Regulation of Pre-T Cell Receptor (pTα-TCRβ) Gene Expression during Human Thymic Development

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Summary

In murine T cell development, early thymocytes that productively rearrange the T cell receptor (TCR) β locus are selected to continue maturation, before TCRα expression, by means of a pre-TCRα- (pTα-) TCRβ heterodimer (pre-TCR). The aim of this study was to identify equivalent stages in human thymocyte development. We show here that variable-diversity-joining region TCRβ rearrangement and the expression of full-length TCRβ transcripts have been initiated in some immature thymocytes at the TCRαβ- CD4+CD8- stage, and become common in a downstream subset of TCRαβ- CD4+CD8+ thymocytes that is highly enriched in large cycling cells. TCRβ chain expression was hardly detected in TCRαβ- CD4+CD8- thymocytes, whereas cytoplasmic TCRβ chain was found in virtually all TCRαβ- CD4+CD8+ blasts. In addition, a TCRβ complex distinct from the mature TCRαβ heterodimer was immunoprecipitated only from the latter subset. cDNA derived from TCRαβ- CD4+CD8+ blasts allowed us to identify and clone the gene encoding the human pTα chain, and to examine its expression at different stages of thymocyte development. Our results show that high pTα transcription occurs only in CD4+CD8- and CD4+CD8+ TCRαβ- thymocytes, whereas it is weaker in earlier and later stages of development. Based on these results, we propose that the transition from TCRαβ- CD4+CD8- to TCRαβ- CD4+CD8+ thymocytes represents a critical developmental stage at which the successful expression of TCRβ promotes the clonal expansion and further maturation of human thymocytes, independent of TCRα.

During thymocyte development in normal mice, rearrangement and expression of the TCRβ chain gene precedes that of the α chain (1). The first TCRβ gene rearrangements are detectable in a subset of CD4-CD8-double negative (DN)1 thymocytes that express IL-2Rα (CD25) (2, 3). However, most rearrangements are out-of-frame at this stage (4). In contrast, the V-D-J rearrangements of the immediate progeny of these cells (i.e., CD25- DN thymocytes) (3) are predominantly in-frame, indicating that cells within the CD25- DN population have been selected on the basis of productively rearranged β chain genes (4). The CD25+ to CD25- transition is accompanied by a strong cellular proliferation, which is consistent with the fact that CD25- DN thymocytes include a high proportion of large cycling cells (5). Proliferating CD25- blasts rapidly acquire (or express already) low levels of CD4 and CD8 and, therefore, represent cells in transit to the CD4+CD8+ double positive (DP) stage (6, 7). At this stage, proliferation comes to an abrupt halt, TCRαβ is expressed for the first time, and DP thymocytes are subsequently selected to develop into mature CD4+ or CD8+ single positive (SP) thymocytes (5, 8, 9). Therefore, the transition from CD25+ to CD25- DN thymocytes appears to be the critical stage at which cells with productive β chain expression gain a selective advantage to mature further, independently of TCRα (3, 4).

Evidence supporting such a regulatory role of the TCRβ protein in early T cell development was originally obtained in SCID mice, and later confirmed in rearrangement (RAG-1 or RAG-2)-deficient mice, and in mice with a mutated TCRβ gene. Both the severe drop in total thymocyte numbers and the blockade at the CD25+ DN stage observed in these animals were fully restored upon introduction of a productively rearranged TCRβ transgene (9-
Evidence for an equivalent pre-TCR complex in human thymocyte development is still lacking. Furthermore, although TCRβ rearrangement also precedes that of TCRα in humans (16), no direct experimental evidence has been provided that resolves the timing of TCRβ and TCRα gene expression during the development of human thymocytes. In the present study we have approached this issue by direct examination of the transcriptional activation of both TCRβ and TCRα genes in individual subsets of TCR-negative thymocytes representative of the earliest human intrathymic stages (17). This analysis allowed us to identify a novel subset of large-sized TCRα/β-CD4+CD8+ cycling thymocytes that express a functionally rearranged TCRβ locus but lack TCRα gene expression. Using cDNA derived from these cells we could identify and clone the human pTα cDNA. Comparison of the developmental regulation of pTα, TCRβ, TCRα, and RAG-1 gene expression provided a clear picture of the maturational progression of early human intrathymic stages and led us to identify the transition from CD4+CD8- to large CD4+CD8+ thymocytes as a key control point in early human T cell development.

Materials and Methods

Cell Samples. Fetal thymus, liver, and bone marrow tissue were obtained from 18–22-wk fetuses after legal termination of pregnancy. Umbilical cord blood samples were collected immediately after delivery. Adult bone marrow was obtained from puncture of the iliac crest of healthy donors. Peripheral blood was obtained from patients subjected to chemotherapy and treatment with G-CSF. CD34+ cells (>99% purity upon reanalysis) were isolated from the indicated sources by Ficoll-Hypaque (Nycomed, Oslo, Norway) centrifugation (18) followed by immunomagnetic sorting with anti-CD34–coupled magnetic beads (Dynabeads, Dynal Corp., Oslo, Norway).

Postnatal thymus samples were removed during corrective cardiac surgery of patients aged 1 mo to 3 yr. Thymocyte suspensions were depleted of small- and medium-sized TCRα/β+ DP thymocytes by centrifugation on stepwise Percoll (Pharmacia LKB, Uppsala, Sweden) density gradients as previously described (19). Large thymocytes recovered from the 1.068 density layer were further depleted (>99% purity) of α/β and γ/δ mature T cells, as well as of B, NK, and myeloid cells, by two rounds of depletion with anti-CD3– and anti-CD19–coated magnetic beads (Dynal), and with anti-CD56 (Leu-19; Becton Dickinson & Co., San Jose, CA and anti-CD11b (MO1; Coulter Corp., Hialeah, FL) mAbs indirectly coupled to sheep anti-mouse IgG–coated magnetic beads (Dynal). CD4+CD8+ thymocytes were magnetically sorted from the remaining pool with anti-CD8–coated magnetic beads (Dynal). CD4+CD8- thymocytes were then isolated from the CD8-depleted pool by treatment with anti-CD4–coated magnetic beads (Dynal). The remaining CD4+CD8- thymocytes were finally sorted into the CD34+CD1+ and CD34+CD1- subsets by treatment with an anti-CD1a (Na1/34) mAb (20) indirectly coupled to magnetic beads (Dynal), as described (21). Mature TCRα/β+ SP cells were isolated from CD1-depleted large thymocytes by treatment with anti-CD3–coated magnetic beads. Conventional TCRα/β+ CD4+CD8- small thymocytes (referred to as DP) were isolated from the 1.09 Percoll density layer by magnetic sorting with anti-CD1a.

Flow Cytometry Analysis. Directly labeled mAbs against the following antigens were used: CD4 (Leu3a-PE), CD3 (HPCA-2-PE), and CD8 (Leu2a-FITC) from Becton Dickinson & Co.: CD1a (T6-RD1 and T6-FITC), and CD71 (T9-FITC) from Coulter Corp.: CD28 (CD28-FITC) from Serotec Ltd., Oxford, UK; and CD44 (CD44-FITC) from Caltag Laboratories (South San Francisco, CA). Unlabeled mAb against CD8 (2ST8-5H7, kindly provided by Dr. E.L. Reinherz, Dana-Farber Cancer Institute, Boston, MA) (22), as well as mAb recognizing monomorphic determinants of TCRα/β (BMA031, generously provided by Dr. R. Kultle, Behngwerke AG, Marburg, Germany) were used in combination with goat anti–mouse FITC- or PE-coupled F(ab)2 Ig (Southern Biotechnology Associates, Inc., Birmingham, AL). Isotype-matched irrelevant mAbs (Caltag Laboratories) were used as negative controls. For detection of cytoplasmic TCRβ, cells were treated with 0.5% saponin (Sigma Chemical Co., St. Louis, MO), incubated with the anti-TCR chain β1 mAb (generously provided by Dr. M. Brenner, Brigham and Women's Hospital, Boston, MA) (23), and labeled with PE-coupled goat anti–mouse IgG1 (Southern Biotechnology Associates, Inc.). Stained cells were analyzed in an Epics XL flow cytometer (Coulter Corp.) as previously described (21). Cell cycle analyses were performed by flow cytometry in cells treated with 0.05% digitonin, washed, and stained with 50 μg/ml of propidium iodide (both from Sigma Chemical Co.).

TCRβ Gene Rearrangement Analysis. TCRβ gene rearrangements were analyzed by using a modification of the genomic DNA-PCR assay described elsewhere (24). Genomic DNA (1 μg) was amplified by PCR for 30 cycles (1 min at 95°C, 2 min at 68°C, and 5 min at 72°C) in 50 μl reaction buffer (Perkin-Elmer Cetus, Norwalk, CT) containing 1.25 U AmpliTaq polymerase (Perkin-Elmer Cetus) and 1 μM each of 5′ and 3′ primers. The primers used to detect DB3, to β3, rearrangements were as follows: 5′DB3 (5′-GGAGGGGACTAGCAGGGAGG-3′); 3′DB3 (5′-ACCACCCAGCTCTCCACGCTCCCG-3′); and 5′β3 (5′-ACCCAGCTCTCCACGCTCCCG-3′) for V-D-J rearrangements. For V-B-specific primers (25) were used in concert with the 5′β3 primer. PCR products (16 μl) were electrophoresed, blotted onto nylon membranes (Zeta Probe, Bio-Rad Laboratories, Hercules, CA), and probed with a 32P-labeled 3′β3 oligonucleotide (5′-GGCTGGAAGGTGGGGAGACGCCCG-3′) located immediately 5′ of the 3′β3 primer used for amplification.
Northern Blot Analysis. Preparations of total RNA (10 μg) isolated as described elsewhere (18) were run on 1% agarose-formaldehyde gels, transferred to nylon membranes, and hybridized as previously described (26) with 32P-labeled cDNA probes corresponding to the TCR Cα (PY1.4) (27) or Cβ (JurT3) (28) regions (kindly provided by Dr. T.W. Mak, The Ontario Cancer Institute, Toronto, ON, Canada). The human p70 cDNA probe was derived in this study (see below). The same blot was subsequently stripped and hybridized with a β-actin probe (26).

Reverse Transcriptase-PCR Analysis. Total RNA (1 μg) was reverse transcribed into cDNA according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany). Equivalent amounts of cDNA among different samples was estimated by reverse transcriptase (RT) PCR carried out for 18, 21, and 25 cycles with β-actin primers, as previously described (18). Titration of cycle number allowed us to perform densitometric analyses (Bio-imaging BAS 1500; Fujifilm, Kanagawa, Japan) under nonsaturating conditions. The β-actin control shown in Fig. 4 A also applies to PCR analyses shown in Figs. 4 B, C, and D. β- and VB degenerate primers (29) used in combination with either Cα or Cβ primers, respectively, enabled the amplification of all known human Vα and VB segments, as described (18, 29). VB-specific transcription was assessed by using Vβ7.2, Vβ13, and Vβ13.1-12-specific primers in concert with a Cβ primer, as previously reported (25). Specific amplifications were detected by Southern blot hybridization with Vβ (27) and Cα (28) cDNA probes.

Amplifications of p70 cDNA were performed for 35 cycles, at an annealing temperature of 55°C, using the following primers: 5'p70, 5'-CAACCCATTCTCTCCGATGGCTCTG-3' and 3'p70, 5'-GGAGAGGTCGAAAGACAGTCG-3'. Amplified products were analyzed by Southern blotting with a cDNA p70 probe derived in this study (see below). RAG-1 transcription was assessed by RT-PCR carried out for 18, 21, and 25 cycles with β-actin primers, as previously described (18). Titration of cycle number allowed us to perform densitometric analyses (Bio-imaging BAS 1500; Fujifilm, Kanagawa, Japan) under nonsaturating conditions. The β-actin control shown in Fig. 4 A also applies to PCR analyses shown in Figs. 4 B, C, and D. β- and VB degenerate primers (29) used in combination with either Cα or Cβ primers, respectively, enabled the amplification of all known human Vα and VB segments, as described (18, 29). VB-specific transcription was assessed by using Vβ7.2, Vβ13, and Vβ13.1-12-specific primers in concert with a Cβ primer, as previously reported (25). Specific amplifications were detected by Southern blot hybridization with Cα (27) or Cβ (28) cDNA probes.

Results

Characterization of a TCR-negative subset of CD4+CD8+ Large Cycling Human Thymocytes. Recent studies have shown that the differentiation of the most immature human T cell precursors, identified as CD34+DN thymocytes (32), proceeds throughout several TCR-negative stages characterized by the progressive loss of CD34 and the sequential acquisition of CD1, CD4, and CD8 (32-34). Thus, human T cell precursors acquire CD4 before CD8 (22, 34) as proposed in the following maturation sequence: CD34+CD1+CD4+CD8→CD34+CD1+CD4+CD8→CD34+CD1+CD4+CD8→CD34+CD1+CD4+CD8→CD34+CD1+CD4+CD8+. The latter subset seems to include the immediate progenitors of the intrathymic subpopulation of small DP thymocytes that express the TCRα/β complex and then differentiate to the SP stage (17).

To identify the developmental stage at which the successful expression of TCRβ may be of selective advantage to human thymocytes independent of the TCRα chain, we examined β and α chain gene status in individual subsets of TCRβ-early T cell progenitors isolated from the postnatal thymus, according to the proposed maturation sequence (17). To this end, large thymocytes recovered from Percoll gradients were depleted of mature T, B, NK, and myeloid cells. As shown in Fig. 1 A, expression of CD4 and CD8 defined three different subsets of TCR-negative thymocytes within the remaining pool: CD4+CD8+, CD4+CD8−, and CD4+CD8−, representing 9.5 ± 4.5%, 29 ± 10%, and 60 ± 10%, respectively. Immunomagnetic sorting allowed the independent isolation of the three subsets that showed >99% purity upon reanalysis (Fig. 1 A).

By forward scatter (FSC) analysis, each TCR-negative sub-
set was shown to include relatively large-sized cells, as compared with the subset of conventional TCRα/β+ DP cortical thymocytes isolated from the Percoll fraction of small cells (Table 1). As expected (32), nearly all cells within the CD4-CD8- pool express CD34, whereas CD1 defines two distinct subpopulations of CD34+CD1+ (52.5 ± 6%) and CD34+CD1- (47.5 ± 6%) thymocytes that were independently isolated (>99% pure) by immunomagnetic sorting (Fig. 1 B). Based on the expression of CD34, CD1, CD4, and CD8 molecules, the four isolated subsets of TCR-negative large-sized thymocytes will be hereafter referred to as CD34+CD1-, CD34+CD1+, CD4+CD8-, and CD4+CD8+.

We next examined in more detail the phenotypic profile displayed by TCRα/β- CD4+CD8+ large thymocytes, as compared with that of common cortical TCRα/β+ CD4+CD8- small thymocytes (Fig. 1 C). It is notable that both subsets coexpressed CD8α and CD8β (Fig. 1 C) and displayed similar expression levels of CD1, CD2, CD4, CD5, and CD7 T-lineage molecules, whereas both lacked expression of CD11b, CD13, CD14, CD16, CD25, CD33, CD56, and CD69 (not shown). However, markers such as CD28, CD44, and CD71 (transferrin receptor), which were weakly expressed on small TCRα/β+ CD4+CD8- thymocytes, were consistently found at higher expression levels on large TCRα/β- CD4+CD8+ thymocytes (Fig. 1 C). More importantly, both subsets were shown to differ dramatically in their cell cycle status. As shown in Table 1, the proportion of TCRα/β- CD4+CD8+ thymocytes engaged in DNA synthesis and mitosis (up to 45%) was much higher than the proportion of the TCRα/β+ CD4+CD8- pool (up to 3%), or any other subset of thymocytes (up to 12%). To avoid confusion, conventional TCRα/β+ CD4+CD8- small thymocytes will be hereafter referred to as DP thymocytes.

**Table 1. Forward Scatter and Cell Cycle Analysis of Early Human Thymocyte Subsets**

| Subset                  | FSC ± SD | Percent cycling cells ± SD | Percent total thymocytes ± SD |
|-------------------------|----------|----------------------------|------------------------------|
| CD4-CD8- TCRα/β-        | 439 ± 20 | 11.0 ± 1.5                 | 0.69 ± 0.3                   |
| CD4+CD8- TCRα/β-        | 404 ± 24 | 10.5 ± 3.6                 | 2.21 ± 1.3                   |
| CD4+CD8+ TCRα/β-        | 422 ± 12 | 42.0 ± 4.5                 | 6.57 ± 4.3                   |
| DP TCRα/β+              | 311 ± 3  | 2.2 ± 0.9                  | 10.10 ± 4.5                  |

Postnatal thymocytes were fractionated into populations enriched for large or small cells on Percoll density gradients. Subsets of TCRα/β- thymocytes and TCRα/β+ DP thymocytes were purified either from the large or from the small cell fractions, respectively. Medium-sized cells including both SP and DP TCRα/β+ thymocytes were not analyzed in this study.

**Figure 1.** Cell surface phenotype of individual subsets of large TCR- human thymocytes. (A) CD4 vs. CD8 expression on Percoll-separated large TCR- thymocytes defines three distinct subsets of CD4+CD8-, CD4+CD8-, and CD4+CD8+ thymocytes. Each subset was reanalyzed for CD4 vs. CD8 expression after immunomagnetic sorting. (B) Expression of CD34 vs. CD1 in the CD4+CD8- population shown in A defines two subsets of CD34+CD1+ and CD34+CD1- thymocytes. Representative profiles of CD34 vs. CD1 expression on both isolated subsets are shown. (C) Expression of TCRβ, CD8β, CD28, CD44, and CD71 on either TCRα/β- CD4+CD8+ large thymocytes shown in A (shaded areas), or TCRα/β+ CD4+CD8+ small thymocytes separated on Percoll gradients (unshaded areas). (Dashed histograms) Background values obtained with isotype-matched irrelevant mAbs.
fragments corresponding to partial rearrangements of $\text{D}_{32}$ to each of the seven $\text{J}_{32}$ ($\text{J}_{32.1-\text{J}_{32.7}}$) gene segments (35). As expected from the physical map, $\text{V}_{32}$-specific primers (25) used in combination with the $3'\text{J}_{32}$ primer enabled the amplification of complete V-D-J rearrangements, but not of germline fragments (35). The resulting PCR products were analyzed by Southern blot hybridization with an internal primer positioned immediately 3' to the $\text{J}_{32}$ cluster (Fig. 2 A).

Partial rearrangements of $\text{D}_{32}$ to $\text{J}_{32}$ were consistently negative in the most early CD34⁺CD1⁻ thymocytes, in which a sharp germline band equivalent to that detected in non-T (K562) cells was observed (Fig. 2 B). A conspicuous germline amplification product was also readily detectable in the two downstream subsets of CD34⁺CD1⁺ and CD4⁺CD8⁻ thymocytes, indicating that they were essentially in germline configuration. However, very faint bands of rearrangement, particularly those corresponding to short-sized products whose amplification is favored by this technique, were eventually detected in both subsets upon prolonged exposure. In contrast, multiple rearrangements of $\text{D}_{32}$ to the $\text{J}_{32.1-\text{J}_{32.7}}$ elements, as extensive as those found in TCRα/β⁺ (DP or SP) thymocytes, were detected at the next developmental stage of TCRα/β⁻ CD4⁺CD8⁺ large thymocytes (Fig. 2 B). Rearrangements involving $\text{V}_{32}$ segments were essentially negative at both the CD34⁺ CD1⁻ and the CD34⁺CD1⁺ developmental stages. Weak bands corresponding to complete V-D-J rearrangements were first detected at the CD4⁺CD8⁻ stage. In contrast, the TCRα/β⁻ CD4⁺CD8⁺ large thymocytes displayed a pattern of multiple rearrangements similar to that observed in more mature TCRα/β-expressing thymocytes. As an example, the Vβ3-Jβ3 rearrangement patterns observed are shown in Fig. 2 C. Identical results were obtained for rearrangements involving Vβ4, Vβ8, and Vβ19 genes (not shown).

Timing of TCRβ and TCRα Gene Transcription during Human Thymic Development.

To next examine the transcriptional activation of TCRα and TCRβ genes during early thymocyte development, total RNA isolated from each individual TCR-negative subset was analyzed by Northern blotting. As shown in Fig. 3, hybridization with a Cβ probe revealed that TCRα/β⁻ CD4⁺CD8⁺ large thymocytes displayed a pattern of abundant 1.3-kb mature and 1.0-kb immature TCRβ transcripts that resembled that found in more mature DP or SP TCRα/β⁻ thymocytes. In contrast, mature transcripts were weakly detected in their immediate precursors (i.e., CD4⁺CD8⁻ TCRα/β⁻ thymocytes) and were absent in the most early CD34⁺CD1⁻ and CD34⁺CD1⁺ thymocytes. All these subsets, however, expressed 1.0-kb immature TCRβ transcripts (Fig. 3), and
low levels of 1.6-kb germline transcripts could also be detected upon prolonged exposure (not shown). Subsequent hybridization with a Cox probe showed that expression of 1.6-kb mature-length TCRα transcripts only occurred in the DP or SP stages, at which the TCRα/β is already expressed on the cell surface (Fig. 3). Expectedly, mature TCRα and TCRβ transcripts were present in Jurkat T cells, but not in JY B cells.

To ascertain unambiguously the expression of mature TCRβ message at the CD4+CD8− stage, as well as to confirm the lack of TCRα transcription in both the CD4+CD8− and the CD4+CD8+ subsets, further studies were performed by using a sensitive RT-PCR technique (18, 29) to detect transcription of all known VB and Vα TCR genes. As shown in Fig. 4 A, the combination of panVB primers with Cox primers revealed that TCRα/β−CD4+CD8+ thymocytes had readily detectable amounts of TCRβ message. There was a higher expression in TCRα/β−CD4+CD8+ blasts, whereas no amplified fragments were detected in CD34+CD1− and CD34+CD1+ thymocytes. Expression of V-D-J-Cβ transcripts at the CD4+CD8− stage was independently confirmed by using Cox primers in combination with VB-specific primers (25). Representative results corresponding to VB2−, VB6−, and VB13-specific amplifications are shown in Fig. 4 B. In addition, pan-Vα primers used in combination with Cox primers confirmed the absence of TCRα transcripts in all thymocyte subsets shown to lack surface TCRα/β (Fig. 4 A). Collectively, our data suggest that, in contrast to mice, the onset of V-D-J TCRβ gene rearrangement and transcription occurs in humans in immature thymocytes already expressing CD4, and is essentially completed during the transition to the next TCRα/β−CD4+CD8+ developmental stage.

**RAG-1 Gene Transcription Is Intrathymically Upregulated during the Transition from the CD34+CD1− to the CD34+CD1+ Stage.** Previous studies in mice have shown that the expression of RAG-1 and RAG-2 genes usefully traces the emergence of TCR gene rearrangement and transcription during T cell development (36). To approach this issue in humans, RAG-1 transcription was analyzed by RT-PCR in the thymocyte subsets previously examined for their TCRβ and TCRα gene status (Fig. 5 A). Comparative

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**Figure 4.** RT-PCR analysis of TCRβ and TCRα gene transcription in early human thymocyte subsets. (A) cDNA samples prepared from the indicated cell sources were amplified by using pan-Vβ and pan-Vα primers in concert with Cβ- or Cox-specific primers, respectively. Amplified products were analyzed by Southern blotting with either Cβ or Cox cDNA probes. Equivalence of cDNA among different samples was assessed by RT-PCR using β-actin primers under nonsaturating conditions (21 and 25 cycles). Sizes of amplified products are indicated at the right (bp). (B) Expression of full-length TCRβ transcripts was analyzed by RT-PCR using Vβ2−, Vβ6−, and Vβ13−specific primers in concert with a Cβ primer. cDNA samples and β-actin controls are identical to those shown in A.

**Figure 5.** Regulation of RAG-1 expression during human intrathymic development. (A) RAG-1 transcription was assessed by RT-PCR and Southern blotting in the indicated cell samples. Sizes of amplified products are indicated at the right (bp). The β-actin control is shown in Fig. 4 A. (B) The relative intensity values of amplifications corresponding to a RAG-1 cDNA titration curve (top) were plotted against the number of RAG-1 cDNA molecules in a logarithmic scale (bottom).
and alignment of the predicted amino acid sequence of human pTc- with
ZO1 glycosilation sites
under accession number U38996. Sites are indicated. (V) Mismatches with respect to a recently reported hu-
thymocytes, coincident with the onset of TCRo~ trancrip-
tion, whereas no message was detected in the most early
ded in positions 31, 91, and 119. Locations of the potential N-linked
residues; (dashes) gaps at the cytoplasmic tail. (7) The three cysteines con-
erved in positions 31, 91, and 119. Locations of the potential N-linked glycosilation sites (CHO) and protein kinase C (PKC) phosphorylation
sites are indicated. (V) Mismatches with respect to a recently reported hu-
mRNA for the cloned human pTc cDNA are available from EMBL/GenBank/DDBJ,
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sites are indicated. (V) Mismatches with respect to a recently reported hu-
mRNA for the cloned human pTc cDNA are available from EMBL/GenBank/DDBJ,
7, transcription of pTa was easily detected in mobilized CD34+ cells from peripheral blood, as well as in CD34+ precursors isolated from umbilical cord blood or from adult bone marrow. More importantly, pTa gene expression also occurred in fetal life in CD34+ progenitors present in the liver at 18 wk of gestation, although it was absent in CD34+ precursors from fetal bone marrow at any developmental age up to 22 wk. These results suggest that transcription of the pTa gene is activated early in ontogeny in CD34+ progenitors, before their entry into the thymus.

**TCRβ Chain Is Expressed in CD4+CD8+ Blasts: Identification of an Immature TCRβ Complex Distinct from the TCRα/β Heterodimer.** The coincident expression of large amounts of TCRβ and pTa transcripts in TCRα/β+ CD4+CD8+ blasts raised the possibility that, at this stage, cells have already been selected by means of a putative human pre-TCR complex consisting of a functional TCRα chain associated with the pTa chain. Flow cytometric analysis shown in Fig. 8 A revealed that cytoplasmic TCRβ was first expressed in a minor proportion of CD4+CD8+ blasts (5-10% in different experiments). Consistent with the prediction in mice of >56% for a population in which cells have been selected on the basis of TCRβ expression (39), cytoplasmic TCRβ protein was found in most (80-90%) TCRα/β+ CD4+CD8+ human blasts (Fig. 8 A). Therefore, nearly all cells at the TCRα/β+ CD4+CD8+ stage have been selected based on productive β gene rearrangement.

To characterize the molecular form of TCRβ chains involved in this selection process, lysates from the TCRα/β+ CD4+CD8+ and CD4+CD8+ thymocytes, as well as from the TCRα/β-expressing Jurkat T cell line, were precipitated with mAbs against constant regions of either the TCRβ (βF1) or the TCRα (αF1) chains, and the immunoprecipitated proteins were then analyzed in Western immunoblots with mAbs to TCRβ or TCRα, respectively. As shown in Fig. 8 B, the anti-TCRβ mAb precipitated three β chain monomeric forms (36-40 kD) from both thymocyte subsets as well as from Jurkat T cells, although a faint signal was observed in the CD4+CD8- thymocytes (Fig. 8 B, left). As expected from previous studies (40), two different bands corresponding in size to the intracellular (70 kD) and the cell surface expressed (80-90 kD) TCRα/β heterodimer were detected in anti-TCRβ precipitates from Jurkat T cells, but were absent in both the CD4+CD8- and the CD4+CD8+ thymocyte subsets. In contrast, a single band, with a slightly lower molecular mass than the 70-kD intracellular TCRα/β heterodimer, was detected in TCRβ precipitates from CD4+CD8+ thymocytes, but was completely absent in CD4+CD8- thymocytes (Fig. 8 B, left). Strikingly, the anti-TCRα mAb failed to immunoprecipitate this TCRβ complex, or any other protein complex, from CD4+CD8- thymocyte lysates, whereas an 80-90 kD TCRα/β heterodimer was precipitated from Jurkat T cells with this reagent (Fig. 8 B, right). Therefore, our data provide evidence that TCRα/β+ CD4+CD8+ blasts express an immature TCRβ complex composed of the TCRβ chain associated with another chain different from TCRα. Although the molecular mass of this complex fits with that expected for a human pTa-TCRβ heterodimer, direct evidence for this notion must...
still await the development of suitable reagents against the human pTα chain.

Discussion

In this study, we have tried to identify the critical stages in early human intrathymic differentiation where most of the developmental decisions are made. In particular, we have focused on the developmental point at which TCRβ may confer on intrathymic precursors a selective advantage to mature further in the absence of TCRα expression. These studies have been greatly facilitated by the observation that the DN to DP transition occurs in humans through a CD4+CD8- intermediate (32-34). Our results show that it is precisely at this CD4+CD8- developmental stage that complete V-D-Jβ gene rearrangement and full-length TCRβ transcription are first detected, although many of these cells still display a TCRβ germline conformation. In contrast, extensive V-D-Jβ gene rearrangement and mature-length TCRβ message are common in TCRα/β- CD4+CD8+ thymocytes, known to represent the immediate progeny of CD4+CD8- cells (32-34). As predicted for a population in which cells have been selected on the basis of successful β gene rearrangement (39), cytoplasmic TCRβ protein was found in nearly all TCRα/β- CD4+CD8+ thymocytes. These data allowed us to propose a model for the early stages of human intrathymic development (Fig. 9) that envisages the CD4+CD8- to CD4+CD8+ transition as the critical point at which human thymocytes are selected based on productive TCRβ expression. According to this model, the onset of V-D-Jβ TCRβ gene rearrangement and transcription occurs relatively late in human thymocyte development, as CD34+CD1+ pro-T cells downregulate CD34 and acquire the CD4 coreceptor molecule, and it is preceded by activation of the recombine machinery (i.e., transcription of RAG-1) during the CD34+CD1- to CD34+CD1+ transition. These results seem in conflict with those recently reported by Ktorza et al. (41) showing RAG-1 and mature TCRβ transcription at earlier intrathymic stages. However, no quantitative analyses were reported in that study to rule out the possibility that amplified transcripts do correspond to downstream cell contaminants.

The proposal that productive TCRβ expression occurs at the CD4+CD8- to CD4+CD8+ transition is further supported by our observation that TCRα/β- CD4+CD8+ thymocytes include a high proportion of cycling cells, an expected finding considering that functional expression of TCRβ results in clonal expansion of murine thymocytes at the equivalent developmental point (5, 7, 8). Based on what is already known in the mouse about the implication of the pTα-TCRβ complex in this maturation step, we performed experiments designed to provide evidence for the existence of an equivalent complex in humans. We identified and cloned a human cDNA highly homologous to the murine pTα (13), with a predicted amino acid sequence that is essentially identical to that recently reported by others as the human pTα chain (37). As described in mice (13), pTα gene expression was also shown to be developmentally regulated in humans. We found that pTα message is highly expressed by both the CD4+CD8- and the CD4+CD8+ TCRα/β- thymocyte subsets, whereas there is a weaker expression in earlier and later stages of development. The coincident expression of TCRβ protein and pTα transcripts (in the absence of TCRα, γ, and δ proteins) in TCRα/β- CD4+CD8+ blasts, strongly suggests that cells in transit to this stage monitor the occurrence of productive TCRβ rearrangements through a human pTα-TCRβ heterodimer. Supporting this possibility, a TCRβ complex of the expected size, and different from the mature TCRα/β heterodimer, was immunoprecipitated from TCRα/β- CD4+CD8+ blasts. However, we have been unable as yet to biochemically resolve this putative pTα-TCRβ heterodimer in reducing gels, probably because the pTα chain escapes detection by current labeling techniques, as reported in mice (12). Though pTα protein expression studies still await the development of suitable anti-pTα reagents, the results reported here allow us to propose that the earliest direct action of the putative human pre-TCR is to induce rapid clonal expansion, and si-
multaneous transition of thymocytes from the CD4⁺CD8⁻ to the CD4⁺CD8⁺ stage.

Analysis on the developmental expression of the human pTα gene provided evidence that pTα is also expressed outside the thymus in CD34⁺ progenitors isolated from distinct adult hematopoietic tissues. More importantly, pTα transcription was also detected early in fetal life in CD34⁺ precursors present in the liver, whereas no pTα expression was found in fetal bone marrow CD34⁺ cells. Although the precursor potential of the latter CD34⁺ subset needs to be analyzed before further conclusions can be drawn, the fact that CD34⁺ fetal liver cells do contain TCRα/β precursor potential (17, 18) may suggest that there is a coincidence of pTα expression and precursor activity for TCRα/β T cells in hematopoietic precursors before their entry into the thymus. Alternatively, pTα-expressing extrathymic CD34⁺ cells may represent recirculating pro-T cells derived from the thymus. This possibility appears very unlikely considering that pTα gene expression has also been found in the bone marrow of athymic mice (38). Finally, we provide evidence that pTα RNA is expressed by the most immature CD34⁺CD1⁻ thymocytes that still have the potential to develop into TCRγδ, dendritic, myeloid, and NK cells (17, 21). Therefore, pTα transcription precedes the onset of TCRβ rearrangement and RAG-1 expression inside the thymus. An intriguing feature of these early intrathymic precursors, also found in their murine counterparts (2, 42), is that they express 1.0- and 1.6-kb germline TCRβ transcripts before the onset of D-J TCRβ rearrangement (our present study). It is believed that TCR gene segments are selectively rendered accessible to the recombinase machinery by developmentally regulated transcription while they are still in their germline configuration (43). Whether the coincident expression of pTα and germline TCRβ transcripts identifies TCRα/β-committed precursors within the CD34⁺CD1⁻ immature pool of thymocytes deserves further investigation. However, given that both the onset of full-length TCRβ transcription and the upregulation of pTα gene expression are downstream events coincident with the acquisition of surface CD4, we favor the view that the CD4⁺/CD8⁻ stage represents the critical point at which intrathymic precursors restrict their developmental potential towards the TCRα/β lineage.

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