Divergent evolution of T cell repertoires: extensive diversity and developmentally regulated expression of the sheep $\gamma\delta$ T cell receptor

Wayne R.Hein and Lisbeth Dudler

Basel Institute for Immunology, Grenzacherstrasse 487, Postfach, CH-4005 Basel, Switzerland

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Sheep $\gamma \delta$ T cells express an unprecedented repertoire of antigen receptors contributed by increased diversity in both variable and constant region gene segments. Variable region diversity results mainly from the utilization of a large family of duplicated V δ genes that have retained two distinct hypervariable segments comparable with the complementarity determining regions present in other antigen receptor V genes. This implies that sheep V δ chains have been intensely selected during evolution, probably at sites involved in ligand recognition. The sheep $\gamma \delta$ heterodimer occurs in at least five isotypic variants formed by the association of a single $C\delta$ segment with one of five functional $C\gamma$ segments, each with distinctive hinge regions. Our analysis also shows that the establishment of a normal peripheral repertoire is both developmentally regulated and dependent on the continual presence of a functional thymus during ontogeny. The existence of an expanded V gene repertoire and multiple receptor isotypes together with the prominence of $\gamma \delta$ T cells in the sheep immune system argues that this lineage of T cells has a more elaborate functional role in this evolutionary pathway.

Key words: γ/δ T cells/gene expression/ontogeny/ repertoire

Introduction

Two distinct types of T cells, distinguished by the surface expression of either an $\alpha\beta$ or $\gamma\delta$ T cell receptor (TCR), develop independently as separate lineages in mammals (Philpott et al., 1992), they account quantitatively for the total pool of peripheral T cells and are the effectors of both cell-mediated immunity and T cell help. Although both lineages use similar mechanisms to diversify their antigen receptors (reviewed by Davis and Bjorkman, 1988; Strominger, 1989), clear differences exist between the repertoire of these two populations in humans and mice. Because there are only a few functional V gene segments at the TCR γ and δ loci, the combinatorial repertoire produced by gene recombination in the thymus is much smaller than for the $\alpha\beta$ TCR, which utilizes large pools of different gene segments (reviewed by Raulet, 1989; Bluestone et al., 1991; Porcelli et al., 1991). Furthermore, the repertoire that is actually available in peripheral tissues is even more severely restricted in the $\gamma\delta$ lineage since cells localizing at specific sites preferentially use certain V gene combinations in their receptors. For example, in humans, 70-90% of the $\gamma\delta$ T cells in blood express the V γ 9 and

V $\delta 2$ gene segments (Triebel *et al.*, 1988a,b; Casorati *et al.*, 1989), and in mice, $\gamma \delta$ T cells at different mucosal surfaces also have distinct and limited receptor diversities (Asarnow *et al.*, 1988; Itohara *et al.*, 1990). In some cases, but not in all, this limited combinatorial repertoire has been partly offset by non-germline encoded diversification at junctional regions and/or by the usage of multiple D elements (Tonegawa, 1983; Lafaille *et al.*, 1989; reviewed by Allison and Havran, 1991). The diversity of $\gamma \delta$ TCRs is thereby confined largely to short junctional regions whereas $\alpha\beta$ TCRs have more widely distributed patterns of variability (Schiffer *et al.*, 1986, 1992).

In the face of such a marked disparity between the repertoire of $\alpha\beta$ and $\gamma\delta$ T cells in humans and mice, it is perhaps hardly surprising that their reported functional properties also differ. While $\alpha\beta$ T cells recognize precisely an enormous range of antigens when presented as processed peptide fragments bound to polymorphic MHC molecules (Davis and Bjorkman, 1988), the potential of $\gamma\delta$ T cells to recognize diverse antigens, and the mechanism involved, remain controversial (reviewed by Matis and Bluestone, 1991; O'Brien and Born, 1991). The effector functions of $\gamma\delta$ T cells are also ill defined and no satisfying consensus has vet emerged about the overall role of these cells in the immune system. These factors, together with the relative scarcity of $\gamma\delta$ T cells in rodents and primates (~5% of blood lymphocytes) have led to the prevailing view that they may have a specialized role in defence against a limited number of antigens, especially at mucosal surfaces (Janeway et al., 1988).

More recent studies in artiodactyls, an order of animals that includes the ruminants and that diverged from the rodent-primate evolutionary stream around 100 million years ago (Novacek, 1992), have revealed a highly discordant representation of the two T cell lineages. In artiodactyls, $\gamma \delta$ T cells form a much larger proportion of the peripheral T cell pool (Hein et al., 1989; Mackay and Hein, 1989; Mackay et al., 1989; Clevers et al., 1990; Hirt et al., 1990). In the blood of young lambs, for example, $\gamma\delta$ T cells are a major lymphocyte population and account for 50-60% of T cells; they are still prominent in the blood of young adults, forming 20-30% of T cells (Hein et al., 1990a). Although some $\gamma\delta$ T cells become localized prominently at mucosal surfaces, a large mobile pool of cells recirculates between the blood, tissue and lymph and is widely disseminated throughout peripheral body compartments (Hein and Mackay, 1991). Chickens also have a high proportion of $\gamma\delta$ T cells (reviewed by Bucy et al., 1991) indicating that this might also have been the case in ancestral mammalian lineages and that during the mammalian radiation, $\gamma\delta$ T cells have been retained as a prominent immune component in some species but lost in others.

The frequency and physiological distribution of $\gamma\delta$ T cells in different animals therefore define a quantitative spectrum ranging between ' $\gamma\delta$ low' species such as rodents and humans on the one hand and ' $\gamma \delta$ high' species such as ruminants and chickens on the other. Current perceptions about the repertoire of $\gamma\delta$ T cells, their evolutionary development, thymic dependency and functional potential are based almost entirely on the analysis of a highly skewed sample of mammalian immune systems, the ' $\gamma\delta$ low' species, which should raise important questions about their wider relevance. The results reported here show that in a species at the other end of this spectrum, $\gamma\delta$ T cells have a significantly greater degree of repertoire complexity, the gene segments involved show hallmarks of ligand-mediated selection and the ontogeny of the peripheral repertoire is strictly regulated by the thymus. These striking molecular contrasts in conjunction with the quantitative differences evident between species argue that a spectrum of $\gamma\delta$ T cell function also exists, ranging from an adaptive but still elusive role in the ' $\gamma\delta$ high' species to probable redundancy in the $\gamma \delta$ low' species.

Results

To examine repertoire diversity, an anchored PCR technique utilizing 3' primers specific for known sheep constant region sequences was used to amplify and clone the $V\gamma$ and $V\delta$ regions being expressed in peripheral $\gamma\delta$ T cells. RNA was prepared directly from spleen or blood lymphocytes recovered ex vivo, thereby avoiding any procedural artefacts that might have been introduced during separation or enrichment of $\gamma\delta$ T cells. A total of 48 V γ clones and 62 V δ clones derived in this way from four fetuses at three stages of development (gestation length = 150 days), from two normal adult animals and from two adults that had been previously thymectomized in utero were fully sequenced (Figure 1 and Table I). We reasoned that this strategy would allow us to obtain an unbiased representation of the diversity within the recirculating peripheral repertoire, to determine whether the available repertoire varied at different stages of ontogeny and to assess the effect of fetal thymectomy on repertoire development.

Primary structure of $V\gamma$ chains

Ten distinct V region segments which could be divided into six families were identified in the 48 V γ clones sequenced (Figure 2). Two families, V γ 2 and V γ 5, containing four and two members respectively, probably arose by recent gene duplication since the V segments within each were highly homologous (~81-97% DNA identity). All other families were represented by single gene segments and the level of DNA identity between families ranged from ~40 to 75%. There were small differences in the lengths of individual V γ segments although all showed conserved codons indicative of immunoglobulin domains. The V γ 6 sequence is somewhat atypical in that the position of the initiation codon (ATG) preceding a long open reading frame predicts an unusually long signal peptide (Figure 2).

With rare exceptions, each V γ segment was rearranged specifically to one of five J γ elements that contain a conserved Gly codon (GGA) and vary slightly in length as shown (see Figure 2 and Table III). The J γ 2, J γ 3 and J γ 5 sequences share 71–79% DNA identity while J γ 1 and J γ 4 are more divergent both from the other J segments and from each other (DNA identity 47–59%).

Fig. 1. Schematic diagram showing the number of thymus-intact (Ti) and thymectomized (Tx) animals examined at different stages in development.

Table I.	Number of peripheral $V\gamma$ and $V\delta$ clones sequenced from
different	stages of development

Developmental stage	Fet	al (da	ays)	Adı	Adult			
	61	117	146	(Total fetal)	Tia	Tx ^b	Total all	
No. animals	2	1	1	(4)	2	2	8	
No. cDNA clones $V\gamma$	8	5	5	(18)	17	13	48	
νδ	5	7	9	(21)	23	18	62	

^aTi, thymus-intact.

^bTx, thymectomized.

Utilization of five diverse C_{γ} segments

By screening cDNA libraries, we have previously identified the sheep $C\gamma 1$ and $C\gamma 2$ gene segments. Southern blot analysis using these probes showed the likely presence of additional $C\gamma$ genes (Hein *et al.*, 1990b). In the present experiments, each amplified $V\gamma$ clone contained ~200 bp of C region sequence from which we identified three new $C\gamma$ segments. The five $C\gamma$ segments were expressed in a developmentally regulated way and each was specifically spliced to distinct sets of rearranged $V\gamma$ and $J\gamma$ segments (see below). The full coding region sequence of each new $C\gamma$ segment was then amplified from cDNA using a strategy of nested priming with appropriate $V\gamma$, $J\gamma$ and 3'UT region primers.

Over the full coding sequence, the five $C\gamma$ segments show 73-84% nucleotide identity and the immunoglobulin-like, transmembrane and cytoplasmic domains generally display well conserved features although the cytoplasmic region of $C\gamma 5$ is more divergent (Figure 3). However, the connecting peptides between the immunoglobulin-like and transmembrane domains are notably heterogeneous, ranging in length from 24 (C γ 5) to 75 (C γ 2) codons (Figure 3A and B; Hein et al., 1990b). Some residues are conserved between all sequences, including the four most membrane-proximal ones (SAYY) and a cysteine residue likely to form a disulphide bridge with the TCR δ chain (Figure 3B). In the more distal region of the connecting peptide, $C\gamma 1$, $C\gamma 2$ and $C\gamma 4$ each contain two additional cysteine residues at conserved positions and there is a five amino acid motif (consensus sequence TTEPP) in four $C\gamma$ regions ($C\gamma 1$ - $C\gamma 4$) (Figure 3B; Hein *et al.*, 1990b).

A large family of V δ genes

The 62 V δ clones sequenced contained 28 distinct V regions forming four families. Twenty-five of the V regions have patterns of sequence homology indicating that they are members of a recently duplicated family, V δ 1 (Figure 4A). The level of nucleotide identity between members of V δ 1 ranged from 79 to 97%. The other families represented by

																								-14				
VG1	(4912B	VG1)									GCC	TGTC	ACATO	TTGC	GAG/	AACCO	CCAG	TCTA	GCTC.	A(CTGTO	CCA	GGC	ATG	TTG	TGG	GCC	CTI
VG2.1	(48395)	VG1)											T-J	A-A-	G		TAG -								A	C	-T-	-C.
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VG2.4	(20138	vg/)	IC.	1117	AGAG	JAGA	ICC I	AGAI	MC II	. می مو	10	,			G		(-1C	L'TAT	r			-0-		C	C		-0-
VG3	(46218	VGI)													-	PPPG	AGCA	CAC-	C-		. GCG(5A			-CA	CCA	CTG	GA-
VG5.1	(46218)	VG2)																		TG	GC	3G			A	c		-C-
VG5.2	(38408)	VG2)							T.	ICTC1	r-c-i	A i			-C	TT ·			G	-CTG	G(3G			A	C		-C-
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								-1	1																16A	16B		
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VG2.1	-C-	T-	C					-C-		-TG		G				ATG	G	A	-AG	A			A-T		AAG	GCA		GT-
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VG4 (4	621BVG	4)																					//	T	-cc	CG -	AGA	CC
VG5.1	-CA	T-	C		G	T		-GG		-TG									• • •	TT -		-T-	A		-GA	AAG	A	• • •
VG5.2	-CA	GT-	C		G	T		-CC	A	- TG										TT-		- T -	A	G	-GA	AAG	A	
VG6	CGC	G-C	C-C	CTC	-GG	GCC	CTC	G-G	T	C-A	GGT	ATC	CAG	CAG	G-G	ATC	-GG	CTG	TCA		CG -	C	G	-TG	GT-		-GG	TCC
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VG2.3		G TCA	T	GT-	GTA	A	GA-			C		• • •			:::		C				• • •	TG-			TTC			
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VG5.1	(GG		G	G		C			C				C	GGG	-CT	-TC					T			T	G		
VG5.2	(GG		G	G		C			C					GGG	-C-	-CC			+		T			T	G		
VG6	G-G (G-T -GA	G	GTG	A	A-G	C	C	c	G-C			-GT	AGG	TC-	G	G-T	C	G			TG-	-T-	CGC	G	-T-		0
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VG1	ATG (cer cee	CGA	CGT	CTT	TTC	TAC	TAC	GAT	GTC	TAC	TAC	TCC	AAG	ATT	GAG	TTC	GAA	TCA	GGA	ATC	GAT	ала	GCA	AAA	TAC	AGT	GTT
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VG3	CAG	TTG	GAG	CAC	G	G-T	T	GTG	ATC	TC-	AC-	ACA	A-T	GCA	GCT	CGA	CAT	C	GT-	GAC	GGG	A-G	C			ATT	GAG	-C#
VG4	GA- (C-C -TG	AA -	A	A-C	C-T	T	GG-	TCA	-CT	A	AGT	-AT		C-G	C	AAA	CCT	AAT	TCC	CGT	TTG	G-G	ATG	G-T	A	-AA	AAG
VG5.1		C		C	-C-	A		T	C	AG-		A	A		cc-	-T-	T	-T-				TG-	GG -	A			CA-	
VG5.2		C		C		A		T	C	AG-		A	A		cc -	-T-	T	-T-				TG-	GG -	A			CA-	0
VG6	CAG	CT	GAG	A-G		C	A-T	CTA	-cc	T-G	-C-	A-G	CGG	G-T	G-G	C	-GG	T	G	-TC	C-A	-GA	GGT	-AT		GT -	C	-C#
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VG3	AGA	A C	T	CG-	ATG	TTC	-CT	-CG	-cc	C	À-G	G-A	AAT	TT -	GTA		X-X	GAA		GTG	C	-TT					-G-	
VG4	A	A				-TC	TTT			C-G	AT-		AAT	C	GTT	-TC	A-G	TCC		GAA	-cc	T				C	TGC	
VG5.1		GC	A		G	T		T	-C-		A	G	G			C	GCA			C	C	-TA				A	A	
VG5.2		GC	A		G			T	-C-		G					C	GCA			C	C	-TA					A	
VG6	GCC	cccc	-GG	-AT	C	- A -		-G-	-CC	A-G	T-C	T-G	AGG	G		-CG	A-G	C		GAG	A	CT-					-C-	
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VG2.3	-GG	G.	AC			-CA	GGC	TGG	G	AAG	-TA	T		-A-	GC -	AC-	-AG	C	-T-					(3)	, te			
VG2.4	-AG	AAGC	TCGCG			-CA	GGC	TGG	G	AAG	-TA	T		- A -	GC -	AC-	-AG	C	-T-					(3)	(ال ف			
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VG4						GGG	C	G	GTA	T	GGT	-AA		T	G	C	A	A	- T -	CCT	C			(J)	35)			
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VG5.2	A-A	C	TC			-CG	GGC	TGG	G	AAG	-TA	т		GA -	GG -	GC-	AA -	A	- T -	G	A	C-T	C	(J(G2)			
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Fig. 2. Nucleotide sequences of sheep $V_{\gamma} - J_{\gamma}$ regions. Dashes indicate nucleotide identity and gaps introduced to maximize alignment are shown as dots. Additional nucleotides at the start of the 5'UT region of $V_{\gamma}6$ are not shown in the alignment but are included in the database entry.

single V segments, V $\delta 2$, V $\delta 3$ and V $\delta 4$, diverge greatly from the V $\delta 1$ group and from each other (DNA identity 42-67%). The V $\delta 1$ coding regions are remarkably uniform in length and contain long stretches of relatively invariant sequence with two main regions of variability around codons 29-31 and 54-57. In Southern blot analysis of genomic DNA digested with several enzymes, a V $\delta 1.1$ probe hybridized to 8-15 bands confirming the presence of a large family of related genes (data not shown).

As in the human and mouse homologue, the sheep TCR δ locus is located on the chromosome within the TCR α locus (Hein *et al.*, 1991). Some V genes are shared between these two loci in humans and mice and may contribute to either an $\alpha\beta$ or $\gamma\delta$ TCR (Takihara *et al.*, 1989; Miossec *et al.*, 1990). We therefore tested whether this might occur with the sheep V δ 1 family by PCR amplification using a primer for the conserved 5'UT region of V δ 1 members (sequence 5'-TCTCAGCTTGAGGCAG-3') in combination with a C α specific primer. Under these conditions, we were unable to amplify any product from peripheral lymphocyte cDNA of two normal 1 year old animals, as assessed by ethidium bromide staining of agarose gels. Control PCR experiments using a combination of the 5'UT V δ 1 and C δ primers gave a prominent amplified band as expected (data not shown).

Comparative alignments of sheep TCR δ clones allowed us to identify three related J δ elements (Figure 4B). In contrast to the V γ -J γ junctions, there appeared to be no particular bias in terms of V δ -J δ combinations.

Homology of mammalian $V\gamma$ and $V\delta$ chains

To assess the evolutionary relatedness of TCR $\gamma \delta$ V regions, amino acid sequences representative of the major functional $V\gamma$ and $V\delta$ families of sheep, humans and mice were compared (Table II). The different $C\gamma$ sequences of these three species show 51-63% amino acid identity. We therefore chose an arbitrary value of 60% identity as being an indicator of likely pairs of homologous sequences. By this criterion, four sheep V regions have clear homologues in either humans or mice. The most striking example is the sheep $V_{\gamma}4$ sequence, which has 74.7% protein identity to mouse $V\gamma 3$. Other examples, and the human or mouse homologue, include $V\gamma3$ (human $V\gamma10$, 60.4%), $V\delta1.1$ (human V δ 1, 69.5%) and V δ 4 (mouse V δ 5, 61.8%; human V δ 3, 65.3%). A number of V δ regions showed 50-60% identity, in some cases to more than one sequence, while the majority of $V\gamma$ regions had no clear homologue in other species. Among the sheep sequences, there are no homologues of the two most frequently expressed human genes, $V_{\gamma}9$ and $V\delta2$ (see Table II).

CG3 CG4 CG5 ACT GGA ACA TAC CTT TGT CTT CTG GAG AAA TTT TTC CCT GAT ATT AAG GTT TAT TGG AAA GAA AAG GAT GGC AAC AGA GCT CTG CG1 CG2 CG3 CG4 $\begin{array}{c} \mathsf{C}_{\mathsf{C}} & \cdots & \mathsf{C}_{\mathsf{T}} & \cdots & \mathsf{C}_{\mathsf{T}} & \cdots & \mathsf{C}_{\mathsf{T}} & \cdots & \mathsf{C}_{\mathsf{T}} & \mathsf{C} & \mathsf{C} & \mathsf{C} &$ CG5 CG1 CG2 CG3 CG4 CG5 CG1 CG4 CG5 CG2 CG3 CG1 CG2 CG3 CG4 CG5 CG1 CG2 CG3 CG4 CG5

 ATC
 A CGI CG2 CG3 CG4 CC5 В IG DOMBIN CG1 CG2 CG3 CG4 CONNECTING PEPTIDE CYT CG1 CG2 CG3 CG4

Fig. 3. (A) Nucleotide and (B) amino acid sequences of three new sheep C_{γ} segments. Sequences are shown from the first residue of the constant region up to the stop codon. The immunoglobulin domain, connecting peptide, transmembrane and cytoplasmic regions are indicated above the amino acid sequences. The positions of conserved cysteine residues found in all chains (\blacktriangle) and additional cysteine residues located in the connecting peptide of the $C_{\gamma}1$, $C_{\gamma}2$ and $C_{\gamma}4$ chains (\bigtriangleup) are indicated. The $C_{\gamma}1$ and $C_{\gamma}2$ sequences were determined previously (Hein *et al.*, 1990b) and are shown for comparison.

Δ		-20
	(4910BVD8)	GACAGATCTCAGCTTGAGGCAGAACTGAGCACATTTGTGCAGGGGGAATCCATGCCTC ATG CCG CTC TCC AGT
VD1 2	(49128004)	GG
VDI 3	(49128004)	GCAGGG
VD1 4	(49128003)	
VD1 5	(49128003)	
VD1 7	(49120003)	
VD1 0	(49120002A)	
VD1 9	(49128/010)	
VD1 10	(491000004)	
VD1.10	(49105004)	
VD1.11	(39400000)	
VD1.12	(46010000)	
VD1.13	(4621BVD3)	
VD1.14	(46210007)	
VD1 19	(40216007)	
VD1 10	(4074000)	
VD1 20	(\$0138003)	
VD1 21	(501 30002)	
VD1.21	(50138004)	
VD1 24	(49749/07)	
VD1 25	(49748007)	
VD1.23	(39400005)	
VD2	(38408707)	
VD4	(4839SVD1) TTC	
	(105550101) 110	
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b1.13 b21.18 b701.19 b701.20 b71.21 b701.22 b701.23 b701.24 b701.25 b703 b704 b705 b705 b707 b708 b709 b701	 T AGC GA CA CA CA CA CA CA	T T T GGA G-T AAA 	G GAG GTC GCA T	 ATG TTC GAT C	A G G AG- ATT AG- ATT 	TAC T-T TCC	-T- -T- -T- GAA -TG GTC ATC	T T T T T T T T T	C C G C C C C C		 G TA- TA- GAC ACC -T	TCT A-C	TA- CA- C TA- C TA- C TCA AC- TCA AC- TCA 	 T T AGT AGC AG- GCC T T T T	GT GT TT GT TCC A-T TCC TTA TTA 	-GT -AT -TC AT- -AT -AT -AT CAA CA- CGT CAA CA- 	GGG GGG CAG CAG G GAT CTG GAT 	T T T T T GCG GCG GAA 			G-C G-C G-T T-T G-T CG- GC GC GC GC GC GC GC GC GC GC GC GC GC	-G- -G- -G- -G- -G- -T TC CGG AA G T T 		GTG AGA 90 TTTC	G G GAA T TGT 	A G A T-T ACT GCT GCT 		
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D. 1. 8 D. 1. 19 D. 1. 19 D. 1. 19 D. 1. 19 D. 1. 19 D. 1. 20 D. 1	 T AGC GA -A CA CA CA CA CA	T T T GGA GT 	GCA GCCA GCCA GCCA GCCA GCCA GCCA GCCA	GAT GAT GAT C C C C C C C C	AAA T G AG AG- AG- AG- AG- AG- T 	TAC T-T TCC C	-T- -T- -T- GAA GTC ATC 	T T T T T T T T T 	C G G C C C TAC AGC 	C GG CTTC C		ATT ATT ATT ATT ATT ATT ATT	TA- CA- C TA- C TA- C CAA AC- TCA TCA C C		GT G-T TT- GT TCC A-T TCC TTA A-G TCC TTA CC- C C	-GT -AT -TC ATTC CAA CCCGT CAA CCCGT G GG GG GG GG GG G-	GGG GGG GGG CAG CAG CAG CAG CAG CAG CAG	T T T T T T GCG GCG GCA 			G-C G-C T-T T-T G-T G-T G-T G-T G-T GC GC GC GC GC GC GC GC GC GC GC GC GC	-G- -G- -G- -G- -G- -G- -G- -G- -G- -G-	 	GTG GTG AGA 90 TTC 		A A GCT TT ACT GCT 		
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1.1.1.3 1.1.1.9 1.1.1.9 1.1.1.9 1.1.1.2 1.1.1.2 1.1.1.2 1.1.2 1.1.2 1.1.2 1.1.2 1.1.3 1.1.1 1.1.2 1.1.1 1.1.2 1.1.1 1.1.2 1.1.3 1.1.4 1.1.5 1.1.1	 T AGG GA A CA	T T GGA G-T AAA C G G G G G G G	G	GAT GAT C GAT C C C C C C C C-	A G G AG- AG- AG- AG- AG	TAC T T T T T T T C C C	T- GAA TG GTC ATC T- GTC	T T T T T T T T T 	C G G C C C TAC AGC C A- AGC 				TA- CA- TA- TA- CAA AC- TCA TCA		G G-T TT G TCC A-T TCC TTA A-G TCC TTA 	-GT -AT -TC -CAA -CC -CC -CC -CC -CC -CC -CC -CC -	GGG GGG GGG CAG GGG CAG GGG CAG G	T T T T T T T T T T T 		-T- -T- A A GA TTT TCT 	G-C G-C G-T T-T T-T G-T G-T G-T G-T G-T G-T G-T	-G- -G- -G- -G- -G- -T- -T- -T- -T- -T-	 	GTIG AGA 90 TTIC 	G G 	A A GCT T-T ACT GCT 		CC CC CC
<pre>D1.13 D1.14 D1.14 D1.14 D1.14 D1.14 D1.14 D1.2 D1.2 D1.2 D1.2 D1.2 D1.2 D1.2 D1.4 D1.5 D1.4 D1.5 D1.4 D1.5 D1.4 D1.5 D1.4 D1.5 D1.4 D1.5 D1.1 D1.1 D1.1 D1.1 D1.1 D1.1 D1.1</pre>	 T AGC GA -A C	T T 	GCA GCA GCA GCA GCA GTC GCA GTC GCA GTC GCA CA GTC GCA GTC T	GAT TTC GAT C C C C C C C C-	A G G G AG- AG- AG- A	TAC T-T TCC T-T TCC	T- -T- -T- GAA -TG GTC ATC TG GTC	T T T T 	C G G C C C C TAC AGC AAGC 				TA- CA- C TA CAA AC- TCA TCA TCA		G G-T TT G-T TCC A-T TCC TCC TTA A-G TCC TCC TCC C C C	-GT -AT -TC -AT -AT -AT -AT -AT -AT -G CAA CCA- CGT G G-G G-G G-G G-G G-G G-G G-G G-G G	GGG GGG GGG CAG CAG CAG CAG CAG CAG CAG	T T T T T T G GCG GCG GAA 			G-C G-C G-T T-T G-T T-T G-T G-T G-T G-T G-T G-T	-G- -G- -G- -G- -T- CGG CGG AAG T T- 		GTTG GTTG AGA 90 TTTC 		A A A TT ACT GCT GCT 		
<pre>D1.13 D1.14 D1.14 D1.14 D1.14 D1.14 D1.14 D1.14 D1.12 D1.12 D1.12 D1.1 D1.2 D1.3 D1.4 D1.5 D1.7 D1.4 D1.5 D1.7 D1.4 D1.5 D1.7 D1.4 D1.15 D1.7 D1.10 D1.11 D1.12 D1.14 D1.15 D1.14 D1.15 D1.14 D1.15 D1.14 D1.15 D1.20 D1.24 D1.24 D1.25 D1.</pre>	 T AGC GA C	T T GGA G-T AAA C C G	G	GAT GAT C C C C C C C C-	A G G G AG- AG- AG- A	TAC TTAC T		T T T T T T T T T- T- T- T- 	C G C C C C C TAC AGC AA 				TA- CA- TA- TA- CAA AC- TCA TCA TCA		G GT TT GT TCC A-T TCC TTA A-G TCC TTA C	-GT -AT -TC -AT -TC -AT -TC -AT -G -G -G -G -G -G -G -G -G -G -G -G -G	GGG GGG GGG GGG CAG GGG CAG GGG G	T T T T T G GCG GCG GCG GCA 		-T	G-C G-C G-T T-T G-T G-T G-T G-T G-T G-T G-T G-T	-G- -G- -G- -G- -G- CGG CGG T T- 		90 TTC 		A GCT GCT 		
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D. 1.3 D. 1.4 D. 1.3 D. 1.4 D. 1.5 D. 1.4 D. 1.5 D. 1.4 D. 1.5 D. 1.4 D. 1.5 D. 1.4 D. 1.5 D. 1.4 D. 1.5 D. 1.1 D. 1.2 D. 1.1 D. 1.1 D. 1.1 D. 1.1 D. 1.1 D. 1.2 D. 1.1 D. 1.1 D. 1.1 D. 1.2 D. 1.1 D. 1.1 D. 1.1 D. 1.2 D. 1.1 D. 1.2 D. 1.1 D. 1.2 D. 1.1 D. 1.2 D.	 T AGC GA C	T T GGA G-T AAA AAA C C C G	GCA GCA GCA GCA GTC GCA GTC GCA 	GAT GAT TTC GAT C C C C C C C C-	A G G AG		-TT- -TG- GAA ATC	T T T T T T T T T 	C G C C C TAC AGC -A- TAC 				TA- CA- TA- TA- TA- TCA AC- TCA TCA TCA TCA TCA TCA		G G-T TT- G TTCC A-T TCC TTA A-G TCC TTA C C C C C C C C C C-	-GT -AT -TC -TC -TC -TC -TC -TC -TC -TC -TC -T	GGGG GGG CAG GG- GAT CTG GAT CTG GAT 	T T T T T T G GCG GCG GCG 			G-C G-C G-T T-T G-T G-T G-T G-T G-T G-T G-T G-T	-G- -G- -G- -G- -T T CGG AAG T T T- T- T- T-		90 TTC 	G G GAA T TGT 	A A 		CC CC
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1.1.1.3 1.1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.3 1.1.4 1.1.5 1.1.6 1.1.1 1.1.2 1.1.3 1.1.1 1.1.2 1.1.1 1.1.2 1.1.1 1.1.2 1.1.1 1.1.2 1.1.1 1.1.1 1.1.1 1.1.2 1.1.3 1.1.1 1.1.1 1.1.2 1.1.3 1.1.1 1.1.2 1.1.2 1.1.3 1.1.1 1.1.2 1.1.2 1.1.2 1.1.2 1.1.2 1.2.2 1.2	 T AGC GA -A C	T T GGA GT AAA AAA G G G G G G G G G G G G G G G 	GCA GCA GCA GCA GCA GTC GCA 	ATG GAT TTC GAT C C C C C C C C C C C C C C C C C C C	A G G G AG- AG- AG- AG- AG-	TAC T-T TCC C C C C C C C C-	-TTTG GAA GTC ATC -TG GTC -TG GTC -TG -TG -TG -TC	T T T T T- ATTT CTG 74A 	C G G C C C TAC AQC 			ATT A-C ATT A-C ATT 	TTA- CA- CA- TA- TA- CAA AC- TCA TCA TCA CAA AC- TCA TCA 		G G-T TT- G TCC A-T TCC TTA A-G TCC TTA C C C C C C-	-GT -AT -TC -TC -TC -TC -TC -TC -TC -TC -TC -T	GGGG GGGG CAG GGAT CTG GAT CTG GAT CTG 	T T T T T T T T G GCG GCG GCG 			G-C G-C G-T T-T G-T G-T G-T G-T G-T G-T G-T G-T	-G- -G- -G- -G- -T- T CGG AAG -T- -T- -T- -T- -T- -T- -T- -T- -T- -T		GTNG AGA 90 TTNC 		A G A TT ACT GCT 		
10.1.3 10.1.3 10.1.3 10.1.3 10.1.3 10.1.3 10.20 10.20 10.21 10.22 10.22 10.23 10.24 10.25 10.2 10.1 10.2 10.3 10.4 10.5 10.4 10.5 10.1 10.1 10.1 10.1 10.1 10.1 10.1 11.1 11.12 11.11 11.12 11.14 11.15 11.20 11.21 11.14 11.15 11.20 11.21 11.22 11.22 11.24 11.25 11.25 12.25 12.25 12.25 </th <th> T AGC GA -A C</th> <th>T T GGA G-T AAA AAA AAA C C C G G G G G G G G G G G G G G C</th> <th>GCA GCA GTC GCA GTC GCA GTC GCA GTC GCA GTC CA GCA GTC GCA GCA GTC GCA GCA GTC GCA GCA GTC GCA GTC GCA GTC GCA GTC GTC GCA GTC GTC GTC GTC GTC GTC GTC GTC GTC GTC</th> <th>GAT TTC GAT GAT C</th> <th>A G G G AG- AG- ATT G G</th> <th>TAC T-T TCC C</th> <th></th> <th></th> <th>C G G C C C TAC AGC C- A- TAC A- A- A- A- </th> <th></th> <th></th> <th>ATT </th> <th>TA- CA- CA- TA- TA- CAA AC- TCA TCA TCA</th> <th></th> <th>G GT TT- G TCC TCC TTA AG TCC TTA AG TCC TTA C C C C C C GG GG</th> <th>-GT -AT -AT -TC -TC -TC -TC -TC -TC -TC -TC -TC -T</th> <th>GGGG GGG CAG CAG GAT CTG GAT CTG GAT </th> <th>T T T T T T T T T T</th> <th></th> <th>T- T- A A TTTT TCT -</th> <th>G-C G-C G-T T-T -GG G-T -GG GCT GC-T -GG GCT </th> <th>-G- -G- -G- -G- -G- -T- CGG T -T- -T- -T- -T- -T- -T- -T- -T- -</th> <th></th> <th></th> <th></th> <th>A TT </th> <th></th> <th>CC </th>	 T AGC GA -A C	T T GGA G-T AAA AAA AAA C C C G G G G G G G G G G G G G G C	GCA GCA GTC GCA GTC GCA GTC GCA GTC GCA GTC CA GCA GTC GCA GCA GTC GCA GCA GTC GCA GCA GTC GCA GTC GCA GTC GCA GTC GTC GCA GTC GTC GTC GTC GTC GTC GTC GTC GTC GTC	GAT TTC GAT GAT C	A G G G AG- AG- ATT G G	TAC T-T TCC C			C G G C C C TAC AGC C- A- TAC A- A- A- A- 			ATT 	TA- CA- CA- TA- TA- CAA AC- TCA TCA TCA		G GT TT- G TCC TCC TTA AG TCC TTA AG TCC TTA C C C C C C GG GG	-GT -AT -AT -TC -TC -TC -TC -TC -TC -TC -TC -TC -T	GGGG GGG CAG CAG GAT CTG GAT CTG GAT 	T T T T T T T T T T		T- T- A A TTTT TCT -	G-C G-C G-T T-T -GG G-T -GG GCT GC-T -GG GCT 	-G- -G- -G- -G- -G- -T- CGG T -T- -T- -T- -T- -T- -T- -T- -T- -				A TT 		CC
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Fig. 4. Nucleotide sequence of sheep (A) $V\delta$ and (B) $J\delta$ segments. Dashes indicate nucleotide identity and gaps introduced to maximize alignment are shown as dots. Only those sequences obtained as full-length V regions are shown. A further four truncated V region sequences have been included in the database entry.

Hypervariable elements within $V\gamma$ and $V\delta$ regions

VD1.21

The translated protein sequences of the full-length V regions obtained (nine V_{γ} and 24 V δ , Figures 2 and 4) were analysed using the formula of Wu and Kabat (1970) to determine the variability of amino acid residues at each position of the two chains (Figure 5). The V δ chains contain two clear peaks of variability around residues 29–31 and 54–57, while other parts of the chain have a relatively invariant framework character. In contrast, comparable hypervariable regions were not obvious in the V γ chains although the three most variable residue positions were broadly clustered towards the N terminus of the mature protein (residues 19–40). The most 3' V region residues and the V–J γ or V–D–J δ junctional regions are also highly variable (Figure 5 and see below).

Regulated development of the peripheral repertoire

A comparison of gene expression in peripheral $\gamma\delta$ T cells at three different stages of fetal ontogeny and in normal adult animals revealed distinct patterns of repertoire development. With the exception of the V γ 2 family of genes, which was expressed at all developmental stages examined, discrete sets of V genes were used in the fetal and adult periphery (Table III). The relative frequency of usage of 'fetal specific' V genes (V γ 4, V γ 5, V γ 6, V δ 2, V δ 1.12–1.18, V δ 2, V δ 3 and V δ 4) also changed with advancing fetal age. V genes detected only in the periphery of normal adult animals included V γ 1, V δ 1.2, V δ 1.4–1.8, V δ 1.10 and V δ 1.11. A few members of the large V δ 1 family of genes were expressed in both fetuses and adults (Table III).

The TCR γ clones derived at all stages of development

	Sheep				Sheep							
	$\frac{1}{V\gamma 1}$	Vγ2.1	V _Y 3	V _γ 4	Vγ5.1	Vγ6		<u>Vδ1.1</u>	Vδ2	Vδ3	Vδ4	
Mouse												
Vγ1.1	_	_	40.4	-	-	—	Vδ1	-		_	-	
$\dot{v_{\gamma 2}}$	-	_	41.4	-	-	-	Vδ2	-	-	-	-	
V ₂ 3	-	_	_	74.7	-	-	Vδ3		_	-	-	
$V_{\gamma}4$	_	-	-	_	_	-	Vδ4	52.7	48.4	-	-	
V~5	41 4	46.4	_	_	44.2	_	Vδ5	-	-	-	<u>61.8</u>	
15							Vδ6	57.4	53.1	40.4	-	
							νδ7	-	-	-	-	
Human												
Ι Vγ2	44.7	50.0	-	-	49.5	-	V δ1	<u>69.5</u>	57.6	41.3	-	
Π V ₂ 9	_	-	-	_	_	-	Vδ2	-	-	-	-	
$III V_{\gamma}10$	_	-	60.4	-	_	-	V δ3	-	-	-	<u>65.3</u>	
$IV V_{\gamma}11$	-	_	_	_	_	_	Vδ4	55.1	57.4	43.4	-	
							Vδ5	_	-	-	-	

Table II. Protein identity among families of functional mammalian $V\gamma$ and $V\delta$ chains

The percentage amino acid identity between compared sequences is shown. Identities >60%, which may indicate homologous genes, are underlined. (-) indicates identity below 40%. The nomenclature and source of human and mouse V region sequences are as follows: mouse V_{γ} and V_{δ} (Raulet, 1989), human V_{γ} (Lefranc and Rabbitts, 1990), human V_{δ} (Hata *et al.*, 1989; Takihara *et al.*, 1989).



Fig. 5. Variability plot of sheep TCR γ and δ chains. The variability of amino acid residues at each position in the two chains was calculated by the method of Wu and Kabat (1970). The signal peptide sequences are underlined.

showed nearly invariant patterns of rearrangement between particular $V\gamma$ and $J\gamma$ segments and splicing to distinct $C\gamma$ regions as shown in Table III. Among the 35 clones sequenced from fetuses and normal adults, only a single exception to this rule was found whereby one adult clone contained the $V\gamma 2.1$ gene segment combined with $J\gamma 1C\gamma 1$ rather than $J\gamma 3C\gamma 3$.

Changes in V gene repertoire after fetal thymectomy

To examine the role of the thymus in the development of the peripheral $\gamma\delta$ repertoire, two fetal lambs were surgically thymectomized *in utero* on days 63 and 73 of gestation. The peripheral repertoire of these animals was then assessed by sequencing a total of 13 V γ and 18 V δ clones when they had reached 6 and 12 months of age respectively.

A number of clear differences were evident between the peripheral TCR γ repertoire of thymectomized (Tx) and thymus-intact (Ti) animals. First, we did not detect any usage of the V γ 1 gene in blood-borne lymphocytes of Tx animals (Table III). Secondly, within the V γ 2 family, the V γ 2.3 segment was expressed predominantly rather than V γ 2.1.

720

Finally, there was an increased frequency (3/13 clones) of V-J-C gene combinations distinct from the rearrangement and splicing patterns seen in Ti animals and a new $J\gamma$ segment $(J\gamma 6)$ joined to $C\gamma 5$ was identified in one clone (see Table III).

The differences between V δ gene usage in Ti and Tx animals were even more striking. Apart from two gene segments, V δ 1.9 and V δ 1.10, the peripheral repertoire of Tx and Ti animals was completely distinct and several new members of the V δ 1 family (V δ 1.19-V δ 1.25) were detected. In addition, the blood of both Tx animals contained $\gamma\delta$ T cells expressing some V δ gene segments (V δ 1.12, V δ 1.13, V δ 3 or V δ 4) that previously had been detected only in the periphery of fetuses (Table III).

$V - J\gamma$ and $V - D - J\delta$ junctional regions

TCR γ and δ clones showed extensive junctional diversity at all developmental stages examined. However, since the genomic sequences of sheep V, D and J gene segments are not yet available, a detailed analysis of these regions is not possible. The comparative lengths of this section of the

Diversity of	the sheep	$\gamma \delta TCR$
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$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Stage of development	Fetal (day	/s)		Adult			
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$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	TCR-ስ							
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	$\frac{10100}{V\gamma 1}$				++++]	Jγ1Cγ1		
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	V _{2.1}	++	+		ر [++++++++		- 	1
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	2.2	+	,		++	$I_{2}I_{2}C_{2}I_{-1/20}$		In/1Co/1 1/12
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	2.3	++	+	+	+	Jy/Cy/1 1/20	+++++++	In 3 Cov1 2/12
$V_{\gamma3}$ ++ +	2.4		+			5 / 5 C / 5 19/20	+	Jy3Cy1 2/12
v_{73} $++$ $+$	V. 2				, ī			1 1 1 1 1 1 1 1 1 1
$V_{\gamma4}$ + $J_{\gamma2}C_{\gamma2}$ $V_{\gamma5.1}$ + $J_{\gamma2}C_{\gamma2}$ 5.2 ++ $J_{\gamma4}C_{\gamma4}$ $TCR-\delta$ $J_{\gamma4}C_{\gamma4}$ $TCR-\delta$ ++++ 12 + 1.3 ++++++ 1.6 +++++ 1.6 +++++ 1.7 + 1.8 +++++ 1.9 + 1.10 ++++ 1.11 ++++ 1.13 ++++ 1.14 ++++ 1.16 + 1.11 ++++ 1.12 + 1.13 ++++ 1.14 + 1.16 + 1.17 + 1.20 + 1.21 + 1.23 + 1.24 + 1.25 + $V62$ + $V62$ +	۷۶			++	+	1.50.5	+	JYOCYS
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	V~4			+		$J\gamma SC\gamma S$		
V_{70} ++ $J_{72}C_{72}$ S_2 ++ $J_{74}C_{74}$ TCR_3 +++ $J_{74}C_{74}$ V_{81} + +++ 12 + +++ 13 +++ +++ 1.3 +++ +++ 1.6 ++ ++++ 1.6 +++++ +++++ 1.9 + +++++ 1.10 +++++ +++++ 1.11 +++++ +++++ 1.16 + +++++ 1.18 + + 1.20 + +++++ 1.21 + ++++++++++++++++++++++++++++++++++++	• / +			I	ر ۲			
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	$\sqrt{\gamma}$ 5.1			+		$J_{\gamma}2C_{\gamma}2$		
V_{76} $+++$ j $J_{74}C_{74}$ $\frac{TCR-\delta}{V\delta l.1}$ + ++++ $l.2$ + ++++ $l.3$ ++ ++++ $l.4$ + ++++ $l.5$ + ++++ $l.6$ ++ ++++ $l.7$ + +++++ $l.8$ +++++ ++++ $l.9$ + +++++ ++++ $l.10$ +++++ +++++ $l.11$ +++++ +++++ $l.12$ + +++++ $l.13$ +++++ +++++ $l.16$ + ++++ $l.16$ + ++++ $l.16$ + ++++ $l.12$ + ++++ $l.20$ + +++++ $l.22$ + +++++ $l.24$ + +++++++++++++ $l.25$ + ++++++++++++++++++++++++++++++++++++	5.2		++		Ľ			
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	Vγ6	+++			J	$J\gamma 4C\gamma 4$		
$V\delta I.$ + ++++ 1.2 + 1.3 ++ 1.4 + 1.5 + 1.6 ++++ 1.7 + 1.8 +++++ 1.9 + +++++ +++++ 1.10 ++++ 1.11 ++++ 1.12 + 1.13 ++++ 1.14 + 1.15 + 1.16 + 1.17 + 1.18 + 1.19 ++++ 1.20 + 1.21 + 1.22 + 1.23 + 1.24 + 1.25 + V δZ + V δZ + ++++++++++++++++++++++++++++++++++++	TCR-δ							
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	<u>Vδ1</u>			+	+++			
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1.2			•	+			
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	1.3			++	+++			
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	1.4				+			
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1.5				+			
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1.6				++			
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1.7				+			
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$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1.9	+			++++		++	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1.10				++		+	
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$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1.24						+	
$\begin{array}{cccc} V\delta 2 & + \\ V\delta 3 & + + & + + + \\ V\delta 4 & + + + & + & + \\ & & & + & + \\ & & & + & +$	1.25						+	
Vδ3 ++ ++++	Vδ2		+					
	Vδ3		+ +				++++	
	Vδ4	+++		+			+	

Each (+) indicates the expression of the particular V gene segment in a cDNA clone. The J_{γ} and C_{γ} segments to which each V_{γ} region was rearranged and spliced is shown.

^aTi, thymus-intact.

^bTx, thymectomized.

molecule are summarized in Table IV. The junctional regions of γ chains were short, containing up to four additional codons between predicted V and J segments. In several clones, codons appeared to have been removed from the ends of participating $V\gamma$ and $J\gamma$ gene segments during rearrangement. There was no clear trend towards differences in junctional length of γ chains at different developmental stages. The junctional regions of δ chains were more variable in length and contained from one to 18 additional codons between identifiable V δ and J δ segments. These regions were longer in normal adult clones than in clones derived from either fetuses or thymectomized adults (Table IV).

Fable	IV.	Average	number	of	codons	at	junctional	regions
	- · ·			•••			Junetional	

	$V\gamma - J\gamma$	$V\delta - (NDN) - J\delta$
Fetus (days)		
61	1.7	7.6
117	1.0	9.7
146	1.4	8.0
Adult		
Ti ^a	1.5	12.0
Tx ^b	2.0	9.3

^aTi, thymus-intact.

^bTx, thymectomized.

Discussion

Our analysis of $\gamma\delta$ TCR diversity in a mammal divergent from primates and rodents indicates that different levels of germline complexity have developed at the TCR γ and δ loci in separate evolutionary pathways. Sheep contain five functional C_{γ} segments, all of which are about equally related to each other by sequence, and, at a lower level, to their human and mouse homologues. Human DNA contains two $C\gamma$ genes which are nearly identical in sequence, apart from differences in length produced by a duplication or triplication of the exon encoding the connecting peptide (Lefranc and Rabbitts, 1985; Buresi et al., 1989). Three of the C_{γ} segments in mice are also closely related and differ at only a few residues, while the fourth $C\gamma$ gene is more divergent (Raulet, 1989). These facts suggest that the five $C\gamma$ genes in sheep are descendants of an ancestral pool that existed before the primate-rodent-artiodactyl evolutionary pathways diverged about 100 million years ago (Novacek, 1992): humans and mice have retained fewer genes from this original pool, some of which have been duplicated since divergence and, in one case in the mouse, mutated to become a pseudogene. At present we do not know whether sheep DNA contains functional $C\gamma$ regions or pseudogenes in addition to the expressed segments detected in the present experiments; future genomic analyses will resolve this issue.

The likely structure of the ancestral mammalian $C\gamma$ genes is less clear. Although all mammalian $C\gamma$ segments have well conserved immunoglobulin-like, transmembrane and cytoplasmic domains, the connecting peptide regions differ markedly both within and between species, perhaps reaching an extreme example in sheep where in some chains the connecting peptide region contains extra cysteine residues and other motifs not present in human and mouse C_{γ} chains. These differences probably arose from the modification of ancestral genes by differential deletion, duplication or triplication of the short exon encoding this region, as occurred in the human $C\gamma^2$ gene (Lefranc and Rabbitts, 1985; Buresi et al., 1989), although the likely sequence of these events remains unclear and may have differed between species. So far, four different C_{γ} transcripts that are similar in structure to the sheep have been detected in cattle (Takeuchi et al., 1992; N.Ishiguro et al., in preparation), indicating that the repertoire features we have detected are likely to be conserved in other artiodactyls.

Important quantitative and qualitative differences have also developed during the evolution of human, mouse and sheep $\gamma \delta V$ gene repertoires. Although all species have a relatively small number of $V\gamma$ and $V\delta$ gene families, there are only a few cases where putative homologous genes are shared between them and the sheep $V\delta 1$ family is unique in that it contains an unusually large number of related members. Moreover, the V δ 1 family has evolved as a separate gene pool that is utilized predominantly, if not exclusively, in $\gamma\delta$ TCRs as shown by the absence of these gene segments in TCR α transcripts. The pattern of variability of amino acid residues in sheep V δ chains allowed the identification of CDR1 and CDR2 regions typical of other antigen receptor V genes. In the $\alpha\beta$ TCR, the CDR3 regions of V α and V β chains are the main sites of interaction with antigenic peptides while the CDR1 and CDR2 segments play a critical role in determining the specificity of MHC recognition (Davis and Bjorkman, 1988; Engel and Hedrick, 1988; Claverie et al., 1989; Hong et al., 1992). By analogy, this suggests that

the sheep V δ germline repertoire has also been shaped by selection processes operating at the level of ligand recognition. Hypervariable regions typical of CDR1 and CDR2 are not obvious in the available human or mouse TCR γ and δ chains, perhaps because there are simply too few sequences (Schiffer *et al.*, 1992), although as in sheep, there is extensive diversity at the CDR3 formed at junctional regions.

The repertoire of rearranged V_{γ} and $V\delta$ gene segments expressed in peripheral $\gamma \delta$ lymphocytes of sheep varied at different stages of fetal development and differed markedly between fetuses and adult animals. In addition, the usage of $J\gamma$ and $C\gamma$ segments varied at different developmental stages due to the nearly invariant pattern detected in $V\gamma - J\gamma$ rearrangements and subsequent splicing to a particular $C\gamma$ segment. The repertoire that is available in the periphery at different stages of development therefore appears highly specialized, differing not only in terms of V gene usage but also in receptor isotype, since the five $C\gamma$ segments have distinct structural differences. As for the human and mouse homologue, sheep have a single $C\delta$ gene (Hein *et al.*, 1990b). It remains to be established whether the different isotypic forms of the receptor associated with the usage of a particular C_{γ} segment are correlated with subsequent patterns of tissue localization or functional properties. A comparable pattern of development of the recirculating peripheral repertoire has not been detected in other animals, although the thymic expression of human and mouse V_{γ} and Vo segments differs between fetuses and adults (Casorati et al., 1989; Lafaille et al., 1989; Krangel et al., 1990; McVay et al., 1991).

Early fetal thymectomy retarded the development of the peripheral repertoire and a number of V δ genes normally expressed only in fetuses remained detectable when Tx animals had reached an adult age. A similar effect was observed in the case of V_{γ} expression; fetal thymectomy abrogated the usage of $V_{\gamma}1$, which was normally expressed first sometime after birth. In addition, the level of $V\delta - D - J\delta$ junctional diversity in clones from adult Tx animals was comparable with the fetal junctional repertoire. Although these features support the notion that the few $\gamma\delta$ T cells able to persist after thymectomy are survivors of early thymic emigrants (Hein et al., 1990a), we cannot exclude the possibility that some of them developed at extrathymic sites. In this context, the increased frequency of unusual combinations of $V\gamma - J\gamma - C\gamma$ segments in thymectomized animals may reflect different levels of control over gene rearrangement and RNA slicing in T cells developing in the periphery as compared with the thymus. It is clear, however, that in addition to being the major source of $\gamma\delta$ T cells in sheep (Hein et al., 1990a), the thymus also profoundly influences repertoire development.

The exact processes that regulate the development of the peripheral repertoire remain unclear. In mice, there is a distinct temporal sequence in the order of V gene expression in thymus (Havran and Allison, 1988). Commencing at around day 14 of fetal development, the V γ 3 gene most 3' to the J segment is preferentially rearranged, and more distal V γ genes rearrange in order over the next week of fetal development. These results have been widely interpreted to imply that successive waves of $\gamma\delta$ T cells exiting from the thymus have distinct patterns of V gene usage. Moreover, because $\gamma\delta$ T cells resident in different body compartments

of adult mice have limited repertoires that correspond to the pattern of V gene rearrangement found in the fetal thymus at different stages, it is believed that the waves of emigrating cells migrate specifically to limited niches in the peripheral immune system (see reviews by Allison and Havran, 1991; Havran *et al.*, 1991). Although there is supporting evidence in the case of $V\gamma3$ expressing cells in the epidermis of mice, which are derived from early fetal thymic precursors (Havran and Allison, 1990), these homing pathways have not been directly demonstrated.

If a similar programme of events occurred in sheep, this could account for both the ordered appearance during gestation of distinct $\gamma\delta$ repertoires in the blood of fetal lambs and the later absence of some gene families in blood due to the sequestering of specific cells in tissue sites. However, it remains to be established whether position-dependent constraints to gene rearrangement persist throughout the longer periods of gestation characteristic of other mammals. Indeed, a recent study showed that at time points between 11 and 22 weeks of human fetal ontogeny, all $V\gamma$ and $V\delta$ families (with the possible exception of V δ 4) were expressed as rearranged genes in the thymus (McVay et al., 1991). Also, by sequencing 11 thymus cDNA clones derived from the two 61 day old fetal lambs included in this study, we detected different patterns of $V\gamma$ and $C\gamma$ gene expression than we did in the periphery at that time (unpublished data). Clearly, these sorts of studies need to be expanded and extended to other times in development, but they suggest that intrathymic selection is also likely to play an important role in shaping the peripheral $\gamma \delta$ repertoire.

Our results also indicate that not all early fetal $\gamma\delta$ T cells have an obligate tendency to home to specific tissue sites during development. When the source of new fetal emigrants was removed by thymectomy, a number of TCR specificities normally available in the periphery at that stage of development did not subsequently become sequestered in tissues but persisted in the blood for extended periods, well into post-natal life. Therefore the absence of expression of particular V genes in late fetal and adult blood does not necessarily reflect intrinsic tissue tropism by specialized subsets of cells but could also result from the continuous replacement of peripheral $\gamma\delta$ T cells by new thymic emigrants that have a competitive advantage in terms of their ability to persist. The homing and migration patterns of $\gamma\delta$ T cells during fetal and post-natal ontogeny, and the factors which regulate the development and distribution of peripheral repertoires, are likely to be complex and require careful study in appropriate physiological systems.

TCR γ and δ clones derived from peripheral lymphocytes at all stages of development contained extensive junctional diversity. The presence of a few additional codons in $V\delta - D - J\delta$ regions in adult animals as compared with fetuses is consistent with similar findings in mouse and human thymus clones and may similarly reflect an increased level of N-nucleotide addition and/or the usage of multiple D elements at the adult stage (Lafaille *et al.*, 1989; McVay *et al.*, 1991). Among the clones sequenced, there was no evidence for the emigration from the fetal thymus of $\gamma\delta$ T cells with invariant canonical junctions analogous to those found in $\gamma\delta$ T cells in the skin and reproductive tract of mice (Asarnow *et al.*, 1988; Itohara *et al.*, 1990).

In summary, our results emphasize that many critical perceptions about $\gamma\delta$ T cells need to be reappraised since

features common to humans and mice cannot always be generalized to all other species. Conclusions about the role that $\gamma\delta$ T cells play in immunity, and the way in which this has evolved, must ultimately be drawn from a far wider evolutionary perspective. To achieve this, it will be important to examine the repertoire and function of these cells in other ' $\gamma\delta$ high' species, including phylogenetically more primitive organisms such as chickens. The present inability of immunologists to formulate a satisfying overall consensus may in no small way reflect the narrow emphasis given to those species in which the $\gamma\delta$ T cell system is arguably devolving towards redundancy.

Materials and methods

Animals and surgical procedures

Healthy White Alpine sheep were obtained from Versuchsbetrieb Sennweid, Olsberg, Switzerland. Fetuses of known gestational age $(\pm 1 \text{ day}, \text{term} = 150 \text{ days})$ were recovered by caesarian section on days 61, 117 and 146 of fetal development. Two fetuses were surgically thymectomized *in utero* as described (Hein *et al.*, 1990a) on days 63 and 73 of gestation and kept under normal husbandry conditions after birth.

Cells

A pooled single-cell suspension of lymphocytes was prepared by finely mincing the spleens of two 61 day old fetuses. Mononuclear cells were isolated from the peripheral blood of a 117 and 146 day old fetal lamb, from two normal adult sheep (1 year old) and from two thymectomized adult sheep (6 months and 1 year old) by centrifugation in Percoll gradients (Miyasaka and Trnka, 1985). Recovered cells were washed several times in phosphate-buffered saline, pelleted and used immediately for RNA extraction.

cDNA synthesis

Total cellular RNA was isolated by the acid phenol method and $\sim 5 \,\mu g$ was used as a template for the synthesis of dT primed single-stranded cDNA using a commercial cDNA synthesis kit (Boehringer Mannheim) according to the supplier's instructions. Single-stranded cDNA was tailed with oligo(dG) using terminal deoxytransferase (Bethesda Research Laboratories). The procedures used throughout followed the detailed description given by Thiesen *et al.* (1990).

Polymerase chain reaction and cloning of V gene transcripts

An anchored PCR technique employing two oligonucleotide primers was used to amplify the expressed TCR V regions (Thiesen et al., 1990). One oligonucleotide was specific for sequences contained in the constant regions of sheep γ and δ chains. A stretch of 35 nucleotides located ~200 bp from the 5' end of the C region that is conserved between the sheep $C\gamma 1$ and C γ 2 sequences (Hein et al., 1990b) was used as the C γ primer. This region lies downstream of a conserved SacI site. The Co primer was complementary to a stretch of sequence 40 bp downstream of a HincII site at the 5' end of the single sheep C δ region (Hein et al., 1990b). The second oligonucleotide primer used in each PCR mixture was complementary to the poly(G) tail as described (Loh et al., 1989) and contained a SacII restriction site. In other experiments, an oligonucleotide primer specific for the constant region of the sheep $C\alpha$ segment (Hein *et al.*, 1991) was used in combination with a primer specific for a stretch of conserved sequence found in the 5'UT regions of the sheep V δ 1 family. The sequences of these primers are as follows (shown 5' to 3'): $C\gamma$, TCACGGTCAGCCAGCTGAGCTTCATGTATGTGTC; Cδ, GTAGAA-CTCCTTCACCAAACAAGCGACGTTTGTC; C α , GAGTCAAAATCG-GTCAACAGGCAGAC; 5'UT Vô1, TCTCAGCTTGAGGCAG; Poly(G), GCATGCGCGGCCGCGGGAGGCCCCCCCCCCCCCCC

Reaction mixtures were denatured by heating to 94°C for 5 min, then subjected to 30 rounds of amplification using a thermo-cycler and commercial kit (Perkin-Elmer Cetus) under the following conditions: 94°C for 30 s, 56°C for 20 s and 72°C for 1 min. Final extension was done at 72°C for 10 min. Amplified DNA fragments were gel purified, eluted using Gene Clean (BIO 101), digested with appropriate restriction enzymes and cloned into compatible Bluescript plasmid vectors. The amplifed V γ fragments were initially cloned into SacII – SacI sites. However, we found on sequencing that many of these were truncated due to the presence of a SacI site in one J γ segment. Subsequently, V γ fragments were blunt-ended and cloned into SacII-EcoRV sites. The amplifed V δ fragments were cloned into SacII-HincII sites.

Cloning of new $C\gamma$ transcripts

The V_{γ} clones generated above contained ~200 bp of the 5' end of the constant region and from these clones we identified three new sheep C_{γ} regions. Each C_{γ} region was spliced to distinct sets of rearranged $V_{\gamma}-J_{\gamma}$ segments and was expressed at specific stages of ontogeny. 5'PCR primers specific for sequences in the V_{γ} and J_{γ} segments that paired to each C_{γ} were synthesized. Since the 3' regions of the sequences of interest were unknown, but might be similar to related genes, we designed four primers that would hybridize to different stretches of the known sheep and bovine 3'UT regions of TCR $_{\gamma}$ transcripts (Hein *et al.*, 1990b; Takeuchi *et al.*, 1992). We then used a two-stage PCR amplification procedure on appropriate samples of cDNA to obtain the full-length coding sequence of the new C_{γ} regions. DNA fragments amplified first with the different $V_{\gamma} - 3'$ UT primer combinations were used as a template for a second amplification using internal $J_{\gamma} - 3'$ UT primers. In this way we were able to amplify specifically the new C_{γ} transcripts which were cloned into Bluescript and sequenced.

Sequencing

Miniprep plasmid DNA (Chen and Seeburg, 1985) was sequenced along both strands using terminal primers and Sequenase (United States Biochemical) according to the maker's instructions. The average length of the cloned fragments was such that the terminal runs overlapped in the central 100-150 nucleotides of the V regions. In a number of cases where ambiguities remained, and as sequence data accumulated, several additional nucleotide primers were designed from known sequences to allow directed internal sequencing runs of individual clones or of different V region families. Sequences were assembled and analysed with the University of Wisconsin Genetics Computer Group (GCG) software run on a VAX computer (Digital, Maynard, MA). Identity between two sequences was determined with the GAP program, which uses the Needleman–Wunsch algorithm.

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