and Bl, five populations could be evaluated by electronic cell sizing with the electronic particle volume analyser (Fig. 1a–c, Fig. 4). The majority of cells in the HMR is represented by two electrophoretically normally distributed populations with mean volumes of 90 ± 15 μm^3 and 125 ± 20 μm^3, both of

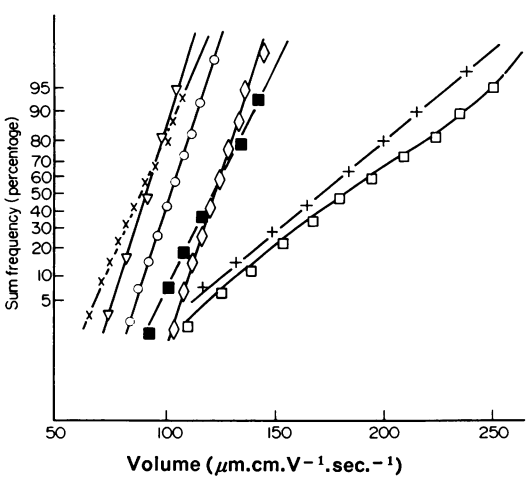

FIG. 4. Volume histograms of electrophoretically separated leucocytes from Thy, LN, Spl and Bl transposed onto Gaussian sum frequency paper. The symbols correspond to those used in Fig. 1a–d and in Table 2.

which groups show Gaussian distribution curves in the volume histograms (Fig. 4). From Fig. 1a–c it can be seen that the cell population with smaller volume possesses higher EPM than that with larger volume. The relative proportions of these cell populations varied in the different organs. Spl and LN possessed more smaller cells than larger cells in the HMR whereas in the Bl the larger cells were more frequent than the smaller cells (Table 2).

In the LMR, the majority of cells fell into two populations with mean volumes of $105 \pm 15\ \mu m^3$ and $125 \pm 15\ \mu m^3$ which were normally distributed in the electropherograms (Fig. 1a–c) and size histograms (Fig. 4). However, in contrast to populations in the HMR, they had identical electrophoretic mobility. In LN and Bl the smaller cells were present in higher numbers than the larger cells, whereas in the Spl, the larger cells were more frequently observed. In the medium EPM range in all three organs a population of larger cells (125–250 μm^3) was detected, which was homogeneous neither in EPM (Fig. 1a–c) nor in size (Fig. 4). These large cells represented 20, 10 and 8 per cent of leucocytes in the Spl, LN and Bl respectively (Table 2).

TABLE 2

PERCENT DISTRIBUTION OF VOLUMETRICALLY AND ELECTROPHORETICALLY DEFINED LYMPHOCYTES IN VARIOUS LYMPHOID ORGANS OF C3H MICE

Cell class (symbols used in figures)	Cell volume (μm^3)	Electrophoretic mobility ($\mu m.cm.V^{-1}.sec.^{-1}$)	Percentage of cells found in:				
			Spleen	Lymph node	Blood	Thymus	Hydrocortisone-treated thymus
(×)	90± 20	$-3{\cdot}20\ ^{-2{\cdot}89}_{-3{\cdot}61}$	28	36	21	2	11
(◆)	125± 20	$-2{\cdot}99\ ^{-2{\cdot}68}_{-3{\cdot}41}$	15	25	35	—	—
(■)	125± 20	−2.48 to −3·41	—	—	—	7	70
(□)	125−250	−2·48 to −3·20	20	10	8	—	—
(○)	105± 15	$-2{\cdot}57\ ^{-2{\cdot}07}_{-2{\cdot}99}$	14	20	22	—	—
(◇)	125± 15	$-2{\cdot}57\ ^{-2{\cdot}07}_{-2{\cdot}99}$	23	9	14	—	—
(●)	175± 55	$-2{\cdot}68\ ^{-2{\cdot}27}_{-3{\cdot}10}$	—	—	—	24	19
(▽)	90± 15	$-2{\cdot}58\ ^{-2{\cdot}27}_{-2{\cdot}99}$	—	—	—	67	—

In the HMR fractions the number of θ-positive cells coincided with the number of cells with mean volumes of 90 μm^3 and 125 μm^3. In the fractions of the LMR the number of cells with mean volumes of 105 μm^3 and 125 μm^3 agreed closely with the number of θ-negative and MBLA-positive cells. The number of cells in the medium EPM fractions with volumes between 125 μm^3 and 250 μm^3 was similar to that of θ- and MBLA-negative cells.

CELL SIZE OF SEPARATED THYMOCYTES FROM NORMAL AND HYDROCORTISONE-TREATED ANIMALS

In the electropherograms of thymocytes approximately 90 per cent of the cells were found in the LMR (Fig. 1d). The analysis of the cell volumes of the separated cells revealed four populations. Two populations each homogeneous in size (Fig. 4) were

located in the EPM range of $-2{\cdot}27$ to $-2{\cdot}99$ μm.cm.V^{-1}.sec.$^{-1}$ (Fig. 1d). One consisted of small cells with a mean volume of 90 ± 15 μm^3 which represent 65 per cent and the other of large cells with a mean volume of 175 ± 55 μm^3 which represent 24 per cent of all thymocytes (Fig. 4, Table 2). In the HMR, two cell populations with mean volumes of 90 ± 20 μm^3 (2 per cent of total thymocytes) and 125 ± 20 μm^3 (7 per cent of total thymocytes) and which follow Gaussian distribution curves in their size histograms could be observed. These populations have also been described in the HMR of the peripheral organs (Fig. 1a–c). Here, however, in contrast to the HMR cells of the periphery, the cells with a mean volume of 125 ± 20 μm^3 seem to be electrophoretically heterogeneous, which became more evident after treatment of the cell donors with 2 mg hydrocortisone per 20 gram body weight over 24 hours. This treatment completely eliminated the 90 μm^3 thymocytes in the LMR, thus changing the distribution curve in favour of the HMR thymocytes (Fig. 5).

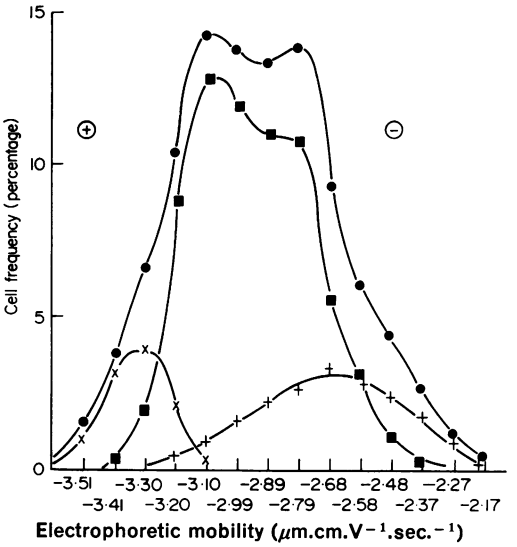

FIG. 5. Electrophoretic distribution profile of thymocytes from hydrocortisone-treated C3H mice. The symbols correspond to those used in Table 2 for the description of cells with defined volumes.

On the basis of Table 2 it is inferred that the hydrocortisone treatment affected not only the 90 μm^3 thymocytes of low EPM but also those with a mean volume of 175 μm^3. Their number decreased in relationship to cells of higher EPM with volumes of 125 μm^3 and 90 μm^3, whereas the numerical relationship between the two latter groups remained unchanged. This behaviour suggests that thymocytes of low EPM are hydrocortisone-sensitive whereas thymocytes of high EPM are more or less hydrocortisone-resistant. Furthermore, the large thymocytes in the LMR seem to be less sensitive than the smaller cells.

ULTRASTRUCTURE OF SEPARATED LEUCOCYTES FROM PERIPHERAL ORGANS AND THYMUS

In the electronmicroscopic investigation of electrophoretically separated cells, main interest centred on the θ-positive cells found in the Thy and in the HMR of LN and Spl.

TABLE 3

MORPHOLOGICAL DETAILS OF THYMOCYTES AND LEUCOCYTES (SPLEEN, LYMPH NODE) IN DEFINED REGIONS OF THE ELECTROPHORETIC DISTRIBUTION PROFILES OBSERVED BY ELECTRON MICROSCOPE

Symbols used in figures	Electrophoretic mobility (μm.cm.V^{-1}.sec.$^{-1}$)	Cell diameter (μm)	Nucleus	Chromatin	Nucleolus	Cell surface	Cytoplasm	Ribosomes	Mitochondria	ER	Inclusion
Thymus											
(▽)	−2·58	5·6	Oval regular	Compact	Absent	Irregular projections	Small	Abundant	Few	Absent	Absent
(+)	−2·68	6·9	Irregular	Compact	Present	Uneven projections	Scanty	Abundant	Few	Absent	Absent
(■)	−2·95	6·2	Irregular deeply invaginated	Prominent	Marginal	Perpendicular excavations	Medium to large	Abundant	Numerous	Well developed	Lysosomes, fat and myelin bodies
Spleen lymph node											
(◇)	−2·57	6·2	Oval regular	Compact	Rare	Uneven projections	Small	Abundant	Few	Absent	Absent
(■)	−2·99	6·2	Oval irregular	Marginal few	Present	Smooth	Large	Abundant	Numerous	Present	
(×)	−3·20	5·6	Oval irregular	Compact	Prominent	Smooth	Small	Abundant	Few	Absent	Rare lysosomes

The cells appearing on the photographic plates were grouped according to cell diameter and morphology and the frequencies in these groups were compared to those determined by electronic cell sizing. The cytological details are summarized in Table 3. In several cases, a clearcut correspondence could be found. In Thy LMR, the 90 μm^3 and 175 μm^3 thymocytes were quite similar in ultrastructure. The major difference was the presence of a nucleolus in the large cells (Table 3) which indicates less activity in the former and a high RNA metabolism in the latter cells. Cytologically distinct from these two cell groups was a group of thymocytes with higher EPM. They showed a more marginally localized chromatin and numerous mitochondria, endoplasmic reticulum and cytoplasmic enclosures were present. Thus they seemed to be metabolically very active cells.

Morphologically similar cells were found in the HMR of LN and Spl which, in contrast to thymocytes, had smooth surfaces. In their structure they differed significantly from cells in the LMR which carry MBLA and surface immunoglobulins, and they were found more frequently in the faster than in the slower HMR fractions. The cells in the faster HMR fraction were distinguished from those in the slower by a smaller cytoplasm, the presence of fewer mitochondria and the absence of endoplasmic reticulum. This type of cell was seldom detected in the Thy. It is most likely that these cells represent the 90 μm^3 population in the HMR of peripheral lymphoid organs. In the medium EPM range of LN, Spl and Bl, which contain the most θ- and MBLA-negative cells, a considerable number of granulocytic cells, monocytes, histiocytes, reticulum cells and macrophages could be identified.

DISCUSSION

Leucocytes from Thy, LN, Spl and Bl were electrophoretically separated by means of free-flow electrophoresis as described elsewhere into bimodal distributions representing ranges of high EPM (HMR) and low EPM (LMR) (Hannig and Zeiller, 1965; Zeiller and Hannig, 1971; Zeiller *et al.*, 1971a, 1972; Zeiller and Dolan, 1972; Nordling *et al.*, 1972). In agreement with these results the separated cells were characterized by the following pattern: in Bl and LN the majority of the leucocytes was found in the HMR, in the Thy 90 per cent of the cells were found in the LMR, whereas in the Spl about equal amounts of cells were found in the HMR and LMR.

The T-cell-specific surface marker θ antigen (Raff, 1969) was detected on the majority of LN, Spl and Bl cells in the HMR fractions only. The B-cell-specific surface marker MBLA (Raff, Nase and Mitchison, 1971) was found on the majority of leucocytes in these organs in the LMR but on <5 per cent of cells in the HMR. All cells in the Thy electropherograms were MBLA-negative but >95 per cent were θ antigen positive.

Indirect immunofluoresence revealed in agreement with Nordling *et al.* (1972) that the distribution pattern of surface immunoglobulin-positive cells was parallel to that of MBLA-positive cells, although the actual number was lower. The considerable overlap of immunoglobulin-positive cells to the HMR, as reported by van Boehmer, Shortman and Nossal (1973) was not observed. θ- and MBLA-negative cells in the medium EPM fractions of peripheral lymphoid organs were considered to be nonlymphoid, since they were not attacked by antilymphocyte antiglobulin (ALG) (Zeiller and Pascher, 1973). On the basis of morphological criteria they were granulocytes, macrophages, histiocytes and monocytes.

This distribution of T and B cells in the electropherograms of peripheral lymphoid

organs was further confirmed through testing of antibody response and cell-bound immunity reactions (Hannig and Zeiller, 1969; Zeiller *et al.*, 1971a, 1972; Nordling *et al.*, 1972). In Thy as well as in peripheral lymphoid organs the immunocompetence of T cells as shown by GvH and helper cell reactivity could only be detected in the fractions of the HMR (Zeiller *et al.*, 1972). However in the Thy, in contrast to peripheral lymphoid organs, most of the θ-positive cells were found in the LMR and were immunoincompetent.

The further investigation of the relationship between these Thy-dependent cells, which differ in negative surface charge, immunocompetence and in their distribution in the different organs, was the main purpose of the present work.

Recently the existence of two thymocyte populations in the Thy was demonstrated: hydrocortisone- and radio-sensitive cells in the cortical region and hydrocortisone- and radio-resistant cells in the medulla (Murray, 1948; Warner, 1964). In contrast to the hydrocortisone-sensitive thymocytes, the resistant thymocytes have a larger cell volume similar to that of LN cells (Schlesinger and Golakai, 1967; Blomgren and Andersson, 1969, 1971), home to LN in a way similar to circulating lymphocytes (Blomgren and Andersson, 1972), are PHA-, MLC-, CML-, and GvH-reactive (though less than peripheral lymphocytes) (Blomgren and Andersson, 1969; Blomgren and Svedmyr, 1971; Elliott *et al.*, 1971), and have lower thymus-specific and higher histocompatibility antigen content on the cell surface (Schlesinger and Golakai, 1967; Lance *et al.*, 1970; Raff, 1971; Leckband and Boyse, 1971). Thymocytes in the LMR show the characteristics of cortical thymocytes (hydrocortisone sensitivity, high θ antigen content, immunoincompetence, small volume) and those in the HMR have the characteristics of medullary thymocytes (hydrocortisone resistance, large volume, low θ antigen content, GvH reactivity). Morphological details of the HMR and LMR thymocytes confirm this classification which Abe and Ito (1970) described.

Investigation of the differentiation pathways of Thy-dependent cells using chromosome markers (Harris and Ford, 1964; Weismann, 1967), radioisotopes (Nossal, 1964; Weismann, 1967; Joel, Hess and Cottier, 1971), and surface antigen markers (Sainte-Marie and Leblond, 1964, 1965; Raff, 1969; Raff and Wortis, 1970) indicated that cortical thymocytes undergo transformation into medullary thymocytes which then emigrate into the periphery. In the course of this development T cells lose some θ and all of their TL and LY surface antigen (Lance *et al.*, 1970; Raff, 1971; Leckband and Boyse, 1971). Our data considered in the light of these facts lead us to suggest the following differentiation sequence: thymocytes of low EPM→ thymocytes of high EPM→ peripheral T cells of high EPM. LMR thymocytes possess the highest θ antigen content which decreases at the same rate as the negative surface charge increases. Peripheral T cells, which are found only in the HMR, had a considerably lower θ content compared to the HMR thymocytes. These differences in θ antigen content strongly support the above-stated thesis.

This sequence of differentiation was also suggested by the quantitative analysis of immunocompetence (GvH reactivity) in the defined groups. GvH reactivity was not found in the Thy LMR, some was found in the Thy HMR and the highest reactivity was found in the HMR of peripheral lymphoid organs. This result is significant since almost all of the cells in the tested groups were θ-positive. Recently it was shown that hydrocortisone-resistant thymocytes (corresponding to the HMR cells) which give rise to only a small MLC reaction were highly reactive when transferred into irradiated recipients and collected and tested 4–5 days later (Mosier and Cantor, 1971).

These experiments may indicate that most of the HMR thymocytes, although they are

still immunoincompetent, already have achieved a sufficiently advanced stage of maturation to allow its completion outside the Thy. That the θ antigen content lies between that of the Thy LMR and of the peripheral T cells also supports this hypothesis.

All of this evidence points to the conclusion that in differentiating T cells an increase in negative surface charge and a decrease of thymus-specific antigen, both of which indicate profound changes in the molecular membrane structure, precede the attainment of immunocompetence by T cells.

By means of electronic cell sizing and electron-microscopic investigation several definite T cell populations could be observed in the electropherograms of Thy and peripheral lymphoid organs. Correlation of their negative surface charge, cell size, morphological details and the above-cited characteristics provided the basis for the construction of a sequence of steps in the maturation of T cells (Table 4).

TABLE 4

PHYSICAL AND BIOLOGICAL PROPERTIES OF T-CELL SUBPOPULATIONS

	Organ distribution				
	Thymus cortex		Thymus medulla	Peripheral lymphoid organs	
Morphology					
Differentiation sequence	T_1 ⟵ - - ⟶ T_2		⟶ T_3	⟶ T_4	- - - ⟶ T_5
Mean electrophoretic mobility	−2·58	−2·68	−2·79	−2·99	−3·22
θ antigen content	+++	+++	++	++/+	+
GvH reactivity	−	−	(+/−)	+	++
Helper cell function†	−	−	+/−	+	+
Hydrocortisone reactivity	++	+	−	−	−
Cell volume (μm^3)	90	175	125	125	90
Metabolic activity	+	++	+++	+++	+

† Data from Zeiller, Holzberg, Pascher and Haning (1972).

The pool of immunoincompetent thymocytes of low negative surface charge contains two cell types, a small, highly hydrocortisone-sensitive cell (T_1) and a rather large, less hydrocortisone-sensitive cell (T_2). The cell types show similar cytological details, except for the presence of a conspicuous nucleolus in the larger cells, which indicates increased RNA and protein metabolism. In the fractions of high EPM a small cortisone-resistant population of medium-sized cells was observed which was bimodal in the electropherograms (T_3 representing the 'slower' and T_4 the 'faster' cells). The GvH reactivity in the T_4 fractions was higher than that in the T_3 fractions. Morphological criteria such as presence of a conspicuous nucleolus, endoplasmic reticulum, numerous mitochondria and cytoplasmic inclusions distinguished them from T_1 and T_2 cells and indicated high metabolic activity. In Thy these cells could be localized in the cortical area around and in the medulla (Abe and Ito, 1970).

Assuming that a decrease in hydrocortisone sensitivity and θ antigen content and an increase in negative surface charge, in metabolic cell activity and lastly in GvH reactivity, all signal progressive thymocyte maturation and differentiation, then a sequence of subsequent steps from T_1 to T_4 thymocytes as outlined in Table 4 is suggested.

Earlier morphological studies on the intact Thy (Sainte-Marie and Leblond, 1964, 1965) and on *in vitro* culture systems (Mandel and Russell, 1971) also suggest the development of large proliferating cortical thymocytes into medium-sized and lastly small medullary thymocytes; these groups were represented in our study by the groups T_2, T_3 and T_4 respectively. Other experiments have given evidence that the large cortical thymocytes are derived from small thymocytes which most likely originate from precursor cells in the bone marrow (Dukor, Miller, House and Allmann, 1965; Blackburn and Miller, 1967). Whether or not these small thymocytes are identical with our T_1 cells is unclear.

Another relationship between large and small cells in the thymus cortex has been shown by studies of involution and regeneration of Thy, induced by hydrocortisone and irradiation treatment. In all of these cases, large cells preceded the recovery of small cortical thymocytes (Blomgren and Révész, 1968; Blomgren and Andersson, 1971). This phenomenon does not necessarily interfere with our thesis, since in regeneration tissues these large cells may be distinct in their commitment from T_2 cells in normal tissues.

In the periphery two different T-cell subpopulations could be detected in the HMR of the various profiles. Besides a lower θ antigen content, one population of relatively lower EPM was, according to the tested parameters, similar to the T_4 cells in the thymus. Consequently, it was called peripheral T_4. A resting T-cell population of highest EPM, highest GvH reactivity, and lowest θ antigen content, which was seldom found in the thymus, was called T_5. Both cells seem to be progeny of thymocytes of medium and high EPM. However, their interrelationship remains unclear. Peripheral T_4 cells could represent (according to their smaller θ antigen content) progeny of T_4 thymocytes which have recently left the thymus (Joel *et al.*, 1971), and which, after further maturation, provide T_5 cells (Mosier and Cantor, 1971). Likewise, peripheral T_4 and T_5 cells could represent two functionally different subpopulations of T cells.

Though this scheme is based on indirect evidence and is therefore more or less theoretical, it may serve as a valuble basis for further investigations. The influence of the thymic environment on this differentiation process, the molecular nature of the changes in surface structure and their significance for the development of immunocompetence of T cells remain unclear, but the electrophoretic separation method employed can make an important contribution to the clarification of these problems in future experiments.

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