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# Physical subpopulations of mouse thymocytes: changes during regeneration subsequent to cortisone treatment

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**Summary.** Thymocytes from adult C3H mice were fractionated on the basis of electrophoretic mobility (EPM) differences by free-flow electrophoresis and the fractions obtained were analysed for size distribution with a Coulter Counter. The data were combined in the form of contour maps (fingerprints) representative of the various physical types of thymocytes. Four thymocyte subpopulations with distinct physical properties were thus characterized and were further shown to differ in their sensitivity to immunosuppressive drugs and in their proliferation rate. $Th_1$ cells possess the slowest EPM and smallest volume (95 $\mu m^3$), are sensitive to cortisone (C) but resistant to cyclophosphamide (Cy) and do not incorporate $^3$[H]-thymidine (TdR) *in vitro*. $Th_2$ cells possess higher EPM and slightly larger volume (103 $\mu m^3$) and are sensitive to both C and Cy. $Th_3$ cells are of still higher EPM, exhibit the largest volume (150–250 $\mu m^3$) of all thymocytes, are sensitive to C and Cy and rapidly incorporate [$^3$H]-TdR *in vitro*. $Th_4$ cells are endowed with fastest EPM and a modal volume of 130 $\mu m^3$ and are resistant to both C and Cy.

The regeneration and fate of these subpopulations were investigated during the period subsequent to cortisone injection. $Th_3$ cells were the first to reappear on day 4–6 following treatment. Thereafter, $Th_2$ and $Th_1$ cells began to rise again and eventually reached levels higher than control by day 12–14 post-treatment. By the same time, $Th_4$ cells, which escaped cortisone lympholytic action became less and less visible on the fingerprint. In fact, administration of a second dose of cortisone by day 8–10 after the first treatment revealed a depletion as well as a physical modification of the C-resistant cell pool. Typical $Th_4$ cells were found again on day 15 after the first cortisone injection. It was only around day 20 that thymus became normally repopulated.

Taken together, these observations indicate that $Th_3$ cells may act as precursors for all 3 other thymocyte subpopulations and that during thymus reconstitution $Th_2$ and $Th_1$ cells are produced at a faster rate than are $Th_4$ cells.

## INTRODUCTION

The lymphocyte population in the mouse thymus has been shown by many investigators to be heterogeneous. Two main thymocyte classes with different sensitivities to corticosteroid treatment have been identified: the corticosteroid-sensitive (CS) pool localized mainly in the thymus cortex and the corticosteroid-resistant (CR) pool confined to the medulla (Ishidate & Metcalf, 1963; Blomgren & Andersson, 1969). Besides functional (Jacobsson & Blomgren, 1972) and antigenic properties (Schlesinger, 1972), physical properties have been proved useful for the characterization of these thymocyte

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classes. Thus, it has been demonstrated that the determination of electrophoretic mobility (EPM) allows easily to distinguish CS thymocytes with a low EPM from CR thymocytes with a higher EPM (Wioland, Sabolovic & Burg, 1972; Dumont & Robert, 1976). Density gradient fractionation studies (Sabolovic & Dumont, 1973; Dumont & Sabolovic, 1973) as well as the investigation of thymus development (Dumont, 1974) further revealed that both thymocyte classes are also themselves heterogeneous with respect to EPM. Another physical parameter by which lymphocyte subpopulations may be characterized is cell volume. Earlier studies by Blomgren & Andersson (1969) have shown that thymus cells from corticosteroid treated mice have a slightly larger volume than cells from untreated mice. Further, Moon, Phillips & Miller (1972), and Droege, Zucker & Jauker (1974) have demonstrated that when used in conjunction with separation methods, electronic cell sizing provides a powerful tool for the investigation of lymphocyte populations.

In the present work, we have associated the measurement of cell EPM with that of cell volume. Thus, thymus lymphocytes from C3H mice were fractionated by free-flow electrophoresis, a procedure which separates cells on the basis of surface-charge differences (Hannig, 1972). The volume distribution of all the fractions were then measured using a Coulter Counter and combined to form a contour map (fingerprint) in which cell EPM and cell volume are the independent variables and cell frequency is the dependent variable (Moon *et al.*, 1972). This procedure was applied to the study of thymocytes from normal mice and cyclophosphamide- or cortisone-treated mice. Moreover, as an attempt to clarify the developmental interrelations between the various thymocyte subpopulations defined by physical parameters, we investigated the regeneration of thymus after its destruction by cortisone (Jacobsson & Blomgren, 1972, 1975; Schlesinger & Israel, 1975).

## MATERIALS AND METHODS

*Mice*

Female mice from strain C3H/He were used. They were 2 months old at the start of the experiments.

*Treatment with cyclophosphamide or cortisone*

Animals were injected intraperitoneally with either cyclophosphamide (Cy, Endoxan-Asta, Laboratoires Lucien, Paris) in a dose of 300 mg/kg of body weight (Dumont & Barrois, 1975) or cortisone acetate (CA, Laboratories Roussel, Paris) in a dose of 200 mg/kg of body weight.

*Preparation of cell suspensions*

After lethal anaesthesia of mice with ether, thymuses were dissected free of adjacent tissues. Care was taken to exclude the parathymic lymph nodes. The organs were then gently disrupted in cold RPMI 1640 medium (Eurobio, Paris) by means of loosely fitted all glass homogenizers (Potter n° 10, VSCI, Paris). Cells were pooled from two to thirty mice depending on the degree of thymus involution. Cell numbers and viability were determined in a standard haemocytometer after trypan blue staining.

*Determination of spontaneous tritiated thymidine uptake*

[$^3$H]-TdR uptake was determined as an index of spontaneous proliferation of thymocytes. Preliminary experiments revealed that *in vivo* labelling by intraperitoneal injection of up to 200 $\mu$Ci/mouse of [$^3$H]-Tdr were inadequate for satisfactory liquid scintillation counting. In order to obtain higher radioactivity yields, dissociated thymocytes were labelled with [$^3$H]-TdR *in vitro*. In a first series of experiments, the cells were incubated with [$^3$H]-TdR (5 $\mu$Ci/ml) for 45 min at 37° in RPMI 1640 medium containing 5% foetal calf serum and then were fractionated with free flow electrophoresis. The cells in the various fractions were precipitated with cold 5% trichloracetic acid (TCA) and the pellets washed in methanol. TCA precipitates were dissolved in 2 ml hyamine and transferred into vials containing 20 ml of scintillation fluid. After 48 h of stabilization in the dark and at 4°, the radioactivity of the vials was measured in a refrigerated scintillation spectrometer (Intertechnique, Plaisir, France).

In a second series of experiments, thymocytes were cultivated for a longer period of time in microtest II plates (Falcon 3040) (Dumont & Robert, 1976). $5 \times 10^5$ cells in 0·2 ml culture medium were labelled with [$^3$H]-TdR (1 $\mu$Ci/well) for 6 h and harvested onto glass fibre filters using a Multiple Automated Sample Harvester (MASH II, Microbiological Associates, Bethesda). Incorporated radioactivity was expressed as the mean of 6 replicate cultures.

*Fractionation of cells by free-flow electrophoresis*

Cells were centrifuged once in RPMI 1640 medium containing 5% foetal calf serum (RS) and transferred into the low ionic strength electrophoresis buffer developed by Hannig (1972). (Composition: 0·4 M Potassium acetate, 0·015 M Triethanolamine, 0·24 M Glycine, 0·011 M Glucose, and 0·03 M Sucrose.) The cell pellet was resuspended in 20 ml RS, diluted by adding 2 × 10 ml low ionic strength buffer, then centrifuged again. The cell pellet was then resuspended in 4 ml low ionic strength buffer and filtered through a loose cotton plug to remove damaged cells. All these steps were performed at 4°. Cell suspension was adjusted to a concentration of 15–20 × $10^6$ cells/ml and immediately fractionated in a Hannig's type free-flow electrophoresis apparatus (Hannig, 1972) (FF5, Desaga, Heidelberg, Germany). The operating conditions were: 93 V/cm, 215 mA, 6°, liquid curtain velocity: 0·2 cm/sec, sample flow rate: 3·75 ml/h. Fractions were collected in siliconized tubes containing 2 ml RS at 4°. Samples to be compared were fractionated under identical conditions on the same day with minimal lag period between each set.

*Cell size analysis*

A Coulter Counter model ZBI (aperture diameter: 100 μm) coupled with a pulse height analyser (C-1000 Channel analyser) were used for determination of cell volume distributions. Spectra of counts versus channel numbers were plotted by an X–Y Recorder (HR-2000) connected to the pulse height analyser. The instrument was calibrated with latex particles (3·66 μm mean diameter). The following settings were used throughout the present study: attenuation: 1, aperture current: 0·177, base channel threshold: 10, window width: 100. For analysis, cells were suspended in 0·15 M NaCl at room temperature. Samples were appropriately diluted so that count did not exceed 2 × $10^4$/ml and examined within 1 min after preparation.

*Construction of the fingerprints*

Successive contour lines of constant cell frequency plotted against cell volume and electrophoretic mobility were constructed as follows:

(a) Cells were fractionated by electrophoresis and the fractions were simultaneously counted and analysed for size distribution with the Coulter Counter. Spectrum of counts versus channel numbers was recorded for each electrophoretic fraction.

(b) The count axis of each of these spectra was then scaled in percent of all cells in all fractions. The channel numbers corresponding to given percentages of all cells recovered could be determined directly on the volume distribution spectrum. The following percentages were arbitrarily taken into account: 0·025, 0·05, 0·075, 0·10, 0·15, 0·20, 0·25 (Fig. 1).

(c) Points corresponding to a same percentage were joined together to form lines of constant relative cell number. The map thus obtained (fingerprint, Moon *et al.*, 1972) represents a two-dimensional pattern of cell volume, cell electrophoretic mobility, and cell concentration.

## RESULTS

### Cellular composition of the normal thymus

Preparative electrophoresis experiments demonstrated the electrophoretic heterogeneity of mouse thymocytes and permitted a study of the size distri-

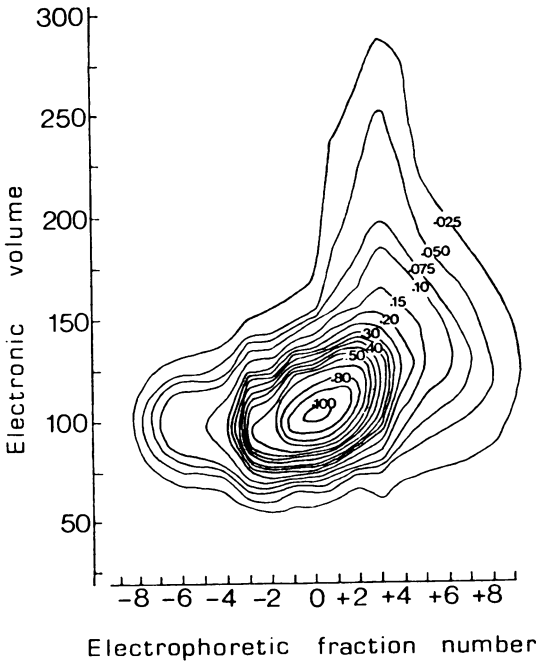


**Figure 1.** Fingerprint of thymocytes from untreated adult C3H mouse. The numbers on the graph indicate the percentage of all cells recovered to which corresponds each curve.

**Table 1.** Physical subpopulations of lymphocytes in the thymus from adult C3H mice

| Thymocyte subpopulation | Relative modal electrophoretic mobility | Modal volume ($\mu m^3$) | Cortisone sensitivity* | Cyclophosphamide sensitivity† |
|---|---|---|---|---|
| $Th_1$ | −2 | 95 | + | − |
| $Th_2$ | 0 | 103 | + | + |
| $Th_3$ | +3 | 150–250 | + | + |
| $Th_4$ | +6 | 130 | − | − |

* 2 Days after injection of cortisone acetate in a dose of 200 mg/kg of body weight.
† 2 Days after injection of cyclophosphamide in a dose of 300 mg/kg of body weight.

bution of thymocytes as a function of their surface-charge. Both physical characteristics were combined to construct a two-dimensional map (fingerprint) representative of the various thymus cell types. Fig. 1 shows such a fingerprint. The bulk of thymocytes exhibited a modal volume of 103 $\mu m^3$ and peaked in the low-mobility range of the fingerprint. This electrophoretic peak was taken as origin (fraction 0). In the fractions with mobility lower than this peak, cells tended to be smaller (modal volume: 95 $\mu m^3$). A subpopulation of larger cells was visible as a tongue extending upwards (volume: 150–250 $\mu m^3$) and peaked at electrophoretic fraction +3. A bulge in the HM region (fractions +3 to +9) made up another subpopulation with modal volume around 130 $\mu m^3$. For the sake of clarity, these thymocyte subpopulations were arbitrarily designated as $Th_1$, $Th_2$, $Th_3$, and $Th_4$ towards increasing EPM (Table 1).

The distribution of cells with a high rate of DNA synthesis was determined after 45 min of *in vitro* labelling with [$^3$H]-TdR. As shown in Fig. 2, incorporated radioactivity was mainly recovered in fractions +2 and +3. This peak of highly labelled cells coincided with that of the larger thymocyte subpopulation. Therefore, this latter subpopulation

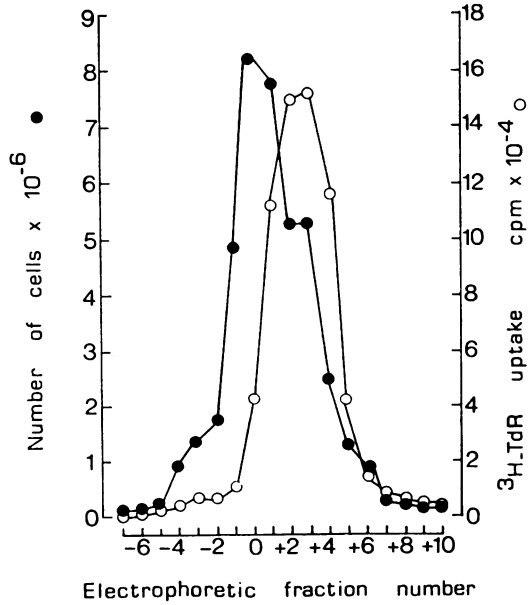


**Figure 2.** Electrophorogram of thymocytes from untreated adult C3H mouse. Cells were incubated for 45 min with 5 $\mu$Ci of [$^3$H]-TdR prior to electrophoretic fractionation. The distribution of all cells (●) and the amount of radioactivity recovered in each fraction (○) are shown.

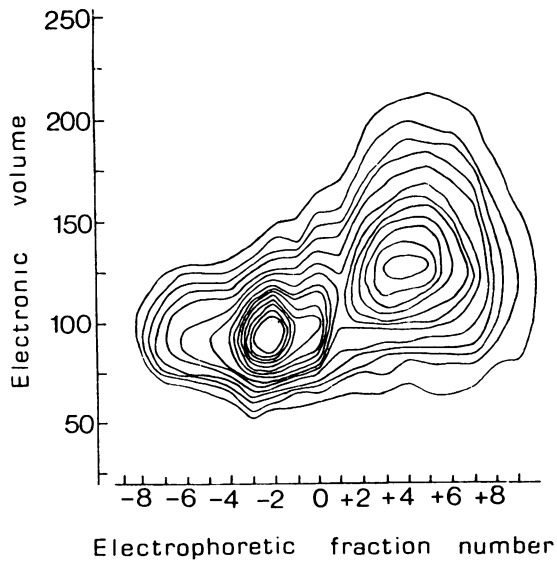


**Figure 3.** Fingerprint of thymocytes from adult C3H mice treated 2 days previously with cyclophosphamide (300 mg/kg of body weight). Outer contour line represents 0·025% of all cells recovered.

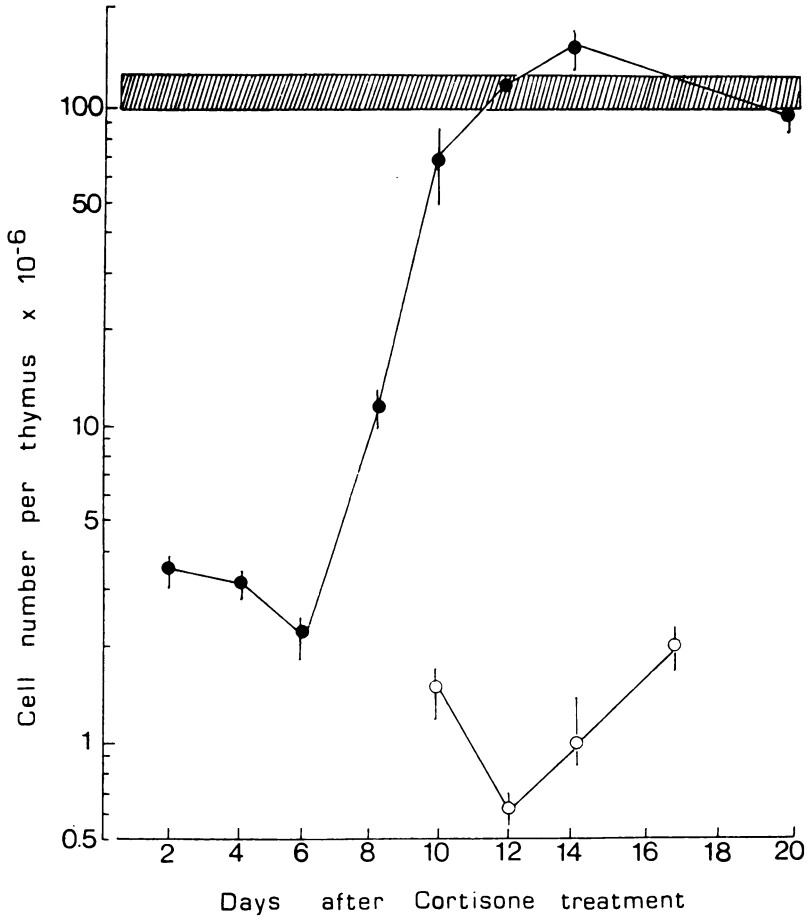


**Figure 4.** Cellularity of the thymus from adult C3H mice at different times after injection of cortisone acetate (200 mg/kg of body weight). Mice received either a single dose of the drug (●) or were injected a second time 2 days before thymus study (○). Each point is the mean ± standard error of 3–6 experiments. The shaded area represents the range of untreated mice.

is likely to represent the pool of highly proliferating cells in the normal thymus.

### Cellular composition of the thymus after cyclophosphamide treatment

C3H mice received a single injection of Cy in a dose of 300 mg/kg of body weight and their thymus cells were studied 2 days later (Dumont & Barrois, 1975). Thymus cellularity was reduced to about 15% of control. Two subpopulations of thymocytes were then clearly distinct on the fingerprint (Fig. 3). The slowest migrating small thymocytes with a modal volume of 95 $\mu m^3$ ($Th_1$) accounted for 60% of Cy-resistant cells and the other cells belonged to fastest migrating $Th_4$ subpopulation. $Th_2$ and $Th_3$ subpopulations were practically absent from the fingerprint.

### Cellular composition of the thymus after cortisone treatment

Two days following cortisone administration, thymus cellularity dropped to 5% of its normal value (Fig. 4). These cells peaked at fraction +6 and possessed a modal volume at 130 $\mu m^3$. This relatively CR thymocyte population appears physically identical to population $Th_4$ identifiable on the

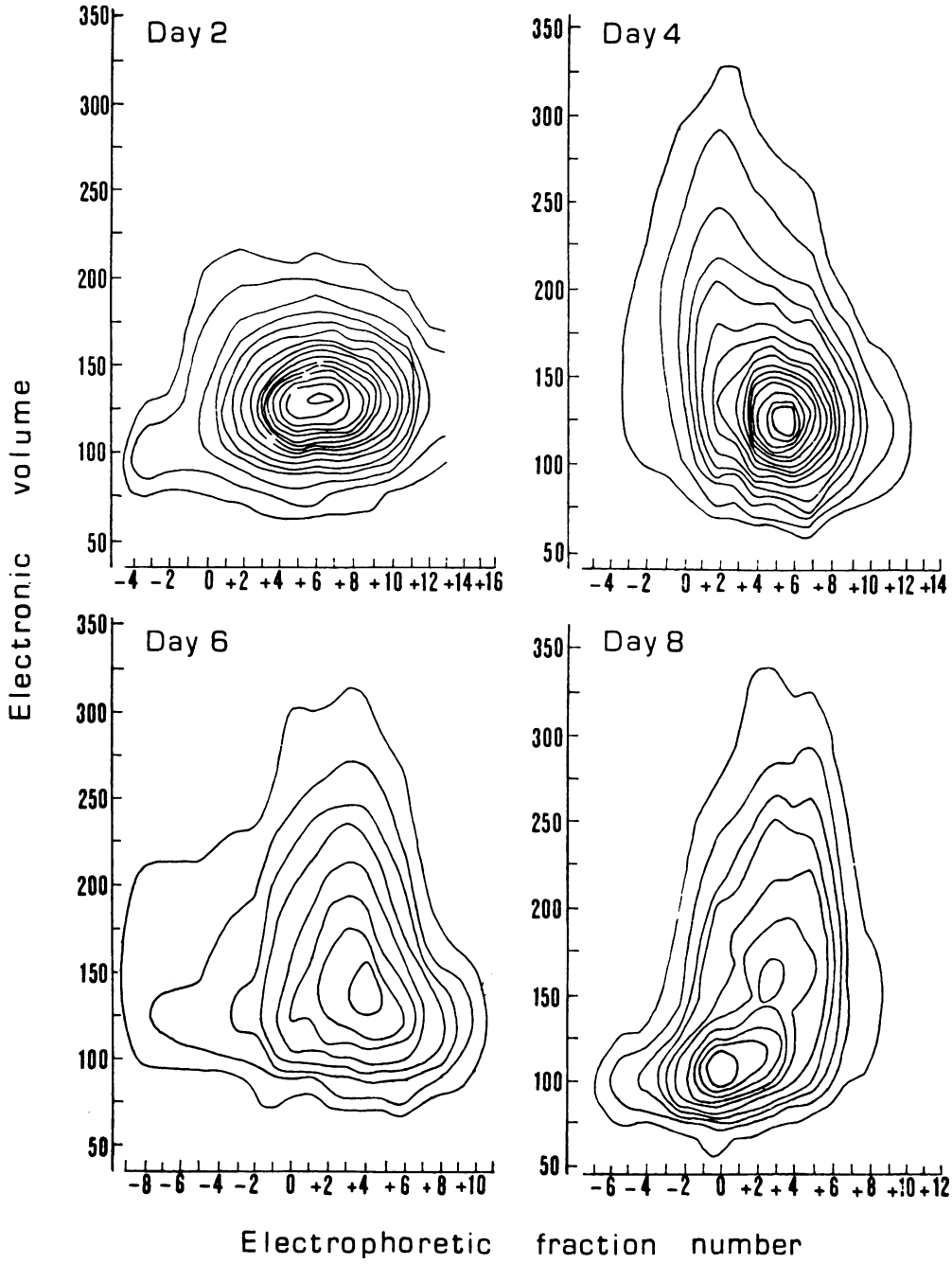


**Figure 5.** Fingerprints of thymocytes from adult C3H mice at different times (2–8 days) after injection of a single dose of cortisone acetate (200 mg/kg of body weight).

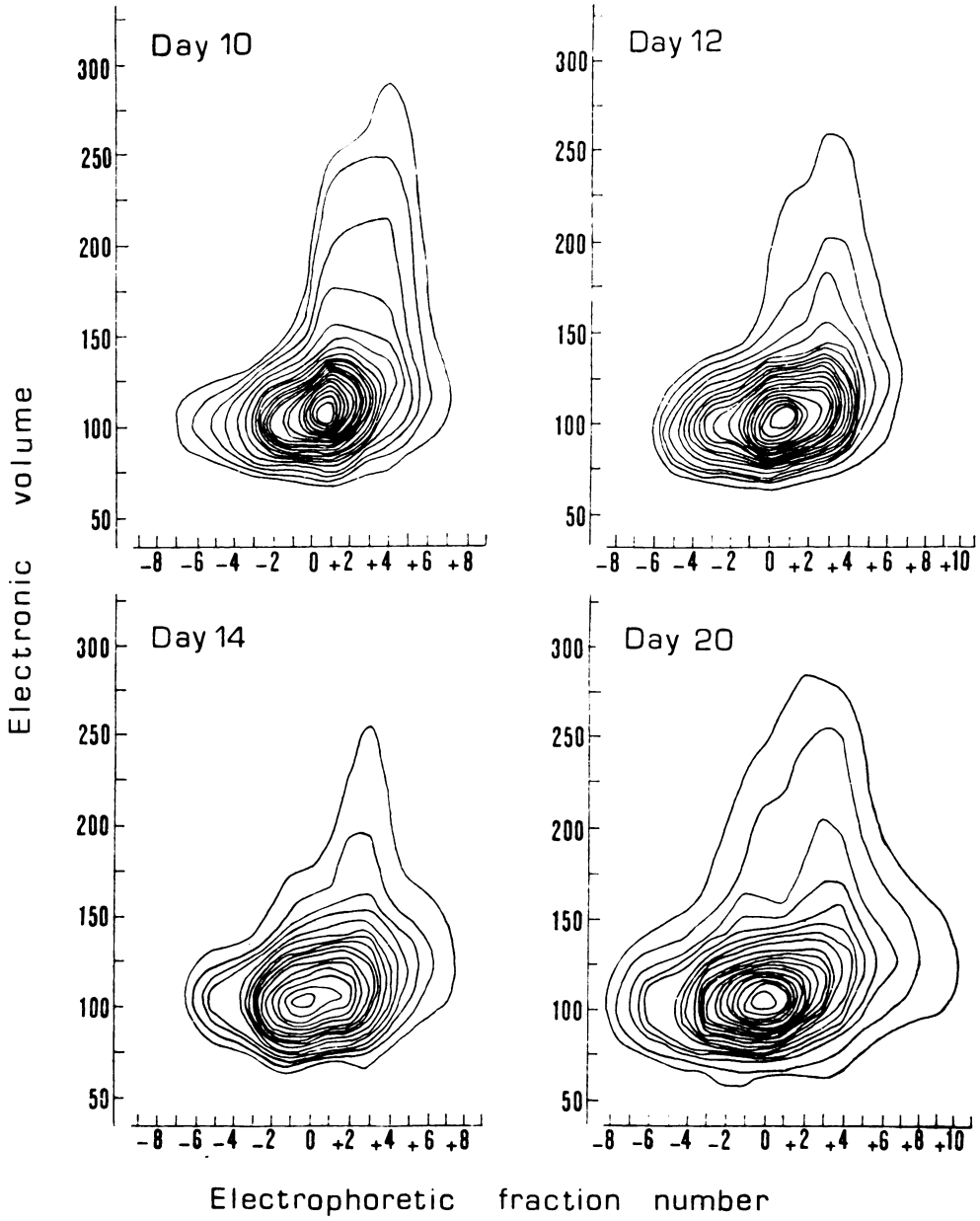


**Figure 6.** Fingerprints of thymocytes from adult C3H mice at different times (10–20 days) after injection of a single dose of cortisone acetate (200 mg/kg of body weight).

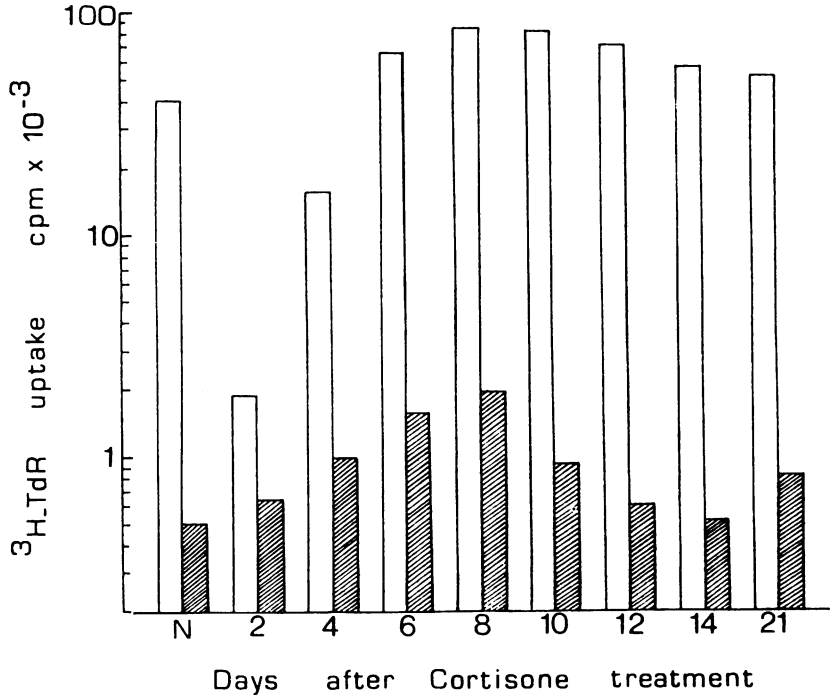


**Figure 7.** Spontaneous *in vitro* incorporation of [$^3$H]-TdR by thymocytes from untreated (N) or cortisone-treated adult C3H mice at different times after treatment. White bars represent [$^3$H]-TdR uptake for the first 6 h of culture. Shaded bars represent [$^3$H]-TdR uptake after 18 h of culture followed by a 6 h [$^3$H]-TdR pulse.

normal fingerprint. Populations 1, 2 and 3 which completely disappear after cortisone treatment correspond thus to CS thymocytes.

### Cell population changes during thymus regeneration

The cellular composition of the thymus was further investigated during the period of regeneration subsequent to destruction by cortisone. Figs 4 and 5 present typical fingerprints at different times after cortisone treatment. Major changes were observed in both the absolute number and the relative proportion of the various physical subpopulations of thymocytes above described (Fig. 4). A first step was marked by the reappearance of $Th_3$ cells which occurred as soon as day 4 following cortisone administration. Such large thymocytes became predominant on day 6 and 8 (Fig. 5). By the same time, the typical CR thymocytes ($Th_4$) were less and less visible on the fingerprint. A second step was characterized by the reappearance of $Th_2$ cells on day 8 post-treatment. At this stage, thymus cellularity started to recover (Fig. 4). Small thymocytes continued to rise on the subsequent days while the proportion of $Th_3$ and $Th_4$ cells tended to diminish (Fig. 6). By day 14, thymus reached a higher cellularity than control and comprised a majority of small elements. Thymus cellularity and fingerprint became again similar to those of normal mice only around day 21.

The capacity to proliferate spontaneously *in vitro* was also studied at various time intervals after cortisone injection. Fig. 7 depicts the extent of [$^3$H]-TdR uptake observed in an experiment in which all thymocyte suspensions were cultured in parallel. While control thymus cells exhibited a high level of spontaneous DNA synthesis for the first 6 h of *in vitro* culture, thymocytes from mice treated 2 days previously with cortisone poorly incorporated [$^3$H]-TdR. However, if the cells had been cultured for 18 h prior to the 6 h pulse with [$^3$H]-TdR, the amount of incorporated radioactivity dropped to values 100-fold lower in control, but only 3-fold lower in cortisone-treated thymocytes. This indi-

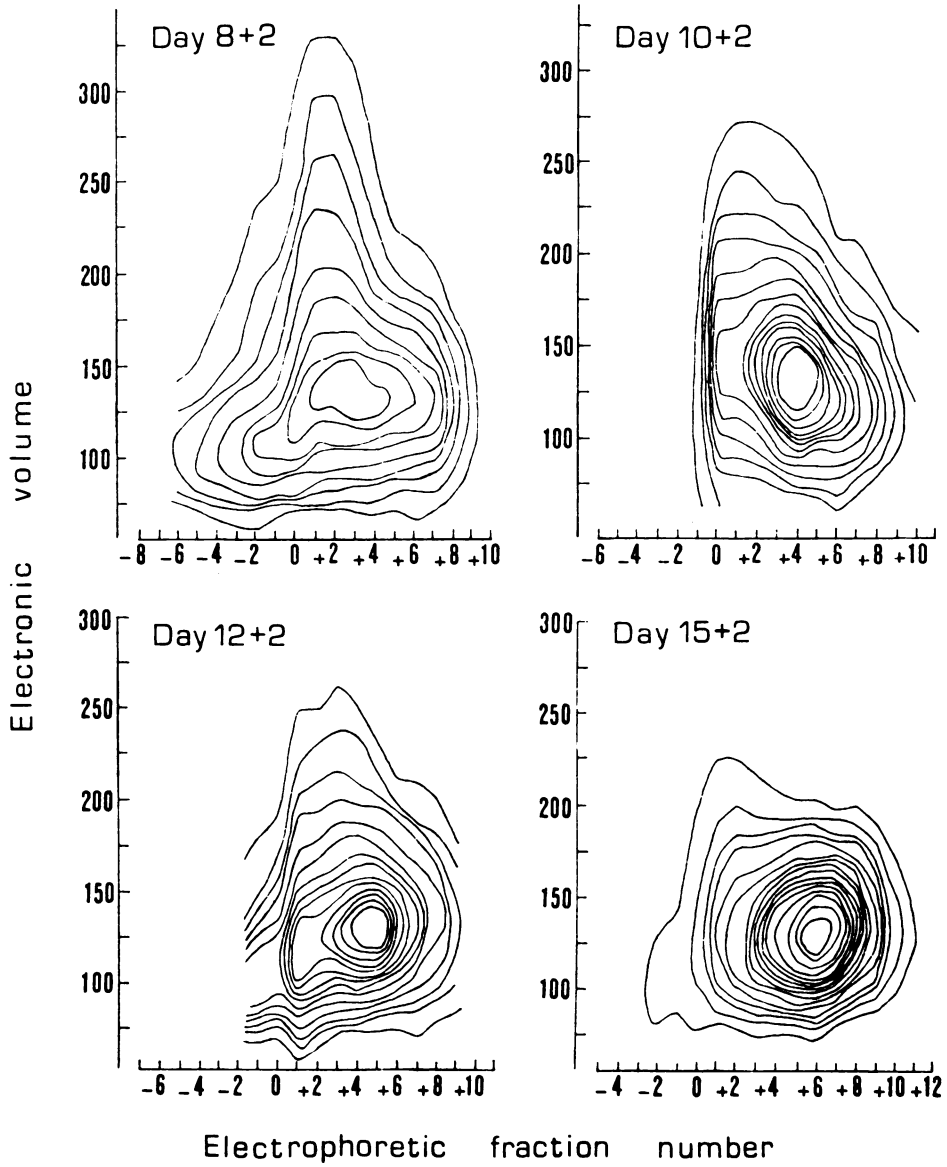


**Figure 8.** Fingerprints of thymocytes from adult C3H mice at different times after a first dose of cortisone followed by a second dose of cortisone 2 days previously.

cates that the cortisone-sensitive cells cannot sustain a proliferative capacity for a long time out of the thymus and probably rapidly die *in vitro*. During the period of thymus regeneration the ability of thymocytes to proliferate in short-term cultures was reconstituted. Already, by day 8 following treatment the rate of spontaneous DNA synthesis extended slightly beyond the control value. The maximal level of DNA synthesis was reached by day 10. Thereafter, there was a return to the normal situation. Interestingly, these rates of [$^3$H]-TdR incorporation paralleled the cortisone-induced disappearance of $Th_3$ cells and their reappearance during the phase of regeneration.

**Regeneration of the cortisone-resistant thymocyte pool**

The above observations suggest that CR cells ($Th_4$) progressively disappear after cortisone treatment or change their physical properties and then are reconstituted at a slower rate than $Th_1$ and $Th_2$ subpopulations. Experiments were conducted to examine this point further. Groups of mice were treated with cortisone, and at various times thereafter, a second injection of cortisone was administered and the thymuses were studied 2 days later. Using such a protocol, both the number and physical properties of the cortisone-resistant thymocytes could be determined in the course of thymus regeneration. Fig. 8 shows the results of typical experiments. By day 10, after the first injection, cortisone-resistant cells accounted for 1·4% of the whole thymocytes. These cells were physically distinct from the CR cells ($Th_4$) recovered from the normal thymus. Thus, they included larger cells and were electrophoretically slower by 3 fractions. By day 12, still fewer cortisone-resistant cells were found which moved however slightly faster. On subsequent days, the cortisone-resistant pool tended to expand and finally by day 17 resembled the typical $Th_4$ subpopulation.

## DISCUSSION

On the basis of differences in drug sensitivity, size and electrokinetic properties, 4 subpopulations of lymphocytes were distinguished in the thymus from adult C3H mice. This confirms and extends previous reports demonstrating the heterogeneity of mouse thymocytes (Sabolovic & Dumont, 1973; Dumont, 1974; Droege & Zucker, 1975). Thus, the existence of two main classes of thymocytes differing in their susceptibility to corticosteroid treatment is a well documented feature (Blomgren & Andersson, 1969, 1971; Dumont & Robert, 1976). In agreement with earlier observations, corticosteroid-sensitive (CS) cells were found endowed with a lower surface-charge than the corticosteroid-resistant (CR) subpopulation (Sabolovic & Dumont, 1973; Dumont & Sabolovic, 1973). Evidence was further obtained that the CS compartment is itself composed of at least 3 electrophoretically distinct cell subsets which were arbitrarily designated as $Th_1$, $Th_2$ and $Th_3$ subpopulations. $Th_1$ and $Th_2$ cells correspond to small thymic lymphocytes and appear physically similar to thymocytes type I and type II described by Droege *et al.* (1974), Droege & Zucker (1975). However, in marked contrast to these authors who defined type II cells as resistant to Cy treatment, we found that $Th_2$ subpopulation exhibits a high Cy-sensitivity. The fact that in these studies a low Cy dose was injected makes it likely the thymus was less affected or already regenerating and might account for the discrepancy. At any rate, it seems clear, from the present observations, as well as from a previous work (Dumont & Barrois, 1975) that the CS pool includes cells with different metabolic status as reflected by different susceptibilities to a sublethal dose of Cy. This drug is known to act primarily on dividing cells (Dewys, Goldin & Mantel, 1970). Then, $Th_1$ cells which are spared by Cy might represent quiescent cells whereas $Th_2$ cells which are destroyed by Cy are probably engaged in the cell cycle. The third type of CS thymocytes, $Th_3$ cells, corresponds to the largest thymocytes detected by the size analyser under the conditions used in the present work. Such a subpopulation of large cells was already noticed by different investigators (Zeiller *et al.*, 1974; Fathman *et al.*, 1975). These large thymocytes were found to possess an average EPM intermediate between those of CS and CR subpopulations. Moreover, they were shown to display intense labelling after an *in vitro* pulse with [$^3$H]-TdR which suggests these cells are actively dividing in the normal thymus. In fact, Fathman *et al.* (1975) observed these large thymocytes were the only cells labelled *in situ* after transcapsular diffusion of administered [$^3$H]-TdR.

The study of thymus regeneration following cortisone treatment provided further information on the developmental relationships between the 4 thymocyte subsets identified. While CR $Th_4$ thymocytes were the only cell type detectable two days after cortisone injection, by day four and six already large $Th_3$ thymocytes began to reappear. This early reappearance of $Th_3$ cells was shown to coincide with recovery of *in vitro* [$^3$H]-TdR uptake which further strengthens the notion that $Th_3$ represent a proliferating pool. Thus, quite in keeping with observations by Blomgren & Andersson (1971), large cells preceded the reconstitution of small thymocytes. This indicates that stem cells derived from the bone marrow (Blomgren & Andersson, 1971) or endogenous to the thymus (Kadish & Basch, 1975) can enter division early after cortisone treatment. By day eight after treatment, $Th_2$ cells

became again visible. It seems likely these cells represent the direct progeny of $Th_3$ cells. At this time, thymus cellularity started to rise indicating an active repopulation of the organ. $Th_1$ cells reappeared only on the subsequent days. This time sequence suggests $Th_1$ cells are themselves derived from $Th_2$ cells. In fact, taken together with their above mentioned Cy-resistance, this observation might lead one to consider that $Th_1$ cells represent the most mature form of cortical thymocytes. However, this would not imply these cells are unable to revert to more active types like $Th_2$ and $Th_3$ and re-enter the generative pool of thymocytes (Bryant, 1972).

While all 3 types of CS cells were clearly seen 10–14 days after the injection of cortisone, CR cells seemed to disappear and reappear at a slower rate. In fact, $Th_4$ apparently decreased in proportion at the same time as large cells regenerated. During that period (day 4 and 6) thymus cellularity kept constant and it is not known whether $Th_4$ cells left the thymus. However, it has been shown that a majority of PHA responsive cells (presumably CR cells) persist in the thymus for long periods (Elliott, 1973, 1977). In contrast, studies by Jacobsson & Blomgren (1972, 1975) have demonstrated that PHA responsiveness decreases during the phase of thymus regeneration. It seems thus possible that $Th_4$ cells might change their physical properties and lose their PHA responsiveness in the process. Indeed, experiments in which a second dose of cortisone was administrated, demonstrated that although, by day 8 after the first injection a significant proportion of thymocytes exhibits cortisone resistance, these cells possessed physical properties different from those of $Th_4$ cells. A regeneration process among the CR compartment might thus be assumed to take place and would be characterized by a progressive increase of surface-charge. These findings fit with previous reports showing an inverse relationship between the production of cortical (CS) thymocytes and of medullary (CR) thymocytes (Jacobsson & Blomgren, 1972). Similarly, it was demonstrated that following cortisone administration the rate of recovery of the number of spleen-seeking thymus cells was more rapid than that of the lymph node seeking cells (Schlesinger & Israel, 1975).

The results of the present study do not allow a distinction between the hypotheses that thymus subpopulations constitute either stages of differentiation of a single cell lineage (Zeiller *et al.*, 1974) or of distinct independent cell lineages (Schlesinger, 1972; Shortman & Jackson, 1974). The cortical pool might differentiate via catenated compartments leading from larger ($Th_3$) to smaller proliferating ($Th_2$) with a terminal compartment of small non-proliferating cells ($Th_1$) (Metcalf, 1966). Alternaatively, as suggested by Droege *et al.* (1974), $Th_1$ and $Th_2$ cells might be the products of two separate lineages. The relationship between CS and CR compartments is not more clear. The observation that their regenerations proceed at different rates might indicate that these two classes of thymocytes are independent. According to Jackson & Shortman (1974) $Th_4$ and CS cells might arise from distinct precursors probably contained in $Th_3$ subpopulation. Indeed, some evidence was presented that most medullary thymocytes are not cortically-derived but are renewed from stem cells of their own (Bryant, 1972). An opposite view, would be that $Th_4$ cells derive from $Th_2$ or $Th_1$ subpopulations but then a mechanism for cell enlargement should be postulated.

## ACKNOWLEDGMENTS

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