EARLY GENETIC EVENTS IN T CELL DEVELOPMENT
ANALYZED BY IN SITU HYBRIDIZATION

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T lymphocytes show a preference for responding to foreign antigen in association with the particular MHC products present in the individual from which they derive, and at the same time, are unresponsive to (tolerant of) combinations of self proteins and self MHC products. Experiments with radiation-induced bone marrow chimeras (reviewed in reference 1) have demonstrated that both the preference for self MHC-restricted recognition of antigen, and self tolerance, are acquired phenotypes that result from selection of T cells with the appropriate receptors.

These same experiments (2) have indicated that the thymus, the organ in which most T cell differentiation occurs, is the major site of this selection process. Recent studies (3–9) have also identified the molecular basis of T cell recognition, demonstrating that specific responses to antigen plus MHC products are dependent on clonally distributed heterodimeric (Ti or α/β) structures. The genes encoding the α/β receptor, and a third related set of rearranging genetic elements (γ), have been cloned and their structures have been delineated (10–14). Therefore, it is now possible to begin to investigate the cellular and molecular basis of T cell repertoire development during the critical differentiation process occurring in the thymus.

In the mouse, committed T cell precursors from the hematopoietic fetal liver first colonize the epithelial thymic rudiment on the 10th or 11th day of gestation (15, 16). These large basophilic cells begin to differentiate in the thymus, and by day 15 have been identified as T cells by the presence on their membranes of the Thy-1 molecule, a pan-T cell marker in the mouse (17). During this interval, there is a rapid increase in the number of cells in the thymus, largely due to intrathymic proliferation (18). The Thy-1⁺ cells are characterized by an absence of the Lyt-2 and L3T4 surface molecules found on mature T lymphocytes (19), low levels of Ly-1 (20), and the presence of receptors for the growth factor IL-2 on a large fraction of the cells (21–23). This dull Ly-1, Lyt-2⁻, L3T4⁺, IL-2-R-bearing phenotype also characterizes the majority of cells found to serve as an intrathymic precursor pool in the adult thymus (24). Between days 15 and 16 of fetal development, the percentage of cells in the thymus with IL-2-R declines,
and thymocytes expressing both Lyt-2 and L3T4 appear (19). These cells have the cell surface phenotype (Lyt-2*,L3T4* or "double-positive") of the bulk of cortical thymocytes in the adult thymus. Cells with the membrane phenotype of mature T cells do not appear until days 17/18, at which time Lyt-2*,L3T4* and Lyt-2-,L3T4+ thymocytes typical of those found in the adult thymic medulla can be identified (19, 25).

Analysis of T cell receptor expression during this differentiation process has been limited to later time points in fetal life (days 14–20). These experiments have shown that β and γ transcripts are present at the earliest time examined (day 14), consistent with the rearrangements observed in the DNA of these cells (26–29). Transcripts from the T cell receptor α locus are first seen in day 17 thymocytes, at which time cell-surface expression of α/β heterodimers can be detected with an anti-Vβ antibody (25, 26, 29). Adult intrathymic precursor T cells resemble day 14–16 thymocytes in possessing β and γ, but not α mRNA (26, 30).

Although these initial analyses of intrathymic T cell differentiation have provided a baseline of information for addressing the issue of T cell repertoire selection, they leave many questions unanswered. To extend our understanding of the expression of T cell receptor–related genes to the earliest times of T cell development, we have begun an analysis of thymocyte differentiation with the technique of in situ hybridization. Using as few as $5 \times 10^5$ cells, this method permits the semiquantitative measurement of mRNA in individual cells, allowing one to determine the distribution of gene expression in heterogeneous thymocyte populations. In the present study we have used probes for Thy-1, and for T cell receptor α, β, and γ genes, to study the induction of Ti gene transcription from the time of entry of the first hematopoietic stem cells into the developing thymus. The results obtained by this approach significantly extend our knowledge of the programmed activation of the T cell receptor loci and reveal an asynchronous expression of Vβ genes during thymic ontogeny.

Materials and Methods

Animals. All studies were done with C57BL/6 (B6) mice. Timed pregnancies were produced by placing breeding pairs together for 3 h in the morning and then checking for vaginal plugs in the females. The day of plugging was designated day 0.

Reagents. Restriction endonucleases and DNA-modifying enzymes were obtained from either Bethesda Research Laboratories (Gaithersburg, MD) or New England Biolabs (Beverly, MA). pGEM plasmid DNA, SP6 polymerase and T7 polymerase, RNasin, and RNase-free DNase were from Promega Biotech (Madison, WI). α-[³²P]thio-UTP and [³H]thymidine were from New England Nuclear (Boston, MA).

Thymocyte Isolation. Fetuses were removed from timed pregnant female mice between 11 a.m. and 3 p.m. on the indicated day, except for the day 12.5 time-point for which fetuses were obtained at 2 a.m. between the 12th and 13th days of gestation. Fetal thymuses were dissected out and single-cell suspensions were prepared from pooled organs by mincing in ice-cold medium followed by filtration through mesh (24). Dull Ly-1, Lyt-2+, L3T4+ cells were prepared from the thymuses of 4–6-wk-old mice using a previously described (24) two-stage antibody and complement–mediated lysis procedure involving anti-Ly-1,2, anti-Lyt-2,2, and anti-L3T4 mAbs.

Hybridization Probes. All probes used for in situ hybridization were cDNA fragments cloned into the polylinker of the pGEM1 plasmid using standard procedures. The β probe, kindly provided by Stephen Hedrick (University of California at San Diego, San
Diego, CA) and Mark Davis (Stanford University, Stanford, CA), was the 86T5 clone, a 640-bp cDNA corresponding to the transcript from a D-J β rearrangement and extending from 5' of the upstream recognition sequences flanking Dβ1 through the end of the Cβ1 coding region (11). The α probe was a 570-bp Nco I fragment including most of Cα and a portion of the 3' untranslated region. It was derived from a cDNA corresponding to the BW5147 α transcript. The γ probe was the full-length cDNA clone 8/10-2γ1.1, generously provided by Tak Mak (Ontario Cancer Institute, Toronto, Ontario, Canada), which includes the Vγ1.2 and J-Cγ2 gene segments (31). The probe corresponding to the V region of the β chain of the 2B4 T cell hybridoma (Vβ3) was prepared by subcloning the 300-bp Eco RI–Sca I fragment from a full-length cDNA (32). The Vβ5.1 probe consisted of a 350-bp Eco RI–Hind III fragment derived from a cDNA clone kindly provided by Dennis Loh (Washington University School of Medicine, St. Louis, MO) (33). The Thy-1 probe was a 600-bp Pst fragment from the Thy-1.1 gene (11).

In Situ Hybridization. In situ hybridization was performed by a modification of the procedure of Harper et al. (34), using high-specific-activity 35S-labeled single-stranded RNA probes. After linearization of the pGEM plasmid DNA containing the appropriate insert, RNA sense or antisense transcripts were synthesized using SP6 or T7 polymerase and a ribonucleotide mix containing 1,200 Ci/mmol α-35S]thio-UTP. A typical reaction contained 4 μl of 5X transcription buffer (1X transcription buffer: 40 mM Tris, pH 7.5; 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl); 2 μl of 100 mM DTT; 0.8 μl RNasin; 4 μl each of 2.5 mM ATP, CTP, and GTP, 1 μl containing 0.05–0.1 μg of linearized, purified template DNA; 7.2 μl of 4X-concentrated [35S]UTP; and 1 μl of SP6 or T7 RNA polymerase. The reaction was incubated for 90 min at 39°C, 1 U/μg template of RNase-free DNase was added, and the reaction was continued for 15 min at 37°C. The labeled RNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended to 2 X 105 cpm/μl in 10 mM Tris, pH 7.5, 0.5 mM EDTA, and 10 mM DTT. All buffers were prepared with diethylpyrocarbonate-treated water.

Cells were prepared for hybridization by applying 5 X 104 to 3 X 105 cells in medium with 10% serum to a precleaned glass slide using a cytocentrifuge run at 500 rpm. The cells were immediately fixed in 4% paraformaldehyde/PBS for 1 min at room temperature, then transferred to 70% ethanol, in which they could be stored for several weeks before use. After removal of ethanol, the slides were rinsed in 2X SSC (1 X SSC: 0.15 M NaCl, 0.015 M sodium citrate), then acetylated with 0.25% acetic anhydride according to Harper et al. (34). This was followed by a 30-min incubation in 0.1 M Tris, pH 7.0, 0.1 M glycine, then dehydration in 70, 80, and 95% ethanol. The slides were briefly air dried, and 10 μl of hybridization mix (2X SSC, 10 mM DTT, 1 mg/ml yeast tRNA, 50% formamide, 2 mg/ml methylated BSA) containing 105 cpm/μl probe were applied. A cover slip was added and sealed with rubber cement, and incubation was carried out at 50°C for 8–12 h. Unhybridized probe was removed with three washes (5 min, 5 min and 1 h) in 2X SSC, 50% formamide at 54°C followed by 30 min of incubation in 2X SSC containing 100 μg/ml RNase A and 1 μg/ml RNase T1 (30 μl under a coverslip in a humidified chamber). After three further washes in 2X SSC, 50% formamide at 54°C, the slides were again dehydrated in ethanol, dried, and coated with NTB2 emulsion (Eastman Kodak Co., Rochester, NY) for autoradiography. Slides were exposed at 4°C for 4–50 d, developed for 2 min in Dektol (Eastman Kodak Co.), and fixed for 5 min in Kodalfix (Eastman Kodak Co.). Counter staining was done with 1% Giemsa for 2 min.

The specificity and sensitivity of this procedure were demonstrated in pilot experiments using class II MHC gene-transfected L cells. Under these conditions, virtually no background was obtained with either sense or antisense class II probes on the parent fibroblasts, while reproducible grain counts proportional to the known class II mRNA content of the cells were obtained from a series of transfectants using the antisense probes. A similar absence of nonspecific hybridization was obtained using T cell receptor (TCR)1 probes on either L cells or B lymphoma cells, and appropriate positive results were obtained using the TCR and Thy-1 probes on a variety of T cell clones and hybridomas whose content of specific mRNA corresponding to each of these genes had been determined previously.

1 Abbreviation used in this paper: TCR, T cell receptor.
Results

Expression of the Thy-1 Gene in Fetal Thymocytes. We first examined the expression in fetal ontogeny of Thy-1, a pan-T cell marker that is expressed at high levels in the thymus (17) and that may be involved in T cell activation (35–37). Cytospun cells from fetal thymuses of different gestational age were analyzed by in situ hybridization with 35S-labeled, single-stranded RNA probes. Fig. 2 shows that Thy-1 mRNA is expressed in 54% of thymocytes on day 12 of gestation, indicating that many of the cells examined in the organ at this early time point are already committed to the T cell lineage. On subsequent days, the percentage of positive cells increased, reaching a maximum of 91% of the population by day 15 and staying at this level through day 17. The remaining 9% of the cells presumably represent nonlymphoid thymic stromal cells. These results are consistent with previous observations using anti-Thy-1 mAbs to detect cell-surface expression of the Thy-1 molecule at day 15 (17), and suggest that the gene is transcribed soon after entry of precursor cells into the thymus, if not before.

Expression of T Cell Antigen Receptor Genes in Fetal Thymocytes. Figs. 3–6 show the time course for expression of TCR genes. As seen in Fig. 3, the TCRγ locus is the first of the TCR-related genes to be transcribed; mRNA was present in 40% of thymocytes at day 12 (compared with 54% Thy-1+ cells). This level increased to ~75% by days 14 and 15. The percentage then began to drop at day 16 and by day 20 was down to 19%. These results imply either that not all Thy-1+ thymocytes simultaneously express TCRγ or that a fraction of thymocytes transcribe a γ species not detected with this probe.

Fig. 4 shows that the TCRβ constant region locus is the next TCR gene to be expressed in ontogeny. On day 12, when γ mRNA was present in 40% of the cells, no TCR Cβ mRNA could be detected. By day 12.5, however, 38% of the cells were expressing low levels of β message, and by day 13, 87% of the thymocytes were positive. Cβ mRNA expression remained at this level throughout the rest of fetal development. The sudden appearance of Cβ mRNA-expressing cells at day 12.5 is most likely due to a rapid wave of gene induction intrathymically in cells already expressing Cγ mRNA. However, it is formally possible that the appearance of β+ cells at day 12.5 represents an influx of precursors already expressing TCRβ message induced prethymically.

An unexpected finding was the bimodal distribution of grain counts in cells expressing β mRNA. From day 14 onward both a high expressor population (~60% of the cells) and a low expressor population (~30% of the cells, containing one-third to one-half as much mRNA) were observed. On day 12.5 only the low expressor population was seen. At present, the basis for the two levels of Cβ
FIGURE 1. In situ hybridization. Example of negative sense-strand hybridization (a) and positive antisense-strand hybridization (b) with TCR C3 probe on day 14 fetal thymocytes prepared as described in Fig. 2.
expression is unknown; however, it is not simply a result of two different cell types in the population expressing different amounts of total mRNA, since other mRNA probes, such as Thy-1 and TCRγ (Figs. 2 and 3), tested on the same cells, did not show this bimodal distribution.

Northern blot analysis of day 15 fetal thymus RNA has shown a predominance of 1.0-kb β transcripts representing D-J joining events before V gene rearrangement (27, 29). It is therefore likely that much of the signal seen in very early (days 12 and 13) thymocytes with in situ hybridization using a Cβ probe derives from transcripts representing either D-J rearrangements or possibly the unrearranged β locus (J-C). To assess when mRNA encoding a fully rearranged V-D-J-C segment is first synthesized, we performed in situ hybridization using probes specific for Vβ3 (Vβ 2B4) (12) or Vβ5.1 (33). Cells expressing the Vβ3 gene segment were first seen on day 14, 1.5 d after Cβ mRNA was first detected (Fig. 5). Only 1% of the cells were positive. This suggests that we were not detecting unrearranged V region transcripts (38), which should be present in most of the cells, but rather, complete V-D-J-C β transcripts. The number of positive cells remained at a low level (~3%) through day 18. In contrast, cells expressing Vβ5.1 were not seen until day 16, indicating that different TCRβ V regions may be expressed asynchronously, similar to what has been observed for Ig V regions (39). Overall, these results are consistent with the notion that the earliest TCRβ
transcription is from an incompletely rearranged or possibly germline gene configuration.

Consistent with previous Northern blot and RNase protection analysis (26, 28), mRNA encoding the constant region of TCRα did not appear until after day 15, at about the time when Lyt-2+, L3T4+ "cortical" phenotype cells are first detectable (Fig. 6). 4% of day 16 fetal thymocytes expressed constant region TCRα mRNA and this percentage increased gradually up until birth (day 20), at which point 40% of the thymocytes had detectable α mRNA. Thus, the earliest point in development at which mature α/β receptor molecules could appear on the T cell surface to participate in thymic selection events is day 16. The relative kinetics of expression of the T cell receptor genes during fetal ontogeny is summarized in Fig. 7.

**TCR mRNA Expression in Adult Dull Ly-1, Lyt-2-, L3T4- Cells.** Although the thymus in an adult mouse begins to involute at puberty, it continues to produce new T lymphocytes throughout the life of the animal (40). The dull Ly-1, Lyt-2-, L3T4- population from adult thymus, which represents <5% of the total cells in the organ, has been shown (24) to contain the adult precursors for all the phenotypically defined thymocyte subsets. Previous studies have demonstrated that this subpopulation expresses mRNA for TCRγ and -δ but not TCRα (26, 30), and that ~50% of these cells express IL-2-R (21). To directly compare this adult precursor population with fetal thymocytes at the single-cell level, we carried out in situ hybridization with most of the probes described above. Fig. 8
Figure 4. In situ hybridization of fetal thymocytes for TCRβ chain expression. Fetal thymocytes from days 12–17 of gestation were evaluated by in situ hybridization with a D-J-C β probe for expression of TCRβ, as described in Fig. 2. Slides were exposed for 5 d. The determination for day 12.5 was done as a separate experiment in which signal on positive control cells was decreased 40% relative to all the other days.

shows that expression of TCRγ, -β, and -α mRNA in dull Ly-1, Lyt-2−, L3T4− adult cells is very similar to that of day 14–16 fetal thymocytes. TCRγ mRNA is expressed in 77% of the cells, TCRα mRNA in almost none of them (1%), and TCRβ mRNA in 79% of the cells. TCRβ shows the same bimodal population distribution as seen in fetal thymocytes after day 12.5.

Discussion

In this report, we have used in situ hybridization to study expression of the Thy-1 and T cell antigen receptor (γ, β, α) genes in developing fetal and immature adult thymocytes. This technique enabled us to examine the expression of these
genes in the first days of fetal thymocyte ontogeny when there are too few cells to analyze by Northern blotting or RNase protection. Also, in situ hybridization permitted an analysis of the distribution of gene expression within the heterogeneous cell populations in the thymus.

As shown in Fig. 7, initiation of TCRα, -β and -γ transcription occurred asynchronously. TCRγ constant region mRNA was present in 40% of the day 12 fetal thymocytes, indicating that this locus is activated either prethymically or immediately upon entry of precursors into the thymus. TCRβ mRNA was found soon after, beginning on day 12.5, and was present in the majority of thymocytes by day 13 of ontogeny. These findings are somewhat surprising because studies on TCRγ and TCRβ gene rearrangements in heterogeneous thymocyte populations and hybridomas made from fetal thymocytes indicated a predominantly germine pattern until day 15 (29, 41). The most likely explanation for these apparently contradictory results is that the constant region–containing transcripts
FIGURE 6. In situ hybridization of fetal thymocytes for TCRα chain expression. Fetal thymocytes from days 13–17 and day 20 of gestation were evaluated for expression of TCRα constant region by in situ hybridization as described in Fig. 2. Slides were exposed for 2 wk.

FIGURE 7. Kinetics of expression of TCR genes. Percent of cells positive for mRNA corresponding to TCR constant regions (derived from Figs. 3, 4, and 6) are plotted for fetal thymic ontogeny.

detected on the earliest days of thymic ontogeny are from germline gene segments that become transcriptionally active just before rearrangement. The identification in thymus RNA of J-C region transcripts of unrearranged TCRγ loci (42) and the presence of transcripts from germline β loci in human leukemias of T cell phenotype (43) provide strong support for this possibility. The occurrence of transcription from unrearranged TCR loci is also consistent with previous work (44) on Ig gene expression in developing B lymphocytes. Early B cell precursors contain "sterile" transcripts corresponding to constant region gene segments from chromosomes on which neither D-J nor V-D-J rearrangements are present. It has been suggested (44) that these transcripts arise from
cryptic promoters after the achievement of an open chromatin configuration before the onset of the rearrangement process.

Additional evidence that the early TCRβ transcripts are from either germline or D-J-rearranged β loci comes from our demonstration that TCRβ (Vβ3) variable-region containing mRNA was not seen until day 14, 1.5 d after the first appearance of constant region transcripts. As only a very small percentage of the total β+ cells contained mRNA homologous to the specific Vβ probe used, the mRNA being detected at this time is most likely to correspond to mature V-D-J-C β transcripts derived from clonally rearranged β loci. This is in contrast to the presence of transcripts from multiple unrearranged V regions detected in pre-B cells (38). If a similar process were occurring in the thymocytes studied here, a much larger fraction of the β+ cells would have been expected to show Vβ hybridization. Alternatively, unrearranged V region transcription might be occurring but at too low a level to be detected by in situ hybridization.

The experiments with Vβ probes also revealed a different time of onset of transcription for the two different V regions studied: Vβ3 appearing on day 14 and Vβ5.1 on day 16. This difference may reflect the arrangement of the Vβ gene segments on chromosome 6, as recent mapping studies (Lindsten, T., and M. Davis, manuscript in preparation) have shown the Vβ3 segment to be one of the most Cβ proximal V regions, and prior work (39) on Vn rearrangement in
pre-B cells has shown that there is a preference for use of the most D-J-C proximal V gene family in such cells.

TCRa mRNA was first detected at day 16, 1 d earlier than was previously seen with Northern blotting. Whether this early mRNA also represents unarranged constant region transcripts remains to be determined. However, the in situ results conclusively show that the Ca locus is not transcriptionally active in a small number of thymocytes before day 16, a possibility that could not be ruled out using Northern blot or RNase protection analysis. Therefore, the appearance of thymocytes with mature α/β receptors at day 17 is not the consequence of expansion of a small subpopulation of cells, and thus, the earliest time at which thymic repertoire selection can occur involving α/β receptors per se is on day 16 of fetal development.

A major issue pertinent to these findings is the nature of the receptors expressed on developing thymocytes that are involved in the early expansion phase between days 13 and 15. Elucidating the nature of this receptor/ligand interaction will help to determine whether this expansion phase is involved in mediating the thymic influence on MHC restriction, so called "positive selection" (45). On days 13–15, before expression of TCRα chain, only a non-α/β receptor structure could be used. TCRγ is a possible candidate for a component of such a receptor. Indeed, we have recently found (46) that the first T3-associated receptor structure to be expressed in the developing thymus is composed of a γ chain disulfide linked to a 45 kD partner that is neither α nor β. Alternatively, early thymocyte expansion may result from stimulation of a nonpolymorphic surface molecule. The early expression (Fig. 2) and activation capabilities of Thy-1 (35–37) and CD-2 (47) make them possible candidates for such a role. Future studies on the surface receptors and activation properties of these immature thymocytes will hopefully be able to distinguish among these possibilities.

Summary

In situ hybridization was used to investigate the expression of T cell receptor (TCR) α, β, and γ mRNAs in developing fetal and adult precursor thymocytes. γ transcription was observed at the earliest time tested (day 12), followed by β 12 h later, and TCRα on day 16. The early β transcripts appeared to be from unarranged or incompletely rearranged (D-J-C) β loci. Vγ region transcription was first detectable on day 14 and transcription of different Vγ genes was induced at different times. These results delineate a scheduled sequence of TCR gene activation, which begins within 1 d after entry of stem cells into the fetal thymus.

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