# The Kinetics of T Cell Antigen Receptor Expression by Subgroups of $CD4^+8^+$ Thymocytes: Delineation of $CD4^+8^+3^{2+}$ Thymocytes as Post-selection Intermediates Leading to Mature T Cells

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**Summary**

Cortical thymocytes from adult mice, separated on the basis of coexpression of CD4 and CD8 or of binding of high levels of peanut agglutinin (PNA), were subdivided according to the level of expression of the T cell receptor (TCR)-CD3 complex. The incidence of dividing cells in the resultant subpopulations was determined by DNA staining. Precursor-product relationships and the timing of TCR-CD3 acquisition were studied using continuous in vivo [$^3$H]TdR labeling and radioautography. The extent of intrathymic selection for TCR specificity in the subpopulations was determined from the incidence of cells bearing V$\beta$6 or V$\beta$17a in different mouse strains.

The majority of dividing $CD4^+8^+$ blast cells expressed extremely low levels of TCR-CD3, indicating that TCR expression and specificity selection generally occurred after division ceased. The [$^3$H]TdR-labeling studies indicated that postdivision TCR expression was rapid, and that those nondividing cortical thymocytes which had not expressed significant levels of TCR by day 1, remained extremely low or negative for their entire 3.6-d lifespan. Small cortical thymocytes which expressed moderate levels of TCR-CD3, were predominantly an unselected population with a lifespan of 3.8 d.

A small subgroup of $CD4^+8^+$ $PNA^+$ cortical thymocytes expressing high levels of TCR-CD3 was identified as a nondividing intermediate between the small cortical thymocytes expressing moderate levels of TCR and mature medullary thymocytes. These intermediates showed a 1-d lag in [$^3$H]TdR labeling, then a 3.4-d transit time. The cell flux through this intermediate subpopulation was $\sim 10^6$ cells/d, similar to the rate of turnover of mature thymocytes; thus, although only 3–4% of thymocytes progressed to this intermediate state, once reaching it most then progressed to full maturity. In accordance with this, the incidence of the V$\beta$ selection markers within the intermediate subpopulation indicated that both positive and negative selection had already occurred. Selection for TCR specificity in the systems studied appeared to take place among $CD4^+8^+$ thymocytes expressing intermediate levels of TCR.

The $CD4^+8^+$ (double-positive) cortical population of the thymus, which strongly binds peanut agglutinin ($PNA^+$),[1] represents the stage of T cell development where the $\alpha/\beta$ form of the TCR and the associated CD3 complex is first expressed (1). The earliest $CD4^+8^+$ thymocytes are a 20% subgroup of rapidly dividing blast cells, which directly generate the 80% nondividing small cortical thymocyte population (1). It has not been clear whether it is the rapidly dividing blasts or their nondividing products that first express significant levels of surface TCR, a point to be considered in this study. Once formed, these $CD4^+8^+$ $PNA^+$ TCR-$CD3^+$ thymocytes are subjected to a stringent selection process, involving positive selection for appropriate interaction with the self-MHC proteins of the antigen-presenting system, and negative selection against reactivity with a variety of self-antigens (2–5). Only 3–4% of all cortical thymocytes survive selection and progress to become mature T cells (6). Our previous kinetic data (6), and that of Penit (7), indicated that most newly formed mature thymocytes differentiate from nondividing thymocytes, rather than from the dividing $CD4^+8^+$ blasts as Guidos et al. have proposed (8). This final maturation involves upregulation of the TCR-CD3 complex on the cell surface, and downregulation of either CD4 or CD8, and

[1] *Abbreviations used in this paper:* BSS, Hepes buffered balanced salt solution; Mls, minor lymphocyte stimulatory locus; PNA, peanut agglutinin; S-phase, phase of DNA synthesis.


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of PNA binding, to produce single positive mature medullary thymocytes; these changes have been shown to depend on interaction with self-MHC (9, 10).

If upregulation of the TCR-CD3 complex precedes the other changes, a distinct transient intermediate should be generated. Such $CD4^+8^+3^{2+}$ and $PNA^+$ $CD3^{2+}$ thymocytes were first detected in the cortex of the human thymus (11, 12), and were proposed as intermediates between double-positive cortical and single-positive medullary cells. At that time it was considered that all cortical thymocytes would progress and mature via this route, a view in conflict with cell kinetic data indicating that most cortical thymocytes behaved as end cells. With the accumulating evidence that a small proportion of $CD4^+8^+$ thymocytes do progress to becoming mature T cells, via a process involving specificity selection, interest in the $CD4^+8^+3^{2+}$ thymocyte as a developmental intermediate has been revived. In separate studies from this laboratory, Hugo, P., R.L. Boyd, G.A. Waanders, H.T. Petrie, and R. Scollay, (manuscript submitted for publication) found such cells to be intermediate between double positive and single positive thymocytes in their pattern of expression of a number of surface markers, and Petrie, H.T., P. Hugo, R. Scollay, and K. Shortman, (manuscript submitted for publication) have observed a sequential appearance of such cells between double-positive and single-positive thymocytes after intrathymic transfer of $CD4^-8^-$ precursors. In work from other laboratories, Guidos and colleagues (8, 13) and Penit (14) have implicated similar cells as developmental intermediates.

In the present study we have used continuous [$^3$H]TdR labeling to determine if the timing of the upregulation of the CD3-TCR complex to produce these $CD4^+8^+3^{2+}$ thymocytes accords with that expected of a developmental intermediate, and to determine if sufficient cells flow through this minor subpopulation for it to be the immediate source of all mature T cells. We have also used the representation of V$\beta$6- and V$\beta$17a-bearing cells within this subpopulation in different mouse strains to determine whether these thymocytes are formed before or after selection of the TCR specificity repertoire.

## Materials and Methods

*Animals.* Most experiments, including the cell cycle and kinetic studies, were performed using specific pathogen-free 4–5-wk-old male CBA CaH Wehi mice. For the TCR specificity selection studies, the mice used were BALB/c and DBA/2 specific pathogen-free females, B10BR and B10G conventionally reared males, or SWR and C57BR conventionally reared females. All were used at 4–6 wk of age. All mice were bred at The Walter and Eliza Hall Institute Animal Facility (Melbourne, Australia), except for the C57BR which were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were left undisturbed for at least 1 wk before removal of thymuses, to allow recovery from any stress of transport or recaging.

*Cell Preparation.* Thymuses were teased into cold, mouse-osmolarity, pH 7.2, Hepes buffered balanced salt solution (BSS) containing 5% FCS, the suspension settled to remove large debris and spun through a layer of FCS to remove fine debris, and the cells then resuspended in cold BSS-FCS.

*[$^3$H]Thymidine Labeling and Radioautography.* Full details have been presented elsewhere (6). The mice, generally three per timepoint per experiment, were given twice-daily (8–9 am and 5–6 pm) injections of 25 $\mu$Ci [$^3$H]TdR (1 Ci = 37 GBq) (Radiochemical Centre, Amersham, UK). This schedule produced no detectable (<10%) loss of thymus weight during the labeling period. Thymuses were removed after 1–5 d of labeling and pooled, cell suspensions prepared, and the thymocyte stained and sorted as described below. The sorted cells were smeared onto gelatin-coated slides, the slides dipped in photographic emulsion (NTB-2; Kodak Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY), and then exposed for 5 wk. Labeled cells were counted as those showing three grains per cell above background. Between 200 and 1,000 cells were counted on each slide, and two to three replicate slides were counted for each timepoint of each experiment.

*Cell Cycle Analysis by Propidium Iodide Staining.* The procedure was the same as that used previously (6). Subpopulations of thymocytes were surface stained and sorted as described below. The cellular DNA of the sorted cells was then stained with propidium iodide using the procedure of Taylor (15). The DNA content of $10^4$ cells was analyzed on a FACScan flow cytometer (Becton Dickinson and Co., San Jose, CA) using the FACScan polynomial DNA compartmental analysis program.

*Monoclonal Antibodies, Fluorescent Reagents, and Staining Procedures.* All hybridomas were grown and the mAbs purified and conjugated in this laboratory. Anti-CD4 was GK1.5 (16), conjugated to allophycocyanin; anti-CD8 was 53-6.7 (17), conjugated to FITC (or for four-color staining was unconjugated protein used with an anti–rat Ig coupled to Texas red second stage [Caltag Laboratories, San Francisco, CA]); anti-CD3 was KT3-1.1 (18) conjugated to biotin; anti-V$\beta$6 was 44-22-1 (19) conjugated to biotin; anti V$\beta$17a was KJ23a (20), conjugated to biotin. The biotin-conjugated antibodies were used with a PE-streptavadin second stage (Caltag Laboratories). PNA was a FITC conjugate (E.Y. Laboratories, Inc., San Mateo, CA), and was used at subagglutinating levels. Immunofluorescent staining procedures were as described previously (6, 21, 22). All staining was at 0–4°C without any prior incubation of cells at higher temperatures. Propidium iodide (Calbiochem, La Jolla, CA) was included at 5 $\mu$g/ml in the final wash to label dead cells.

*Flow Cytometric Analysis and Cell Sorting.* Sorting on the basis of two fluorescent colors was carried out on a modified FACS II instrument (Becton Dickinson and Co.). Sorting on the basis of three fluorescent colors, and flow cytometric analysis on the basis of one to four fluorescent colors, was carried out on a FACStar Plus instrument (Becton Dickinson & Co.). Cells were passed through a 25-gauge needle just before analysis, to reduce the incidence of doublets. Dead cells were excluded on the basis of low-angle light scatter and bright propidium iodide staining. Where noted, the additional low-angle light scatter gate was used to exclude the large, dividing blast thymocytes, but to include in the "small" category all cells of the size of small cortical thymocytes and most of those of the size of mature medullary thymocytes, as detailed previously (21). Analyses were based on 25,000–100,000 cell files.

## Results

*Surface Expression of the TCR-CD3 Complex by $CD4^+8^+$ Thymocytes.* Unseparated thymocyte suspensions and gated

$CD4^+8^+$ thymocytes both displayed three distinct surface levels of the TCR-CD3 complex on fluorescent staining with anti-CD3 (Fig. 1), anti-TCR-$\alpha/\beta$ (not shown), or specific anti-V$\beta$ chain mAb (Fig. 1), as others have also observed (23, 24; Hugo, P., R.L. Boyd, G.A. Waanders, H.T. Petrie, and R. Scollay, manuscript submitted for publication). The major very low fluorescence peak we will term TCR-$CD3^-$, even though when sensitive staining and analysis procedures were used this peak was displaced from the background second stage staining reagent controls. This displacement of the entire first peak from the background was also seen on staining with an anti-TCR-$\alpha/\beta$ mAb, and with some V$\beta$-specific mAb where only a small proportion of the thymocytes would be potentially positive. Such very low level staining of all cells might be due to very low levels of surface TCR-CD3, but might also represent nonspecific binding or an irrelevant cross-reactivity. We have not attempted to distinguish these alternatives. The second major peak of cells with moderate fluorescence we will term TCR-$CD3^+$. The third, numerically smaller, high fluorescence peak we will term TCR-$CD3^{2+}$. The thymocytes of this latter peak expressed the same levels of the TCR-CD3 complex as mature peripheral T cells, and the majority of these cells were mature, $CD4^-CD8^+$ or $CD4^+CD8^-$, medullary thymocytes.

However, as well as the mature medullary thymocytes, a minor subgroup of $CD4^+8^+$ thymocytes also expressed high levels of surface TCR-CD3 (Fig. 1). We will term these cells TCR-$CD3^{2+}$ as well, even though their peak fluorescence was sometimes marginally below that of mature cells, and extended into the TCR-CD3 "intermediate" zone. By sorting

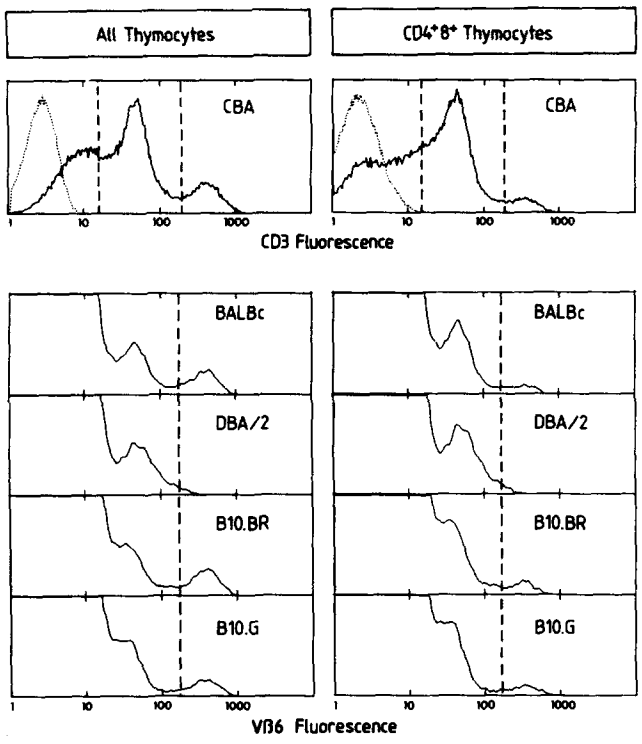


**Figure 1.** The level of expression of the TCR-CD3 complex by thymocytes of different mouse strains. Thymus cell suspensions were stained in one fluorescent color with either anti-CD3 or anti-V$\beta$6 alone (*left*), or in three fluorescent colors with these reagents and anti-CD4 plus anti-CD8, with gating for $CD4^+8^+$ cells (*right*). The broken lines indicate the usual gates for defining the $CD3^-$, $CD3^+$, and $CD3^{2+}$ populations. Note that the "$CD3^-$" group may have expressed extremely low levels of the TCR-CD3 complex.

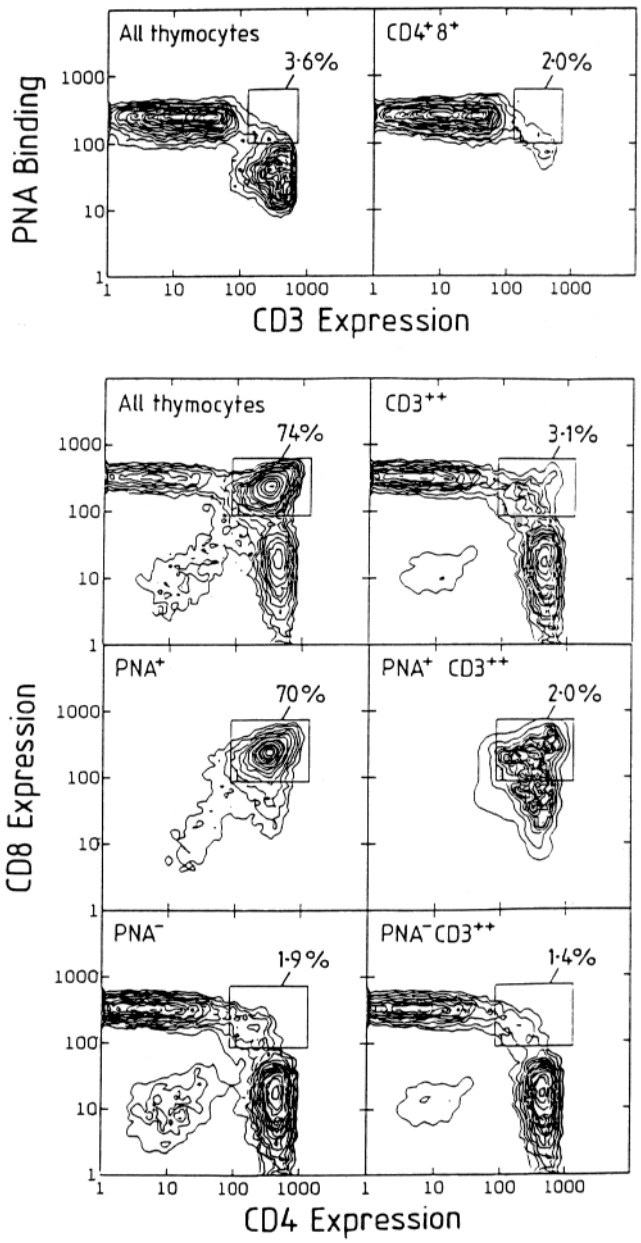


**Figure 2.** Fluorescent four-color crosscorrelation analysis for the extent of correspondence between $PNA^+$ $CD3^{2+}$ and $CD4^+8^+$ $CD3^{2+}$ thymocytes. One example of the analysis of CBA thymocytes is given. The gating for $CD3^{2+}$ was as in Fig. 1. The proportion of total thymocytes within the gates indicated for this particular experiment is shown on each two-parameter contour plot.

and visual inspection it was verified that the majority of these were single cells, not doublets. Low-angle light scatter analysis indicated that the majority were slightly larger than the typical small cortical thymocyte, but smaller than mature thymocytes (data not shown). This $CD4^+8^+3^{2+}$ subpopulation was the putative intermediate transit cell to be studied in our detailed kinetic experiments.

*Binding of PNA by $CD4^+8^+CD3^{2+}$ Thymocytes.* Cortical $CD4^+8^+$ thymocytes bind high levels of $PNA^+$, and this also serves to distinguish them from mature, medullary thymocytes which bind only very low levels ($PNA^-$) (21). When thymocytes were stained with PNA and CD3, a distinct group of $PNA^+CD3^{2+}$ thymocytes was also seen, similar in number to the $CD4^+8^+3^{2+}$ cells (data not shown). To determine if these were identical subpopulations, thymocytes were stained in four fluorescent colors with anti-CD4, anti-CD8, anti-CD3, and PNA, and the various markers cross-correlated (Fig. 2). It was clear that with the reasonably tight gates used that there was extensive correspondence between the $CD4^+8^+3^{2+}$ and the $PNA^+CD3^{2+}$ subpopulations (65% in Fig. 2 and higher in other experiments), and this correspondence increased if the gates were widened. However, with the gates shown in Fig. 2, some $CD4^+8^+3^{2+}$ thymocytes would be classed as $PNA^-$, and some $PNA^+$ $CD3^{2+}$ thymocytes would be classed as $CD4^+8^+$. Assuming these groups all represented transit states en route to mature T cells, the results would fit a model whereby $CD4^+8^+$ $PNA^+$ thymocytes lost CD8 before PNA binding en route to becoming $CD4^+8^-PNA^-$ T cells, but lost PNA binding before CD4 en route to becoming $CD4^-8^+PNA^-$ T cells.

*Do Dividing $CD4^+8^+$ Thymocytes Express Surface TCR?* To determine the relationship between cell division and TCR-CD3 surface expression, thymocytes were separated into various categories according to CD4, CD8, and CD3 expression, and the cell cycle status of the sorted subpopulations was then determined by propidium iodide staining for DNA content. As Table 1 demonstrates, most of the dividing $CD4^+8^+$ cells were clearly within the $CD3^-$ category, although only about half of all $CD3^-$ cells were dividing. However, a low level of division was noted within the $CD3^+$ group. This indicated that, at least for the majority of thymocytes, TCR-CD3 was first expressed at moderate levels on the cell surface after the cells ceased dividing. These observations were checked using low-angle light scatter as a measure of the incidence of large blast cells (21). The incidence of such "blast" cells was 3.3-fold lower in $CD4^+8^+3^-$ cells compared to $CD4^+8^+3^+$ cells. This shows the same tendency as the cell division rate estimates, but was less than the fivefold differences of Table 1. This suggests that there were some $CD4^+8^+3^+$ thymocytes that had ceased division but were still relatively large in size.

The small subgroup of $CD4^+8^+$ thymocytes expressing very high levels of TCR-CD3 showed the same low level of cycling cells as mature single positive thymocytes (Table 1). Note that this level of cells synthesizing DNA [S-phase cells], although low, was not negative; this is in accordance with our earlier studies (6) showing a few dividing cells occurred among the mature thymocytes of adult mice. Overall, these results supported the view that the transit from $CD4^+8^+$ cortical cells to mature thymocytes in adult mice normally does not involve cell division.

**Table 1.** *Cell Cycle Analysis of Thymus Populations*

| Population | Percentage of all thymocytes | Percentage of population in: S | $G_2+M$ | $G_o+G_1$ |
|---|---|---|---|---|
| $CD4^+8^+$ $CD3^-$ | 38 | 20 ± 2 | 4 ± 3 | 76 ± 4 |
| $CD4^+8^+$ $CD3^+$ | 44 | 4 ± 1 | 1 ± 1 | 95 ± 2 |
| $CD4^+8^+$ $CD3^{2+}$ | 3 | 3 ± 2 | 1 ± 1 | 96 ± 3 |
| $CD4^+8^-3^{2+}$ and $CD4^-8^+3^{2+}$ | 12 | 4 ± 1 | 1 ± 1 | 95 ± 2 |

Populations of thymocytes from CBA mice were isolated by three-color fluorescent staining and sorting, as in Fig. 1, using the CD3 gates illustrated in Fig. 1. Note that the "$CD3^-$" group may have expressed extremely low surface levels of TCR-CD3. Low-angle light scatter gates were used to exclude dead cells and debris, but in contrast to Fig. 3, the large sized blast cells were not excluded. The cellular DNA of the sorted subpopulations was then stained with propidium iodide, and the cell cycle stage distribution then calculated from the DNA distribution profiles. Results are the means (± SEM) of three separate experiments.

*[$^3$H]TdR Labeling Kinetics of Cortical Thymocytes Expressing Various Levels of TCR.* To determine the post-cell division precursor-product relationships between $CD4^+8^+PNA^+$ thymocytes expressing various levels of the TCR-CD3 complex, we followed the rate of accumulation of labeled cells within each subpopulation under conditions of semi-continuous [$^3$H]TdR administration. The rate of loss of unlabeled cells then indicated the turnover rate of the subpopulation. These experiments involved repeated administration of [$^3$H]TdR to mice, isolation of thymus subpopulations at various times by cell sorting, then radioautographic analysis of the sorted cells. This approach was previously used by us (6) to demonstrate the high production rate and fast turnover of $CD4^+8^+$ thymocytes and the low production rate and slow turnover of mature $CD4^+8^-3^{2+}$ and $CD4^-8^+3^{2+}$ thymocytes. This, combined with the observed lag in the accumulation of label into the mature cells, led us to conclude that their immediate precursors were nondividing, small cortical thymocytes. In the present study we have extended our kinetic analysis by making three subdivisions of CD $4^+8^+PNA^+$ thymocytes according to surface CD3 level, as shown in Fig. 1. We chose PNA binding as the separation marker for the $CD4^+8^+PNA^+$ population, since the subsets expressing various CD3 levels could then be isolated by two-color rather than three-color sorting; only a two-color sorting instrument was continuously available on the daily basis required for this kinetic series. To direct attention to postmitotic events, the $PNA^+$ thymocytes were gated on the basis of light scatter to exclude the larger dividing blasts; such light scatter gating gives a good discrimination between dividing and nondividing thymocytes (21). This gating also

served to exclude some immature, large sized $CD4^-8^-$ thymocytes which would otherwise be included within the $PNA^+$ category, and to exclude any cell doublets or aggregates. However, we included within this "small $PNA^+$" fraction cells up to the medium size of mature thymocytes; the majority of $CD4^+8^+PNA^+$ $CD3^{2+}$ thymocytes fell within these scatter gates.

The small $PNA^+$ thymocytes showed the overall labeling pattern expected from our previous studies (6) on $CD4^+8^+$ thymocytes: a very short lag (about equivalent to one cell cycle), then a linear labeling pattern, suggesting an orderly sequence of cell generation, fixed residence time, then cell exit, with virtually complete turnover of the population by 4 d (Fig. 3). This contrasted with the prolonged lag, the slow turnover and the nonlinear labeling pattern obtained with mature thymocytes, as reported previously (6) and as shown only for the first 5 d in Fig. 3. The labeling pattern of the $PNA^+$ $CD3^-$ and $PNA^+$ $CD3^+$ cells was similar to that of $PNA^+$ small cells in general, but the $CD3^-$ cells appeared to have a shorter initial lag and consistently showed a slightly faster turnover than the $CD3^+$ cells; this small difference in turnover was also found in four other kinetic experiments each using different mouse strains (data not shown). The important point, however, is that both subpopulations gave a strictly linear accumulation of labeled cells, with no evidence for a shift of cells with time from the $CD3^-$ to the $CD3^+$ category. This indicates that from the first measured point (1 d) onwards, the small, nondividing $CD3^-$ cells remained $CD3^-$ rather than becoming $CD3^+$. We conclude that expression of the TCR-CD3 complex occurs very rapidly after cell division ceases, and that those cells which are still within the $CD3^-$ gate by day 1 are fated never to express higher levels of surface TCR.

In contrast to the $PNA^+$ $CD3^+$ cells, a very distinct and consistent lag of 1 d was obtained for the labeling of the small group of $PNA^+$ $CD3^{2+}$ thymocytes (Fig. 3). This indicates they were not derived directly from dividing cortical blasts, as were the $CD3^-$ and $CD3^+$ subsets, but from a nondividing precursor population. After this lag, the accumulation of labeled cells was rapid and close to linear, with most cells labeled by day 4; this indicates that only 3 d was required for transit through the $PNA^+$ $CD3^{2+}$ cell compartment. If the labeling kinetics of this $PNA^+$ $CD3^{2+}$ subset is compared to that of small $PNA^+$ $CD3^+$ cortical cells, and that of the mature single positive thymocytes (Fig. 3), the data support the concept that the $PNA^+$ $CD3^{2+}$ cells are intermediates, derived from only a small proportion of the small $PNA^+$ $CD3^+$ cells, and en route to becoming mature single positive thymocytes.

*Cell Flow through Thymocyte Subpopulations.* The view that $CD4^+8^+$ $PNA^+$ $CD3^{2+}$ cells were the immediate precursors of mature thymocytes was further checked by calculating the daily cell flux through the different subpopulations, based on the numerical size of each subpopulation and the turnover times calculated from Fig. 3 and from our previous studies (6). These results are summarized in Table 2. Only 7% of the daily production of $CD4^+8^+$ $PNA^+$ $CD3^+$ small cortical thymocytes, or 3.5% of the total daily production of all small cortical thymocytes, appeared to upregulate the TCR-CD3 complex and become $CD4^+8^+$ $PNA^+$ $CD3^{2+}$ inter-

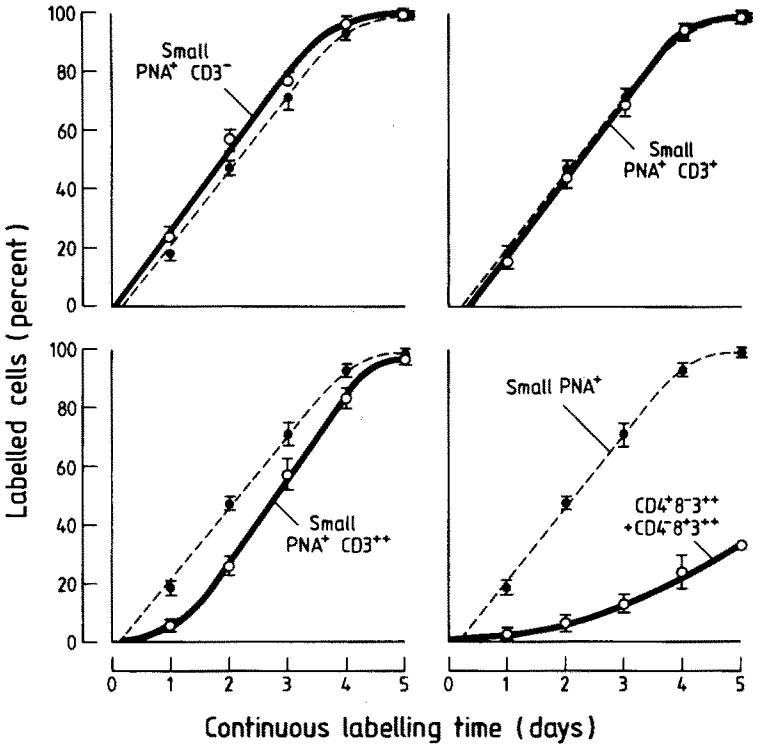


**Figure 3.** Kinetics of in vivo accumulation of labeled thymocytes during semi-continuous [$^3$H]thymidine administration. CBA mice were used. In most experiments cells were labeled in two fluorescent colors for PNA binding and CD3 expression using the gates of Figs. 1 and 2; additional low-angle light scatter gating was used to exclude large dividing blast cells and so produce "small" (small + medium) thymocytes. In the case of the pooled mature single positive thymocytes, cells were labeled with CD4, CD8, and CD3 as detailed elsewhere (6), but were not gated for cell size. The results for the sorted total small $PNA^+$ fraction, not separated on the basis of CD3 expression, is given as the broken line on each graph to facilitate comparison.

**Table 2.** *Cell Production and Turnover in Thymus Populations*

| Population | Percentage of all thymocytes | Lifespan or transit time (d) | Calculated turnover (cells/d/thymus) |
|---|---|---|---|
| $PNA^+$ $CD3^-$ | 37 | 3.5 | $10.6 \times 10^6$ |
| $PNA^+$ $CD3^+$ | 46 | 3.8 | $12.1 \times 10^6$ |
| $PNA^+$ $CD3^{2+}$ | 2.8 | 3.4 | $0.8 \times 10^6$ |
| $CD4^+8^-3^{2+}$ and $CD4^-8^+3^{2+}$ | 12 | 13.3 | $0.9 \times 10^6$ |

The turnover times were calculated from the data of Fig. 3, except for mature thymocytes where the more extended kinetic data of reference 6 were used. In this case the mean transit time was calculated from the 7.5% per day turnover in the near-linear central part of the labeling curve; this is an average value, since the intrathymic residence time of individual mature thymocytes is variable. The relative numbers of each subpopulation in the thymus represent the mean values for the experiments of Fig. 2. The mouse thymus is assumed to contain $10^8$ thymocytes, close to the mean for the strain used.

mediate cells. However, the number of these putative intermediate thymocytes generated by this process each day was very close to the final daily rate of generation of mature thymocytes.

*Positive and Negative Selection for Vβ6 in the $CD4^+8^+3^{2+}$ Subpopulation.* The small proportion of $CD4^+8^+$ thymocytes that finally mature into T cells is believed to reflect extensive selection for TCRs of appropriate specificity. Since according to the above calculations virtually all of this loss of cortical thymocytes occurs before the formation of the $CD4^+8^+$ $PNA^+$ $CD3^{2+}$ cells, these should be a post-selection population resembling mature thymocytes in TCR repertoire. The effects of selection on subpopulations of normal mouse thymocytes can be monitored by changes in the incidence of cells expressing particular Vβ gene products (5). We first determined the relative representation of Vβ6 in the CD $4^+8^+3^{2+}$ subpopulation, using the approach developed by MacDonald and colleagues (25, 26). In mice expressing products of the minor lymphocyte stimulatory locus ($Mls^a$), Vβ6-bearing cells are deleted within the thymus before the mature thymocyte stage, so providing a model for negative selection. In mice expressing IE class II MHC gene products (but not $Mls^a$), Vβ6-bearing cells are present at relatively high frequency among mature but not among cortical thymocytes, so providing a model of positive selection. The distribution of Vβ6 TCR expression by thymocytes of the mouse strains used to assess negative and positive selection is shown in Fig. 1; $V\beta6^-$, $V\beta6^+$, and $V\beta6^{2+}$ cells were readily discriminated and quantitated. By comparing these values to similar determinations of the total incidence of TCR-bearing cells (from anti-CD3 staining), the relative incidence of Vβ6 among the thymocyte was calculated.

By comparing results from the $Mls^a$, DBA/2 mice with that of the two $Mls^b$ strains (Table 3 and Fig. 1), it was clear that negative selection against $Mls^a$–reactivity was not detectable at the TCR-$CD3^+$ stage, and was extensive by the $CD4^+8^+$ TCR-$CD3^{2+}$ stage; deletion appeared to take place at a TCR-CD3 "intermediate" stage. By comparing results from the $IE^-$, $Mls^b$, B10G mice with those from the two $IE^+$, $Mls^b$ strains, it appeared that positive selection was not detectable at the TCR-$CD3^+$ stage and was evident and statistically significant ($p = .01$–$.02$) by the $CD4^+8^+$ TCR-$CD3^{2+}$ stage (Table 3). Positive selection effects were not immediately apparent from the Vβ6 distributions alone (Fig. 1), since the incidence of $CD4^+8^+$ $CD3^{2+}$ cells varied considerably between the strains and the relevant parameter was the incidence of Vβ6 relative to that of other TCRs, as monitored by CD3 expression (Table 3). Since the differences in Vβ6 incidence being measured were relatively small, even within the mature population, it was not possible to judge whether such positive selection was complete or only partial.

*Negative Selection for Vβ17a in the $CD4^+8^+3^{2+}$ Subpopulation.* It was possible that clonal deletion within the thymus

**Table 3.** *Selective Changes in Vβ Expression among $CD4^+8^+$ Thymocytes*

| Mouse strain | $CD4^+8^+$ thymocytes | | | | | | $CD4^+8^-$ and $CD4^-8^+$ mature thymocytes ($V\beta6^{2+}/CD3^{2+}$) |
|---|---|---|---|---|---|---|---|
| | $CD3^+$ | $CD3^{2+}$ | $V\beta6^+$ | $V\beta6^{2+}$ | $V\beta6^+/CD3^+$ | $V\beta6^{2+}/CD3^{2+}$ | |
| | (%) | (%) | (%) | (%) | | | |
| BALB/c ($Mls^b$, $IE^+$) | 51 ± 3 | 5.6 ± 0.8 | 4.0 ± 0.5 | 0.62 ± 0.02 | 0.08 ± 0.01 | 0.114 ± 0.013 | 0.116 ± 0.014 |
| DBA/2 ($Mls^a$, $IE^+$) | 50 ± 4 | 5.4 ± 0.1 | 3.1 ± 0.3 | 0.10 ± 0.05 | 0.06 ± 0.01 | 0.022 ± 0.007 | 0.003 ± 0.001 |
| B10BR ($Mls^b$, $IE^+$) | 49 ± 3 | 4.1 ± 0.4 | 2.8 ± 0.2 | 0.40 ± 0.05 | 0.06 ± 0.01 | 0.099 ± 0.008 | 0.098 ± 0.005 |
| B10G ($Mls^b$, $IE^-$) | 53 ± 3 | 4.8 ± 0.4 | 3.1 ± 0.2 | 0.36 ± 0.04 | 0.06 ± 0.01 | 0.076 ± 0.006 | 0.057 ± 0.006 |

Thymocytes from 5-wk-old mice were stained with anti-CD4, anti-CD8, and anti-CD3, or with anti-CD4, anti-CD8, and anti-Vβ6, as detailed in Fig. 1. After flow cytometric analysis of 100,000 cells, the proportions of cells in each population were determined, using the TCR-CD3 gates illustrated in Fig. 1. The results are the means (± SEM) of eight determinations (B10BR and B10G) or of three determinations (BALB/c and DBA/2) each determination being on a pool of two to four thymuses. The ratios were determined for each sample, and the results are the means (± SEM) of these individual ratios. The separated groups of $CD4^+8^-$ and $CD4^-8^+$ mature thymocytes were pooled for these TCR-CD3 analyses, to provide an appropriate comparison with the double positive thymocytes.

**Table 4.** *Selective Changes in Vβ17a Expression among CD4⁺8⁺ thymocytes*

| Mouse strain | CD4⁺8⁺ thymocytes | | | | | | CD4⁺8⁻ and CD4⁻8⁺ mature thymocytes |
|---|---|---|---|---|---|---|---|
| | CD3⁺ | CD3²⁺ | Vβ17a⁺ | Vβ17a²⁺ | Vβ17a⁺/CD3⁺ | Vβ17a²⁺/CD3²⁺ | Vβ17a²⁺/CD3²⁺ |
| | (%) | (%) | (%) | (%) | | | |
| SWR (IE⁻) | 51 ± 1 | 3.9 ± 0.1 | 1.5 ± 0.1 | 0.19 ± 0.01 | 0.030 ± 0.001 | 0.048 ± 0.005 | 0.099 ± 0.007 |
| C57BR (IE⁺) | 57 ± 1 | 5.7 ± 0.2 | 1.9 ± 0.2 | 0.08 ± 0.03 | 0.032 ± 0.003 | 0.014 ± 0.004 | 0.005 ± 0.001 |

The results were obtained as in Table 3, with anti-Vβ17a substituted for anti-Vβ6. Results are the means of three determinations, each on a pool of three thymuses.

according to Mls$^a$–reactivity was not a general model for negative selection, since presentation of this self-antigen has unusual features (27). The findings were therefore checked using the model of Kappler et al. (5) of deletion of thymocytes bearing Vβ17a in certain IE⁺ mouse strains. In C57BR, IE⁺ mice, where V7β17a-bearing cells have been deleted from mature thymocytes, the incidence of cells bearing high levels of Vβ17a was also low in the CD4⁺8⁺3²⁺ subpopulation compared to that in SWR, IE⁻ mice (Table 4). Again, the deletion was not apparent among the CD4⁺8⁺ thymocytes bearing low levels of CD3 or Vβ17a. This indicated that a loss of self-reactive thymocytes by deletion before the CD4⁺8⁺ PNA⁺ CD3²⁺ stage was common to both the Mls$^a$–dependent and the IE–dependent models (5, 25, 26) of tolerance induction.

## Discussion

*Cell Division, TCR Expression, and Specificity Selection.* Our cell cycle analysis results, when combined with our earlier findings (6), lead us to the generalization that in adult mice most dividing CD4⁺8⁺ thymocytes express either no surface TCR, or only extremely low levels, and that conversely most thymocytes expressing moderate to high levels of surface TCR are not dividing. This implies that the cells being subjected to positive selection, the precursors of mature T cells, are mainly nondividing small cortical thymocytes. A crucial piece of evidence in support of this argument is the lag in the entry of DNA precursors into the mature thymocyte population, indicating most of the immediate precursors are nondividing cells (6, 14). Our interpretation differs from that of Guidos et al. (8, 13), who claim that CD4⁺8⁺ blasts express a low and significant level of TCR-CD3, and are directly subject to positive selection. Their blasts were apparently defined by light scatter rather than cell cycle status and from our results they may have included large but nondividing cells. If only blasts were subject to positive selection, our results would imply they must still pass through a nondividing stage lasting several days before becoming mature cells. It is simpler to suppose that positive selection normally begins to take effect when the surface TCR-CD3 level is in the readily detected, medium-expression range, a stage when the majority of cells are nondividing small thymocytes.

A further implication of our generalization is that positive selection itself is a one-for-one process, and does not involve selective clonal proliferation. Again, the low incidence of dividing cells among mature thymocytes and the lag in their labeling with DNA precursors supports this concept. Similar conclusions have been reached by von Boehmer and Kisielow (38) studying TCR-transgenic mice.

Our generalization is clearly not completely accurate, however, since a few dividing CD4⁺8⁺ cells were CD3⁺, a few mature thymocytes were labeled with [$^3$H]TdR by 1 d, and a low but definite incidence of cycling cells was found in the mature thymocyte population, as we have noted previously (6). Recently, Ceredig (28) has shown that in the postnatal thymus, as opposed to the adult thymus, a high proportion of single positive and CD3²⁺ mature thymocytes are dividing cells. It is not clear whether this represents a selective antigen-MHC driven expansion or some more general process for increasing T cell numbers. Some residue of this mature thymocyte expansion process may persist even in 4–6-wk-old specific pathogen-free mice, and explain the few dividing mature cells we observe.

*Are the CD4⁺8⁺3²⁺ Thymocytes a Dividing Population?* In a recent study, Penit (14) concluded, on the basis of indirect staining procedures rather than direct isolation, that the CD4⁺8⁺3²⁺ intermediate subpopulation of adult mice included a high incidence of dividing cells. Our calculations indicate that cell expansion at the level of CD4⁺8⁺3²⁺ thymocytes would result in overproduction of cells, and this would imply further cell loss and cell selection after this stage. However, our data, and our interpretation of them, is in conflict with that of Penit (14). He found that 7% of all CD3²⁺ thymocytes to be in S-phase, in contrast to the 3–4% we measure; the reasons for this significant difference are not clear, but the study of Ceredig (28) discussed above may provide some guidance. The important point is that we have now directly isolated this minor CD4⁺8⁺3²⁺ subpopulation and found only a low incidence of dividing cells, if anything lower than that of mature thymocytes. Thus we conclude that this intermediate subpopulation does not rep-

resent a special phase of cell expansion, although it may be subject to the same postmaturation expansion process observed for mature thymocytes in the postnatal thymus (28).

*The Fate and Significance of CD4⁺8⁺PNA⁺CD3⁻ Small Cortical Thymocytes.* The small thymocyte population, which showed no (or very low) levels of surface TCR-CD3, showed a strictly linear accumulation of labeled cells in the continuous [³H]TdR uptake experiments, similar to the population expressing low, but definite levels of TCR-CD3, and similar to small cortical thymocyte in general. Thus, it behaved as an independent group of cells, rather than as the precursor of the cells expressing low levels of TCR-CD3 (if they were all precursors they would have shown a faster initial labeling, with a corresponding lag in the labeling of the TCR-CD3⁺ group). Since we have concluded that for most cells the TCR-CD3 complex is expressed at moderate levels only after division ceases, it follows that some nondividing TCR-CD3⁻ cells in the normal thymus must represent a transit state and be precursors of the TCR-CD3⁺ group. We assume this state is very transient, and that the moderate level of TCR-CD3 characteristic of CD4⁺8⁺ thymocytes is acquired rapidly, in <1 d, our first kinetic timepoint. In fact, by extrapolation the first appearance of labeled cells did appear to be several hours faster in the CD3⁻ than in the CD3⁺ group (Fig. 2). The cells that fail to express significant levels of TCR-CD3 by 1 d appear to remain in that state until the end of their 3.5-d intrathymic lifespan, when we assume they die in a programmed manner, as shown in the model of Fig. 4. The simplest explanation is that these cells represent the failure of the TCR-gene rearrangement process, failures that may be due to nonfunctional rearrangements that produce no transcript, or to TCR $\alpha$ and $\beta$ chain peptide products that failed to associate or migrate to the cell surface.

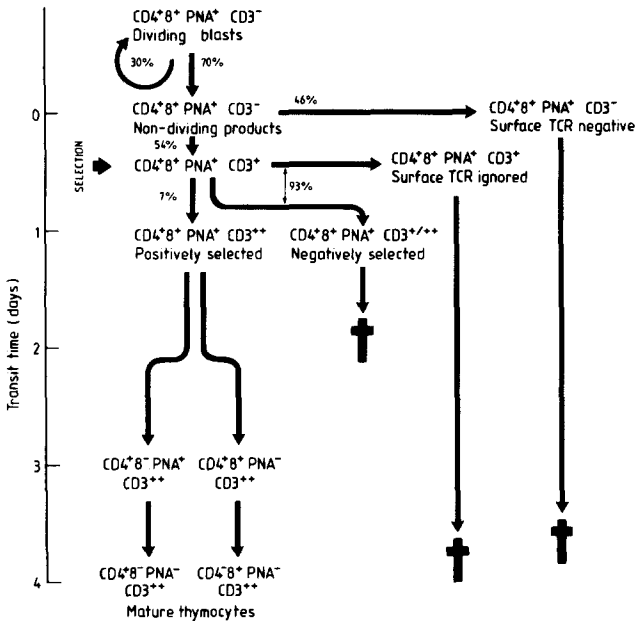


**Figure 4.** A model representing the late stages of T cell development in the thymus. The proportions of cells entering each pathway is generally based on data from this study, except for the figure of 30% of blasts reentering division which is from Baron and Penit (37). The transit times are from this study, with the cross indicating the end of the intrathymic lifespan, presumed to be by intrathymic death. Note that loss by negative selection could not be separated from loss by "neglect", so the proportion of cells undergoing negative selection could not be estimated and the timing of deletion is a guess.

*The Fate and Significance of CD4⁺8⁺PNA⁺CD3⁺ Small Cortical Thymocytes.* We assume that the small cortical thymocytes expressing moderate levels of the TCR-CD3 complex represent the population from which mature T cells are selected, either directly, or after some preliminary step producing correct coupling to CD3 (29) or upregulation of surface TCR (30). The flow through this population each day is in great excess over the daily production rate of mature thymocytes; we estimate only 7% of these cells (or 3–4% of all cortical thymocytes) ever become mature T cells. The labeling kinetics is therefore dominated by the 93% reject cells. Are these rejects simply ignored by the selection system, or are they actively destroyed after TCR engagement with self-antigens associated with self-MHC? We have no way of separately tracing these different groups of rejects. However, since we suspect from our results that most of the cells about to undergo active negative selection express intermediate levels of TCR, and such cells are relatively infrequent, we guess (as shown in Fig. 4) that only a small proportion of cells are eliminated by negative selection, and that this elimination is relatively rapid. The simplest way to explain the linear labeling kinetics of the CD4⁺8⁺ PNA⁺ CD3⁺ group is to assume that most of these cells are ignored by the selection process and die a programmed death after 3.8 d (Fig. 4). These TCR-CD3⁺ cortical cells consistently showed a small increase in average lifespan, compared to the TCR-CD3⁻ group. This might be the result of some of these cells receiving some form of positive signal via the TCR, a signal sufficient to slightly prolong the life of the cell but insufficient for a complete switch to the stable mature state.

*CD4⁺8⁺ PNA⁺ CD3²⁺ Thymocytes as Maturation Intermediates.* The 1-d lag in labeling of the small PNA⁺ CD3²⁺ thymocytes with [³H]TdR, and their 3-d transit time, supports the hypothesis that they are development intermediates between the cortical cells expressing moderate TCR levels and mature thymocytes, the latter having been shown to display a 4-d lag before the maximum rate of labeling (6). The observation that neither of these lag phases is absolute (some cells being labeled by 1 d in both cases) may have two explanations: first, a few dividing CD4⁺8⁺ PNA⁺ blasts express moderate levels of surface TCR and may enter the selection process early; second, a few dividing cells are seen among both the CD4⁺8⁺ CD3²⁺ group and the mature single positive thymocytes, and these would generate some rapidly labeled products.

However, the identification of a subpopulation as a developmental intermediate does not demonstrate that all development flows via that route. A good example is the transit from CD4⁻8⁻ to CD4⁺8⁺ thymocytes, where an immature CD4⁻8⁺ intermediate was found and assumed to be an obligatory developmental step (31–33); however, we later

demonstrated by [$^3$H]TdR labeling studies that the flux through this population was inadequate to explain the production of all $CD4^+8^+$ thymocytes (34) and an alternative $CD4^+8^-$ immature intermediate has now been well documented (35). Thus, it is important that the cell flow through the $CD3^{2+}$ cortical population (calculated to be $0.8 \times 10^6$ cells/d) appeared adequate to account for the daily production of mature thymocytes (calculated to be $0.9 \times 10^6$ cells/d), which in turn is very close to the measured rate of export of mature T cells from the thymus to the periphery (calculated to be $10^6$ cells/d [36]). These figures have a further implication: since there is not detectable excess of cells at the $CD4^+8^+$ $PNA^+$ $CD3^{2+}$ stage, most specificity selection must precede this population.

Our static multiparameter surface phenotype analysis suggests that the $CD3^{2+}$ cortical intermediate population does not lose all cortical markers simultaneously, but rather in the order CD8, then PNA binding, then CD4. In retrospect it would have been preferable to define the central intermediate population as being both $PNA^+$ and $CD4^+8^+$, since this should have caught all cells in transit to maturity, before differential loss of any cortical-type markers. However, this would have demanded five parameter sorting (including light scatter), not freely available at the time of these experiments. A tighter definition of the intermediate population could also involve gating for an intermediate level of TCR-CD3 between our $^+$ and $^{2+}$ categories; according to Guidos et al. (13) the TCR-CD3 expression of the intermediates is a little below that of mature thymocytes, although we find extensive overlap. A sequence of developmental intermediates presumably could be isolated representing progressive changes towards one or the other of the fully mature single-positive thymocytes (Fig. 4); a static analysis of this type using the CD3, CD4, and CD8 markers has been performed by Guidos et al. (13). Overall, it seems likely these developmental intermediates are seen as distinct subpopulations only because, in the absence of a rapid dilution by cell division, the loss of surface markers (such as CD4 or CD8 or the PNA receptor) by membrane or protein turnover takes longer than the gain of new markers (such as CD3 or TCR) by synthesis.

*The Developmental Stage of Positive and Negative Selection.* Our deduction, based on cell numbers and transit times, that most intrathymic repertoire selection must be over by the time the $CD4^+8^+$ $PNA^+$ $CD3^{2+}$ subpopulation is formed, was verified by assessing the incidence of cells bearing $V\beta6$ or $V\beta17a$. Extensive negative selection based on $Mls^a$ or IE reactivity had been affected before formation, or at the time of formation, of these intermediate cells. Although the narrow "window" available for detecting positive selection limited precision in the system used, the results indicate that positive selection also preceded formation of these intermediates. Overall, it appeared, for the particular model systems chosen, that selection was affected on cells bearing a level of surface TCR intermediate between the "low" and the "high" levels designated by the gates in Fig. 1. Selection at such an intermediate TCR expression level has also been proposed by Hugo, P., R.L. Boyd, G.A. Waanders, H.T. Petrie, and R. Scollay (manuscript submitted for publication) and by Guidos et al. (13), although clearly the stage of negative selection can vary depending on the selecting antigen and the TCR expressed (10, 38). Our data also indicate that the selection process was largely complete within 1 d of first TCR expression (the time taken to form the $CD4^+8^+$ $CD3^{2+}$ population), a relatively rapid and efficient process in terms of the 4-d cortical cell lifespan. These data on normal mice provides an important baseline for assessing the process of specificity selection in transgenic mouse models, where changes due to selection are more easily measured, but where the timing and level of TCR expression could be abnormal.

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