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Immunology

Kinetics of mature T-cell development in the thymus

(T-lymphocyte differentiation/thymus selection/thymus subpopulations)

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ABSTRACT **We have reexamined the balance between cell birth, cell maturation, and cell death in the thymus by labeling dividing thymocytes and their progeny *in vivo* with [^{3}H]-thymidine, isolating clearly defined subpopulations by fluorescence-activated cell sorting, and determining the distribution of label by autoradiography. When mature thymocytes were precisely defined (as CD4$^+$CD8$^-$ CD3$^+$ or CD4$^-$CD8$^+$ CD3$^+$) and separated from immature single positives (CD4$^+$CD8$^-$ CD3$^-$ and CD4$^-$CD8$^+$ CD3$^-$), a lag was observed in the rate of entry of [^{3}H]thymidine into mature cells. Thus, many of the mature thymocytes appear to derive from a small nondividing cortical thymocyte pool, rather than originating directly from the earliest dividing CD4$^+$CD8$^+$ blasts. There was little evidence for cell division during or after mature thymocyte formation, suggesting a one-for-one differentiation from cortical cells rather than selective clonal expansion. The rate of production of mature single positive thymocytes agreed closely with estimates of the rate of export of mature T cells from the thymus and was only 3% of the rate of production of double-positive cortical thymocytes. This was compatible with a stringent selection process and extensive intrathymic cell death and suggested that no extensive negative selection occurred after the mature cells were formed.**

The majority of CD4$^+$CD8$^+$ (double positive) cortical thymocytes are believed to die in the thymus after a short life-span (1–5). However, some do mature into CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ (single positive) medullary thymocytes through a process of positive selection by self major histocompatibility complex (MHC) antigens (6–15). The number entering the mature state is reduced by a process of negative selection, which eliminates cells with excessive direct reactivity with self-MHC and self-antigens (8, 9, 12, 13, 16–19). Cortical thymocytes that are not rescued by the positive selection process presumably also die intrathymically of neglect.

Kinetic studies on T-lymphocyte development in the thymus originally presented the paradox of a very rapid rate of thymocyte birth (equivalent to one-third of all thymocytes per day) (1, 4) compared to a slow rate of export to the periphery (equivalent to 1% of all thymocytes per day) (3, 4). The conclusion from the balance sheet was that most cells born in the thymus die in the thymus. A series of studies comparing the relative retention in the thymus of iododeoxyuridine and thymidine after their incorporation into thymocyte DNA had seemed to provide direct evidence for this intrathymic death (19–21); however, deiodination, rather than cell death and nucleoside reutilization, appears to have been the real explanation of these results (22). Since the balance sheet aspects remain the primary argument for extensive intrathymic death, it is important to recheck the evidence for thymocyte overproduction and to determine whether it is the mature or the immature subpopulations that are involved.

The uptake of label into the DNA of cells in the S phase of the cell cycle shortly after injection of mice with [^{3}H]-thymidine can be used to estimate the incidence of dividing cells in a thymus subpopulation. The accumulation of labeled cells during semicontinuous administration of [^{3}H]thymidine can be used to measure the rate of accumulation of nondividing products of this cell division, the progressive decrease in the number of unlabeled cells reflecting the exit from the nondividing thymus subpopulation by death, migration, or differentiation. This approach had been used in this laboratory (2) to demonstrate that the cells of "cortical phenotype" were generated at a very much faster rate than the putative end-product cells of "medullary phenotype." In those experiments, the medullary phenotype cells appeared to label without any lag, evidence that they were not derived directly from the small nondividing cortical thymocytes; it was proposed that they came either from the dividing cortical blast cells (as some recent experiments have also suggested) (23) or from a population of dividing cells that were detected within the medullary phenotype population itself. However, the marker available at the time of these experiments (the level of surface Thy 1 antigen) separated thymocytes into only two general groups, and nowadays would be considered inadequate to distinguish the multiple subpopulations within the thymus. The group previously considered as mature medullary cells would have included some early, Thy 1^{low} precursor cells and would have pooled together the separate CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ lineages. We have therefore repeated experiments of this general type but using the surface markers CD4, CD8, and CD3 to define precisely the subpopulations used for kinetic analysis. The simultaneous use of at least three markers proved to be essential, the conclusions differing significantly from the earlier studies.

MATERIALS AND METHODS

Mice. Male 5-wk-old specific pathogen-free CBA CaH WEHI strain mice from the Hall Institute animal facility were used throughout.

Thymocyte Subpopulations. Full details of the isolation procedures are given elsewhere (24). Briefly, the following monoclonal antibodies were used: anti-CD4, GK1.5; anti-CD8, 49.11.1 for cytotoxic treatment and 53.6.7 for fluorescent labeling and magnetic bead depletion; anti-CD3, 145-2C11; anti-HSA (heat stable antigen), B2A2. CD4$^+$CD8$^+$, CD4$^+$CD8$^-$, and CD4$^-$CD8$^+$ cells were obtained by two-color fluorescent staining and sorting with a modified FACS II instrument. CD4$^-$CD8$^-$ cells were obtained by cytotoxic antibody and complement treatment followed by magnetic bead depletion. CD4$^-$CD8$^+$ CD3$^+$ and CD4$^+$CD8$^-$ CD3$^+$ cells were obtained by first depleting thymocytes bearing the

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Abbreviations: MHC, major histocompatibility complex; HSA, heat stable antigen.

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unwanted marker as described above and then sorting after two-color fluorescent staining of the two positive markers. $CD4^-CD8^+$ HSA^- and $CD4^+CD8^-$ HSA^- cells were prepared by depletion with anti-HSA, followed by two-color fluorescence staining and sorting for CD4 or CD8. The purity of the sorted fractions on reanalysis was in the range 95–99%.

[^{3}H]Thymidine Incorporation and Autoradiography. For measuring the incidence of dividing cells, 100 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (22 ml/mmol; Radiochemical Centre, Amersham) was injected intraperitoneally 1 hr before removal of the thymuses. For continuous labeling studies, 25 μCi of the same [^{3}H]thymidine was injected intraperitoneally twice daily. After separation of the subpopulations, cells were centrifuged through serum, resuspended in 5 μl of serum, hand-smeared onto gelatin-coated slides, and rapidly dried. The slides were fixed (89% methanol/1% acetic acid/10% water), dried, and dipped in autoradiographic emulsion (NTB-2; Kodak). After 4 weeks exposure, the slides were developed, lightly stained with Giemsa, and mounted using glass coverslips and dibutyryl polystyrene xylol. Labeled cells were scored as those with 3 grains per cell above background; 1000 cells were counted on each of two duplicate slides.

Propidium Iodide Staining and DNA Analysis. Cells in the sorted fractions were stained for DNA content by the procedure of Taylor (25), and 10^4 stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson). The red fluorescence histogram was then analyzed by the FACScan polynomial DNA compartmental analysis program.

RESULTS

Rates of Cell Division. The incidence of dividing cells in the major thymus subpopulations was assessed by two separate procedures—[^{3}H]thymidine uptake after a short pulse, which labels S-phase cells, and the distribution according to DNA content, which gives an estimate of the proportions in different phases of the cell cycle (Table 1). The results of the two methods were generally in agreement and in accordance with previous estimates that ≈20% of all thymocytes were actually dividing cells and ≈80% were nondividing product cells. A similar distribution was found amongst the major group of $CD4^+CD8^+$ thymocytes, indicating that ≈90% of all dividing cells in the thymus were $CD4^+CD8^+$. These dividing cells are known to be large cortical blast cells, the majority population of small cortical thymocytes being virtually all nonproliferating cells (1, 2, 4, 5). Although dividing cells were enriched in the minor $CD4^-CD8^-$ fraction, this accounted in total for <10% of all dividing cells in the thymus.

When single positives were isolated using just the CD4 and CD8 markers, both types had a significant incidence of dividing cells, although less than other fractions (Table 1). This result seemed in agreement with our earlier data (2). However, we (24) and others (23, 26, 27) have since demonstrated the existence of a subpopulation of $CD4^-CD8^+$ thymocytes that are not mature cells but are large outer-cortical cells that lack CD3 and express very high levels of HSA (recognized by the monoclonal antibodies B2A2, M1/69, or J11d). We have isolated this subset and found it contains a high proportion of dividing cells (24). Less well recognized is an apparently similar minor subpopulation of immature cells that are $CD4^+CD8^-$, which lack CD3 and many of which express high levels of HSA. This subpopulation represents ≈15% of all $CD4^+CD8^-$ thymocytes in our CBA mice, but it is variable in other strains. We have found this subpopulation to be enriched in the outer cortex and to contain dividing cells (unpublished data). Accordingly, we applied additional markers to exclude both of these types of "immature single positives." The mature cells were sorted as $CD3^+$ or, alternatively, were selected as being HSA^-. The former procedure allowed collection of all mature cells, whereas the latter procedure sampled only ≈30%, since some mature thymocytes express HSA at a level that is low but still sufficient to be eliminated by this procedure (24). When these extra criteria were applied, the apparent incidence of dividing cells in all mature thymocyte fractions fell markedly (Table 1). The low level finally measured in the $CD3^+$ and the HSA^- fractions may have been due to cross-contamination (up to 5% in these samples) and to inherent errors in the staining analysis (due to doublets and debris). When taken at face value, the data suggest there are still some dividing cells in the mature population, but the incidence of these is clearly very low.

Continuous Labeling Studies. To follow the flow of labeled DNA from the dividing into the nondividing product cells, [^{3}H]thymidine was injected twice daily for various periods. The rate of loss of unlabeled cells could then be used to determine the rate of cell turnover in the subpopulations. Labeling levels reached a plateau at ≈95% for all the populations studied (Fig. 1 *Top* and *Bottom*), showing that the injection and exposure protocols effectively labeled most newborn cells in the thymus and that even the slowly labeled thymocyte subpopulations turn over completely within 2–3 weeks. It was important that counts on the thymocyte suspensions showed that there was less than a 10% drop in the total cells recovered per thymus at any time after injection, indicating that neither radiation damage from [^{3}H]-thymidine nor the stress of the procedure had significantly perturbed the steady state of the young adult mouse thymus populations. Both the total thymus cells and the isolated majority population of $CD4^+CD8^+$ cortical thymocytes showed a rapid and near-linear accumulation of labeled cells (Fig. 1 *Top*). The results indicate that the major product of cell division within the thymus is the nondividing $CD4^+CD8^+$

Table 1. The incidence of dividing cells in thymus subpopulations

Thymus subpopulation	% of all thymocytes	[^{3}H]Thymidine uptake, % labeled	Propidium iodide staining analysis: % S phase	% G_2 + M phase	% G_0 + G_1 phase
All thymocytes	100	12 ± 1	11 ± 3	3 ± 1	86 ± 3
$CD4^+CD8^+$	86	11 ± 1	13 ± 3	3 ± 1	84 ± 3
$CD4^-CD8^-$	2.7	27 ± 3	30 ± 4	6 ± 3	64 ± 5
$CD4^-CD8^+$	3.1	5 ± 1	15 ± 1	2 ± 1	83 ± 1
$CD4^-CD8^+$ $CD3^+$	2.3	1	4 ± 2	1 ± 1	95 ± 2
$CD4^-CD8^+$ HSA^-	0.8	1	2 ± 1	1 ± 1	97 ± 1
$CD4^+CD8^-$	8.2	5 ± 1	7 ± 1	2 ± 1	91 ± 1
$CD4^+CD8^-$ $CD3^+$	7.7	1	4 ± 1	1 ± 1	95 ± 1
$CD4^+CD8^-$ HSA^-	2.5	1	2 ± 1	1 ± 1	97 ± 1

Where standard deviations are given, results are the means of three experiments; single experiments are shown otherwise. Each experiment was on a pool of four to six thymuses.

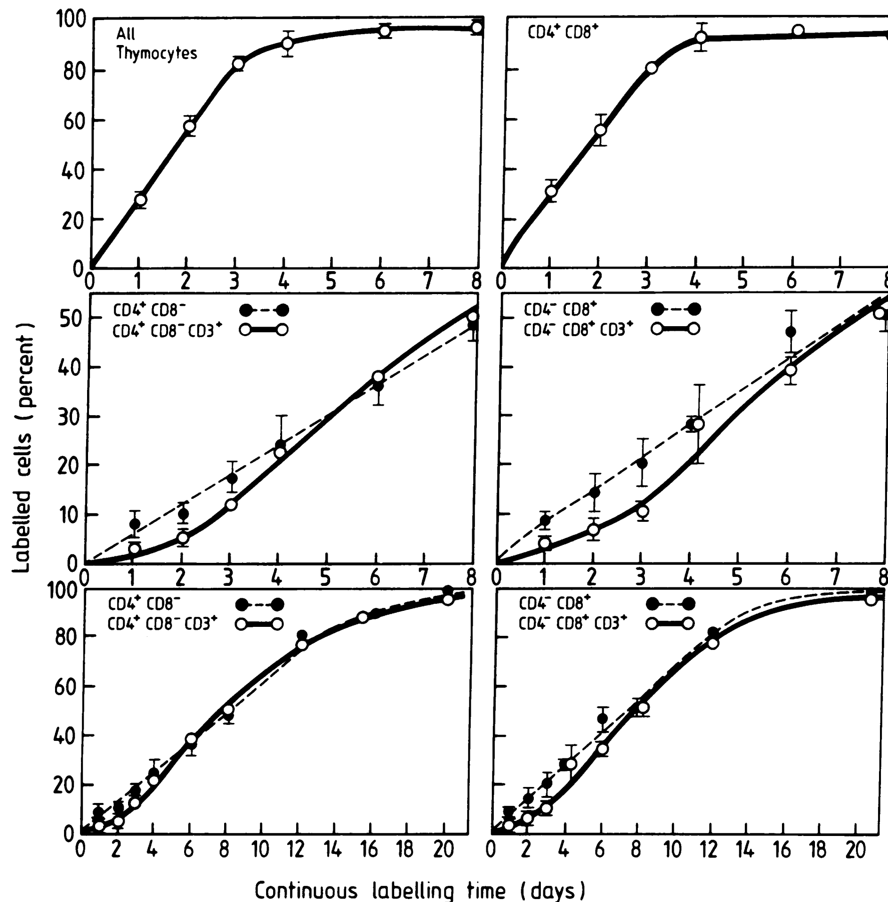

FIG. 1. The kinetics of accumulation of labeled cells in thymus subpopulations during semicontinuous [^{3}H]thymidine administration. Single positive thymocytes were purified either on the basis of the markers CD4 and CD8 alone (dashed lines), or on the basis of CD4, CD8, and CD3 (solid lines) to select only the mature cells. Where standard deviations are given, points are the means of three to five experiments; single experiments are shown elsewhere. Each experiment was on a pool of four to six thymuses, and values are based on counts of 1000 cells on each of two separate slides. (*Middle*) Kinetics of the crucial early times in detail. (*Bottom*) The same data as above but the full time course with approaching saturation is shown.

small cortical thymocyte and that these have a mean intrathymic life-span of 3.5 days after the last cell division in their generation. These results on directly isolated cortical cells agree closely with earlier estimates (2) in which less stringent markers were used and the behavior of cortical cells was estimated by an indirect procedure.

The flow of label into the single positive subpopulations was followed initially by using cells isolated on the basis of only two markers, CD4 and CD8 (Fig. 1). The results were in accordance with these populations having a slow generation rate and a slow turnover compared to the cortical cells. No lag was detected in the accumulation of labeled cells. Thus, both the $CD4^-CD8^+$ and the $CD4^+CD8^-$ subpopulations appeared to behave like the medullary phenotype population previously isolated on the basis of the level of Thy 1 expression (2). However, as noted above, these fractions must have contained a small proportion of immature single positives in rapid cell division, and this must have caused a false high estimate of mature cell labeling at the earlier time points. When these immature cells were removed by introducing the third marker CD3 as an additional criterion for maturity, the overall slow labeling kinetics was still obtained (Fig. 1 *Bottom*). However, an important difference was that both the $CD4^-CD8^+$ $CD3^+$ and the $CD4^+CD8^-$ $CD3^+$ subpopulations then showed a definite lag in the accumulation of labeled cells at the crucial early time points (Fig. 1 *Middle*). A χ^2 test showed that the $CD3^+$ values differed significantly from the no lag straight line expectation ($P = 0.01$–0.001) for both the 1- and 2-day points and for both subpopulations. Such a lag indicates that a major proportion of the mature cells were differentiating from a nondividing precursor cell.

DISCUSSION

The kinetics of mature T-cell development in the thymus has also been examined by Penit (28), who used bromodeoxyuridine to label the DNA of dividing cells and fluorescence microscopy as the detection system. This DNA precursor shows less reutilization problems than [^{3}H]thymidine, so the fate of a single pulse could be followed, but possible problems due to debromination (22) and interference with cell differentiation (29) must now be considered. In Penit's study the subpopulations were not isolated for direct examination, their labeling kinetics being calculated indirectly by subtraction after using one additional fluorescent color for phenotype analysis. Mature single positives were not segregated on the basis of CD3 staining. Penit's results indicated a 2-day lag in the entry of labeled cells into the $CD4^+CD8^-$ subpopulations, in general agreement with our findings for $CD4^+CD8^-$ $CD3^+$ mature cells. Presumably, interference by the immature $CD4^+CD8^-$ subset was not such a problem in the mouse strains used. However, no lag was seen in the entry of labeled cells into the $CD4^-CD8^+$ group. We suggest this was because the $CD4^-CD8^+$ $CD3^-$ immature cells obscured the result for the mature cells, much as we observed. Our results showing very similar generation kinetics for both mature thymocyte lineages suggest they originate from a common precursor by a similar selection process, in line with current models.

The presence of a lag in the entry of labeled cells into both subpopulations of mature thymocytes, when they are properly defined, suggests that mature cells are not all immediately derived from the earliest dividing cortical blasts. Many, if not all, must come from a nondividing precursor population. The most obvious candidate is the large pool of small nondividing $CD4^+CD8^+$ thymocytes. However, it should be noted that the lag observed in Fig. 1 was not an absolute one lasting 3.5 days, as would be expected if $CD4^+CD8^+$ cells were required to complete their intrathymic life-span before the choice between death or selection to maturity. Some mature cells were indeed labeled even by 1 day. At present we cannot tell whether these were derived from the dividing $CD4^+CD8^+$ blasts, from the earliest $CD4^+CD8^+$ small thymocytes, or from the few dividing cells we appeared to find in the $CD3^+$ single positive fractions (Table 1). If we assume they derive from $CD4^+CD8^+$ precursors, the results fit a model in which cells are selected at random from the entire pool of $CD4^+CD8^+$ thymocytes, regardless of their intrathymic age. The earliest blast cells should then be less depleted of selectable cells than the small cortical thymocytes. This, and their capacity for some further expansion, may explain why the $CD4^+CD8^+$ blasts give detectable mature products one day after intrathymic transfer but that small $CD4^+CD8^+$ thymocytes do not (23). However, there is an alternative model. Selection might act only on the $CD4^+CD8^+$ blasts, producing a special subpopulation of small nondividing $CD4^+CD8^+$ thymocytes already destined to mature but not yet expressing the mature surface phenotype: such cells would account for the labeling lag. The time taken to acquire high levels of CD3, or to lose CD4 or CD8, could produce such a subpopulation.

The average life-span of the mature single positive cells in the thymus, after allowing for the lag of $\approx$1 day in their labeling kinetics, is 12 days for both subpopulations. However, the accumulation of labeled cells (and loss of unlabeled cells) did not have the linear "first in first out" kinetics of the $CD4^+CD8^+$ subpopulation (Fig. 1 *Bottom*); the curves obtained suggest that exit of mature cells from the thymus is a stochastic process, the actual residence time of individual cells being very variable. In absolute terms, the rate of production of mature cells was calculated to be 3.4% that of the $CD4^+CD8^+$ cortical thymocytes, or equivalent to $\approx$1% of the thymus total cell number per day. This production rate is the same as the measured rate of export of cells from the thymus to the periphery as determined by the fluorescein isothiocyanate labeling method (3). This is in clear contrast to the rate of production of small cortical $CD4^+CD8^+$ thymocytes, which is far in excess of this export rate. In conjunction with the fact that recent thymus emigrants include few, if any, $CD4^+CD8^+$ cells (30), the data clearly support the view that the excess production within the thymus is confined to the $CD4^+CD8^+$ cells and that these die intrathymically.

These cell production and turnover figures have some implications for the process of specificity selection within the thymus. The low level of dividing cells we find in the mature thymocyte subpopulations, and the fact that many develop from nondividing cells, makes it unlikely that positive selection in the thymus is like antigen-selective clonal expansion in the periphery; the results suggest a more direct one-for-one differentiation process. The results also suggest that once they are generated there is little loss of mature single positive cells by death within the thymus and that any numerically significant negative selection occurs before the mature single positive phenotype is developed or during the actual process of development. It is tempting to guess that the measured 3% rate of selection of cortical thymocytes to maturity, and to export, represents the overall efficiency of the process of forming a T-cell antigen receptor that is structurally sound, restricted to self-MHC, but not directly self-reactive. However, it is possible that the system is redundant at several levels, and that not all potentially selectable cells are processed to the mature T-cell state. It will be of interest to compare these overall rates of cell birth, maturation, and death in the normal mouse thymus with those of cells bearing one particular selectable T-cell receptor, such as found in transgenic animals (9, 12, 15).

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