Lineage Relationships and Developmental Kinetics of Immature Thymocytes: CD3, CD4, and CD8 Acquisition In Vivo and In Vitro

By Howard T. Petrie, Patrice Hugo, Roland Scollay, and Ken Shortman

From The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

Summary

T lymphocytes develop in the thymus from immunologically naive bone marrow precursors. Based on T cell receptor rearrangement and transcription, and thymic reconstitution potential, we have deduced a developmental sequence among immature thymocytes, before the acquisition of the lineage markers CD3, CD4, and CD8. In the current study, we have followed the ontogenic progression of the latter stages in this sequence, using two different systems: (*a*) in vivo, by direct injection into the thymus of nonirradiated, congenic recipients; and (*b*) in vitro, using culture medium without mitogens or cytokines. In vivo, the less mature Pgp-1$^-$ interleukin 2 receptor α–positive (IL-2Rα^+) CD3$^-$4$^-$8$^-$ subset (also heat-stable antigen high) requires 3 d before becoming predominantly IL-2Rα^- CD3^{lo}4$^+$8$^+$ typical cortical-type cells, and at least 5 d before the appearance of any mature single-positive cells (CD3^{hi}4$^+$8$^-$ or CD3^{hi}4$^-$8$^+$). However, these Pgp-1$^-$ IL-2Rα^+ precursors do not differentiate further in unstimulated culture. The more mature Pgp-1$^-$ IL-2Rα^- CD3$^-$4$^-$8$^-$ subset becomes primarily CD3^{lo}4$^+$8$^+$ within 1 d after transplantation, and some mature single-positive progeny are evident by day 3. By 5 d, most of these Pgp-1$^-$IL-2Rα^- precursor cells have become CD3^{hi}, and have lost or are downregulating either CD4 or CD8. In culture, these Pgp-1$^-$ IL-2Rα^- cells also acquire high levels of CD4 and CD8 within 1 d, and low levels of CD3 by 2 d. However, they do not progress further to mature single positives in vitro, and most of them die by day 3. These experiments directly confirm our previously proposed developmental sequence, and demonstrate the kinetics of T lymphocyte production in a low-stress, steady-state environment.

T lymphocytes are generated in the thymus, where immunologically naive bone marrow–derived precursors undergo a series of differentiative events, including TCR gene rearrangement and expression, MHC restriction (positive selection), and induction of tolerance (negative selection). These events result in the production of mature T lymphocytes expressing the TCR/CD3 complex, as well as either the CD4 or CD8 accessory surface molecules. During their intrathymic maturation, thymocytes can be divided into distinct populations on the basis of surface expression of these same surface markers (1). The least mature cells in the thymus are thought to be CD3$^-$4$^-$8$^-$ and to express high levels of the heat-stable antigen (HSA^{hi})[1] (2), although current evidence indicates that very early thymocytes express low levels of CD4 (Wu, L., R. Scollay, M. Egerton, M. Pearse, Spangrude, and K. Shortman, manuscript submitted for publication). Distinct subsets have previously been described among these immature CD3$^-$4$^-$8$^-$ precursors to TCR-α/β^+ mature T lymphocytes (3, 4). Based upon thymic reconstitution kinetics, proliferative potential, cell cycle status, and TCR gene rearrangement and expression, we have proposed a developmental sequence for these immature cells (5). Progression along this sequence is marked by the loss of phagocytic glycoprotein 1 (Pgp-1, or CD44) expression, and transient expression of the p55 IL-2R (IL-2Rα, or CD55). Although our data supported this model of development, direct evidence of the sequential maturation of these subsets has been lacking.

To provide direct evidence for the precursor/product relationships of these subsets, and to determine the kinetics of appearance of downstream progeny, we have analyzed the differentiation of the most mature CD3$^-$4$^-$8$^-$ subsets, namely the Pgp-1$^-$ IL-2Rα^+ and Pgp-1$^-$ IL-2Rα^- cells. Such differentiation has been studied in two systems. In the first, purified thymocytes were injected intrathymically into nonirradiated congenic recipients differing at the Thy-1 locus, and the donor-derived cells were analyzed at various intervals for expression of CD3, CD4, and CD8. The use of nonir-

[1] *Abbreviations used in this paper:* HSA, heat-stable antigen; Pgp-1, phagocytic glycoprotein 1.

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radiated recipients allowed the close approximation of the steady-state thymus, with normal autocrine factor production and competition for environmental niches. In the second system, purified thymocytes were cultured in simple medium, and likewise analyzed for development at periodic intervals. These experiments have enabled us to directly demonstrate the precursor/progeny relationship between these two immature subsets, their subsequent progression through other nonmature intermediates, and the overall kinetics of the production of mature T lymphocyte progeny.

Materials and Methods

Mice. Male 4–6-wk-old C57BL/Ka/Thy-1.1/Lb (Thy-1.1$^+$; donor phenotype) or C57BL/Ka (Thy-1.2$^+$; recipient phenotype) mice, bred and maintained under specific pathogen-free conditions at the Walter and Eliza Hall Institute, were used throughout the experiments described.

Preparation and Enrichment Thymocyte Subsets by Depletion. Additional detail for staining and depletion procedures are given elsewhere (4). Freshly removed thymuses were pressed through wire mesh screen, washed in mouse-isotonic Hepes-buffered RPMI 1640 containing 1% FCS, and resuspended at one thymus per 10 ml in the same medium containing the appropriate antibodies for cytotoxic depletion: 17A2, anti-CD3; 172.4, anti-CD4; or D9, anti-CD8. After 40 min of incubation on ice, 3 ml of young rabbit serum was added as a source of complement, along with DNase (10 μg/ml) to prevent cellular aggregation. The suspension was brought to 37°C and then incubated for an additional 20 min. Dead cells were removed by density centrifugation on metrizamide (4). The remaining cells were treated with a second mAb against each antigen: KT3, anti-CD3; GK1.5, anti-CD4; or 53.6, anti-CD8. After washing, cells were mixed for 25 min with sheep anti-rat antibody–coated paramagnetic beads (Dynabeads, Dynal Corp., Oslo, Norway) at a ratio of six beads/cell, and a final volume of 10^8 cells/ml. The beads and adherent cells were then removed using a strong magnet. Cells purified in this manner were >98% depleted for the antigens of interest, as determined by direct staining, or by staining with anti-Ig second stage reagent. Subsets of CD3$^-$4$^-$8$^-$ thymocytes were further purified by cell sorting as described below.

Cell Staining and Sorting. Details of sorting and staining procedures are given elsewhere (4). Antibodies used for staining were as follows: IM781, anti-Pgp-1; PC-61, anti-IL-2Rα; M1/69, anti-HSA; KT3, anti-CD3; GK1.5, anti-CD4; 53.6, anti-CD8; 19F12, anti-Thy-1.1. These antibodies were either directly conjugated to FITC, PE, or allophycocyanin (APC), or were conjugated to biotin for second stage staining with FITC, PE, APC, or Texas Red streptavidin conjugates. Propidium iodide was used to discriminate dead from live cells. Analysis and sorting were performed using a FACStar^{plus} flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Intrathymic Transfer. Purified populations of donor (Thy-1.1$^+$) cells were washed and resuspended at 2–5 × 10^7 cells/ml in balanced salt solution containing 5% FCS. Intrathymic injections were performed using a new method, which eliminated the need for thoracic surgery. Recipient mice (Thy-1.2$^+$) were anesthetized by intraperitoneal injection with ketamine/xylazine, and a 4–5-mm midline incision was made in the skin over the sternum. Without opening the thoracic cavity, one or both thymic lobes were injected with 10 μl of cells, by intercostal injection between the third and fourth ribs, 3 mm from the midline, using a glass syringe and 30 gauge needle. At least two mice were injected for each cell type and time point. Due to the small size of the incision in the skin, sutures or staples were not necessary to close the wound.

Analysis of Donor Progeny after Intrathymic Transfer. After intrathymic transfer into nonirradiated recipients, the chimeric thymus consists primarily of thymocytes arising from the recipient. To enrich for cells of the donor phenotype, thymocytes of the recipient were depleted as described above, using anti-Thy-1.2 antibody (clone 30H12) for the complement-mediated lysis. No second antibody treatment was used before paramagnetic bead depletion. Depleted cells were stained for cells of the donor phenotype and the other antibodies of interest, and analyzed as described above, with gating for donor phenotype (Thy-1.1$^+$) cells.

Cell Culture. Purified cell populations were resuspended at 10^6 cells/ml in mouse-isotonic RPMI 1640, containing 10% FCS and 5 × 10^{-5} M 2-ME. 200 μl was dispensed to wells of 96-well flat-bottomed tissue culture plates (CoStar, Cambridge, MA). Plates were incubated in humidified 10% CO_2 in air at 37°C. After the desired incubation, cells were harvested, stained, and analyzed as described above.

Results

Intrathymic Development of CD3$^-$4$^-$8$^-$ Pgp-1$^-$ IL-2Rα^+ Cells. To directly determine the precursor potential and developmental kinetics of the immature thymocytes in our previously proposed sequence (5), we purified individual subsets, injected them intrathymically into congenic recipients, and allowed them to mature for various periods. The intrathymic maturation of Pgp-1$^-$ IL-2Rα^+ cells is shown in Fig. 1. 1 d after transfer, most of these cells (>90%) were still CD4$^-$8$^-$ and CD3$^-$, although a few percent of CD3^{lo}4$^+$8$^+$ cells were evident. By 3 d post-transfer, ~60–70% of donor-derived cells were CD4$^+$8$^+$ and CD3^{lo}. This correlated well with IL-2Rα expression, as 75–80% of Pgp-1$^-$ IL-2Rα^+ donor cells had become IL-2Rα^- by this time, while ~15–20% remained IL-2Rα^+ (Fig. 2). Significant numbers of CD3$^-$4$^-$8$^+$ cells, which are proposed transitional intermediates, were present; a few percent of the equivalent CD3$^-$4$^+$8$^-$ cells were also found, although not in sufficient number to appear on contour plots. After 5 d of development, ~90% of the cells derived from Pgp-1$^-$ IL-2Rα^+ precursors were still CD4$^+$8$^+$. However, ~5% were CD3^{hi}; these cells were either CD4$^+$8$^-$, or were downregulating CD8 en route to becoming CD4$^+$8$^-$. No CD4$^-$8$^+$ mature cells were yet seen by day 5.

Intrathymic Development of CD3$^-$4$^-$8$^-$ Pgp-1$^-$ IL-2Rα^- Cells. We have previously proposed that the Pgp-1$^-$ IL-2Rα^- subset of CD3$^-$4$^-$8$^-$ thymocytes is slightly more mature than the Pgp-1$^-$ IL-2Rα^+ subset (5). Consequently, Pgp-1$^-$ IL-2Rα^- cells were expected to exhibit slightly faster maturation kinetics than the Pgp-1$^-$ IL-2Rα^+ cells. Such findings are demonstrated in Fig. 1. Within 1 d after transfer, the majority of Pgp-1$^-$ IL-2Rα^- thymocytes (~75%) had become CD4$^+$8$^+$; most were CD3$^-$, but ~25% were CD3^{lo}. About 5–10% of donor-derived cells were either CD3$^-$4$^-$8$^+$ or CD3$^-$4$^+$8$^-$ transitional intermediates at this point, although again the density distribution of these cells was not sufficient to appear on contour plots. By 3 d post-transplantation, most of the cells derived

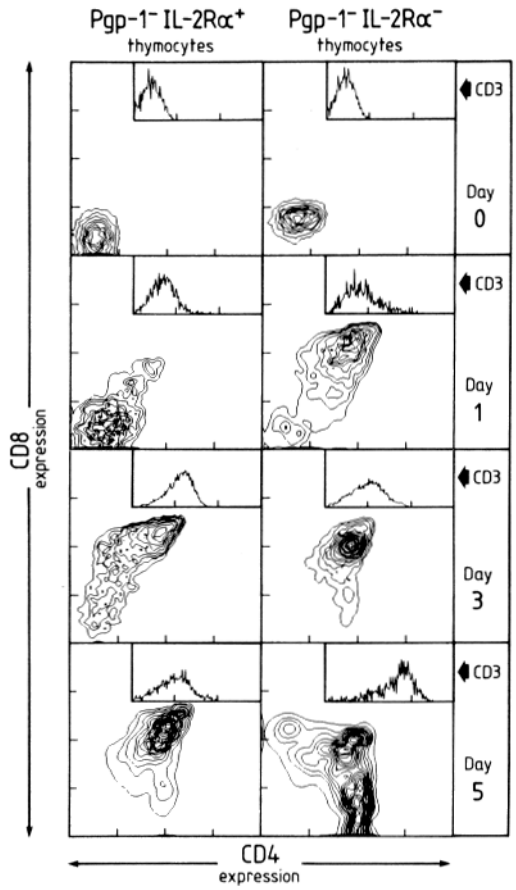

Figure 1. Intrathymic maturation of subpopulations of immature thymocytes. CD3⁻4⁻8⁻ thymocytes were prepared and separated into Pgp-1⁻ IL-2Rα⁺ and Pgp-1⁻ IL-2Rα⁻ subsets, and then injected intrathymically into nonirradiated, congenic recipients. Their developmental progeny was then analyzed at various intervals, by cytotoxic depletion of host cells and immunofluorescence staining of donor cells. The results shown are four-color flow cytometric analyses, with gating for donor-type (Thy-1.1⁺) cells only. Contour plots represent CD4/CD8 levels, with CD3 levels shown as the respective histogram insets. Scale intervals represent log-decade fluorescence intensity.

from Pgp-1⁻ IL-2Rα⁻ precursors were CD3^{lo}4⁺8⁺ typical cortical thymocytes. However, a small proportion (~5%) were CD3^{hi}, representing the first appearance of mature CD4⁺8⁻ thymocytes. Within 5 d after transplantation, essentially all the progeny of Pgp-1⁻ IL-2Rα⁻ cells had become CD3^{hi}, and significant proportions of both CD4⁺8⁻ (40%) and CD4⁻8⁺ (15%) were present. Thus, the overall patterns of development of the Pgp-1⁻ IL-2Rα⁺ and Pgp-1⁻ IL-2Rα⁻ subsets were strikingly similar, with the former subset being about 2 d less mature.

CD3, CD4, and CD8 Acquisition In Vitro. To determine whether similar maturational events could be observed in culture, these same CD3⁻4⁻8⁻ subsets were purified and incubated in standard culture medium without added mitogens or growth factors. The Pgp-1⁻ IL-2Rα⁺ subset failed to proliferate or differentiate further in culture, consistent with our previous findings using CBA mice (6). However, the Pgp-1⁻ IL-2Rα⁻ subset did progress, as is shown in Fig. 3. Within 3 h, these cells showed detectable increases in both CD4 and CD8 expression, and most cells expressed high levels of both markers within 24 h of culture. However, CD3 levels increased more slowly, with a slight change at 12 h, and still detectable progression between 24 and 48 h. However, CD3 acquisition was arrested at the CD3^{lo} stage, after which cell viability decreased dramatically. Cell viability was ~70% by 24 h, and to 30–40% by 48 h. Most cells were dead within an additional day in culture. The phenotypic maturation of these cells was paralleled by a decrease in cell size (data not

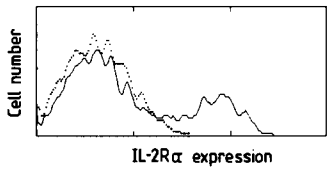

Figure 2. Downregulation of IL-2Rα expression by CD3⁻4⁻8⁻ Pgp-1⁻ IL-2Rα⁺ thymocytes. Cell surface expression of IL-2Rα was determined on donor-type cells 3 d after intrathymic injection of Pgp-1⁻ IL-2Rα⁺ cells (*solid line*) or Pgp-1⁻ IL-2Rα⁻ cells (*dotted line*) into nonirradiated recipients. Scale intervals represent log-decade fluorescence intensity.

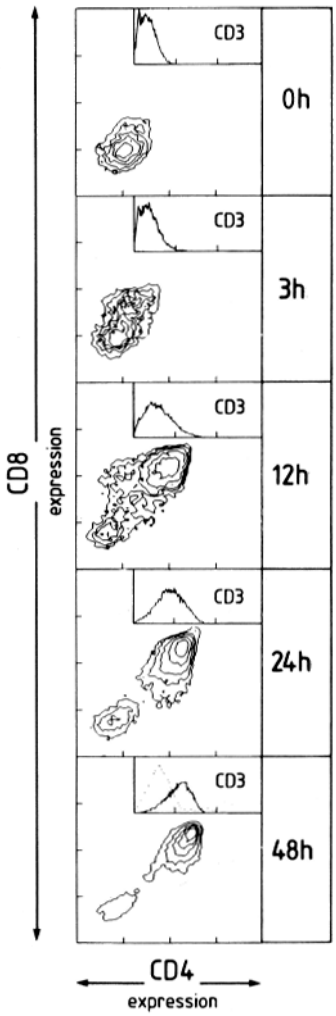

Figure 3. Maturation of CD3⁻4⁻8⁻ Pgp-1⁻ IL-2Rα⁻ thymocytes in vitro. Purified cells were cultured in simple medium without stimulation, and CD3, CD4, and CD8 expression on viable cells was examined at various time intervals. Viability decreased proportionally with time (>90% at 12 h; 75% at 24 h; 45% at 48 h) and most cells were dead by day 3. Scale intervals represent log-decade fluorescence intensity.

shown), and the result was the production of typical small cortical $CD3^{lo}4^+8^+$ cells by day 2. Both $CD3^-4^+8^-$ and $CD3^-4^-8^+$ transitional intermediates were observed, although these were not present in sufficient numbers to appear in contour plots. The appearance of these cells in culture, especially the $CD3^-4^-8^+$ subset, is in contrast to our previous data using CBA mice (6). This difference is presumably the result of mouse strain variation (7).

Discussion

To follow the normal developmental progression of immature thymocyte precursor cells, we purified subpopulations of these cells, and injected them intrathymically into nonirradiated, congenic recipients. The use of nonirradiated mice allows the normal maturation of injected cells in a near steady-state thymus, in contrast to irradiated models of thymic reconstitution, where autocrine factor production and absorption, and competition for microenvironmental niches, are disrupted by massive cytodepletion. Further, the administration of donor cells by intercostal injection significantly reduces the stress-induced thymic atrophy associated with standard approaches to thymic transplantation, which require thoracic surgery and wound closure. Thus, the results obtained with this new method should closely approximate the normal events associated with intrathymic T cell development. Using this system, we observed a chronologically defined maturation of purified immature thymocytes into nonmature $CD3^{lo}4^+8^+$ cortical cells, and then into mature $CD3^{hi}$, $CD4^+8^-$, or $CD4^-8^+$ thymocytes. The kinetics of maturation indicate that Pgp-1^- IL-$2R\alpha^+$ precursor cells are less mature than Pgp-1^- IL-$2R\alpha^-$ precursor cells, taking ~2 d longer to reach an equivalent stage of development. The progressive loss of IL-$2R\alpha$ expression by the former, and the virtual similarity of their developmental products, indicates that they are sequential stages on a single developmental pathway leading to mature T lymphocytes, as we have previously suggested (5). $CD3^-4^-8^-$ Pgp-1^- IL-$2R\alpha^-$ precursor cells rapidly become $CD3^-4^+8^+$ (1 d), and then $CD3^{lo}4^+8^+$ cortical-type cells (2–3 d), both in vivo and in vitro. However, the less mature $CD3^-4^-8^-$ Pgp-1^- IL-$2R\alpha^+$ precursors, while perfectly capable of giving rise to all downstream progeny in vivo, do not progress further in vitro, either in unstimulated culture (6) or in the presence of IL-2 or other mitogenic stimuli (data not shown). This thymic dependence suggests that Pgp-1^- IL-$2R\alpha^+$ cells represent an important control point in thymocyte differentiation, requiring the appropriate signals (e.g., cytokines, accessory cell surface molecules) to progress further. In addition, while Pgp-1^- IL-$2R\alpha^-$ cells differentiate to mature progeny within 5 d in vivo, they die within 3 d at the $CD3^{lo}4^+8^+$ cortical stage in vitro. Ostensibly, it is positive selection events that rescue maturing thymocytes from preprogrammed cell death (8), and allow them to become $CD3^{hi}$ (9). In addition, it has been shown that in the absence of positive selection, differentiation is arrested at the $CD3^{lo}4^+8^+$ stage (8, 10). Since Pgp-1^- IL-$2R\alpha^-$ precursors die at this stage in culture, where positively selecting elements are likely to be absent, our data suggest that positive selection must occur ~3–5 d after downregulation of IL-$2R\alpha$ on immature thymocytes.

The development of $CD3^{lo}4^+8^+$ thymocytes from $CD3^-4^-8^-$ precursors has been suggested to occur via an "immature single-positive" intermediate stage, i.e., $CD3^-4^+8^-$ (7, 11) and/or $CD3^-4^-8^+$ (12, 13) cells. We have observed both of these transitional intermediates, both in vivo and in vitro, during the course of these experiments. However, we generally find them to be in the minority, and because of this and other kinetic data (14; and Petrie, H., and P. Hugo, manuscript in preparation), we believe them to be nonobligatory intermediates in the generation of $CD3^{lo}4^+8^+$ cells, intermediates that have randomly acquired higher levels of either CD4 or CD8 en route to expressing both. In vitro, both of these immature single-positive subsets behave almost identically to the $CD3^-4^-8^-$ Pgp-1^- IL-$2R\alpha^-$ subset, with regard to the spontaneous acquisition of CD3, CD4, and CD8 (Petrie, H., and P. Hugo, manuscript in preparation). This similarity is supported by molecular analysis of TCR gene rearrangement and mRNA expression (M. Pearse, personal communication). We have recently found, by cytofluorometric and molecular analysis, that the subset described here as Pgp-1^- IL-$2R\alpha^-$ $CD3^-4^-8^-$ actually expresses low levels of both CD4 and CD8 molecules (15). Low-level expression of CD4 and CD8 on an immature thymocyte subset with similar biochemical and functional properties has been proposed by others (16). The similarities between the Pgp-1^- IL-$2R\alpha^-$, $CD3^-4^+8^-$, and $CD3^-4^-8^+$ subsets, and their rapid and spontaneous progression to $CD3^-4^+8^+$ and $CD3^{lo}4^+8^+$ in

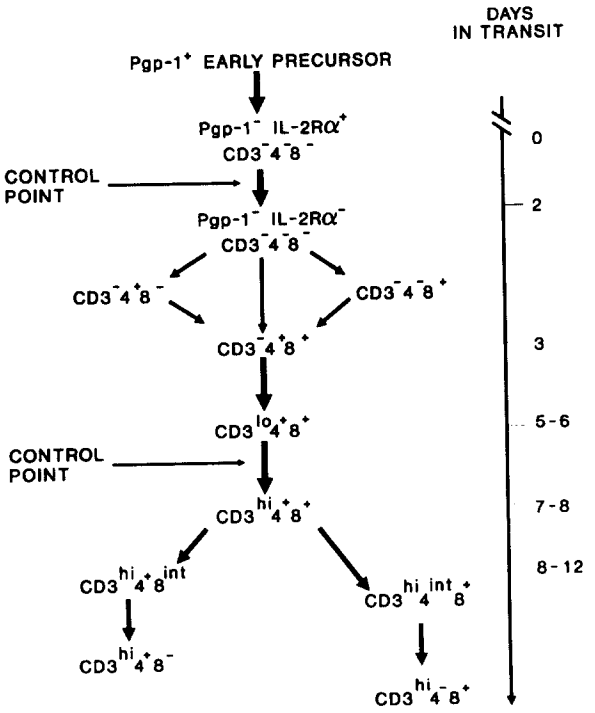

Figure 4. Timing of the developmental sequence for intrathymic maturation of TCR-α/β^+ thymocytes.

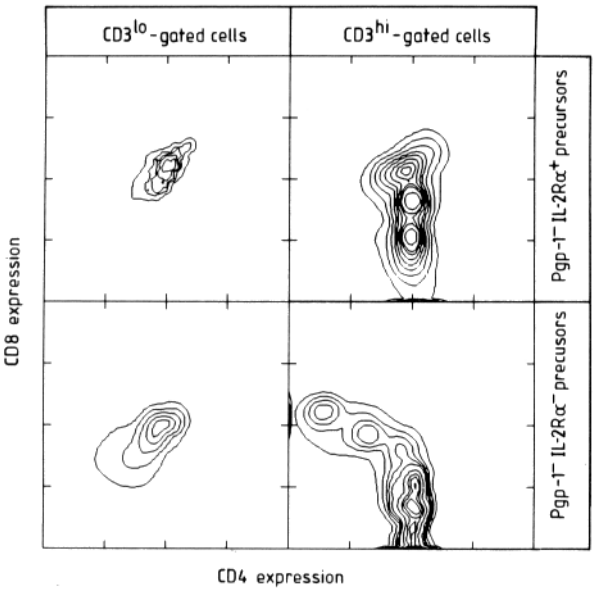

Figure 5. $CD3^{hi}4^+8^+$ transitional intermediates during intrathymic maturation of immature cells. Donor-type thymocytes were analyzed 5 d after injection of $CD3^-4^-8^-$, Pgp-1^- IL-2Rα^+, or Pgp-1^- IL-2Rα^- starting populations into nonirradiated recipients. Donor-derived thymocytes were additionally gated for $CD3^-/^{lo}$ cells (*left panel*) or $CD3^{hi}$ cells (*right panel*). Scale intervals represent log-decade fluorescence intensity.

vitro, lead us to propose that all of these subsets are in fact a single population in transition, with each phenotype representing a portion of the spectrum of CD3, CD4, and CD8 surface expression (Fig. 4).

We have also noted, during the course of the in vivo studies, another transitional intermediate during thymocyte maturation, namely, the $CD3^{hi}4^+8^+$ cell type (Fig. 5). Such a subset has recently been observed by others (11, 17, 18), and it appears to be the immediate precursor of single-positive mature cells (18; Hugo, P., R. L. Boyd, G. A. Waanders, H. T. Petrie, and R. Scollay, manuscript submitted for publication, and Shortman, K., D. Vremec, and M. Egerton, manuscript in preparation). The levels of CD4 and CD8 on these cells are, however, distinct from those found on typical cortical $CD4^+8^+$ cells, in that most $CD3^{hi}$ double positives express lower levels of one or the other marker. After 5 d of intrathymic development, the progeny of both the Pgp-1^- IL-2Rα^+ and the Pgp-1^- IL-2Rα^- $CD3^-4^-8^-$ subsets included some of these $CD3^{hi}$ cells, although in different proportions (Fig. 5). In both cases, all $CD3^-/^{lo}$ progeny expressed typical high levels of both CD4 and CD8. The $CD3^{hi}$ cells, however, included wells with a spectrum of CD4 and CD8 levels. The more mature Pgp-1^- IL-2Rα^- subset, which clearly produced both $CD4^+8^-$ and $CD4^-8^+$ mature thymocytes by day 5, also included distinct $CD4^+8^{int}$ and $CD4^{int}8^+$ cells. The less mature Pgp-1^- IL-2Rα^+ cells, which produced $CD4^+8^-$ but not $CD4^-8^+$ mature cells by day 5, also gave rise to $CD4^+8^{int}$ cells, but not (at this stage) their $CD4^{int}8^+$ equivalents. The production of $CD4^+8^-$ cells is known to precede that of $CD4^-8^+$ cells in a variety of systems (19– 22). We now believe the $CD3^{hi}4^+8^+$ subset to be the immediate precursor of mature thymocytes, and to be in the process downregulating either CD4 or CD8.

The studies described here directly confirm the developmental relationships of the latter two stages in our previously proposed $CD3^-4^-8^-$ thymocyte sequence (5, 20), as well as the kinetics of their subsequent development through sequential intermediates and into mature cells. Guidos et al. (22) have previously shown that $CD3^-4^-8^+$ and $CD3^{lo}4^+8^+$ cells, shown here as transitional intermediates between immature and mature thymocytes, give rise to mature cells in a similar nonirradiated intrathymic model. Their studies show these intermediates to be slightly faster in the rate of production of single-positive mature cells than our most mature $CD3^-4^-8^-$ subset. Our data confirm their findings, and extend them to include even earlier precursors. In addition, the combination of in vivo and in vitro studies has allowed us to define critical control points in this developmental process, where further growth and differentiation are substantially dependent upon the influence of the thymic microenvironment.

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Address correspondence to Howard T. Petrie, Lymphocyte Differentiation Unit, The Walter and Eliza Hall Institute of Medical Research, Post Office, The Royal Melbourne Hospital, Victoria 3050, Australia.

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