Multiple Rearrangements in T Cell Receptor α Chain Genes Maximize the Production of Useful Thymocytes

By Howard T. Petrie,*§ Ferenc Livak,§‡ David G. Schatz,§‡ Andreas Strasser,* I. Nicholas Crispe,§ and Ken Shortman*

*From the *Walter and Eliza Hall Institute of Medical Research, Melbourne, 3050 Victoria, Australia; and ‡Howard Hughes Medical Institute, §Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06510*

Summary

Peripheral T lymphocytes each express surface T cell receptor (TCR) α and β chains of a single specificity. These are produced after random somatic rearrangements in TCR α and β germline genes. Published model systems using mice expressing TCR α and/or β chain transgenes have shown that allelic exclusion occurs conventionally for TCR-β. TCR α chain expression, however, appears to be less strictly regulated, as endogenous TCR α chains are often found in association with transgenic TCR β chains in TCR α/β transgenic mice. This finding, coupled with the unique structure of the TCR α locus, has led to the suggestion that unlike TCR β and immunoglobulin heavy chain genes, TCR α genes may make multiple rearrangements on each chromosome. In the current study, we demonstrate that the majority of TCR$^-$, noncycling thymocytes spontaneously acquire surface expression of CD3/TCR. Further, we show that cultured immature thymocytes originally expressing specific TCR α and β chains may lose surface expression of the original TCR α, but not β chains. These data provide evidence that not only must multiple rearrangements occur, but that TCR α gene rearrangement continues even after surface expression of a TCR α/β heterodimer, apparently until the recombination process is halted by positive selection, or the cell dies. Sequential rearrangement of TCR α chain genes facilitates enhanced production of useful thymocytes, by increasing the frequency of production of both in-frame rearrangements and positively selectable TCR α/β heterodimers.

T lymphocytes develop in the thymus, where they become self-tolerant, self-MHC restricted, and functionally competent (for reviews see references 1–3). Most peripheral T cells bear on their surface a heterodimeric receptor composed of TCR α and β chains, expressed in conjunction with the invariant CD3 protein complex. TCR α and β chain proteins are produced after somatic rearrangements in germline gene sequences encoding multiple members of V, J, and in the case of TCR-β, D gene regions (for a review see reference 4). Such rearrangements are random, which accounts for much of the diversity in the TCR repertoire. One liability of this mechanism, however, is that a large proportion of such rearrangements (two out of three at each locus) result in out-of-frame configurations. Thus, many T lymphocytes are believed to perish intrathymically as the result of failed rearrangements.

During differentiation, immature thymocytes pass through a series of developmental stages that can be defined phenotypically (1). The most numerically predominant of these (~80% of all thymocytes) expresses both CD4 and CD8 coreceptor surface molecules. These CD4$^+$8$^+$ (double positive [DP][1]) cells can be further subdivided on the basis of CD3/TCR surface expression levels (see Fig. 1). The most mature of these (about 1–2% of DP) express high levels of CD3/TCR α/β, and represent cells that have undergone positive selection, but have not yet downregulated either CD4 or CD8 (5–7). Such CD3^{hi}DP arise from a group of DP cells characterized by low level expression of CD3 and TCR α/β. These CD3^{lo}DP represent 60–70% of all DP cells, and are believed to be the population of cells subject to positive and negative selection (1). The remainder of DP cells (~30%) apparently lack CD3 and TCR expression (CD3$^-$DP), and have been thought to represent two types of cells; those currently rearranging TCR α and β genes, and those that have failed to generate in-frame rearrangements in either TCR α or β genes (5, 8).

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive; RAG, recombination activating gene.

Although the fate of DP cells is central to the process of T cell development, their direct study has been plagued by two inherent characteristics of this cell type. First, DP cells are short-lived in vivo, and even more so in vitro (9), thus severely restricting the types of investigations that may be performed. However, by far the most limiting factor is that death among DP cells is accelerated by treatment with anti-CD3/TCR antibodies, presumably by mimicking the signals involved in negative selection (10–12). Since DP subsets can be distinguished only on the basis of surface CD3/TCR levels, any attempt to segregate DP subsets induces their rapid death. These obstacles have now been overcome through the use of mice expressing transgenic *bcl-2* (13–15). In such mice, T cell development, function, number, and ratios are essentially normal (15). However, DP cells from these mice are remarkably resistant to the forms of death characteristic of DP cells from normal mice, including preprogramed death and death induced by CD3/TCR engagement with antibodies (15). Consequently, we are now able to examine aspects of DP cell development using purified DP subsets.

In the current study, we show that the vast majority of DP thymocytes successfully rearrange and express TCR α and β chain genes, contrary to expectations based upon single and random rearrangement events on each allele at each locus (for a discussion, see references 4 and 8). Further, we show that noncycling thymocytes already expressing cell surface CD3/ TCR α/β molecules also express the recombination activating genes RAG-1 and -2. These cells not only continue to rearrange TCR α genes, but can also replace existing surface TCR α chains with newly formed ones, thus maximizing their chances for successful positive selection. The implications of these findings regarding thymocyte selection, survival, and death are discussed in the context of a substantially revised model of intrathymic T cell development.

Materials and Methods

Mice. All mice used for these studies were bred and maintained under specific pathogen-free conditions. C57BL/6 mice for RAG expression studies were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice expressing transgenic *bcl-2* (15) and/or H-Y-specific, H-2D^b–restricted TCR genes (16) were bred at the Walter and Eliza Hall Institute of Medical Research.

Cell Staining and Sorting. Single cell suspensions of fresh or cultured thymocytes were stained and sorted as described (5, 9, 17, 18). Mature CD4$^-$8$^+$ transgenic TCR α/β^+ T cells were sorted from the LNs and spleen of transgenic mice after depletion of CD4$^+$ and B cells using antibodies and complement (9, 17, 18). Antibodies used for depletion, staining, and sorting were as follows: anti-CD3, clone KT3; anti-CD4, clone GK1.5; anti-CD8, clone 53–6.7; anti-TCR β, clone H57-597; anti-TCR α transgene, clone T3.70; and an antibody detecting transgenic TCR β chains, clone F23.1. Antibodies were either directly conjugated to fluorescein (anti-CD8, anti-TCR α transgene), or to allophycocyanin (anti-CD4), or were conjugated to biotin and used with a PE-streptavidin as a second stage. Sorted cells were always >99% pure. Cultured cells were restained using the same antibody/fluorochrome combinations used in sorting. Cell sorting and analysis were performed using a FACStar Plus® cell sorter (Becton Dickinson & Co., Mountain View, CA).

Cell Culture. Purified populations of thymocytes were cultured in RPMI 1640 medium made isotonic to mouse serum, and containing 5–10% fetal bovine serum and 5×10^{-5} M β-ME. Cultures were incubated at 37°C in humidified incubators containing 10% CO_2/90% air. Both before and after culture, cell viability and number were determined by light microscopy, using eosin as a viability dye.

RAG Expression. RNA was extracted and purified from purified populations of thymocytes using guanidium isothiocyanate/acid phenol as described (19). RNA was electrophoresed on 1% formaldehyde/1.2% agarose gels. The amount of RNA loaded in each lane was the total amount isolated from $\sim 1.2 \times 10^6$ cells, as determined by flow cytometer and hemacytometer counting. Confirmation of equal loading of RNA between lanes was obtained by ethidium bromide staining of the gel after electrophoresis. Electrophoresed RNA was transferred to nitrocellulose filters, and Northern blot hybridization was carried out using a random-primed α-[^{32}P]dCTP-labeled 1.4-kb ApaI fragment of murine RAG-1 cDNA as a probe (20). Blots were exposed, imaged, and quantitated using a PhosphorImager® scanner (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Spontaneous Acquisition of Surface CD3/TCR α/β by Noncycling CD3$^-$DP Thymocytes. Since small cortical CD3$^-$DP cells are nondividing (21) and apparently TCR$^-$ (see Discussion), it has been assumed that these were likely to represent the large pool of end-stage products of failed rearrangement attempts. However, when cultured in simple medium without additional stimuli, most CD3$^-$DP thymocytes from *bcl-2* transgenic mice rapidly acquired typical cortical surface levels of CD3 (Fig. 2). About 80% of noncycling CD3$^-$DP, and 90% of cycling CD3$^-$DP blasts, expressed low level CD3 by day 2 of culture (Table 1). No cells ex-

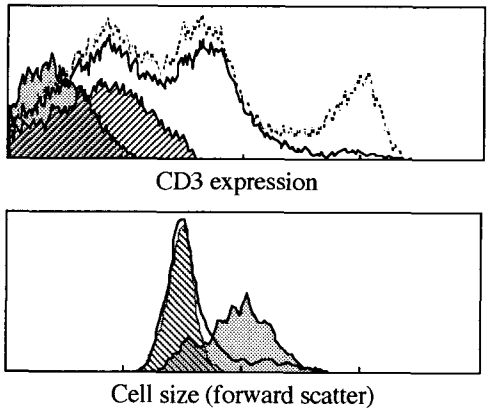

Figure 1. CD3/TCR levels and cell size of various thymocyte subpopulations. (*Top*) (*Dotted line*) unseparated thymocytes; (*solid line*) gated CD4$^+$8$^+$ thymocytes; (*diagonal hatching*) sorted CD3$^-$DP (containing small and blast cells); (*grey fill pattern*) unstained thymocytes. (*Bottom*) (*Solid line*) Total CD3$^-$DP thymocytes; (*diagonal hatching*) sorted small (noncycling) CD3$^-$DP; (*grey fill pattern*), sorted CD3$^-$DP blast cells. CD3 expression is shown on a four-decade $\log_{10}$ fluorescence intensity scale. Cell size (forward scatter) is shown on a linear scale.

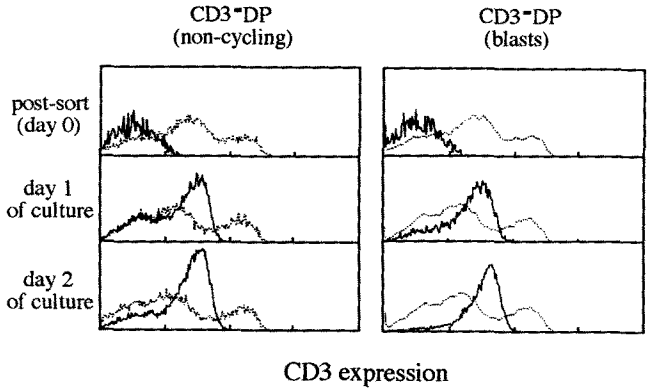

Figure 2. Spontaneous CD3 acquisition by *bcl-2* transgenic CD3⁻DP cells in culture. When cultured in simple medium, most CD3⁻DP cells, including the noncycling component, rapidly acquire typical immature levels of cell surface CD3, as well as TCR α/β (see text). (*Solid lines*) Staining profiles for sorted, cultured cells. (*Dotted lines*) Same staining antibodies on fresh unseparated thymocytes. Results shown are typical for two to five experiments for each cell type or culture period (see Table 1). Fluorescence levels are shown on a four-decade $\log_{10}$ fluorescence intensity scale.

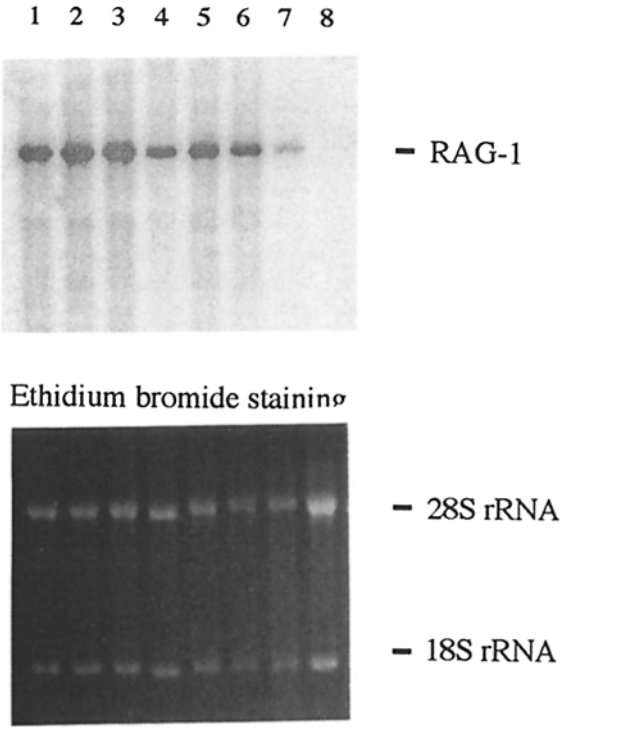

Figure 3. Northern blot analysis of RAG-1 mRNA expression by developmentally segregated subsets of immature thymocytes. Virtually all nonpositively selected subsets of DP thymocytes express significant levels of RAG-1 messenger RNA, suggesting that they are capable of continued TCR gene rearrangements. Samples are as follows: Lanes (*1*) unseparated thymocytes; (*2*) total DP thymocytes; (*3*) total CD3⁻DP thymocytes; (*4*) small (noncycling) CD3⁻DP thymocytes; (*5*) total CD3^{lo}DP thymocytes; (*6*) small (noncycling) CD3^{lo}DP thymocytes; (*7*) CD3^{hi}DP thymocytes; (*8*) mature thymic SP cells (CD3^{hi}4$^+$8$^-$ and CD3^{hi}4$^-$8$^+$). Results shown are typical of two to three experiments for each cell type.

pressing these levels of CD3 were present in the post-sort reanalysis (Fig. 2, top), demonstrating that such surface expression represents bona fide acquisition of CD3. TCR β chain expression profiles were indistinguishable from those of CD3 in all experiments (data not shown). Since mAbs to TCR α chains are not available, TCR α chain expression could not be demonstrated. However, CD3/TCR β expression at these levels is not known to occur in the absence of TCR-α, suggesting that these are TCR α/β heterodimers. The slightly higher levels of CD3/TCR seen after culture, when compared with freshly isolated thymocytes (Fig. 2),

Table 1. *Spontaneous CD3 Acquisition by bcl-2 Transgenic CD3⁻4⁺8⁺ Cells in Culture*

Cell type	Culture time	Total culture viability	Viable cell recovery (% of input viable cells)	Number of experiments	Percent cells in CD3^{lo} range
	d	%			
	0	80 ± 4*	–	5	7 ± 3‡
CD3⁻DP	1	71 ± 1	64 ± 5	3	60 ± 7
(noncycling)	2	52 ± 5	49 ± 15	5	81 ± 2
	0	89 ± 7	–	4	9 ± 3
CD3⁻DP	1	95 ± 5	173 ± 35	2	72 ± 7
(blasts)	2	93 ± 1	146 ± 6	2	92 ± 2
	0	87 ± 3	–	2	96 ± 1
CD3^{lo}DP	1	69 ± 4	71 ± 11	2	94 ± 3

* Mean ± SE.
‡ Limits of CD3^{lo} range determined empirically using the staining profile of unseparated thymocytes.

are due to both increased levels of autofluorescence, and up-regulation of surface expression caused by disengagement from the thymic milieu (22). This is also seen in cultured $CD3^{lo}$DP cells (data not shown), and does not reflect true maturational change. No proliferation occurred among noncycling $CD3^-$DP cells in culture, as total cell numbers did not increase, and no [^{3}H]thymidine incorporation was observed (data not shown). Cell number among cycling $CD3^-$DP blasts increased by about 50% in vitro, probably representing one cell division among a proportion of cells already in mitosis at the time of isolation.

The Relationship of RAG-1 RNA Expression to Normal Thymocyte Maturation. The high frequency of CD3/TCR acquisition by noncycling $CD3^-$DP thymocytes was surprising, as these cells were expected to represent the large proportion of cells failing productive TCR gene rearrangements. Further, these findings suggested that such cells must be capable of continued TCR gene rearrangement, and should therefore express indicators of V(D)J recombinase activity. One of the best indicators of such activity is expression of RAG-1 and -2 (20, 23). Whereas the proteins encoded by these genes have not as yet been demonstrated to mediate V(D)J recombination directly, recombination is not known to occur in the absence of RAG expression (23, 24). Consequently, we assayed developmentally segregated subsets of thymocytes for RAG expression by Northern blotting (Fig. 3). Confirming the above findings of CD3/TCR acquisition by noncycling $CD3^-$DP, we found that small noncycling $CD3^-$DP thymocytes from normal C57BL/6 mice expressed high levels of RAG-1 message. RAG-2 message was also present in all subsets at lower, but proportional, levels (data not shown), as expected (23). Isolated RNA from equal numbers of cells, as determined by FACStar® (Becton Dickinson & Co.) and hemacytometer counting, was loaded in each lane, as is confirmed by ethidium bromide staining of the gel before transfer to nitrocellulose (Fig. 3). Similar populations of cells from *bcl-2* transgene mice also expressed RAG-1 message, albeit at a reduced level (data not shown). The reasons for this reduced expression are not clear. However, thymocytes from *bcl-2* transgene mice also express lower levels of other mRNAs on a per cell basis (Petrie, H., and F. Livak, unpublished observations) suggesting that this is not a specific downregulation of recombination-associated activity in these cells.

In addition to expression in $CD3^-$DP cells, we found significant levels of RAG-1 mRNA among immature cells already expressing CD3/TCR α/β (i.e., $CD3^{lo}$DP). This has previously been shown using mice expressing transgenic TCR genes (25), and has been suggested by the downregulation of RAG expression after anti-CD3 treatment (26). Our data extends these results by demonstrating that RAG expression persists even among TCR-expressing, noncycling thymocytes from normal mice. Clearly, mature single positive (SP) thymocytes do not express detectable RAG activity (Fig. 3), suggesting that positive selection may downregulate active recombination, as suggested (25, 27). Also consistent with recently published data using TCR transgenic mice (25), we found low level, but detectable, RAG-1 message among positively selected $CD3^{hi}$DP cells. Such expression may represent residual message in cells that have recently undergone positive selection. It is possible that inhibition of recombination may be mediated at the protein level in positively selected cells, thus negating the potentially disruptive effects of any residual RAG message.

Replacement of Transgenic TCR α Chains with Endogenous TCR α Chains by Nonselected Thymocytes in Culture. The expression of RAG mRNA by immature thymocytes already expressing in-frame rearrangements (i.e., $CD3^{lo}$DP cells), as shown above, suggested that in the absence of positive selection, TCR-expressing thymocytes might continue to rearrange TCR genes, in an attempt to express new TCRs that may be positively selectable. If this were true, new TCR chains should eventually replace existing ones on the surface of nonselected thymocytes. Using $CD3^{lo}$DP thymocytes from normal mice, this possibility cannot be assessed, as no single TCR α chain specificity exists in sufficient numbers for sorting, and in any case, the simple act of sorting by TCR expression would induce death, as described earlier (see introductory section). We have overcome these obstacles by using mice expressing single TCR α and β chains of known specificity, as well as the *bcl-2* transgene to prevent death induced by anti-TCR antibodies used in cell sorting. Mice expressing transgenic TCR α and β chains specific for the male H-Y antigen presented in the context of H-$2D^b$ were chosen for this purpose (16). TCR transgenic mice were bred onto the H-$2D^d$ (DBA/2) genetic background, and were crossed with *bcl-2* transgenic mice also bred onto DBA/2, such that positive selection would not occur in the offspring. Further, only female mice were used as sources of thymocytes, such that negative selection events were also avoided. Transgene TCR α/β^{lo}DP cells were isolated from such mice, and cultured for 4 d in simple culture medium. Cultured cells were then restained and analyzed for transgenic TCR α and β chain surface expression using the same antibodies used in sorting, which recognize the transgenic TCR α and β chains. As is shown in Fig. 4 *a*, about 25–30% of initially TCR α/β transgene–positive cells had apparently lost most TCR α transgene expression. Proportional losses of transgenic TCR β chain expression were not seen. Neither transgenic TCR α nor β chains were lost from the surface of TCR α/β transgene–positive mature peripheral T cells from (H-Y TCR transgenic X *bcl-2* transgenic) C57BL/6 female (i.e., positively selecting, nonnegatively selecting) mice after a similar culture period (Fig. 4 *b*), demonstrating that the loss of transgenic TCR α chains seen among immature cells is not an artefact induced by culture. The most likely explanation for these findings is that in nonpositively selected thymocytes, TCR α genes continue to rearrange, even after surface expression of TCR α/β heterodimers. It is interesting to note that the population of cells showing loss of the TCR α transgene does not appear to be completely negative for transgene expression, suggesting that newly formed (in this case, endogenous) TCR α chains may simply compete with existing ones for available TCR β chains. Thus, it follows that in the

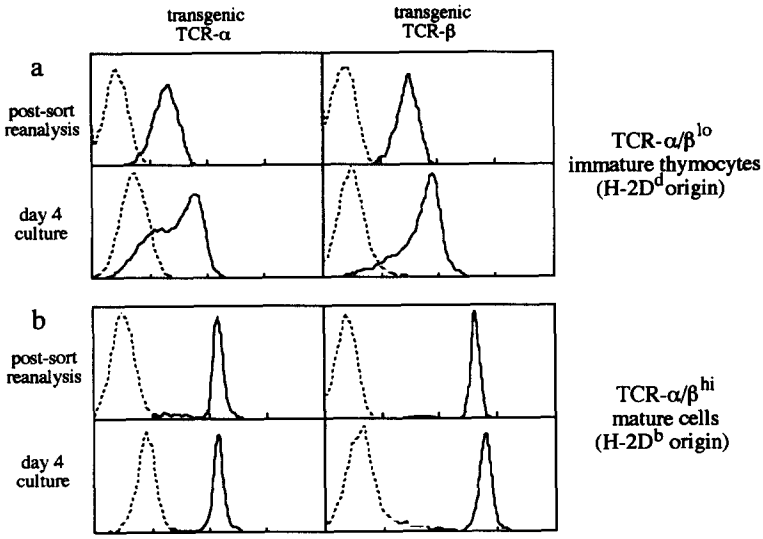

Figure 4. Loss of surface TCR α chains by transgenic CD3^{lo}DP thymocytes in vitro. Thymocytes expressing CD4, CD8, and low (immature) levels of transgenic TCR α and β chains were isolated from (TCR α/β transgenic X *bcl-2* transgenic) F_1 mice expressing the nonpositively selecting, nonnegatively selecting background (H-2D^d females). Mature T cells expressing CD4 or CD8 and high (mature) levels of transgenic TCR α and β chains were isolated from the LN and spleen of (TCR α/β transgenic X *bcl-2* transgenic) F_1 mice expressing the positively selecting, nonnegatively selecting background (H-2D^b females). A significant proportion of immature TCR^{lo}DP thymocytes display loss of transgene TCR α expression within a few days of culture (*a*) (*Solid lines*) Stained fresh or cultured thymocytes; (*dotted lines*) unstained fresh or cultured thymocytes. No loss of TCR α or β transgene expression was observed in cultures of mature T cells (*b*). Essentially identical results were obtained in three to four separate experiments for each cell type. TCR expression levels are shown on a four-decade $\log_{10}$ fluorescence intensity scale.

event an existing in-frame joint is replaced by an out-of-frame one, existing TCR α surface expression may persist for some time, depending on the half-life of the TCR α/β surface complex. This is consistent with the findings shown in Table 1, demonstrating that cultured CD3^{lo}DP do not downregulate surface CD3 expression after culture.

Discussion

Several of the experiments described herein involve the use of transgenic model systems, however, we believe that our results are applicable to unmanipulated animals as well. Numerous experiments validating the usefulness of the TCR transgenic mouse model used here have been described (see references 2, 6, 16, 25, and 28 for a partial bibliography). The *bcl-2* transgenic mice were more recently established (13–15). However, in all studies published to date (13–15, 18), the vast majority of data suggest that despite their profound resistance to cell death, most developmental phenomena occur normally in such mice. Further, the use of these transgenic models has facilitated the execution of experiments not possible using cells from normal mice due to inherent limitations, such as death induced by anti-CD3/TCR antibodies, or very low frequencies of individual TCR α chain specificities.

Our results support a number of conclusions. First, the proportion of thymocytes that eventually express TCR α/β surface molecules is much higher than can be predicted by classical models. Assuming that TCR α and β genes rearrange independently, that each allele rearranges only once, and that rearrangements of each locus ceases after an in-frame rearrangement on one allele, each cell would have an ~30% chance of producing an α/β pair. Even assuming that TCR β chains are already productively rearranged in thymocytes that rearrange TCR α chain genes (see below), only a 56% success rate for TCR α/β assembly can be predicted (see reference 4 for a discussion of these estimates, using TCR β genes as an example). To achieve the high frequency of success shown here (i.e., 80–90%; see Fig. 2 and Table 1), a significant number of immature thymocytes must carry out multiple rearrangements of at least one of their TCR α alleles. Assuming that V_α-J_α rearrangement occurs exclusively by DNA deletion (as opposed to inversion), each successive rearrangement would involve V_α gene segments located further 5′ and J_α gene segments located further 3′ in the locus. Such a mechanism has been previously proposed by others (for a review, see reference 4), but has not been directly demonstrated.

Since survival of DP cells in our cultures was <100% (Table 1), it was possible to suggest that the high rate of CD3/TCR acquisition by CD3$^-$DP reflects the survival of those cells making in-frame rearrangements, and selective death of the remainder. However, no differences in survival rates have been noted between CD3$^-$ and CD3^{lo} DP cells in culture (Table 1, and additional data not shown), suggesting that this is not the case. Further, the demonstration of RAG expression by noncycling CD3$^-$DP cells also supports the concept that these cells are actively rearranging TCR genes. RAG expression by non-TCR-transgenic, noncycling cells already expressing surface TCR α/β suggests that TCR gene rearrangement continues in all nonmature (i.e., nonpositively selected) thymocytes. The finding that RAG expression is downregulated in CD3^{hi} cells suggests that positive selection may be required before TCR gene rearrangement finally stops. This is consistent with recently published data (27), showing that RAG expression is high in immature TCR transgenic thymocytes when the positively selecting MHC element is absent, but is downregulated when positive selection can occur.

Because two out of three TCR gene rearrangements are expected to produce out-of-frame joints, multiple sequential TCR gene rearrangements would be an effective mechanism for maximizing the proportion of potentially useful thymocytes. However, successful surface expression of productively rearranged antigen receptors is not sufficient for T cell maturation. Rather, thymocytes expressing TCRs exhibiting self-MHC restriction capacity are chosen from a pool of thymocytes

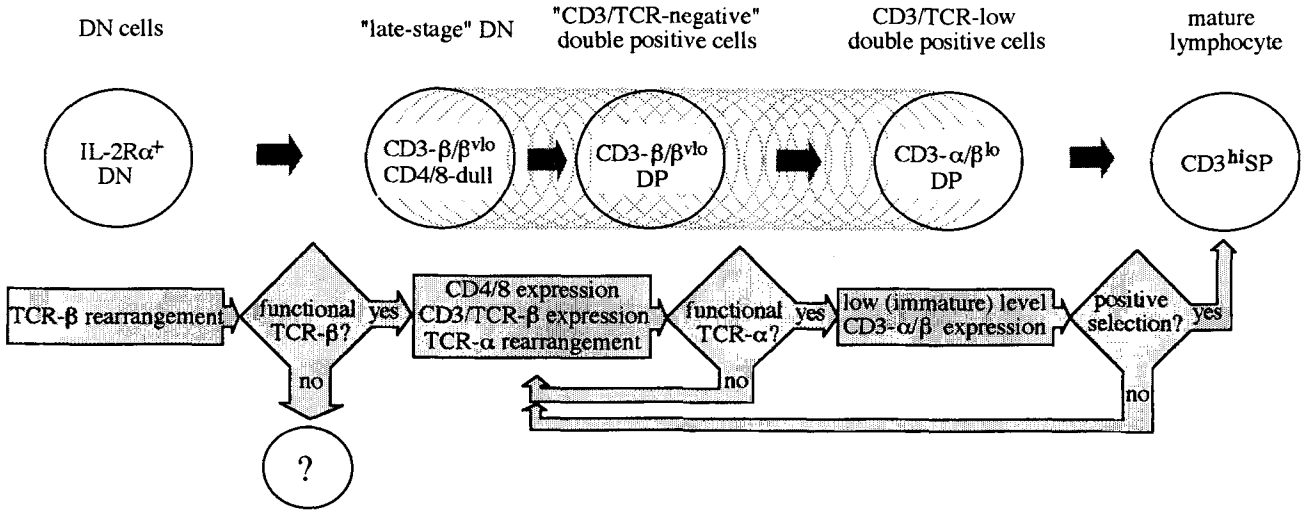

Figure 5. A model for T cell development, showing the relationship of TCR α and β gene rearrangement to the progression through various developmental stages. The developmental stages shown have been characterized and/or described previously (1, 9, 28, 35). The spontaneous transition from "late-stage" DN (18) to CD3^{lo}DP is also shown, as has been described (17, 36, 37). CD3^{lo}DP cells that fail positive selection and continue TCR α gene rearrangements do not revert to a TCR β/β^{lo} state, but may replace existing TCR α chains with newly formed ones if subsequent rearrangements are in-frame. (*Vlo*) very low and (*lo*) low (typical cortical) levels, as described (17, 28, 35–37).

that express randomly generated TCRs. Our data suggest that the increased likelihood for survival imparted by multiple rearrangements is further amplified by continued TCR α gene rearrangements among nonselected cells, even those already exhibiting surface expression of in-frame TCR gene products. Cells that initially express a nonselectable receptor continue to rearrange TCR α loci, and can express TCR α chains of a new specificity, providing that at least one subsequent rearrangement is in-frame. Consequently, it appears that thymocytes maximize their chances for positive selection by continually making new receptor combinations until positive selection occurs. Successive TCR α gene rearrangement would lead to deletion of the existing in-frame V_α-J_α joint in nonpositively selected CD3^{lo}DP if such a rearrangement occurs on the same allele, thus preventing the cell from expressing two different TCR α chains. However, rearrangement on the other allele may result in two in-frame rearrangements in the same cell, as has been reported in T cell hybridomas (25, 29). In this case, an alternative mechanism for regulation of surface expression must exist, such as differences in the affinities of the two TCR α chains for pairing with TCR β (25). In any case, an out-of-frame rearrangement of a previously in-frame allele may not necessarily result in the immediate loss of surface TCR, as discussed above.

For several reasons, it appears likely that TCR β chain genes are already rearranged in cells actively rearranging TCR-α. First, thymocyte development is arrested at the CD4$^-$8$^-$ (double negative [DN]) stage in RAG-deficient mice (24, 30), suggesting that at least some TCR rearrangement is required for progression to the DP stage. Second, thymocytes from TCR β chain–deficient mice also exhibit developmental arrest at the DN stage (31), whereas TCR α–deficient mice have normal numbers of DP thymocytes (31, 32). This suggests that DP cells are likely to have undergone TCR β but not necessarily TCR α gene rearrangement. Third, the introduction of a rearranged TCR β (but not TCR α) transgene into SCID mice (28) or RAG-1–deficient mice (31) induces the development of DP cells, suggesting that the presence of an in-frame TCR β gene (or its product) allows progression to the DP stage. Finally, essentially all DP cells from TCR α–deficient mice express in-frame TCR β gene rearrangements (33), showing that in-frame TCR β gene rearrangements occur before the DP stage. Thus, progression from the DN to the DP stage appears to be dependent upon successful TCR β but not α gene rearrangement. Rearrangement of TCR-β at the DN stage would impart significant biological benefits. Since DN cells represent only about 2% of all immature thymocytes, and since about 56% of these would make productive rearrangements in TCR-β after single rearrangements on both alleles (4), the number of immature thymocytes with failed TCR-β rearrangements would be <1% of all immature cells produced. Further, successful assembly of an α/β heterodimer by a DP cell would thus be dependent solely upon the success of TCR α gene rearrangement. Therefore, about 56% of all DP would have in-frame TCR α and β genes after one recombination event on each TCR α allele. Each successive rearrangement of both TCR α alleles would yield in-frame TCR α genes in 56% of the remaining cells, bringing the total number of cells with productive rearrangements in both TCR α and β genes to over 80% after just two successive TCR α rearrangements. Thus, the amount of waste in the thymus is likely to be significantly less than previously thought. Further, a dead-end population, representing cells with out-of-frame rearrangements, may not exist to any significant extent. Other mechanisms of cell death are certain to persist, however, such

as failure to produce any in-frame rearrangements, failure to produce a positively selectable TCR α/β pair, or production of a self-reactive TCR leading to negative selection.

Evidence is mounting that after productive rearrangement, TCR β chains may be expressed on the cell surface as homodimers at very low levels, in conjunction with CD3 (28, 34, 35). This could explain why the most mature DN cells appear to express very low levels of CD3/TCR-β (17, 36, 37), and why $CD3^-$DP cells do not appear to be entirely CD3/TCR-β-negative (Fig. 1). It is interesting to note that rearrangement may proceed at certain susceptible loci without affecting others (i.e., TCR-β but not -α, or the reverse), suggesting the presence of locus-specific regulatory components of the recombination process. If TCR β rearrangement does precede that of TCR-α, as most evidence suggests, (24, 28, 30–33) specific regulation would in most cases be necessary to prevent the deletion of productively rearranged TCR β genes while TCR α rearrangement is proceeding.

It is now possible to propose a significantly revised scheme for intrathymic T cell development (Fig. 5). TCR α chain gene rearrangement proceeds in a sequential manner, as described above. Once an in-frame rearrangement occurs, TCR α can pair with preexisting TCR β chains and become expressed on the cell surface, making the cell available for selection. However, rearrangement of TCR-α does not stop, and continues in nonselected cells until positive selection occurs, or the cell dies. This model also suggests the possibility that one mechanism for the finite lifespan of DP cells may be the progressive exhaustion of all or most TCR α V or J gene segments. Experiments are currently underway to address this possibility.

The authors thank Drs. Al Bothwell, Adrian Hayday, and Charles Janeway for critical review of the manuscript; Drs. Alan W. Harris and Suzanne Cory for *bcl-2* transgenic mice; Dr. Harold von Boehmer for TCR transgenic mice; and Rebecca Kydd for technical assistance.

This work was supported by the Australian National Health and Medical Research Council (K. Shortman), by a Cancer Research Institute Investigator Award (I. N. Crispe), by the Howard Hughes Medical Institute (F. Livak and D. G. Schatz) and by fellowships of the Leukemia Society of America and the Swiss National Science Foundation (A. Strasser).

Address correspondence to Dr. Howard T. Petrie, Memorial Sloan-Kettering Cancer Center, Immunology Program, 1275 York Avenue, New York, NY 10021.

Received for publication 30 November 1992 and in revised form 25 March 1993.

References

1. Shortman, K., M. Egerton, G.J. Spangrude, and R. Scollay. 1990. The generation and fate of thymocytes. *Sem. Immunol.* 2:3.
2. Kisielow, P., and H. von Boehmer. 1990. Negative and positive selection of immature thymocytes: timing and the role of ligand for alpha/beta T cell receptor. *Sem. Immunol.* 2:35.
3. Fowlkes, B.J., and D.M. Pardoll. 1989. Molecular and cellular events of T cell development. *Adv. Immunol.* 44:207.
4. Malissen, M., J. Trucy, E. Jouvin-Marche, P.-A. Cazenave, R. Scollay, and B. Malissen. 1992. Regulation of TCR-α and -β gene allelic exclusion during T-cell development. *Immunol. Today.* 13:315.
5. Shortman, K., D. Vremec, and M. Egerton. 1991. 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 cells. *J. Exp. Med.* 173:323.
6. Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmer. 1991. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell.* 66:533.
7. Penit, C. 1990. Positive selection is an early event in thymocyte differentiation: high TCR expression by cycling immature thymocytes precedes final maturation by several days. *Int. Immunol.* 2:630.
8. Kronenberg, M., G. Siu, L.E. Hood, and N. Shastri. 1986. The molecular genetics of the T cell antigen receptor and T cell antigen recognition. *Annu. Rev. Immunol.* 4:529.
9. Petrie, H.T., M. Pearse, R. Scollay, and K. Shortman. 1990. Lineage relationships and developmental kinetics of immature thymocytes: CD3, CD4, and CD8 acquisition in vivo and in vitro. *J. Exp. Med.* 172:1583.
10. Finkel, T.H., J.C. Cambier, R.T. Kubo, W.K. Born, P. Marrack, and J. Kappler. 1989. The thymus has two functionally distinct populations of immature α/β T cells: one population is deleted by ligation of α/β TCR. *Cell.* 58:1047.
11. Smith, C.A., G.T. Williams, R. Kingston, E.J. Jenkinson, and J.J.T. Owen. 1989. Antibodies to CD3/T cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature (Lond.).* 337:181–184.
12. Shi, Y., R.P. Bissonette, N. Parfrey, M. Szalay, R.T. Kubo, and D.R. Green. 1991. In vivo administration of monoclonal antibodies to the CD3/T cell receptor complex induces cell death (apoptosis) in immature thymocytes. *J. Immunol.* 146: 3340.
13. Strasser, A., A.W. Harris, D.L. Vaux, E. Webb, M.L. Bath, J.M. Adams, and S. Cory. 1990. Abnormalities of the immune system induced by dysregulated *bcl-2* expression in transgenic mice. *Curr. Top. Microbiol. Immunol.* 166:175.
14. Sentman, C.L., J.R. Shutter, D. Hockenbery, O. Kanagawa, and S.J. Korsmeyer. 1991. *bcl-2* inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell.* 67:879.

15. Strasser, A., A.W. Harris, and S. Cory. 1992. *bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. *Cell.* 67:889–899.
16. von Boehmer, H., and P. Kisielow. 1990. Self-nonself discrimination by T cells. *Science (Wash. DC).* 248:1369.
17. Petrie, H.T., M. Pearse, R. Scollay, and K. Shortman. 1990. Development of immature thymocytes: initiation of CD3, CD4, and CD8 acquisition parallel down-regulation of the interleukin-2 receptor alpha chain. *Eur. J. Immunol.* 20:2813.
18. Petrie, H.T., R. Scollay, and K. Shortman. 1992. Commitment to the T cell receptor α/β or γ/δ lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. *Eur. J. Immunol.* 22:2185.
19. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual (Second Ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 7.12–7.15.
20. Schatz, D.G., M.A. Oettinger, and D. Baltimore. 1989. The V(D)J recombination activating gene, RAG-1. *Cell.* 59:1035.
21. Egerton, M., R. Scollay, and K. Shortman. 1990. Kinetics of mature T-cell development in the thymus. *Proc. Natl. Acad. Sci. USA.* 87:2579.
22. Nakayama, T., C.H. June, T.I. Munitz, M. Sheard, S.A. McCarthy, S.O. Sharrow, L.E. Samelson, and A. Singer. 1990. Inhibition of T cell receptor expression and function in immature CD4-positive CD8-positive cells by CD4. *Science (Wash. DC).* 249:1558.
23. Oettinger, M.A., D.G. Schatz, C. Gorka, and D. Baltimore. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science (Wash. DC).* 248:1517.
24. Shinkai, Y., G. Rathbun, K.-P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, and F.W. Alt. 1992. RAG-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell.* 68:855.
25. Borgulya, P., H. Kishi, Y. Uematsu, and H. von Boehmer. 1992. Exclusion and inclusion of α and β T cell receptor alleles. *Cell.* 69:529.
26. Turka, L.A., D.G. Schatz, M.A. Oettinger, J.J.M. Chun, C. Gorka, K. Lee, W.T. McCormack, and C.B. Thompson. 1991. Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor cross linking. *Science (Wash. DC).* 253:778.
27. Brandle, D., C. Muller, T. Rulicke, H. Hengartner, and H. Pircher. 1992. Engagement of the T cell receptor during positive selection in the thymus down-regulates RAG-1 expression. *Proc. Natl. Acad. Sci. USA.* 89:9529.
28. Kishi, H., P. Borgulya, B. Scott, K. Karjalainen, A. Traunecker, J. Kaufman, and H. von Boehmer. 1991. Surface expression of the beta T cell receptor (TCR) chain in the absence of other TCR or CD3 proteins on immature T cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:93.
29. Malissen, M., J. Trucy, F. Letourneur, N. Rebai, D.E. Dunn, F.W. Fitch, L. Hood, and B. Malissen. 1988. A T cell clone expresses two T cell receptor α genes but uses one α/β heterodimer for allorecognition and self-MHC restricted antigen recognition. *Cell.* 55:49.
30. Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell.* 68:869.
31. Mombaerts, P., A.R. Clarke, M.A. Rudnicki, J. Iacomini, S. Itohara, J.J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M.L. Hooper, and S. Tonegawa. 1992. Mutations in T cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature (Lond.).* 360:225.
32. Philpott, K., J. Viney, G. Kay, J. Rastan, E. Gardiner, S. Chae, A. Hayday, and M.J. Owen. 1992. Lymphoid development in mice congenitally deficient in TCR-α/β cells. *Science (Wash. DC).* 256:1448.
33. Mallick, C.A., E.C. Dudley, J.L. Viney, M.J. Owen, and A.C. Hayday. 1993. Rearrangement and diversity of T cell receptor β chain genes in thymocytes: a critical role for the β chain in development. *Cell.* 73:513.
34. Punt, J.A., R.T. Kubo, T. Saito, T.H. Finkel, S. Kathiresan, K.J. Blank, and Y. Hashimoto. 1991. Surface expression of a T cell receptor β chain in the absence of TCR α, δ, and γ proteins. *J. Exp. Med.* 174:775.
35. Groettrup, M., A. Baron, G. Griffiths, R. Palacios, and H. von Boehmer. 1992. T cell receptor (TCR) β chain homodimers on the surface of immature but not mature α, γ, δ chain deficient T cell lines. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2735.
36. Nikolic-Zugic, J., and M.W. Moore. 1989. T cell receptor expression on immature thymocytes with in vivo and in vitro precursor potential. *Eur. J. Immunol.* 19:1957.
37. Nikolic-Zugic., J., M.W. Moore, and M.J. Bevan. 1989. Characterization of the set of immature thymocytes which can undergo rapid in vitro differentiation. *Eur. J. Immunol.* 19:649.