Developmentally regulated fetal thymic and extrathymic T-cell receptor $\gamma\delta$ gene expression

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The $\gamma\delta$ T-cell receptor (TCR) is the first TCR to be expressed in ontogeny in all vertebrates in which it has been examined thoroughly. Murine $\gamma\delta$ cell-surface protein is detected by the fourteenth day of gestation. In this work, the activation of $\gamma\delta$ RNA has been studied. Data indicate that the first TCR protein to appear in the thymus is encoded by γ genes that are activated after cells colonize the thymus. However, the sequential appearance of different $\gamma\delta$ TCR proteins during thymic ontogeny cannot be readily explained by differential temporal activation of $V\gamma$ genes in the thymus. There are distinct patterns of γ and δ gene expression during fetal liver development and in the fetal gut (or tissue associated with it). Cells apparent in the liver of mice at birth express $\gamma\delta$ cell-surface protein, but they disappear from the liver very soon afterward. One $V\gamma$ gene is rearranged and expressed prethymically. In addition, γ gene expression is detectable in the livers of newborn athymic mice. Together, these observations indicate a thymic-independent pathway of activation of TCR genes.

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The majority of mature peripheral T cells bear the T-cell receptor (TCR) $\alpha\beta$ complex, which recognizes foreign antigen in the context of self-major histocompatibility complex (MHC) molecules (Allison et al. 1982; Hedrick et al. 1984; Dembic et al. 1986; Marrack and Kappler 1986; Saito et al. 1987). A minor subset of T lymphocytes (Bank et al. 1986; Brenner et al. 1986; Lew et al. 1986; Nakanishi et al. 1987; Pardoll et al. 1987) express a TCR heterodimer composed of γ and δ subunits (Saito et al. 1984a,b; Hayday et al. 1985b; Chien et al. 1987). $\alpha\beta$ and $\gamma\delta$ lymphocytes appear to represent distinct sublineages of T cells (Reilly et al. 1986; von Boehmer et al. 1988; Winoto and Baltimore 1989).

Although immunological specificities for some $\gamma\delta$ T cells have recently been described, reactivity to antigen both in the presence and in the absence of class I or II MHC gene products has been reported (Bonneville et al. 1989a; Haregewoin et al. 1989; Holoshitz et al. 1989; Janis et al. 1989; O'Brien et al. 1989; Vidovic et al. 1989). It is therefore unclear both how the response of $\gamma\delta$ T cells to antigen is genetically restricted and whether an MHC-guided "education" of $\gamma\delta$ cells occurs during their development. Polymorphic proteins encoded by the MHC have been reported to effect inactivation of reactive $\gamma\delta$ T cells in transgenic mice, in one case by intrathymic deletion (Dent et al. 1990), and in another case

by peripheral inactivation (Bonneville et al. 1990). The generality of these observations has yet to be determined.

The expression of immunologically functional TCR molecules on the surface of a T cell requires the coordination of $(CD3)$ genes (Ohashi et al. 1985; Brenner et al. 1986; Borst et al. 1987). This complex array is assembled during ontogeny with a complete $\gamma\delta$ TCR being the first TCR detectable on the surface of murine thymocytes, by day 14 of gestation (Bluestone et al. 1987; Nakanishi et al. 1987; Pardoll et al. 1987; Havran and Allison 1988; Sowder et al. 1988). At this time and throughout fetal thymic development, murine thymocytes express TCR polypeptides encoded by distinct pairs of γ and δ genes (Fig. 1; Cron et al. 1988; Havran and Allison 1988; Ito et al. 1989). Early expression of a $V\gamma5$, $V\delta1$ TCR gives way to predominant expression of a $V\gamma6$, $V\delta1$ TCR later in ontogeny, and subsequently to $V\gamma4$, $V\delta5$, $V\delta6$, or $V\delta7$ TCRs around the time of birth (Havran and Allison 1988; Ito et al. 1989). This pattern extends into the adult periphery (Jones et al. 1986; Kuziel et al. 1987; Maeda et al. 1987; Havran et al. 1989; Kyes et al. 1989; Takagaki et al. 1989).

However, the developmental activation of the TCR genes that accompanies both early and selective expression of $\gamma\delta$ proteins has not been well studied during

Figure 1. The organization of the TCR1- γ and TCR1- δ gene families. (A) The TCR1- γ genes in the mouse are represented as gene clusters. The nomenclature is based on that suggested by Heilig and Tonegawa (1986). The relative positions along the chromosome are shown, but distances are not proportional; breaks in the lines that connect gene segments represent unmapped distances or incompletely characterized regions (λ). Vertical lines represent leaders; shaded boxes represent V- and J-region exons; solid boxes represent C-region exons. Arrows indicate direction of transcription (5'→3'). Typical gene rearrangements are represented by lines connecting V-gene segments to corresponding J-C segments. The C3 gene is a pseudogene (Hayday et al. 1985b). (B) Representation of the δ -gene locus located between the TCR α chain V and J genes. The precise number of V δ genes has not been determined. Note that joining of V and J at the α -chain locus effectively deletes the δ locus on that chromosome.

normal embryonic development, as compared to the study of hybridoma tumor lines cultivated in vitro. Several questions concerning the regulation of TCR gene loci during development can be asked. For example, are TCR genes activated only after cells colonize the thymus? Is fetal $\gamma\delta$ TCR gene expression limited to the thymus? Does the expression of TCR proteins directly mirror TCR gene activity? It is already known, for example, that V γ 1 or V γ 2 genes can be expressed as productively rearranged RNA without assimilation of the products into TCR polypeptides (Heilig and Tonegawa 1986; Iwamoto et al. 1986). In addition, TCR β chains in isolation of TCR α chains, can be expressed as cell-surface molecules in immature T cells (von Boehmer et al. 1988), possibly mediating allelic exclusion of TCR β -chain gene rearrangement. It seems reasonable that early activation of rearranged TCR γ or δ genes could do likewise.

In the experiments described here, we analyzed the expression of murine TCR genes during T-cell ontogeny, up to and including birth. We demonstrate that (1) rearrangement and expression of different V γ genes is differentially and developmentally controlled, (2) intrathymic expression of rearranged γ genes is not predictive of translation and assembly of cell-surface polypeptides, (3)

there is expression of γ and δ genes in fetal liver and gut preparations, (4) there is expression of TCR $\gamma\delta$ protein in the liver of mice at birth, (5) cells expressing TCR γ genes in the newborn liver are not solely immigrants from the thymus, and (6) the $\gamma\delta$ -expressing cells in the newborn liver are lost within several days after birth.

Results

Approach taken

To analyze the developmental activation of TCR γ genes, procedures were required that allow detection of transiently expressed rearranged genes in a low percentage of cells in fetal tissue. The polymerase chain reaction (PCR; Saiki et al. 1986; Roth et al. 1988; Kyes et al. 1989) was chosen for its high sensitivity. By demanding amplification between a primer from a V gene segment and a primer from the C gene segment, the technique detects expression of V-J-C rearrangements as opposed to germ line genes or intermediate rearrangements that can confuse other analyses of gene expression, such as RNase protection across a promoter. The authenticity of the PCR products detected was determined by hybridization to J-segment probes that lie internal to the primers used and that would not detect expression of unrearranged V genes. In some instances of V γ 4-, V γ 5-, and V γ 7-directed amplification, PCR fragments have been cloned and sequenced and confirmed as predicted products of rearranged genes (Kyes et al. 1989; S. Kyes and A.C. Hayday, in prep.). The PCR analyses were performed on cDNA to RNA from thymus, gut, and liver of newborn mice and of fetal mice aged at 11, 12, 12.5, 13, 15, 17, and 19 days, as judged by date of vaginal plug (day 0), and morphology of fetal limb development (Rugh 1968). Mostly transcripts of rearranged V4-V7 γ genes (Fig. 1; Kyes et al. 1989) were analyzed, but expression of some TCR δ genes was also examined. In control experiments, the expression of tubulin and *pax-1* (Deutsch et al. 1988) was analyzed: The results (Fig. 2, and discussed below) were entirely consistent with the known expression patterns of these genes.

To judge approximately how many cell-equivalents of TCR RNA were present in a population, PCR signals from populations were quantitated by comparison to signals derived by amplification of different concentrations of substrate cDNA from a cloned cell line, as described in the legend to Figure 2. Quantitations could be performed with different sets of primers (Fig. 2a,b). An approximately linear proportionality existed across an initial substrate concentration range of 10^4 -fold. The hybridization signals were also proportional to the amount of product run on the gel, after amplification of a constant amount of cDNA (Fig. 2c). Irrespective of the primers used, no products were detected by hybridization if no cDNA was included in the amplification reactions, whether or not the reagents were UV-treated (Fig. 2d). That equivalent amounts of total cDNA were available to the PCR primers was confirmed by the capacity to amplify equivalent amounts of (ubiquitously expressed) tubulin product from each sample (Fig. 2f).

Figure 2. PCR controls. (a–e) Southern analysis of PCR products. Exposure times; (a–c, and e) 16 hr; (d) 4 days. (a) V γ 6 gene expression in 33BTE 67.1 and in 17-day fetal thymus. Equivalents of 1 μ l of cDNA (random hexamer primed) were amplified with V6 (sense) and C1L (antisense) primers in 25- μ l reactions; 10 μ l of products was electrophoresed, transferred to nitrocellulose, and hybridized with 32 P-labeled J1 probe (also used for b–d). (Lane 0) Undiluted cDNAs; (lanes –1 through –4) 10-fold dilutions of 33BTE 67.1 cDNA into a constant amount of cDNA negative for murine TCR gene expression, derived from FR3T3 rat fibroblasts. Arrow marks correct position for V6 PCR product (889 bp). (b) V γ 6 gene expression in 33BTE 67.1 and in 19-day fetal gut: Equivalents of 1 μ l of cDNA (random hexamer primed), 10-fold diluted into FR3T3 cDNA, were amplified with V6 (sense) and C1S (antisense) primers; products were of expected size (350 bp). (c) Adult thymus and kidney, V γ 5 gene expression. (Thy) 10 of 25 μ l of adult thymus V5-C1S PCR product; (1/10 Thy) 1 μ l of adult thymus PCR product; (K) 10 of 25 μ l of adult kidney PCR product. Correct size is 165 bp. (d) γ PCR signals from 13-day brain and from samples with no cDNA added. (V4–V7) no cDNA: 1 μ l of 1 $\times$ mock cDNA synthesis reaction added to each reaction mix; expected sizes were 255, 165, 350, and 255 bp, respectively. (V7) V7–C1S PCR products of B + UV, which is 13-day brain, random hexamer-primed cDNA, PCR mixture UV-treated (310 nm, 20 min) before addition of cDNA and *Taq* polymerase; no cDNA + UV (UV-treated); no cDNA –UV (not UV-treated). (e) *pax-1* gene expression: Thymus at 14, 15, and 17 days and 2 weeks after birth, analyzed for *pax-1* gene expression by PCR (directed by *pax-1*-specific primers) followed by Southern transfer and probing with 32 P-labeled *pax1* cDNA. (f) Tubulin gene expression: ethidium bromide-stained gel of tubulin PCR products (primers for human tubulin, correct size $\sim$ 300 bp); (lanes 1–3) cDNA to three different RNA samples used in these analyses; (lane 4) λ BstEII size markers.

Parallel analyses of γ gene expression in fetal liver cells were carried out by hybridization in situ (Jones et al. 1988; Carding et al. 1989), and by cell-surface protein analysis. These analyses independently confirmed the data derived by PCR. For some analyses, tissues from several mouse strains were analyzed. Frequently, preparations were derived from two independently maintained mouse colonies (one in the United States and one in the United Kingdom). No significant differences in the patterns of gene expression were noted between the two colonies.

Expression of TCR $\gamma\delta$ genes early in thymic development

The earliest appearance of $\gamma\delta$ TCR polypeptides has been reported to be that of V γ 5-, V δ 1-encoded TCR on day-14 thymocytes (Havran and Allison 1988; Ito et al. 1989). Consistent with this, analysis of rearranged V5 gene expression shows a band of appropriate size in day-13

thymus RNA (Fig. 3a), which could not be readily detected in either day-12 or day-12.5 thymus RNA. Rearranged V δ 1 is also expressed at day 13 (Fig. 3c). Neither V δ 3 nor V δ 5 is expressed at comparable levels until later in ontogeny (Fig. 3c).

Although in some thymic samples V γ 5 gene expression on day 13 preceded that of V γ 4 and V γ 6 by 1 day, this was not generally so; and in many analyses expression of all four rearranged V γ genes examined was clearly evident on day 13 (Fig. 3a,b). Conspicuously, V γ 7 expression was evident throughout thymic ontogeny, including that on day 12 (Figs. 3b and 4c).

After day 14, roughly comparable PCR signals were detected for all four rearranged V γ genes examined. Irrespective of V gene usage, the γ gene signals detected on day 19 were significantly stronger than those detected on day 13 [(Fig. 3a,b) starting with equivalent amounts of cellular RNA and checking for equivalence of cDNA synthesized (Fig. 2f)]. The V6-directed signal in the day-19 thymus was equivalent to between 0.1 and 1% of the signal from a T-cell hybrid, 33BTE-67.1 (Happ et al.

Figure 3. PCR analysis of expression of rearranged TCR γ/δ genes in the developing thymus. Internal / probes labeled with ^{32}P were used to detect PCR products generated by amplification of cDNA (synthesis directed by C1S- γ and C-S- δ antisense oligonucleotides) using (the same) C-segment oligonucleotides, in combination with oligonucleotides specific for the V γ gene (a and b) or the V δ gene (c) segments. Amplified products were electrophoresed and transferred to nitrocellulose, as described in the legend to Fig. 2. By comparison with coelectrophoresed size markers, the amplified fragments migrated at approximately the sizes predicted for the bona fide products. [Occasionally doublets were seen, as has been reported before for the analysis of TCR $\gamma\delta$ -genes by PCR (Kyes et al. 1989; Takagaki et al. 1989).] The days of gestation at which the RNA samples were extracted are indicated. Before cDNA synthesis, RNA was quantitated, as described in Methods.

1989), that expresses homogeneously a V γ 6-based TCR (Fig. 2a). The continued expression of V γ 5 in the thymus on day 19 is in contrast to the precipitous drop in absolute numbers of thymocytes expressing the V γ 5-based TCR occurring prior to day 18 (Havran and Allison 1988). The continued expression of V γ 5 may now be confined to a small number of V γ 5 TCR(+) cells that express higher levels of the RNA and/or be predominantly attributable to a different cell population (see Discussion).

The patterns of γ gene expression are unlikely to be determined by some artifactual property of the fetal tissue RNAs. Comparable results were obtained with numerous independent preparations of RNA from equivalent stages. Furthermore, the gene *pax-1*, implicated as active in thymic development by hybridizations in situ to thymus sections (Deutsch et al. 1988), shows an en-

Figure 4. PCR analysis of expression of rearranged TCR γ genes in fetal tissue. (G) Gut; (L) liver; (B) brain. V-gene segments for which amplification oligonucleotides were specific and days of gestation on which RNA was extracted are indicated. Comparable quantities of RNA were used for cDNA synthesis, as described in the legends to Figs. 2 and 3, and in Methods, and comparable amounts of cDNA were amplified by PCR. Gel loading, and exposure to film was as for Fig. 2a–c. However, in b, higher amounts of V5 PCR products were loaded in an attempt to detect signal in day 11 to 12.5 gut and liver.

tirely different expression pattern, peaking at days 14 and 15 (Fig. 2e), entirely consistent with the results of expression in situ (Deutsch et al. 1988).

Extrathymic $\gamma\delta$ gene expression in fetal mice

Two distinct patterns of TCR γ gene expression were detectable in fetal liver and gut. In the first pattern,

shown most clearly by V γ 5, increasing expression in fetal gut and liver occurs subsequent to expression of the gene in fetal thymus (Fig. 4a) and is detected first in the gut (day 17). Transcripts encoding rearranged V δ genes are also detected in fetal liver and gut. Again, the V δ 1 expression parallels that of V γ 5 and, as in the thymus, is detected before that of V δ 3 (Fig. 4a).

The extrathymic expression of the TCR transcripts may be attributable, at least in part, to cells expressing $\gamma\delta$ TCR polypeptides that have emigrated from the thymus. Consistent with this, by 2 weeks after birth, there is very little V γ 5 gene expression in the gut, paralleling the decrease in V γ 5 gene expression in the thymus after birth (Kyes et al. 1989). The expression of V γ 6 in day-19 fetal gut was equivalent to between 0.01 and 0.1% of that detected with the V γ 6(+) cell line, 33BTE 67.1 (Fig. 2b).

The second pattern of extrathymic expression is entirely different, as shown by the V γ 7 gene (Fig. 4a–c). Rearranged V γ 7 is expressed in both gut and liver at day 11 (the earliest time examined), prior to the time at which the developing thymic lobes are colonized by T-cell progenitors (Metcalf and Moore 1971). In liver, gut, and thymus, expression can be detected at time points throughout ontogeny. V γ 7 expression was not detected in fetal brain (Fig. 4c; also see Fig. 2d) or in embryonal stem cells (data not shown). In the newborn liver, expression of all V γ genes declines dramatically within 3 days of birth (data not shown, but see below).

Expression of γ genes in the fetal liver at day 12 to 13 was also demonstrated by hybridization in situ (Jones et al. 1988; Carding et al. 1989) (Fig. 5; Table 1), which indicated that γ -expressing cells comprised between 0.1 and 1% of cells in day-12 to day-13 fetal liver. This per-

centage increased to between 1% and 8% at birth, although there was a reproducible drop around day 15 (data not shown; L. McVay, S.Kyes, S.R. Carding, and A.C. Hayday, in prep.). The dramatic rise in the capability to detect V5(+) cells between day 15 and day 18 (Table 1) was entirely consistent with the PCR patterns seen (Fig. 4a). The number of grains per cell also increased as ontogeny progressed. By analysis in situ, no cells that expressed detectable levels (>15–20 copies of mRNA per cell) of TCR α or β RNA were detected (Table 1). (These data do not exclude very low “level per cell” expression of TCR α or β genes.) The analyses in situ also failed to detect any V γ 2-expressing cells. V γ 2 can be expressed in either $\gamma\delta$ or $\alpha\beta$ cells (Saito et al. 1984a,b).

Hybridization in situ showed an emergence of cells expressing RNA for the δ chain of CD3 and of cells expressing TCR δ genes (Table 1). This coincided with the appearance of V γ 5 RNA expression, raising the possibility that γ gene expression late in ontogeny in the fetal liver might translate into the production of a V γ 5-based TCR protein, akin to the first TCR detected in the thymus.

TCR1 protein expressed extrathymically at birth

To determine whether the γ and δ gene expression detected in the liver was translated into protein, serological examination was made of liver cell suspensions freshly obtained from newborn (0.5–12 hr after birth) mice. CD3(+) cells were identified by fluorescence-activated cell sorting (FACS) analysis and by UV microscopy (data not shown) using two independently derived antibodies directed against CD3 [the hamster antibody

Figure 5. TCR $\gamma\delta$ gene expression in fetal liver. Liver cells were obtained from 12-day fetal and 20-day newborn (obtained within 6 hr of birth) BALB/c mice. Cytochrome preparations of liver cells were hybridized with either a sense (s) or antisense γ constant (C1) region-specific, V γ 5-specific, CD3-specific (T3) or, δ constant region-specific (δ) 35 S-labeled RNA probes. After 10 days of autoradiography, the slides were developed and counterstained, and representative fields from three independent experiments were photographed.

Table 1. TCR gene expression in fetal liver during gestation

TCR RNA probe	Percent fetal liver cells positive at gestational age (day)			
	(BALB/c)		(nu/nu)	
	15	18	20	20
γ -V γ -C1,2 (as)	0 ^a	1.0	6.6	6.9
γ -V γ -C1,2 (s)	0	0	0	0
γ -V2 (as)	0	0	0	—
γ -V5 (as)	0.06	1.2	7.6	—
γ -V5 (s)	0	0	0.01	—
δ -C (as)	0	0.8	7.1	5.2
δ -C (s)	0	0	0	0
T3(δ) (as)	ND	1.3	6.8	ND
β -C (as)	0	0	0	0
α -C (as)	ND	0	0	0

Cytocentrifuge preparations of liver cell suspensions were prepared from carefully staged embryos of different gestational ages. Slides were hybridized with various ³⁵S-labeled RNA probes, synthesized in either the antisense (as), or sense (s) (control) orientation, and specific for constant (C) or variable (V) gene segments of the γ -TCR and for other TCR chains. Over 1000 cells were counted from each of four independent experiments to provide the data presented above. Cells overlaid with >5 grains per cell were considered positive.

^an < 0.05%.

145-2C11 (Leo et al. 1987) and the rat antibody YCD3-1 (Portoles et al. 1989)]. These cells were enriched for by antibody-dependent magnetic separation techniques, and were reanalyzed for anti-CD3 by FACS (Fig. 6). In different experiments, between 35 and 70% of the enriched population was CD3(+). A significant fraction of the CD3(+) cells from newborn mice expresses a cell-surface $\gamma\delta$ TCR that uses V γ 5 protein, as detected with antibody 536 (Havran and Allison 1988). This observation has been made consistently with preparations of newborn liver cells obtained from several strains of mice (BALB/c, CBA/Ca, C57BL/6, and B10.BR) and from nonobese diabetic mice. CD3 and associated peptides have also been immunoprecipitated from ¹²⁵I-labeled membrane proteins of newborn liver cells and resolved on two-dimensional "off-diagonal" gels (data not shown). Several preparations of normal hamster immunoglobulin and rat antibody isotype matched to the rat YCD3-1 antibody [e.g., the anti-CD4 monoclonal antibody GK1.4 (Dialynas et al. 1983)] were not reactive with any newborn mouse liver cell preparations (Fig. 6). Such data indicate that CD3(+) cells are CD4(-), and that the cells are not staining nonspecifically, for example, through cell-surface immunoglobulin or Fc receptors. The cells were also Thy-1(-) and CD8(-) (Fig. 6). In contrast, the CD3(+) cells expressed CD45, an antigen found on virtually all hematopoietic cells. Analyses of the enriched cells by hybridization in situ showed that the majority of the TCR gene expression detected in fetal liver populations is concentrated in CD3(+) cells (Table 3).

The kinetics of expression of V γ 5 cells in the liver of

neonatal mice were studied by use of immunogold-conjugated secondary antibodies in conjunction with a silver enhancement reagent, to increase the sensitivity of detection of positive cells. Results reveal that the frequency of V γ 5 TCR(+) cells in the liver of neonatal mice rapidly decreases between 1 and 3 days after birth (Table 2). This corresponds with the rapid postpartum decline in TCR γ gene expression (discussed above). At 6 days after birth, some CD3(+) cells can be detected again (Table 2), but preliminary data suggest that these cells are likely to be $\alpha\beta$ T cells contaminating the preparations.

γ Gene expression in fetal liver of athymic mice

The expression of TCR γ genes was examined late in the gestation of fetal nude mice, which do not develop a lymphoid thymus during embryogenesis (Jordan et al. 1977). By hybridization in situ, approximately comparable numbers of day-20 (newborn) mouse liver cells expressing TCR γ mRNA were detected in both euthymic and athymic mice bred from two independent mouse colonies (one in England and one in the United States; Table 1). PCR analysis of newborn mouse liver RNA showed clear expression of V γ genes (of V7 in particular), although the levels of V5 expression in the athymic mice were generally lower than those detected in congenic euthymic mice (Fig. 7). These observations demonstrate that late in ontogeny, cells expressing rearranged γ genes (particularly V7) develop in the fetal liver in the absence of a thymic influence.

Cytokine production by cells of newborn mouse liver

We have shown previously that fetal thymocytes display constitutive expression of the genes for growth factors IL-2 and IL-4 at set times during early thymic development (Carding et al. 1989, S. Carding et al., in prep.). These factors may be used for the proliferation and differentiation of immature cells in the thymus. If the liver of newborn mice is to provide an environment to support the growth and differentiation of $\gamma\delta$ T cells, it should be possible to identify the expression of T-cell growth/differentiation factors within cells of the liver. To distinguish synthesis of these factors from passive accumulation, specific probes were hybridized in situ to detect lymphokine mRNA. Analysis by hybridization in situ of neonatal liver cell populations revealed expression of several cytokine and cytokine receptor genes. In particular, CD3(+) cells (see above) were enriched in cells expressing IL-2 and the IL-2 receptor (Table 3).

Discussion

PCR, hybridization in situ, and cell-surface labeling have been used to analyze the activation of $\gamma\delta$ TCR genes during murine ontogeny. The major conclusions concerning the expression patterns seen are qualitative.

The $\gamma\delta$ TCR is the first to appear during the development of the thymus in all organisms in which it has

Figure 6. Cell-surface phenotype of CD3⁺-enriched liver cells from newborn mice. CD3⁺, FITC-labeled cells were stained with phycoerythrin-conjugated anti-CD4, anti-CD8, or anti-CD45 antibodies. V γ 5⁺ cells were identified by staining with the antibody 536, a secondary, biotinylated anti-hamster antibody and streptavidin–phycoerythrin.

been examined thoroughly (Haars et al. 1986; Bluestone et al. 1987; Nakanishi et al. 1987; Pardoll et al. 1987; Havran and Allison 1988; Sowder et al. 1988). For this and other reasons, the receptor has been termed TCR1 (Janeway et al. 1988; Sowder et al. 1988; Kyes et al. 1989). The first murine TCR polypeptide expressed is encoded by V γ 5, V δ 1 genes. We found that the expres-

sion of the V γ 5 gene begins in cells after they have colonized the thymus. This is consistent with the notion that pluripotent stem cells of the fetal liver colonize the thymus, wherein T-cell-specific gene expression is induced in them. Some days after V γ 5 (and V δ 1) gene expression can be detected in the thymus, the expression of these genes can be detected in the fetal liver and gut.

Table 2. Cell-surface expression of T-cell antigens in neonatal liver cells

T-cell antigen	Percent cells positive at neonatal age (day)				
	1	2	3	4	6
CD3	10.1 $\pm$ 2.1	1.1 $\pm$ 0.3	0.3 $\pm$ 0.1	0.1 $\pm$ 0.1	1.7 $\pm$ 0.6
V γ 5	8.6 $\pm$ 2.1	0.4 $\pm$ 0.1	0.1 $\pm$ 0.1	0 ^a	0
Thy-1	0	0	0	0	0.4 $\pm$ 0.1
CD8	0	0	0	0	0.2 $\pm$ 0.1

The frequency of BALB/c liver cells expressing T-cell surface antigens was determined by using either an anti-rat (for the anti-CD8 and anti-Thy-1 antibodies) antibody conjugated to gold particles or a secondary biotinylated anti-hamster antibody with a tertiary monoclonal anti-biotin antibody conjugated to gold particles (for anti-CD3, and anti-V γ 5). Gold-labeled cells were visualized microscopically, after incubation with a silver enhancement reagent. Isotype-matched antibodies or immunoglobulin-containing nonimmune serum were used as controls. Determinations of frequency of all positive cells were made from three independent preparations of neonatal BALB/c liver cells.

^a<0.1% positive cells were detected.

Figure 7. PCR analyses of TCR γ gene expression in fetal liver of athymic and euthymic mice, as indicated. The $V\gamma$ gene segments for which amplification oligonucleotides were specific are indicated. Exposure: (V5) 16 hr; (V7) 48 hr.

The order of appearance of transcripts in the different tissues is suggestive of a precursor/product relationship. That is, $\gamma\delta$ T cells that develop in the fetal thymus migrate to the liver and gut as ontogeny proceeds. This is supported by the observation that within 2 weeks of birth, $V\gamma 5$ and $V\gamma 6$ gene expression declines dramatically both in the thymus and in the gut.

However, our data also show that expression of at least one other member ($V7$) of the rearranging γ gene family is normally activated early in fetal liver and gut development, prior to the colonization of the thymus (Moore and Owen 1967; Metcalf and Moore 1971). This observation is significant in that it demonstrates, albeit at low levels, that TCR gene rearrangement and expression occur during normal development without an absolute requirement for the thymus. Earlier, it was considered that rare gene rearrangement events may occur in the germ line (Hayday et al. 1985a). The role of the

thymus may be to catalyze gene activation and rearrangement and to provide the cellular and hormonal interactions that effect "selection" of cells bearing appropriate receptors, even in the $\gamma\delta$ cell lineage. Furthermore, the very early activation of $V7$ in the gut and liver, as well as in the thymus, may be a molecular reflection of some aspect of the common endodermal contribution to these tissues.

Rearranged $V\gamma 7$ gene expression continues in liver and gut tissue throughout fetal ontogeny and, consistent with this, is readily detectable in athymic mice at birth (Fig. 7). This is significant in that it can potentially explain the capacity to detect $\gamma\delta$ T cells that express $V\gamma 7$ in the gut tissue of some young, adult athymic mice (Takagaki et al. 1989; S. Kyes and A.C. Hayday, unpubl.). Adult athymic mice have also been shown to develop small numbers of T cells late in life (Pardoll et al. 1988). The pattern of extrathymic activation of $V7$ expression that is demonstrated here makes it plausible that even in normal mice, $V7$ -expressing (and potentially other) $\gamma\delta$ T cells develop independent of the thymus. This issue can be explored by thymectomy. The implication is that such T cells develop in the periphery of mice without passing through thymic selection. This raises the issue of whether peripheral stromal cells express restricting elements able to positively select $\gamma\delta$ T cells (Benoist and Mathis 1989; Berg et al. 1989).

The differences in extrathymic expression shown by $V\gamma 5$ and $V\gamma 7$ are also significant in their demonstration that not only are the closely linked $V\gamma$ genes distinct in their coding sequences (Garman et al. 1986; Pelkonen et al. 1987) but that they may also be distinguished by the regulation of their transcriptional activities. Indeed, the DNA sequences that directly precede the RNA initiation points of the $V\gamma 2$, $V\gamma 5$, and $V\gamma 6$ genes are quite dissimilar from each other over some hundreds of nucleotides (L. McVay and A.C. Hayday, unpubl.).

Within the thymus, however, the progressive expression of $\gamma\delta$ TCR polypeptides encoded by discrete pairs of γ and δ V -gene segments is not well reflected by differential transcriptional activation of the γ genes. That is, the total thymus γ gene expression does not parallel that in $CD3(+)$ -sorted thymocytes. What, therefore, controls the expression of discrete $\gamma\delta$ TCR proteins? Our data (Fig. 3) suggest that rearranged $V\delta$ genes are expressed selectively; $V\delta 1$ is expressed early (a parallel pattern to $V\gamma 5$), whereas $V\delta 3$ and $V\delta 5$ genes are not. These data are consistent with the schedule of TCR δ rearrangement (Iwashima et al. 1988). This V -gene-dependent expression is not a reflection of activation of the most proximal V - D elements but may indicate that some $V\delta$ genes have regulatory signals facilitating early expression, whereas other $V\delta$ genes may be more like $V\alpha$ genes, among which they are situated, and may be restricted to late fetal expression. It is possible that the $\delta\alpha$ locus is the "timekeeper" of TCR expression. By this model, rearranged and expressed γ and β genes, respectively, are used in the formation of TCRs according to a schedule determined by the regulated expression of δ and α genes, respectively.

Table 3. Gene expression in populations of liver cells isolated from newborn mice (percent cells positive)

Probe	Isolated cell populations	
	CD3 ⁺	CD3 ⁻
TCR		
CD3	69	5
C γ	67	3
C α	1	1
Cytokines		
IL-2	49	2
IL-4	12	7
IFN- γ	29	36
TNF β	37	18
IL-2 receptor	49	8

CD3⁺ and CD3⁻ cells were isolated from the livers of newborn mice and analyzed for TCR- and cytokine-specific gene expression by hybridization in situ, as described in Methods. In all cases, control probes transcribed in the sense orientation failed to produce any detectable hybridization signal.

However, this locus is unlikely to be the sole regulator. Cell-surface $V\gamma 6$, $V\delta 1$ expression does not occur early in thymic ontogeny, despite the fact that expression of both genes is detectable at day 13. Similarly, expression of $V\gamma 5$ and $V\delta 1$ genes that occurs late in ontogeny (day 19) also does not translate into cell-surface expression of the corresponding TCR. Throughout ontogeny, the thymus is mostly populated by immature thymocytes that will never express CD3–TCR levels characteristic of mature T cells. As the thymus increases in size during ontogeny, the absolute number of such cells increases. Hence, although the expression of certain γ and δ genes may be attributable to mature, CD3(+) $\gamma\delta$ cells, the broad spectrum of $\gamma\delta$ gene expression detected by our analyses throughout most of fetal ontogeny may be attributable to immature thymocytes. These cells may develop from three distinct waves of thymic immigrants (Sowder et al. 1988). If cells in each wave are induced to rearrange and express the γ genes in a quasi-random fashion, it is perhaps not surprising that expression of a broad set of γ genes is detectable throughout fetal ontogeny. As thymic development proceeds, the $\gamma\delta$ -expressing thymocytes that will form mature CD3(+) cells may be influenced by changing selective mechanisms expressed by the thymic matrix. Alternatively, the $\gamma\delta$ gene expression may represent abortive events in $\alpha\beta$ cells.

Although this cannot be ruled out, such a breadth of abortive $\gamma\delta$ gene usage in $\alpha\beta$ cells has not been noted previously, and recent data suggest that $\gamma\delta$ gene expression (with the possible exception of $V\gamma 2$) should be silenced in $\alpha\beta$ T cells (Bonneville et al. 1989a). Furthermore, when PCR analyses were made of lymphocytes from adult peripheral tissue, the patterns of gene usage in each case corresponded well with the predominant $\gamma\delta$ protein usage, for example, $V5$ in the skin, $V6$ in reproductive epithelia, and $V7$ in the gut (S. Kyes and A. Hayday, unpubl.). Because gut tissue contains many $\alpha\beta$ T cells (Kyes et al. 1989), the major difference between those adult tissues and the thymus is probably the presence of an excess of immature T cells in the thymus. This further implicates those cells as the source of promiscuous γ gene expression.

In the newborn liver, at least some of the signals detected in the studies reported here are attributable to CD3(+), $\gamma\delta$ (+) cells. Our studies demonstrate that the newborn liver appears to be a conspicuous site of $\gamma\delta$ protein expression on CD45⁺, Thy.1⁺, CD4⁺, and CD8⁺ cells. The expression of the gene for the T-cell-specific growth factor, IL-2, together with the gene for at least one chain of the IL-2 receptor, suggests that these $\gamma\delta$ T cells may possess some T-cell effector functions. Remarkably, the cells have disappeared almost entirely from the liver within 3 days of birth; their fate is unresolved. The murine fetal liver is hematopoietic during embryogenesis, but this activity also stops within several days of birth, possibly because dramatic changes in the liver extracellular matrix render maintenance of lymphoid cells difficult. (We regard the possibility that the $\gamma\delta$ gene expression detected in newborn liver is a

manifestation of maternal thymic lymphocyte infiltration as unlikely because in the analysis of athymic mice, the mothers were inbred, homozygous nude animals and were anatomically athymic.) At this stage, it is not possible to speculate constructively on the potential hazards of extrathymic $\gamma\delta$ T-cell differentiation. It is clear that $\gamma\delta$ T cells are also present in the fetal livers of humans (Aparicio et al. 1989; Carding et al. 1990; L. McVay, S.R. Carding, S. Kyes, and A.C. Hayday, in prep.). It will be interesting to determine how many details of murine $\gamma\delta$ gene activation are conserved in other vertebrates.

Methods

Animals

Tissues were dissected from fetal CBA/Ca, BALB/c, C57BL/6, B10.BR, or NOD mice. The date of detection of a vaginal plug was counted as day zero (0). Gestational age was confirmed by morphological evaluation of limb bud development (Rugh 1968).

Antibodies

All antibodies used in these studies were isolated from either hybridoma culture supernatant or from *nu/nu* mouse ascitic fluid after passage over, and elution from, a Sepharose–protein G column (Pharmacia) used according to the suppliers' instructions. Purified antibodies were either biotinylated or conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin for staining. The anti-CD3 antibodies used were YCD3-1 and the 145-2C11 hamster antibody (Leo et al. 1987), generously provided by Dr. J. Bluestone (Chicago, IL). The rat GK1.5 (Dialynas et al. 1983), M1/9.3.4HL.2 (Springer et al. 1978), and 20-10-5S (Auchincloss et al. 1982) antibodies were used to detect CD4, CD45, and Thy-1 T-cell-surface antigens, respectively. The anti-CD8-specific antibody was supplied by Becton Dickinson. The hamster monoclonal antibody 536 (Havran and Allison 1988) was used to detect $V\gamma 5$ polypeptides and was the kind gift of Dr. J.P. Allison.

Cell isolation

CD3⁺ and CD3[−] cells were isolated from the livers of newborn mice with the MACS magnetic cell sorter (Miltenyi Biotec GmbH, Bergisch-Gladbach, FRG). Briefly, a suspension of liver cells was incubated with the biotinylated rat anti-mouse CD3 antibody YCD3-1 (Portoles et al. 1989), followed by saturating amounts of fluorescein–streptavidin and superparamagnetic beads conjugated to biotin. CD3⁺ cells were then isolated by passage over a separation column placed in a magnetic field. The column was washed to remove nonbound cells and flushed with PBS after removal from the magnetic field to remove bound cells. CD3[−] cells were collected in the column effluent. The purity of the eluted cells was verified by flow cytometry on a FACS 440 (Becton Dickinson).

FACS analysis was performed. One million CD3⁺ cells isolated from the livers of newborn mice (which were FITC-labeled; see above) by the MACS cell separator, were incubated with saturating amounts of phycoerythrin-conjugated anti-CD45, -CD4, or -CD8 antibodies in staining buffer (PBS, 5% fetal calf serum, 0.1% Na₂S₂O₃) on ice for 30 min. Cells were washed twice with staining buffer, resuspended in fixation buffer [PBS, 1% paraformaldehyde (pH 7.3)], and analyzed on a FACS 440. For the detection of cells expressing $V\gamma 5$ protein, cells were incubated with antibody 536 and a secondary biotin-

ylated goat anti-hamster immunoglobulin antibody (Vector Labs), followed by strepavidin-phycoerythrin as described above.

Hybridization in situ

Mononuclear cell suspensions were obtained from fetal liver by dispersing the tissue gently in PBS and performing centrifugation on a Ficoll-Hypaque cushion. Hybridization in situ was carried out on cytocentrifuge preparations of mononuclear cells, as described previously (Jones et al. 1988; Carding et al. 1989).

Hybridization probes

All probes used for hybridization in situ were cDNA fragments inserted into the polylinker site of pGEM-3 or pGEM-4 plasmids (Promega) by standard procedures. The $\gamma\delta$ -TCR probes used in this study have been described previously (Kyes et al. 1989). The C β probe was a 650-bp *EcoRV*-*ScaI* fragment derived from cDNA pHDS11 (Saito et al. 1984a); the C α probe was derived from pHDS58 (Saito et al. 1984b), and the CD3 probe was provided by Dr. Cox Terhorst. The IL-2, IL-4, IFN- γ , and IL-2 receptor probes have been described previously (Carding et al. 1989). The TNF β probe, kindly provided by P. Gray, was a 600-bp *KpnI*-*PvuII* cDNA derived from a genomic TNF β clone. After linearization of pGEM vector containing the appropriate insert, ³⁵S-labeled RNA sense or antisense transcripts were synthesized with SP6 or T7 RNA polymerase (Kyes et al. 1989). To detect γ and δ gene PCR products, internal *J* probes were used, after labeling with [³²P]dCTP with Klenow enzyme.

cDNA preparation

Total cellular RNA was isolated from fetal and adult tissues (Chirgwin et al. 1979) in the presence of equal amounts of carrier yeast tRNA (100 μ g/ml). The integrity of mRNA was verified by optical density, ethidium bromide staining after electrophoresis on agarose gels, and Northern hybridization. Ten micrograms of total RNA was used to synthesize cDNA with random hexamer primers or 120 pmoles of C γ 1 oligonucleotide (Heilig and Tonegawa 1986) or C δ oligonucleotide. Equivalent amounts of cDNA could be estimated by comparable capacity to act as substrate for amplification directed by tubulin gene-specific primers.

PCR

cDNA to total RNA was used in 25-cycle PCR amplifications, according to Roth et al. (1988) and Kyes et al. (1989). PCR primers were gene-specific and are described in Kyes et al. (1989). In addition, a further primer specific for the 3' end of C γ 1 was synthesized, so that any TCR gene analysis could be performed by coupling *V* primers either to our original C γ primer (C1S) to yield short products or to the new 3' C primer (C1L) to yield longer products. An analogous set of gene-specific primers were used to amplify TCR δ genes. Unique primers were also synthesized for *pax-1* and tubulin gene studies. PCR products were electrophoresed on 2% agarose gels, transferred to nitrocellulose, and hybridized with random-primed, ³²P-labeled *J* region-specific probes, except where stated otherwise in the figure legends. Amounts of product analyzed in specific cases are referred to in the appropriate figure legends.

Immunogold histochemistry

Cytocentrifuge preparations of liver cells from newborn and neonatal mice were incubated with anti-CD3-, anti-CD8, anti-Thy-1, or anti-V γ 5 antibodies and either anti-rat (for the anti-CD8 and anti-Thy-1 antibodies) antibodies conjugated to gold particles (Janssen Life Science Products, Piscataway, NJ) or a biotinylated anti-hamster antibody for the detection of CD3 and V γ 5 antibody-reactive cells in conjunction with a gold-conjugated monoclonal antibiotin antibody (British Biotechnology Ltd., Oxford, England). Cell preparations were washed with PBS at pH 7 and incubated with a silver enhancement reagent (Janssen) to visualize positive cells by light microscopy.

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