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Influence of site of expression on the fetal γδ T-cell receptor repertoire

SUSAN KYES, WILLIAM PAO, AND ADRIAN HAYDAY

Departments of Biology and Immunobiology, Yale University, New Haven, CT 06511

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ABSTRACT **The sequences of productive T-cell γ/δ receptor transcripts were compared in different murine fetal tissues. Differences from tissue to tissue suggest that the sequence repertoires are at least in part the products of selection, presumably through interaction of T cells bearing the γδ receptor with fetal self-ligands.**

Theoretically, the outcome of a differentiation program can be limited "mechanistically," reflecting restricted differentiation potential of a precursor cell, and "selectively," reflecting the dominant influence on the program of heterogeneous cells at the site of differentiation. The degree to which mechanism and selection influence differentiation is a major issue in vertebrate developmental biology. For lymphocytes that will bear the T-cell receptor composed of α and β chains (αβ TCR), it has been established that interactions of immature cells with stromal cells of the thymus in large part select which thymocytes will be exported to the peripheral immune system as mature T cells (1–6). T cells bearing the αβ TCR (αβ T cells) recognize polymorphic ligands—hence, the utility of a system that selects for maturation of thymocytes with appropriate ligand-binding specificities. However, the generality of such a system is unclear. T cells that bear the γδ TCR (γδ T cells) also develop substantially (although seemingly not uniquely) in the thymus (7). It is not clear, however, what γδ T cells generally recognize *in vivo*. Nor is it clear that the γδ TCR repertoire is ordinarily a product of selection. To examine this, we have compared the γδ TCR repertoires in different fetal tissues directly *ex vivo*. We reasoned that if the repertoire diversity is the same in all tissues, it is presumably a product of mechanistically restricted stem cell capability, whereas if the repertoires are different in different tissues, then they are at least in part results of interactions at different sites—that is to say, tissue-specific selection events.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c mice were obtained from The Jackson Laboratory and bred in the Yale University Animal Facility. Fetal mice were staged, and tissues were prepared as described (7). All experiments with animals were carried out in American Association for the Accreditation of Laboratory Animal Care-accredited facilities under a protocol awarded to A.H. by the Yale Use of Animals Committee.

cDNA. Sequences were derived after polymerase chain reaction (PCR) amplification of specific mRNA, as described in our previous analyses (7, 8). In this study, the RNA was harvested in each case from dissected tissues of complete prenatal or immediately postnatal litters. Each RNA preparation was checked for its integrity by gel electrophoresis and staining (by a modification of the formaldehyde gel technique that is applicable to 1-μg samples of total RNA) and by the capacity of the cDNA corresponding to the RNA to efficiently amplify β-tubulin gene fragments between β-tubulin primers (7). Intact RNA samples from two or three independent litters were then pooled to provide the substrate for reverse transcription and amplification. Pooling avoids skewing of the data by properties unique to one mouse or one mouse litter. Gut, liver, and thymus were sampled from mice of the same litters.

PCR. Amplification was attempted between variable gene (*V*) segment primers and constant gene (*C*) segment primers. In this way, all rearrangements to a particular *V* gene segment could be detected and compared, irrespective of the diversity gene (*D*) and joining (*J*) segments recombined to the *V*. This was especially relevant for the δ-chain gene analyses (see Table 2). The primers were: $V_\gamma 5$, GAGGATCCCGCTTGGAAATGGATGAGA; $C_\gamma 1$, (*i*) AAATTGTCTGCATCAAGTCT and (*ii*) CCACCACTCGTTTCTTTAGG; $V_\delta 1$, TTTAGTATACAAGCTAAATAGC; and C_δ, (*i*) AGGGTAGAAATCTTTCACCAGACA and (*ii*) CTCATGTCAGCCCACCTTAA. For each *V*, two sets of amplifications were made with different *C* segment primers to confirm by further means the authenticity of the products detected.

Sequence Analysis. PCR products were cloned into pGemini vectors (Stratagene). All γ/δ(+) recombinant clones from a transformation experiment were sequenced, except for those that arose on plates very close to one another and therefore were potentially cross-contaminated. For each repertoire, clones were sequenced from more than one ligation experiment. Entire insert sequences were determined by using flanking and internal primers to drive dideoxynucleotide reactions, as described before (8). The sequence repertoires were compared one with another statistically by using the Wilcoxon two-sample rank test, with adjustment for tied scores. This test is appropriate because it allows determination of the probability of a null hypothesis that two samples are derived from the same population, without making a hypothesis *a priori* about the nature of the population.

RESULTS AND DISCUSSION

Murine $V_\gamma 5$–$C_\gamma 1$/$V_\delta 1$–C_δ is the predominant TCR expressed early in fetal thymic development (9, 10). The γ chains are encoded by an essentially monomorphic rearrangement of $V_\gamma 5$–$J_\gamma 1$ (10). The junction is a simple one (Table 1), with no N region nucleotide contribution. This is characteristic of "fetal-type" joins formed early in ontogeny (10, 12, 13). The recurrent γ-chain gene rearrangement is termed the "canonical" γ sequence. There is likewise a canonical fetal-type $V_\delta 1$–$D_\delta 2$–$J_\delta 2$ rearrangement (Table 2, sequence 1). Fetal thymocytes that express $V_\gamma 5$–$V_\delta 1$ chains can give rise to peripheral dendritic epithelial cells (DECs) of postnatal skin (14). DEC populations have essentially the same fetal-type γδ

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Abbreviations: TCR, T-cell receptor; α/β or γ/δ TCR, TCR composed of α and β or γ and δ chains; γδ T cell, T cell bearing γδ TCR; DEC, dendritic epithelial cell; V, variable; C, constant; D, diversity; J, joining.

Table 1. DNA sequences of germ-line and rearranged murine $V_\gamma5$–$J_\gamma1$ genes at the junctions of the two gene segments

$V_\gamma5$	$J_\gamma1$	Source	% of prod. rearr.	Material sequenced	Ref.
TGT GCC TGC TGG GAT CT	AT AGC TCA GGT TTT	Germ line	N.A.	DNA	
TGT GCC TGC TGG GAT	AGC TCA GGT TTT	DECs	18/21	DNA	11
			12/12	RNA	*
TGT GCC TGC TGG GAT	AGC TCA GGT TTT	Fetal thymus	26/27	DNA	10
TGT GCC TGC TGG GAT	AGC TCA GGT TTT	19-day gut	19/20	RNA	*
TGT GCC TGC TGG	AGC TCA GGT TTT	19-day gut	1/20	RNA	*
TGT GCC TGC TGG GAT	AGC TCA GGT TTT	17-day gut	6/7	RNA	*
TGT GCC TG	T AGC TCA GGT TTT	17-day gut	1/7	RNA	*
TGT GCC TGC TGG GAT	AGC TCA GGT TTT	Fetal liver	9/15	RNA	*
TGT GCC TGC TGG GAT CT	T AGC TCA GGT TTT	Fetal liver	4/15	RNA	*
TGT GCC TGC TGG GAT CT	C AGC TCA GGT TTT	Fetal liver	1/15	RNA	*
TGT GCC TGC TGG GAT C	AT AGC TCA GGT TTT	Fetal liver	1/15	RNA	*

The frequency with which the described sequence was reported among productive rearrangements of these segments in each sampling group is shown as % of prod. rearr., and 19-day and 17-day tissues denote days of fetal gestation. Not shown are the sequences of seven frameshift rearrangements determined in this study (three from 17-day gut and four from fetal liver); however, see text. N.A., not applicable.
*Data are from this study.

TCR sequence repertoire as that of fetal thymocytes (11, 15): the $V_\gamma5$ and $V_\delta1$ rearrangements are mostly canonical.

We recently found that murine $V_\gamma5$–$C_\gamma1/V_\delta1$–C_δ transcripts are also the most abundant γδ TCR transcripts in mid-to-late fetal gut and liver (ref. 7; Fig. 1), and $CD3^+$ cells that bear the $V_\gamma5/V_\delta1$ receptor and that have many properties of conventional thymocytes can be clearly demonstrated (albeit at low levels) in the livers of late fetal and newborn mice (7). Indicative of the specificity of these results, $V_\gamma5$–$C_\gamma1$ transcripts were not detected in either total fetal brain or explanted embryonal stem cell RNA (ref. 7; A.H., unpublished data). To determine whether the $V_\gamma5$–$C_\gamma1/V_\delta1$–C_δ TCR repertoires in the extrathymic fetal tissues are the same as those found in the fetal thymus and skin (described above), the structures of rearranged transcripts in various tissues were determined (as described in *Materials and Methods* and in refs. 7, 8, and 16).

By comparison with analysis of amplified, rearranged DNA used to elucidate the fetal thymus and skin repertoires (10, 11), the analysis of amplified transcripts has the advantage of restricting data output to that on expressed genes. Indeed, by using this approach to analyze DEC cells derived *de novo* from adult murine epidermis, 12 of 12 $V_\gamma5$ sequences were canonical (Table 1). Fifty-seven additional cDNA sequences were then determined *de novo* from the murine fetal and newborn extrathymic transcripts shown in Fig. 1.

Sequences of cDNAs revealed that the $V_\gamma5$ repertoire from 19-day fetal gut was essentially the same as the fetal thymus repertoire (Table 1). All 20 cDNAs sequenced from 19-day gut were fetal type (see above), all were productively rearranged, and all but one were canonical (Table 1). Similarly, from 17-day gut, all but one productively rearranged cDNAs were fetal-type and were canonical (Table 1). The similarity of the fetal gut and fetal thymus $V_\gamma5$ repertoires is striking. This repertoire was previously thought to be largely restricted to fetal thymus and skin (14). Since gut $V_\gamma5$–$V_\delta1$ expression is only detected in ontogeny after it has commenced in the thymus (Fig. 1 and ref. 7), the fetal gut cells expressing the $V_\gamma5$ rearrangements may have come from the thymus. If so, those cells in normal mice do not home exclusively from thymus to the skin. Alternatively, γ-chain gene rearrangement can occur extrathymically and gut γδ T-cell populations do develop in mice with only rudimentary thymi (7, 17–19). Hence, it is possible that the gut rearrangements were generated *in situ*, in which case γδ chain development in the gut apparently follows the same course as in the thymus.

$V_\gamma5$ transcripts from late fetal and newborn liver were also exclusively fetal-type. The canonical sequence was the most common $V_\gamma5$ rearrangement, but by contrast to fetal thymus, fetal gut, or skin, its representation among productive rearrangements was unprecedentedly low (only 9 of 15 or 60%) (Tables 1 and 3). Because of this observation, a Wilcoxon

Table 2. Comparison of the junctional DNA sequences of rearranged (rearr.) murine $V_\delta1$, $D_\delta2$, and J_δ in skin and in fetal thymus and liver

Rearr. junctional sequence	$V_\delta1$	$D_\delta2$	J_δ	Source	Ref.
—	TCA GAT AT	ATC GGA GGG ATA CGA G	C TCC TGG . . .*	Germ line	
1	TCA GAT (canonical)	ATC GGA GGG A	GC TCC TGG . . .*	Skin, fetal thymus	10, 11
2	TCA GAT (most frequent)	ATC GGA GGG A	GC TCC TGG . . .*	Late fetal liver	†
3	TCA GAT (reiterated)	ATC GGA GGG ATA CGA GCT	ACC GAC . . .‡	Skin, fetal thymus	10, 11
4	TCA GAT (3/8)	ATC GGA GGG ATA CGA GCT	GAC . . .‡	Late fetal liver	†
5	TCA GAT (1/8)	ATC GGA GGG ATA C CT	ACC GAC . . .‡	Late fetal liver	†

All the productive joins derived by amplification from liver by using $V_\delta1$–$C_\delta1$ primers are shown and are compared with two sequences that are reiterated in fetal thymus and skin. All joins are fetal type—that is, the sequences can be attributed entirely to a combination of germ-line DNA and palindromic nucleotides.
*$J_\delta2$ sequences.
†This study.
‡$J_\delta1$ sequences.

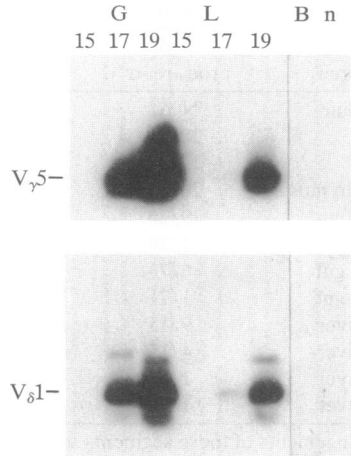

FIG. 1. Amplification products of transcripts of rearranged $V_\gamma 5$ and $V_\delta 1$ genes in fetal gut (lanes G) and liver (lanes L). RNA from fetal tissue was converted to cDNA, which was amplified by using a $V_\gamma 5$ primer in combination with an antisense $C_\gamma 1$ primer or a $V_\delta 1$ primer in combination with an antisense C_δ primer. The products were electrophoresed in a 1.6% agarose gel and blotted to nitrocellulose, and a major band of the predicted size was detected with a radiolabeled J_γ or J_δ probe internal to both primers (7). Lanes 15, 17, and 19 denote days of fetal gestation on which tissue was extracted for RNA. The comparability of cDNA preparations was assessed by their capacity to support equivalent amplification between tubulin gene primers (7). Specificity control lanes: B, fetal brain RNA; n, no cDNA added to the amplification reaction.

two-sample rank test (with adjustment for tied scores) was applied to test the null hypothesis that the fetal liver sequences were a sampling of an identical repertoire to that sampled by the thymus, skin, and gut sequences. The results are statistically significant and are shown in Table 3. In every case, there is less than 1 chance in 25 that the fetal liver repertoire is a sampling of an identical population to that in the other tissues. Indeed, the value of $P = 0.04$ derives from a comparison of the 15 fetal liver cDNA sequences with 21 DEC genomic DNA sequences, in which instance there is no evidence that the noncanonical joins were even expressed. When transcript populations were compared (e.g., fetal liver with 20 fetal gut sequences), the probability of identity between the repertoires is <1:175.

What of the noncanonical, productive fetal liver sequences? The 40% of productive rearrangements that were noncanonical comprised three different cDNA sequences, but strikingly they were all one codon larger than the canonical sequence (Table 1). Indeed, two of the noncanonical cDNA sequence sets encode the same protein sequence. Hence, in all but a single case, the γ-chain amino acids encoded by these novel, noncanonical junctions were the same. Therefore, the expressed liver repertoire differs from the repertoires expressed in fetal thymus, in skin, and in fetal gut by virtue of reduced frequency of the canonical γ-chain sequence, and, in its place, a productive coding rearrangement (*i*) that has not been described in previous analyses *in vivo*, (*ii*) that is different by one amino acid from the canonical sequence, and (*iii*) that is reiterated in a third of all cases (Tables 1 and 3).

$V_\gamma 5$ is expressed in cells that express rearranged $V_\delta 1$ genes. To examine whether the late fetal liver $V_\delta 1$ sequence repertoire might also be distinguishable from other $V_\delta 1$ repertoires, the sequences of eight productively rearranged $V_\delta 1$ cDNAs were examined. All were fetal type (Table 2, sequences 2, 4, and 5). All examples of $V_\delta 1$–$D_\delta 2$–$J_\delta 2$ were canonical (Table 2, sequence 2). However, there was an equally high frequency of productive $V_\delta 1$–$J_\delta 1$ rearrangements transcribed, among which a previously unreported sequence was again reiterated (Table 2, sequence 4). It was different by one codon from a productive $V_\delta 1$–$J_\delta 1$ rearrangement that was reiterated in 4 of 7 productive $J_\delta 1$ joins in the fetal thymus, and 3 of 7 productive $J_\delta 1$ joins in the skin (Table 2, sequence 3).

Hence, the fetal liver δ-chain gene repertoire is also similar to but distinct from the analogous repertoire in the fetal thymus. $V_\gamma 5/V_\delta 1$ gene rearrangement and expression have been demonstrated in the late fetal and newborn livers of euthymic mice and of mice with only rudimentary thymi (7). However, expression in the former is higher than in the latter, suggesting that the liver expresses γδ-chain gene rearrangements in thymus-dependent and thymus-independent cells (7). The cells from the thymus might be responsible for most of the canonical γδ gene joins in the liver repertoire, in which case the repertoire of γδ joins developed in the liver *in situ* would be even more different from the γδ repertoire of the fetal thymus and fetal gut.

In summary, the $V_\gamma 5/V_\delta 1$ repertoires are different at different sites in the fetal mouse. The fetal gut repertoire is essentially the same as that of the fetal thymus, but the repertoire in similar-age liver is distinct by two main criteria: (*i*) statistically lower frequency of the canonical $V_\gamma 5$ joins and (*ii*) reiteration of novel joins (in particular, a novel $V_\gamma 5$ join in 33% of cases). Some of these novel joins—in particular the rearrangements of $V_\delta 1$ to $J_\delta 1$—might be expressed only in immature cells and not necessarily be translated into functional cell-surface receptors. Irrespective of this, the finding in the liver of a more diverse sequence repertoire that includes novel productive rearrangements makes the homogeneity of the repertoires of the fetal thymus and gut more striking.

The factor(s) that determines the predominance of the canonical joins in the fetal thymus and gut may be "mech-

Table 3. The frequency of reiterated $V_\gamma 5$ sequences among productively rearranged genes in different murine tissues

Tissue	Sample size*	Frequency of canonical $V_\gamma 5$ sequence, %	Statistical probability of identity between sample and fetal liver	Frequency of next most common $V_\gamma 5$ sequence, %	Material sequenced	Ref.
Skin	21	86	0.0401	S	DNA	11
Fetal thymus	27	96	0.0014	S	DNA	10
17-day gut	7	82	†	S	RNA	‡
19-day gut	20	95	0.0057	S	RNA	‡
Late fetal liver	15	60	—	33	RNA	‡

Statistical probabilities were derived by application of the Wilcoxon two-sample rank test (see text). S denotes a singleton in cases where no sequence other than the canonical one occurred more than once in the sample analyzed.
*Number of productively rearranged genes analyzed.
†Sample size insufficient for statistical analysis.
‡Data from this study.

anistic" or "selective." There is clearly some mechanistic contribution to the fetal γδ TCR repertoires. Early fetal thymic gene rearrangements are usually simple gene fusions with limited diversity both in mice and in humans (10, 12, 13), and nonproductively rearranged sequences are often highly reiterated [e.g., $V_\gamma 6$ and $V_\delta 1$–$D_\delta 2$–$J_\delta 1$ rearrangements in the fetal thymus (10)]. Furthermore, four of four out-of-frame (unselectable) $V_\gamma 5$–$J_\gamma 1$ junctions that our analysis detected in the fetal liver were all the same—one nucleotide short of the canonical join (data not shown). It might be argued then, that the novel joins found in the liver reflect a mechanistic, extrathymic stem cell capability to generate more diverse rearrangements. However, this is not likely since the liver γ- and δ-chain gene rearrangements are of extremely simple fetal type like those in the early fetal thymus, are not very diverse in the way that late fetal and early postnatal thymic rearrangements are diverse (10), and, reflecting this, invariably differ from the canonical sequence by just one codon.

Instead, the data best fit a model in which thymic and extrathymic fetal gene rearrangements are mechanistically restricted, but the mechanism alone can generate a broader repertoire than the "homogenized" repertoires of the fetal thymus and gut. Hence, in normal mice *in utero*, the γδ TCR repertoire is influenced by additional factors that vary according to the site of expression. In the thymus, the influencing factor(s) causes cells that rearrange and express canonical γδ joins to dominate the repertoire above the contribution of cells, mature or immature, that express other junctions. Since fetal tissues are regarded as being isolated from major foreign antigen challenge, the influencing factors are possibly self antigens in those tissues. This is further evidence in support of the original hypothesis that at least some γδ cells interact with self-ligands (20). The data presented here imply that the influence extends to selecting γδ junctional sequences. This is consistent with the hypothesis of Lafaille *et al.* (21) and with those authors' data that $V_\gamma 5$ junctions can be varied in fetal thymi *in vitro* by manipulations that probably interfere with thymocyte–stromal interactions through the TCR (22, 23). In conclusion, distinct γδ repertoires are shown to be a feature of different fetal tissues *in vivo*, leading us to believe that within the γδ lineage, repertoires are shaped in the fetal mouse by tissue-specific selection events. The fact that the early fetal human thymus, unlike that of the mouse, is dominated by simple but not homogeneous joins (13) suggests that parallel influencing factor(s) may not be operating in the same way in both classes of animals. This could correlate with the fact that humans may not make parallel use of early fetal thymic cells, since their adult repertoires, by contrast to those of the mice, do not seem to include populations of cells with monomorphic receptors derived from early in ontogeny.

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