Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1614–1618, March 1989
Immunology

A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor

(T-cell differentiation/thymus)

MARTIN PEARSE, LI WU, MARK EGERTON, ANNE WILSON, KEN SHORTMAN, AND ROLAND SCOLLAY

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

Communicated by J. F. A. P. Miller, October 26, 1988

ABSTRACT **Precursors of all T-lineage cells are found in the population of thymocytes that lacks the CD4 and CD8 surface markers. These "double-negative" thymocytes are heterogeneous and can be divided into discrete subpopulations based on their expression of other surface markers. We have determined the relative maturity of these subpopulations based on the extent of rearrangement and expression of their T-cell receptor genes, their cell cycle status, and their thymus reconstitution capacity. Within the subpopulation of double negatives expressing high levels of the heat-stable antigen, the additional markers phagocytic glycoprotein 1 (Pgp-1) and interleukin 2 receptor (IL-2R) can be used to define the sequence IL-2R$^-$ Pgp-1$^+$ → IL-2R$^+$ Pgp-1$^-$ → IL-2R$^-$ Pgp-1$^-$, which occurs before the expression of CD4 and CD8. Transient expression of the IL-2R marks an important developmental point in the sequence just prior to a burst of cell proliferation and a loss of thymus reconstitution ability. The earliest cells in this sequence are already partially rearranged for genes in the $C_\beta 1$ region. IL-2R expression marks a second wave of T-cell antigen receptor of β-chain gene rearrangement and the initiation of T-cell antigen receptor α- and β-chain gene expression.**

Recent evidence (1–3) suggests mature CD4 or CD8 single-positive cells (CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$) are generated from double-negative cells (CD4$^-$CD8$^-$) via the numerically dominant double-positive (CD4$^+$CD8$^+$) stage. Whether they can also arise directly from the double-negative stage remains obscure. An important implication of the latter suggestion is that there should exist among the double negatives distinct precursors capable of giving rise to only single positives (one or both types) or only double positives. Four markers that allow further useful subdivision of the double-negative thymocytes are the T-cell antigen receptor (TCR) and the associated CD3 complex (4–8), phagocytic glycoprotein 1 (Pgp-1) (9–11), heat-stable antigen (HSA) recognized by the monoclonal antibodies (mAbs) B2A2, M1/69, and J11d (10, 12–15), and the 55-kDa chain of the interleukin 2 receptor (IL-2R) recognized by mAbs such as PC.61 (9–12, 15–17).

To establish the relative maturity of the CD4$^-$CD8$^-$ thymocyte subpopulations defined with these antibodies, we have compared them for the rearrangement and expression of their TCR genes, for their cell cycle status, and for their thymus-reconstituting activity. Previous studies in this area have given discordant results (9, 14, 15, 18), which we now attribute, in part, to the use of insufficient surface markers to delineate all separate subpopulations involved. Our present studies use six surface markers in all, which makes the description of subpopulations unwieldy but is necessary to resolve the discrepancies. We deduce that within the main group of HSA^{++} double negatives there exists a series of distinct stages prior to expression of CD4 and CD8.

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MATERIALS AND METHODS

Mice. Male 5- to 6-wk-old mice (CBA/CaH, C57BL/Ka, C57BL/Ka Thy-1.1) were maintained under pathogen-free conditions at the Walter and Eliza Hall Institute animal facility.

Preparation of Subpopulations of CD4$^-$CD8$^-$ Thymocytes. Subsets of CD4$^-$CD8$^-$ thymocytes were obtained >99% pure by complement-mediated depletion and fluorescence-activated cell sorting as described in detail (10, 19). The starting populations in this study were the CD4$^-$CD8$^-$ or CD4$^-$CD8$^-$ IL-2R$^-$ populations prepared by depletion with anti-CD4 (mAb GK1.5), anti-CD8 (mAb 49.11.1), and in some cases anti-IL-2R (mAb 7D4). The recovered cells were stained in one or two colors as detailed elsewhere (10), using fluorescein-conjugated anti-HSA (M1/69) alone or together with either biotin-conjugated anti-IL-2R (PC.61) or anti-Pgp-1 (1M7.8.1), with a phycoerythrin-streptavidin second stage. The stained samples were analyzed or sorted using a modified (14, 19) FACS II (Becton Dickinson). The purity of the sorted cells was always assessed by reanalysis, and contamination by any other active subset was always <3% and usually <1%. The reconstitution expected from the measured contamination levels cannot account for the measured activities.

RNA and DNA Hybridization Analysis. High molecular weight DNA and total cytoplasmic RNA were prepared directly from the same sorted population of cells (and from the other control populations indicated) as described (20). To determine TCR β-chain gene rearrangement *Hin*dIII-digested DNA was probed with a ^{32}P-oligolabeled C_β-region probe (86T1) (21). RNA was separated on a 1% agarose/formaldehyde gel. Hybridizations were initially performed with the C_β region probe (described above), following which the filters were stripped and reprobed for the TCR α chain (Pα8) (22). Hydridization conditions for Northern (RNA) and Southern blots were as described elsewhere (5).

Assays of Precursor Activity by Intrathymic Injection. Subpopulations of CD4$^-$CD8$^-$ thymocytes from 5-wk C57BL/Ka donor mice were injected intrathymically into γ-irradiated (750 rads; 1 rad = 0.01 Gy) Thy-1 congenic recipients. At various times cell suspensions were made from the recipient thymuses (and lymph nodes) and immunofluorescent stained in three colors, using fluorescein-conjugated anti-Thy-1 (donor type), phycoerythrin-conjugated anti-CD4 (GK1.5), biotin-conjugated anti-CD8 (53.6), and coumarin-avidin. Flow cytometry was then used to determine the total donor type Thy-1$^+$ cells per lobe and to analyze these for CD4 and CD8 expression as well as for size by low-angle light scatter. Values as low as 0.5% donor cells can be easily assessed in this system.

Cell Cycle Analysis by DNA Staining. The DNA content of thymocytes, based on propidium iodide uptake, was deter-

Abbreviations: TCR, T-cell antigen receptor; HSA, heat-stable antigen recognized by the monoclonal antibodies B2A2, M1/69, and J11d; IL-2R, interleukin 2 receptor; mAb, monoclonal antibody.

mined by the method of Taylor (23). Labeled cells (10,000) were analyzed for DNA content on a FACScan (Becton Dickinson) flow cytometer, and the proportion of cells in the G_0/G_1, G_2 + M, and S phases of the cell cycle were determined using the FACScan DNA polynomial compartmental analysis program.

RESULTS

$CD4^-CD8^-$ Thymocyte Subpopulations. The few IL-$2R^+$ cells in the adult mouse thymus are concentrated within the $CD4^-CD8^-$ population (16)—more precisely, within the $CD4^-CD8^-$ $CD3^-$ HSA^{++} subpopulation (10). An example of flow cytometric analysis of CBA strain double-negative thymocytes stained for HSA and IL-2R is given in Fig. 1*a*; this shows the clear division of the HSA^{++} cells into 48% (±14%) IL-$2R^+$ and 52% IL-$2R^-$ components. Similar results were obtained with C57BL/6 and C57BL/Ka mice except that the proportion of HSA^- cells was lower and 62% of HSA^{++} cells were IL-$2R^+$.

Pgp-1 generally shows a reciprocal distribution to HSA on adult mouse $CD4^-CD8^-$ thymocytes (10), but a small proportion of the HSA^{++} cells (9.5 ± 1.5%) are also Pgp-1^+. None of these HSA^{++} Pgp-1^+ cells are IL-$2R^+$. An example of a separation of these few HSA^{++} Pgp-1^+ cells from a preparation of $CD4^-CD8^-$ IL-$2R^-$ adult CBA thymocytes is given in Fig. 1*b*; note that cells bearing both these markers spread over a wide zone and so may be heterogeneous. Similar proportions of HSA^{++} Pgp-1^+ cells were found in C57BL/Ka thymuses. In contrast, in the day-14 embryonic thymus, 86% of $CD4^-CD8^-$ cells were found to be HSA^{++} Pgp-1^+ (data not shown); this proportion dropped rapidly to 16% at day 16, suggesting a transition from HSA^{++} Pgp-1^+ to HSA^{+++} Pgp-1^-. Thus, although the proportion of HSA^{++} Pgp-1^+ cells appears insignificant compared with the HSA^- Pgp-1^+ group in adult mice, it may represent a developmentally important subpopulation.

Subpopulation TCR-Gene Rearrangement. We have determined the status of TCR genes in $CD4^-CD8^-$ thymocyte subpopulations by the direct analysis of cells sorted as in Fig.1 , avoiding any form of expansion of the cells in culture because of the risks of selection. The extent of rearrangement of TCR β-chain genes in double-negative thymocyte subpopulations from adult CBA mice can be assessed from Fig. 2 *a* and *b*, in which *Hin*dIII digests were hybridized with a C_β probe. The germ-line configuration (kidney samples in Fig. 2) gave two bands, one at 9.4 kilobases (kb) including $C_\beta1$, $J_\beta1$, and $D_\beta1$, and one at 3.0 kb, including $C_\beta2$ (21). Microdensitometry readings on both kidney and bone-marrow samples gave an average absorbance ratio of 2.1:1 for the germ-line 9.4: 3.0-kb bands. Rearrangement reduced the intensity of the 9.4-kb band but did not affect the 3.0-kb band, which therefore served as an internal control. Extensively rearranged cells, such as thymocytes (Fig. 2) and peripheral T cells gave absorbance ratios of <0.3:1 for the 9.4:3.0-kb bands. An estimate of the extent of β-chain gene rearrangement in the various subpopulations was made on the basis of microdensitometry readings on the original autoradiographs.

The population of $CD4^-CD8^-$ thymocytes overall contained a mixture of rearranged and germ-line forms of the β-chain gene (Fig. 2 *a* and *b*), a consequence of the cellular heterogeneity of this population. In agreement with our earlier findings (5), germ-line forms were concentrated in the subpopulations expressing low levels of HSA. However, the HSA^{++} subgroup also included a proportion of cells with TCR β-chain genes in germ-line configuration. Within this HSA^{++} subgroup the subpopulation of IL-$2R^-$ cells (see Fig. 1*a*) had more β-chain gene rearrangement than the IL-$2R^+$ cells (Fig. 2*a*). Very similar results were obtained with the equivalent subpopulations from C57BL/Ka mice. This result indicated that the IL-$2R^-$ subpopulation was more mature, or further downstream, than the IL-$2R^+$ subpopulation, in contrast to the conclusion of previous thymus reconstitution studies (15).

One possible reason for this contradiction could have been the heterogeneity of the HSA^{++} IL-$2R^-$ subpopulation of double negatives as revealed by Pgp-1 expression (Fig. 1*b*). Accordingly, we sorted the major well-defined HSA^{++} IL-$2R^-$ Pgp-1^- group and the minor, more diffuse HSA^{++} IL-$2R^-$ Pgp-1^+ group and examined these for β-chain gene rearrangement (Fig. 1*b*). The minor IL-$2R^-$ Pgp-1^+ subpopulation contained a high proportion of β-chain genes in germ-line configuration, more than was found in the IL-$2R^+$ subpopulation. The major IL-$2R^-$ Pgp-1^- subpopulation was almost completely rearranged. The three subpopulations of HSA^{++} double negatives can now be arranged in an order of maturity based on degree of the β-chain gene rearrangement, as summarized in Fig. 3.

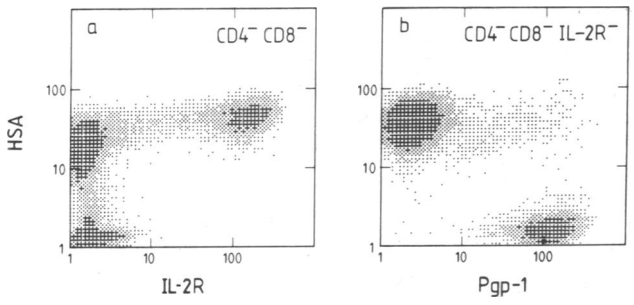

FIG. 1. Separation of subpopulations of $CD4^-CD8^-$ thymocytes by two-color immunofluorescence staining and flow cytometry. Two separate experiments using CBA mice are shown, typical of six (*a*) or three (*b*) experiments undertaken. Note the minor but definite subpopulation of HSA^+ Pgp-1^+ cells among $CD4^-CD8^-$ IL-$2R^-$ thymocytes.

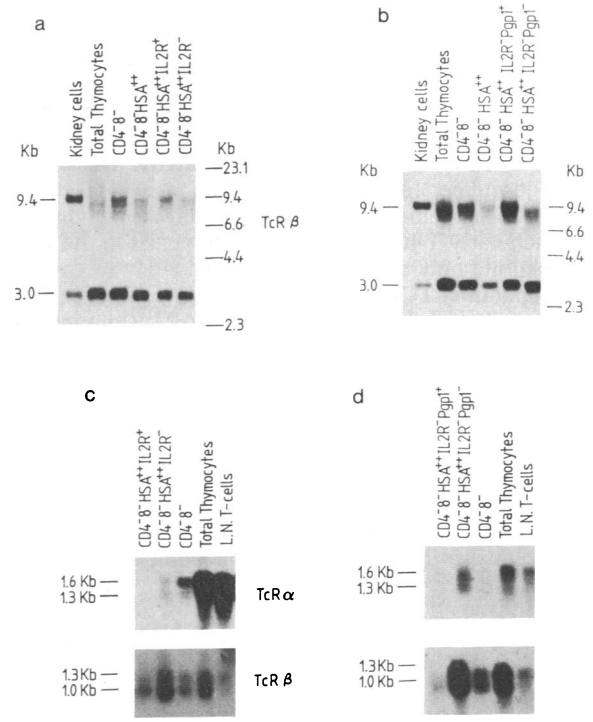

FIG. 2. TCR β-chain gene rearrangement (*a* and *b*) and α-chain and β-chain mRNA expression (*c* and *d*) by subpopulations of $CD4^-CD8^-$ thymocytes. The subpopulations of $CD4^-CD8^-$ thymocytes were isolated as outlined. Lymph node (L.N.) T cells were obtained by depleting suspensions of all immunoglobulin-bearing cells with anti-immunoglobulin-coated magnetic beads (Dynal) (19).

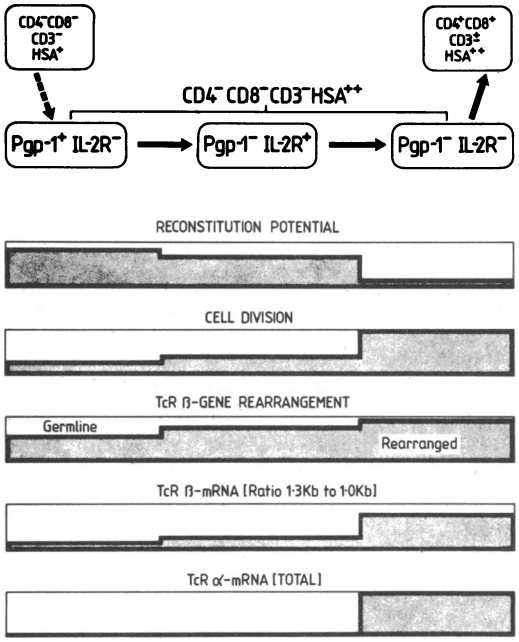

FIG. 3. Relative maturity of subpopulations of $CD4^-CD8^-$ thymocytes and a proposed developmental sequence. Reconstitution potential is expressed as donor-type cells in the recipient thymus at the peak reconstitution time for that subpopulation per 10^5 cells injected; scale is logarithmic from 10^4 to 10^8 cells. Cell division is expressed as % cells in cycle on a linear 0–100% scale, assuming 60% cells in S + G_2 + M = 100% dividing cells. TCR β-chain gene rearrangement is on a linear 0–100% scale, the values based on comparison of intensity of the 9.5-kb band to that of the 3.0-kb band (Fig. 2). Ratio of 1.3-kb to 1.0-kb TCR β-chain mRNA is on a linear scale from 0 to 5:1. Level of TCR α-chain mRNA is expressed on a linear 0–100% scale with the highest level recorded set at 100%.

Because the least mature subpopulation we have delineated within the $CD4^-CD8^-$ HSA^{++} group is already >50% rearranged at the $C_\beta 1$ locus, we are seeking still earlier T-lineage cells, especially among the subpopulations expressing lower levels of HSA. However, we have not yet isolated a subpopulation with early precursor activity and predominantly germ-line configuration β-chain genes, a result in apparent disagreement with the report of Trowbridge *et al.* (24). Such early T-lineage cells probably exist in the thymus but constitute only a tiny subpopulation.

Subpopulation TCR mRNA Expression. The order of relative maturity of the $CD4^-CD8^-$ HSA^{++} subpopulations based on β-chain gene rearrangement was confirmed by studies of TCR α- and β-chain mRNA expression, as shown in Fig. 2 *c* and *d* and summarized in Fig. 3. Northern (RNA) analysis using the C_β probe detects both a mature 1.3-kb and an immature 1.0-kb message. The ratio of the 1.3-kb to the 1.0-kb band then gives an index of maturity. Proportionally more of the 1.3-kb β-chain message occurs in the $IL\text{-}2R^-$ subpopulation, and proportionally more of the 1.0-kb β-chain message occurs in the $IL\text{-}2R^+$ subpopulation (Fig. 2*c*); in addition, the absolute level of β-chain message was much higher in the $IL\text{-}2R^-$ subpopulation. When the $IL\text{-}2R^-$ fraction was subdivided on the basis of Pgp-1 expression as in Fig. 1*b*, the proportion of mature β-chain message was further elevated in the $IL\text{-}2^-$ $Pgp\text{-}1^-$ subpopulation, whereas the trace amount of β-chain message from $IL\text{-}2R^-$ $Pgp\text{-}1^+$ subpopulation was predominantly the 1.0-kb form (Fig. 2*d*).

Clear-cut results in accordance with all the previous data were also obtained using a C_α probe (Fig. 2 *c* and *d*). There was abundant α-chain message in the $IL\text{-}2R^-$ $Pgp\text{-}1^-$ subpopulation, but little, if any, in the $IL\text{-}2R^+$ or $IL\text{-}2R^-$ $Pgp\text{-}1^+$ subpopulations.

Subpopulation TCR Surface Expression. Although $CD4^-$ $CD8^-$ thymocytes showing surface expression of TCR and CD3 complex are found in the adult mouse thymus, the majority of these are among the HSA^- cells (4–7, 12). These cells lack T-cell precursor activity (9) and thus appear to be off the mainstream of early T-cell development that we are attempting to track. A small proportion (6 ± 3%) of the HSA^{++} thymocytes does express surface TCR. However, we (5) and others (25) have found that the majority (but not all) of these cells express the $\gamma\delta$ rather than the $\alpha\beta$ form of the TCR. Assuming the $\gamma\delta$-bearing cells to be a separate lineage, it seems likely that the $\alpha\beta$ TCRs appear on the cell surface about the same time as CD4 and CD8.

Precursor Activity. In view of the apparent contradictions between our results and the thymus reconstitution data of Shimonkevitz *et al.* (15), we extended our own earlier studies on thymus reconstitution (9) to include all subpopulations resolved in Fig. 1 and to include cells expressing various levels of HSA. The precursor potential of the double-negative thymocyte subpopulations was assessed by direct intrathymic injection, as described elsewhere (9). A sample of the results is shown in Table 1. The time points presented are chosen to emphasize the kinetic differences between subpopulations. We emphasize that all active subpopulations produced progeny that included $CD4^+CD8^+$ cells and eventually both $CD4^+CD8^-$ and $CD4^-CD8^+$ mature cells, although in some cases the mature cells appeared as late as day 20. This finding suggests that none of the subpopulations are restricted in their potential and that all are components of the same developmental stream.

Other workers (14, 15) had previously found the precursor activity of the double-negative thymocytes to be within the group expressing high levels of HSA (HSA^{++}). As shown in Table 1 experiment A, our work agrees with this, but we also find significant activity in the subpopulation that expresses low levels of HSA (HSA^+) and lacks CD3. This subpopulation was not delineated in our previous study because the HSA^+ cells were not separated from the cells completely negative for HSA (HSA^-), which we now find have very little reconstitution activity. The HSA^{++} subpopulation gave good reconstitution, which was already substantial by day 7 and progressed to small double positives and mature single positives by day 14. The HSA^+ population, on the other hand, gave poor reconstitution at day 7 but good reconstitution at day 14. The progeny of these HSA^+ precursors were always less mature than the progeny of the HSA^{++} precursors; in other experiments the HSA^+ precursors were even slower in their developmental kinetics than in experiment A of Table 1. On a cell-for-cell basis the HSA^+ subpopulation was more active than the HSA^{++} subpopulation, and when these data were corrected to account for the presence of the inactive $CD3^+$ cells (12) (60% of the HSA^+ but only 6% of the HSA^{++} fractions), the HSA^+ $CD3^-$ subpopulation could be seen as a rich source of early precursors. However, because the HSA^{++} subpopulation was much larger, it accounted for the greater part of the total thymus precursor activity. Overall, this result confirms the observation (15) that all T-precursor cells in the thymus bear HSA, but identifies a new subgroup of early precursors expressing low levels of this surface antigen.

The subpopulations of HSA^{++} double-negative thymocytes defined by expression of IL-2R and Pgp-1 (as in Fig. 1) showed marked differences in their thymus reconstitution capacity. We found most reconstitution activity was within the $IL\text{-}2R^+$ cells, although the $IL\text{-}2R^-$ cells were also active (Table 1 experiment B, where the numbers injected were in proportion to their thymic representation). The $IL\text{-}2R^-$ cells gave delayed reconstitution kinetics similar to the HSA^+ cells, suggesting the $IL\text{-}2R^-$ subpopulation was less mature than the $IL\text{-}2R^+$ subpopulation. Thus, the apparent contra-

Table 1. Thymus reconstitution capacity of subpopulations of $CD4^-CD8^-$ thymocytes

$CD4^-CD8^-$ subpopulation injected	Cells injected per lobe, $\times 10^{-3}$	Day of assay	Donor cells recovered per lobe, $\times 10^{-3}$	Subset representation among donor-derived cells, %					
				Large $CD4^-CD8^-$	Large $CD4^-CD8^+$	Large $CD4^+CD8^+$	Small $CD4^+CD8^+$	Medium $CD4^+CD8^-$	Medium $CD4^-CD8^+$
Experiment A									
HSA^-	46	7	<3						
		14	350	<1	3	17	78	2	<1
HSA^+	43	7	20	30	<1	20	50	<1	<1
		14	3100	<1	3	13	82	2	<1
HSA^{++}	100	7	770	2	2	18	78	<1	<1
		14	5200	<1	4	8	79	7	2
Experiment B									
HSA^{++} IL-$2R^+$	40	7	1480	3	5	23	69	<1	<1
		14	2150	<1	3	5	73	16	3
HSA^{++} IL-$2R^-$	25	7	50	12	2	17	68	<1	<1
		14	1000	<1	4	15	77	3	<1
Experiment C									
HSA^{++} IL-$2R^-$	100	7	80	89	2	3	6	<1	<1
Pgp-1^+		11	4400	3	4	21	72	<1	<1
HSA^{++} IL-$2R^-$	170	7	190	<1	2	22	75	<1	<1
Pgp-1^-		11	60	<1	1	7	54	31	7

Data are from three separate experiments, each representative of two to four similar experiments. The preparation, staining, and sorting of subpopulations are described. In C57BL/Ka mice 10% of cells were HSA^- (a peak of cells showing no staining above background), 84% were classed as HSA^{++} (a peak of cells showing very bright staining—at least as bright as small cortical thymocytes), and 6% were classed as HSA^+ (a broad spread of cells between these peaks, showing low-level staining). Overlap zones between these fractions were rejected. Mature medium single positives were distinguished from other subsets by size (medium rather than large or small), Thy-1 level (low rather than high), and CD4 or CD8 level (high rather than low), as described in detail elsewhere (9).

diction between the results of molecular analyses and the results from the intrathymic-transfer precursor assay was also obtained in our laboratory.

These contradictions would be resolved if the numerically small IL-$2R^-$ Pgp-1^+ subpopulation (defined as relatively early by the molecular criteria) had all the thymus reconstitution activity, and the numerically larger IL-$2R^-$ Pgp-1^- subpopulation with extensively rearranged TCR genes had little thymus reconstitution activity. When tested by intrathymic transfer (Table 1 experiment C), this hypothesis was confirmed. The IL-$2R^-$ Pgp-1^+ subpopulation gave, as predicted, good precursor activity with relatively delayed reconstitution kinetics. In contrast, the more numerous IL-$2R^-$ Pgp-1^- subpopulation gave measurable but poor reconstitution, even though more cells were transferred, and the little reconstitution that was obtained was very rapid.

Table 2. Cell cycle status of subpopulations of $CD4^-CD8^-$ thymocytes

$CD4^-CD8^-$ thymocyte subpopulation	Cells, %		
	G_1/G_0	S	G_2 + M
C57BL/Ka strain			
All $CD4^-CD8^-$	63	32	5
HSA^-	95	3	2
HSA^- $CD3^-$	95	3	2
HSA^+ $CD3^-$	95	4	1
HSA^{++} IL-$2R^+$	81	16	3
HSA^{++} IL-$2R^-$	52	44	4
HSA^{++} IL-$2R^-$ Pgp-1^+	82	13	5
HSA^{++} IL-$2R^-$ Pgp-1^-	41	52	7
CBA strain			
All $CD4^-CD8^-$	61	32	7
HSA^-	97	2	1
HSA^- $CD3^-$	99	1	0
HSA^{++} IL-$2R^+$	79	17	4
HSA^{++} IL-$2R^-$	44	47	9
HSA^{++} IL-$2R^-$ Pgp-1^+	86	12	2
HSA^{++} IL-$2R^-$ Pgp-1^-	46	50	4

Cell Cycle Analysis. The $CD4^-CD8^-$ subpopulations were stained with propidium iodide and the proportions of cells in G_0/G_1, G_2 + M, and S phases of the cell cycle were determined by flow cytometric analysis (Table 2). All subpopulations expressing low levels of HSA, including the HSA^+ $CD3^-$ cells with precursor activity, contained very few cells in cycle. Among the HSA^{++} group the IL-$2R^-$ Pgp-1^- subpopulation contained the highest proportion of dividing cells. The IL-$2R^+$ subpopulation included some cells in cycle but fewer than the IL-$2R^-$ Pgp-1^- group. As summarized in Fig. 3, the $CD4^-CD8^-$ subpopulations appear to form a sequence based on intensity of cell proliferation, with the putative most mature, HSA^{++} IL-$2R^-$ Pgp-1^- subpopulation showing a rapid proliferation rate similar to that of $CD4^+CD8^+$ cortical blasts, the cells we consider to be immediately downstream in the developmental pathway. Lack of detectable precursor activity in this IL-$2R^-$ Pgp-1^- subpopulation may be related to their rapid rate of proliferation, which may have exhausted much of their capacity for further expansion in a recipient thymus.

DISCUSSION

The information on the rearrangement and expression of the TCR genes, the thymic reconstitution data, the cell cycle data, and the overlaps in surface phenotype among the double-negative thymocyte subpopulations, all point to the developmental sequence shown in Fig. 3 as the simplest unifying model. Although the order of the subpopulations correctly depicts the relative maturity of the cells by the criteria we have used, the sequence remains to be established by a direct precursor–product approach; more complex, branched, or multipathway models cannot at present be excluded. In particular, the earliest T-lineage cell in the sequence requires closer investigation. We assume it is a minor thymocyte subpopulation that is $CD4^-CD8^-$ $CD3^-$, which expresses low levels of HSA and which also expresses low levels of Thy-1 and bears the SCA-1 antigen, to link with the phenotype of the bone-marrow cell recently shown to have thymic reconstitution potential (26).

The finishing point of the sequence proposed in Fig. 3 is a $CD4^+CD8^+$ (double-positive) cortical thymocyte, which also has the surface phenotype HSA^{++} $IL\text{-}2R^-$ $Pgp\text{-}1^-$. All double-negative populations listed in the sequence produce double-positive cells on intrathymic transfer (Table 1), and we have found that the most mature subpopulation in the sequence ($IL\text{-}2R^-$ $Pgp\text{-}1^-$) will transform into double-positive cells on short-term culture (A.W. and K.S., unpublished data). Progression to the $CD4^+CD8^+$ cortical cell may be via a $CD4^-CD8^+$ $CD3^-$ HSA^{++} (immature single-positive) intermediate (9, 12, 19), but at present we lack the TCR gene rearrangement and precursor activity data needed to assess the developmental status of this subpopulation. The $CD4^+CD8^+$ products of this sequence are initially large dividing cells, which then produce small nondividing $CD4^+CD8^+$ cells, the majority of which die within the thymus (9, 27, 28).

Although the proposed sequence is only a fragment of the total developmental process leading to mature T cells, it presents some central issues for consideration. First, the transient expression of IL-2R at the midpoint of this sequence marks the initiation of a burst of cell proliferation and of TCR gene rearrangement and expression and precedes loss of the capacity to reconstitute a thymus. Cell proliferation initiated at this point may simply flow on without further stimulus to the $CD4^+CD8^+$ blast cells and so be the basis of most extensive cell generation within the thymus. The nature of the signal that initiates these central events can now be sought by culture studies on some earlier, precycling subpopulations. Although the IL-2R itself might be involved, evidence to date suggests otherwise. Cells responsive to IL-2 and mitogens are found among double-negative thymocytes (16, 29), but these are predominantly in the HSA^-, $CD5^+$, $CD3^+$, and $IL\text{-}2R^-$ subpopulation (29, 30), which is apparently off the developmental mainstream. The double-negative thymocytes bearing IL-2R antigen do not respond to interleukin 2 in culture (8), and, as Table 2 and previous data (22, 23) demonstrate, the most actively proliferating subpopulations are $IL\text{-}2R^-$.

Second, a very striking finding is that the earlier subpopulations in the sequence, before the main burst of cell division, are already $\approx$50% rearranged at the $C_\beta 1$ locus. We do not know whether these subpopulations are a mixture of fully rearranged and unrearranged cells or whether they represent a uniform group of cells rearranged on just one chromosome. Support for the latter concept of individual early T cells with partially rearranged β-chain genes comes from the Abelson virus-transformed early T-cell lines of Cook and Balaton (31), some of which show both germ-line and rearranged forms of the gene $C_\beta 1$. This raises the possibility that the TCR repertoire is generated in two waves of gene rearrangement rather than in a single burst, and that the three subpopulations of HSA^{++} $CD4^-CD8^-$ thymocytes are components of the second wave. Despite the technical difficulties involved in handling still smaller cell numbers from subpopulations now representing, at most, 0.1% of all thymocytes, it is clearly important to identify the earliest T-precursor cell in the thymus and to determine the stage at which the first TCR gene rearrangements occur.

The techniques used in this study do not approach the question of the $\gamma\delta$-expressing cells, so these cells are not included in our model.

Finally, as presented, Fig. 3 is a sterile pathway. A deliberate omission from the sequence is the most important thymic function, the development of single-positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) TCR-bearing mature T cells. These most probably are derived from *some* $CD4^+CD8^+$ cells at the end of the sequence, as our studies on thymus reconstitution (9) and recent studies on the process of immunological tolerance in the thymus (1–3) all suggest. However, this mechanism remains obscure; all or some of these cells could be produced via developmental pathways that branch off at an earlier stage.

Angela D'Amico and Nella Fisicaro provided skilled technical assistance. Financial support was from the U.S. National Institutes of Health Grant AI-17310, the C. H. Warman Research Fund, and the National Health and Medical Research Council, Australia.

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