Available online at www.sciencedirect.com
ScienceDirect
Molecular Immunology 44 (2007) 3155–3161
Molecular Immunology
www.elsevier.com/locate/molimm

Highly diverse TCR δ chain repertoire in bovine tissues due to the use of up to four D segments per δ chain

Ildiko Van Rhijn [a,*], Rachel Spiering [a], Mieke Smits [a], Marja T.M. van Blokland [c], Roel de Weger [c], Willem van Eden [a], Victor P.M.G. Rutten [a], Ad P. Koets [b]

[a] *Department of Infection and Immunity, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands*
[b] *Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands*
[c] *Department of Pathology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*

Received 4 January 2007; accepted 1 February 2007

Abstract

Tissue-specific distribution of γδ TCRs with limited TCR diversity is a common phenomenon in species with a low percentage of γδ T cells like humans and mice. We set out to investigate whether this is also the case in cattle (*Bos taurus*), a species with high percentages of γδ T cells. Using a method that was independent of variable (V) segment-specific primers, we generated 65 unique TCR δ chain sequences. We found no evidence for preferential use of certain Vδ segments in lymph node, skin, spleen, small intestine, large intestine, and blood. The δ chain CDR3 length distribution was very wide in each tissue, which was confirmed by spectratyping. The highly variable CDR3 length was due to the use of up to four diversity (D) segments by one bovine δ chain. Human and murine δ chains contain only one or two D segments. The five functional Dδ segments that we describe here were identified at cDNA and genomic level, and are the first ruminant D segments described. Fourteen TCR δ chain sequences used novel Vδ1 segments, and one expressed a novel member of the Vδ3 family. The number of known functional Vδ segments in cattle including these new ones is 42 now, but the total number may be much higher. A high number of Vδ segments in combination with the use of up to four out of five D segments, and the possibility of using non-template encoded (N) nucleotides on either side of these, makes the potential bovine δ chain repertoire much bigger than any known TCR chain. This situation is quite different from the situation in humans and mice, and suggests that the differences between γδ high and γδ low species in distribution, diversity, and function of γδ T cells may be substantial.

Keywords: γδ T cell receptor; *Bos taurus*; Spectratyping

1. Introduction

Species can be classified into two groups according to the frequency of γδ T cells in their T cell population. Sheep, cattle, pigs, chicken, and rabbits belong to the γδ T cell high group. The γδ low group is represented by humans, guinea pigs, dogs, and mice. In γδ high species up to 60% of the T cell population consists of γδ T cells. For the γδ high species that were studied, the number of TCR δ chain variable (Vδ) segments is much higher than in humans and mice, representing γδ low species (Antonacci et al., 2005). In γδ low species, γδ T cell subsets show a tissue-specific distribution. In humans and mice this is illustrated by the tissue specific distribution of Vδ segments. Human Vδ1 positive T cells are found in intestinal tissue and Vδ2 mainly in blood. Human intestinal Vδ1 expressing cells are oligoclonal (Chowers et al., 1994; Holtmeier et al., 1995). Mice have a skin-associated γδ T cell population expressing a very limited TCR repertoire using Vγ5 and Vδ1 (Asarnow et al., 1988) and a population in the reproductive tract and tongue that preferentially uses Vγ6 and Vδ1 (Itohara et al., 1990). The physiological functions of the different γδ T cell subsets is not known, but their different tissue localization is thought to reflect differences in function.

Ruminant γδ T cells share some features that distinguish them from γδ T cells of other species like the expression of members of the WC1 family of scavenger receptors, which have no counterpart in humans or mice (O'Keeffe et al., 1999) and a constant (C) γ segment that has an extended hinge region

* Corresponding author. Tel.: +31 30 2534608; fax: +31 30 253555.
E-mail address: i.vanrhijn@vet.uu.nl (I. Van Rhijn).

0161-5890/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2007.02.003

with additional cysteine residues (Hein and Mackay, 1991). The function of ruminant γδ T cells and the stimuli they react to are largely unknown.

V segments of TCR are grouped in interspecies families, or subgroups, with >75% nucleotide identity. The bovine TCR γ locus, containing 6 Cγ segments and 11 Vγ segments has been well characterized (Herzig et al., 2006a). The genomic organization of the bovine and ovine αδ loci is less well characterized. The current version of the bovine genome (Btau_3.1, available at http://pre.ensemble.org) has big gaps in the αδ locus, and has many contigs containing Vδ segments that have not been assigned to a chromosome yet. Rearranged bovine δ chain sequences have been obtained from peripheral blood (Ishiguro et al., 1993), skin (Hein and Dudler, 1997), and thymus (Massari et al., 2000). Most of the sequenced ovine and bovine Vδ segments are closely related and belong to one big family, the Vδ1 family. Only a few members of the other three known Vδ families have been sequenced (Herzig et al., 2006b). The number of functional Vδ segments in humans is 8–10, including the TRAV/DV genes. Mice have 20 functional Vδ segments in their genome. The number of ovine Vδ segments is known to be at least 40 (Antonacci et al., 2005). Sheep and cattle have three highly homologous Jδ segments (Hein and Dudler, 1997; Massari et al., 2000). Dδ segments had not yet been identified in ruminants, but we report the bovine Dδ segments in this paper.

Because most knowledge about γδ T cell subset distribution concerns γδ low species, we set out to determine the usage of TCR δ V, D, and J segments and CDR3 sequences in different tissues in cattle, a γδ high species. Tissue specificity of γδ T cell subsets has been studied in cattle based on the expression of molecules like CD8, E-selectin, or WC1 (Blumerman et al., 2006; Hedges et al., 2003; Machugh et al., 1997; Wilson et al., 1999). Using antibodies that probably recognize epitopes on the TCR γ chain, a cell population enriched in spleen was identified (Wilson et al., 1998). In cattle, there is some evidence for tissue specific distribution of γ chain, but not at all for δ chain (Hein and Dudler, 1997; Ishiguro et al., 1993).

To obtain an unbiased collection of TCR sequences we chose for a method that circumvents the use of V segment-specific primers by using circularized templates and primers that were located on the C segment of the δ chain only. It is likely that not all bovine Vδ families are known, and this method enables the amplification of unknown segments. In addition, spectratyping was performed to determine CDR3 length distribution using primers that amplify δ chains that use the most common Vγ and Vδ segments.

2. Materials and methods

2.1. Animals

Prescapular lymph node, small intestine, large intestine, spleen, and peripheral blood drawn from the jugular vein were collected from a Holstein-Frisian crossbred bull of 3 months of age. At the time of tissue collection and blood sampling, the animal showed no clinical signs of disease. γδ T cells that originate from the skin were obtained using a pseudoafferent lymph cannulation method (Van Rhijn et al., 2007) and used without further purification. PBMC were prepared using a standard Ficoll density gradient.

2.2. RNA isolation, cDNA synthesis, and circularization

Of each tissue, 3×10^7 cells were used to isolate RNA with the Qiagen RNAEasy kit, followed by first strand cDNA synthesis with Multiscribe reverse transcriptase. A previously described method to synthesize second strand cDNA (Uematsu, 1991) was used in a modified form. In short, 16 μl *E. coli* ligase buffer (Invitrogen), 7.5 μl 4 mM DNTP, 6 μl 0.1M DTT (Gibco-BRL), 30 U *E. coli* DNA ligase (Invitrogen), 18 U *E. coli* DNA polymerase I (Invitrogen) and 2.5 U rnase H (New England Biolabs) were added to the cDNA in a total volume of 125 μl water and incubated for 2 h at 16 °C. 1 U T4 polymerase (Invitrogen) was added and incubated for 5 min at 16 °C to blunt the ends. DNA was cleaned and brought to a volume of 13 μl with a DNA clean and concentrator kit (Zymo Research). For the circularisation, 3.15 μl 5 × T4 buffer (Gibo-BRL) and 1.6 U T4 ligase (Gibo-BRL) were added to 11 μl cDNA eluate and incubated overnight at 16 °C.

2.3. PCR, cloning, and sequence analysis

Fig. 3 shows the location of the primers used for PCR on circularized δ chain cDNA. PCRs were performed with PFU Turbo polymerase (Stratagene) according to the protocol of the manufacturer under the following cycling conditions: an initial denaturation of 7 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at a primer-specific annealing temperature, 1 min at 72 °C, followed by a final elongation step of 7 min at 72 °C. Nested PCR was performed with the forward primer S (5′-CCCAAACGGTTTTCCTTTAAAGACAACAAACC) and reverse primer R (5′-CCTTCACCAAACAAGCGACGTTTGTCC) at an annealing temperature of 58 °C, followed by PCR with the forward primer F (5′-GCCTGATGAAAGACAGAAGTCCTGGG) and reverse primer E (5′-ACAAAAACAGATGGGCTGGCAGCAGG) at an annealing temperature of 61 °C. Circularized as well as linear cDNA were used in the PCR procedure in order to be able to distinguish between specific PCR products and background products. PCR products were cut from an agarose gel, purified, and ligated in a Topo4blunt vector which was used to transform one shot Top10 cells (Invitrogen). Vector DNA of single colonies was sequenced by Baseclear (Leiden, Netherlands). The obtained sequences were compared to the NCBI databases (www.ncbi.nlm.nih.gov/BLAST). The Translate Nucleic Acid Sequence Tool was used (http://biotools.umassmed.edu) for translation into amino acids. All alignments were performed with ClustalW (align.genome.jp).

2.4. Spectratyping

Forward primers specific for Vγ segments (nomenclature according to Herzig et al., 2006a): TRGV1 (5′-ATGTTGTGGGCCCTAGTGCTGC) TRGV3 (5′-ATGTCACCATTGGAA-

GCATTCACATTTTTC); TRGV7 (5′-GCCRTTGTGCACTG-GTATCAAGAG) were used with reverse primer A (5′-GAAGGAAGAAAMAYAGTGGGCTTGGG), labeled at the 5′ end with 6-carboxyfluorescein (FAM), that anneals to a region that is conserved in the six known bovine Cγ segments at an annealing temperature of 53 °C. A conserved primer that amplifies all Vδ1 family members (5′-TCTGAGCTTGAGGC-AGAACT) (Ishiguro et al., 1993) was used in combination with reverse primer E (5′-ACAAAAACAGATGGGCTGGC-AGCAGG) labeled at the 5′ end with 6-carboxyfluorescein (FAM), at an annealing temperature of 53 °C. PCR products were run on a 310 ABI Prism Genetic Analyzer and analysed with Genotyper software (Applied Biosystems, Foster City, CA). The GeneScan-500 Rox Size standard-labeled molecular weight standard (Applied Biosystems) served as a reference. For each combination of tissue and primer set, a duplicate set of PCR amplification and spectratyping was performed.

3. Results

3.1. Vδ and Jδ usage in different tissues

We sequenced a total of 154 cDNA clones of δ chains with the complete CDR3 region and at least part of the V segment, all of which were in frame. The sequences were derived of six different tissues from a Holstein-Frisian crossbred bull of 3 months of age. Of the cDNA clones, 65 were unique rearrangements and 89 were redundant. Part of the redundant clones probably originated from the same RNA template and may be over-represented due to preferential amplification by PCR. For this reason we excluded redundant clones from further analysis when they originated from the same PCR. Table 1 shows the CDR3 sequence and the V, D, and J segment usage of the non-redundant cDNA clones. Most published bovine TCR δ chain sequences have been generated using a universal primer that amplifies all members of the Vδ1 family. In fact, the majority of the known bovine TCR δ chain sequences belongs to the Vδ1 family. The known non-Vδ1 TCR δ chain sequences have been generated with Vδ2, Vδ3, or Vδ4 specific primers (Herzig et al., 2006b). The use of a Vδ family-specific primer allows a representative study among the members of a certain Vδ family, but not among the different Vδ families. To circumvent this problem we applied a method which only uses primers in the δ constant chain, which is free from bias towards certain Vδ segments introduced by Vδ segment specific primers. In addition, our method is suitable to pick up unknown Vδ families. Based on a percentage of identity to known Vδ segments belonging to those Vδ families between 75 and 97%, 49 of the 65 non-redundant clones represented the Vδ1 family, three belonged to the Vδ3 family, and 13 sequences did not reach far enough in the V segment to allow for a unequivocal identification. Fourteen cDNA clones used 11 different Vδ1 segments that had not yet been shown to be expressed as cDNA. Because the previously identified bovine Vδ segments had been numbered Vδ1.1–Vδ1.26 (Hein and Dudler, 1997; Ishiguro et al., 1993), these novel ones were numbered Vδ1.27–Vδ1.37. Accordingly, in addition to the single known bovine Vδ3 segment (Herzig et al., 2006b), one novel Vδ3 segment was found, which we named Vδ3.2. We found no evidence for preferential use of certain Vδ segments in lymph node, skin, spleen, small intestine, large intestine, and blood. All three known bovine Jδ segments were represented. Consistent with results from other groups, Jδ1 and Jδ3 were predominant. We found only two cases of Jδ2 usage.

3.2. CDR3 sequences reflect the expression of a very diverse repertoire in all tissues studied

CDR3 lengths of the δ chains that we sequenced had a length of 10–29 amino acids, counting the residues between the second cysteine and the FGXG motif. We did not find any tissue-specific CDR3 consensus sequences or motifs. To confirm the wide CDR3 length distribution that we observed in δ chain sequences from the different tissues by an independent technique, we performed spectratyping. An advantage of this technique is that it takes the complete repertoire that is present in a tissue into account, and is independent of the sample that is taken by picking cDNA clones for sequencing. Because Vδ1 family members are by far the most abundant, and because all known members of the Vδ1 family can be amplified with one forward primer (Ishiguro et al., 1993), spectratyping using this primer covers almost all Vδ chains present in a specific tissue. In all tissues studied, a wide Vδ1 CDR3 length distribution was observed (Fig. 1). The observed CDR3 length distribution correlated very well with the lengths that we observed by sequencing cDNA clones. The fact that there were no predominant peaks suggests the absence of highly expanded T cell clones. Spectratyping was also performed for γ chains, of which preferential tissue distribution of V segments has been reported in cattle. Because Vγ1, Vγ3, and Vγ7 could be amplified from most tissues, spectratyping was limited to these segments. Fig. 1 shows that the γ chain repertoire is much more limited in CDR3 length variation than the δ chain repertoire, and, contrary to the δ chain results, tends to show differences between tissues.

3.3. Identification and usage of five bovine Dδ segments

Alignment of the nucleotide sequences of the published δ chain CDR3 sequences and the ones we describe here, revealed five potential Dδ segments. We considered a stretch of a minimum of six nucleotides identical to a certain D segment to reflect the use of that D segment. The genomic sequences of the five potential Dδ segments that we found based on the CDR3 sequences could be located in the right order on bovine chromosome 10 (Btau_3.1, available at http://pre.ensemble.org) before the Jδ and Cδ segments, flanked by recombination signal sequences consisting of nonamer-12 bp spacer-heptamer and heptamer-23 bp spacer-nonamer sequences (Fig. 2). Of the bovine δ chains that were analyzed, some use four D segments, many use three D segments, but also δ chains that use two, one, or no stretches of six or more nucleotides corresponding to a D segment were found (Table 1). This situation is different from humans and mice, which have been reported to use a maximum of two D segments. The D segments were used in different

Table 1
V segment usage and δ chain CDR3 sequences

Tissue of origin	CDR3 sequence			V segment	GenBank accession no. V segment	D segments	J segment	CDR3 length
	V	N and D segments	J					
Large intestine 1	ALW	RSVPGGGGWYAGGVRTGIR	NPLIFGKGTYLNVEPE	Vδ1.11	D16113	Dδ1, Dδ3, Dδ5	Jδ3	26
Large intestine 2	ALR	WGVH	ETDKLIFGKGTRLIVEPK	Vδ1.2	D13656	Dδ1	Jδ1	13
Large intestine 3	ALR	WGVH	ETDKLIFGKGTRLIVEPK	Vδ1.33	EF175171	Dδ1	Jδ1	13
Large intestine 4	ALR	AGIYGRMGYA	DKLIFGKGTRLIVEPK	x	x	Dδ2, Dδ5	Jδ1	17
Large intestine 5	VLR	ARGSGYYVGY	ETDKLIFGKGTRLIVEPK	Vδ1.27	XM_588974	Dδ3, Dδ5	Jδ1	19
Large intestine 6	ALP	LRTWTGGTTGKK	TDKLIFGKGTRLIVEPK	Vδ1.34	EF175172	Dδ1	Jδ1	20
Large intestine 7	ALG	ERRAGLGVTYGDT	TDKLIFGKGTRLIVEPK	Vδ1.35	EF175173	Dδ1, Dδ4, Dδ5	Jδ1	21
Large intestine 8	ALR	DWAWTGGFTYKQ	NPLIFGKGTYLNVEPE	Vδ1.9	D13658	Dδ1, Dδ4	Jδ3	19
Large intestine 9		ERQRWDLLQ	NPLIFGKGTYLNVEPE	x	x	Dδ2	Jδ3	≥13
Large intestine 10		DWGTY	NPLIFGKGTYLNVEPE	x	x	Dδ1, Dδ4	Jδ3	≥9
Small intestine 1	TLS	EAQ	NPLIFGKGTYLNVEPE	Vδ1.16	U73383	-	Jδ3	10
Small intestine 2	ALS	DYKGTSK	ETDKLIFGKGTRLIVEPK	Vδ1.2	D13656	-	Jδ1	16
Small intestine 3	ALR	DNK	TDKLIFGKGTRLIVEPK	Vδ1.3	D16112	-	Jδ1	11
Small intestine 4	ALR	DNK	TDKLIFGKGTRLIVEPK	Vδ1.2	D13656	-	Jδ1	11
Small intestine 5	AVS	ASISQRWTGVGFPGNVRWDTQGD	PLIFGKGTYLNVEPE	Vδ3.2	EF175174	Dδ1, Dδ2, Dδ4, Dδ5	Jδ3	29
Small intestine 6	ALR	WGVH	ETDKLIFGKGTRLIVEPK	Vδ1.2	D13656	Dδ1	Jδ1	13
Small intestine 7	ALC	EPRGYYNVL	NPLIFGKGTYLNVEPE	x	x	Dδ3, Dδ4	Jδ3	16
Small intestine 8	ALR	EIQRPWGTTWD	NPLIFGKGTYLNVEPE	x	x	Dδ3, Dδ5	Jδ3	18
Skin 1	LLV	RWDTQ	TDKLIFGKGTRLIVEPK	Vδ1.28	XM_586316	Dδ5	Jδ1	13
Skin 2	AVR	EGQRRGWTGIYNGGIRGY	ETDKLIFGKGTRLIVEPK	Vδ1.10	D13661	Dδ1, Dδ5	Jδ1	27
Skin 3	ALW	RGAPS	ETDKLIFGKGTRLIVEPK	Vδ1.36	EF175175	-	Jδ1	14
Skin 4	ALH	DGWWTGGFGYGMG	TDKLIFGKGTRLIVEPK	x	x	Dδ1, Dδ3, Dδ5	Jδ1	21
Skin 5	ALW	ERTGLGWDLLRDK	NPLIFGKGTYLNVEPE	Vδ1.19	U73386	Dδ1, Dδ2, Dδ5	Jδ3	20
Lymph node 1	ALN	LQRYG	ETDKLIFGKGTRLIVEPK	Vδ1.3	D16112	-	Jδ1	14
Lymph node 2	VLR	SQRTGGFTSSGRY	ETDKLIFGKGTRLIVEPK	Vδ1.37	EF175176	Dδ1, Dδ2, Dδ5	Jδ1	22
Lymph node 3	ALS	DYRQRWTGWDLPVGYRTVGQ	NPLIFGKGTYLNVEPE	Vδ1.3	D16112	Dδ1, Dδ2, Dδ3, Dδ5	Jδ3	27
Lymph node 4	ALR	WGVH	ETDKLIFGKGTRLIVEPK	Vδ1.2	D13656	Dδ1	Jδ1	13
Lymph node 5		YVGY	NPLIFGKGTYLNVEPE	x	x	Dδ5	Jδ3	≥8
Lymph node 6	AVT	YVGYK	TDKLIFGKGTRLIVEPK	x	x	Dδ5	Jδ1	13
Lymph node 7	AVR	DTAVGFTEVRGRS	DKLIFGKGTRLIVEPK	Vδ1.20	U73387	Dδ2, Dδ5	Jδ1	20
Lymph node 8	AVR	ELGQAYWGGIYELRVGP	TDKLIFGKGTRLIVEPK	Vδ1.21	U73388	Dδ1, Dδ2	Jδ1	25
Spleen 1	ALW	ELRALDWGIYEGSS	SWDTRQIFFGAGTKLFVEPQ	Vδ1.29	XM_605207	Dδ1, Dδ2	Jδ2	25
Spleen 2	ALH	WTINGGIA	LIFGKGTYLNVEPE	Vδ1.23	U73390	Dδ1, Dδ5	Jδ3	13
Spleen 3	ALW	DQVGSYTTL	TDKLIFGKGTRLIVEPK	Vδ1.25	U73392	Dδ5	Jδ1	17
Spleen 4	ALR	YSVGLGSGIYGPVGYVRKYD	TDKLIFGKGTRLIVEPK	Vδ1.30	XM_583486	Dδ1, Dδ2, Dδ3	Jδ1	28
Spleen 5	AVR	ERVLLDWDSAVPSGRTRIR	TDKLIFGKGTRLIVEPK	Vδ1.20	U73387	Dδ2, Dδ4	Jδ1	27
Spleen 6	ALW	SVRRWTGINVRGIRG	PLIFGKGTYLNVEPE	Vδ1.31	XM_599828	Dδ1, Dδ4, Dδ5	Jδ3	21
Spleen 7	TLS	ERTLGPNVRW	ETDKLIFGKGTRLIVEPK	Vδ1.16	U73383	Dδ1, Dδ4	Jδ1	19
Spleen 8	ALR	ASGTAVGYQRTVGYA	DKLIFGKGTRLIVEPK	Vδ1.14	U73381	Dδ3, Dδ4, Dδ5	Jδ1	22
Spleen 9	ALW	ERRAGLGVTYGDT	TDKLIFGKGTRLIVEPK	Vδ1.37	EF175176	Dδ1, Dδ4, Dδ5	Jδ1	21
Spleen 10	AVT	YVGYK	TDKLIFGKGTRLIVEPK	x	x	Dδ5	Jδ1	13
PBMC 1	ALF	FLWTRHRGIPY	ETDKLIFGKGTRLIVEPK	Vδ1.32	XM_604310	Dδ1, Dδ5	Jδ1	20
PBMC 2	ALQ	RTLDWGFESGWDTR	ETDKLIFGKGTRLIVEPK	Vδ1.37		Dδ1, Dδ5	Jδ1	23
PBMC 3	TLS	NVGYGY	ETDKLIFGKGTRLIVEPK	Vδ1.18	U73385	Dδ5	Jδ1	15
PBMC 4	ALS	NVGYGY	ETDKLIFEKGTRLIVEPK	Vδ1.18	U73385	Dδ5	Jδ1	15
PBMC 5	ALH	DGGPRTPRSY	ETDKLIFGKGTRLIVEPK	Vδ1.6	D16115	-	Jδ1	19
PBMC 6	ALH	AAVGYYVRGISY	ETDKLIFGKGTRLIVEPK	x	x	Dδ3, Dδ4, Dδ5	Jδ1	21
PBMC 7	AVR	ELESFYVGLGDLRAQNNGTN	ETDKLIFGKGTRLIVEPK	Vδ1.20	U73387	Dδ1, Dδ2	Jδ1	29
PBMC 8	ALR	DRIVGLWDLRGVLR	NPLIFGKGTYLNVEPE	Vδ1.9	D13658	Dδ1, Dδ2, Dδ3	Jδ3	21
PBMC 9	ALS	DWGVR	ETDKLIFGKGTRLIVEPK	Vδ1.3	D16112	Dδ1	Jδ1	15
PBMC 10		ERQRWDLLQ	NPLIFGKGTYLNVEPE	x	x	Dδ2	Jδ3	≥13
PBMC 11	ALC	ELVNAALIYEVGYYGGDTH	PLIFGKGTYLNVEPE	Vδ1.8	D16116	Dδ2, Dδ3, Dδ5	Jδ3	25
PBMC 12	PRP	QSRPQAGYLSGSVAMC	SWDTRQIFFGAGTKLFVEPQ	x	x	-	Jδ2	27
PBMC 13	ALS	AADWIYEDTWD	NPLIFGKGTYLNVEPE	x	x	Dδ1, Dδ5	Jδ3	18
PBMC 14	ALH	DGGGIFGIK	NPLIFGKGTYLNVEPE	Vδ1.23	U73390	Dδ5	Jδ3	16
PBMC 15	ALC	EYSPDWPNGVQ	NPLIFGKGTYLNVEPE	Vδ1.26	U73393	Dδ1	Jδ3	18
PBMC 16	ALP	EAYPGGPYIQY	ETDKLIFGKGTRLIVEPK	Vδ1.23	U73390	Dδ1	Jδ1	20
PBMC 17	ALS	GPGGLGTR	TDKLIFGKGTRLIVEPK	Vδ1.7	D16114	Dδ1	Jδ1	16
PBMC 18	SLS	EYSVRWTGDVTWDT	PLIFGKGTYLNVEPE	Vδ1.16	U73383	Dδ1, Dδ5	Jδ3	20
PBMC 19	AGT	ASGGFQ	NPLIFGKGTYLNVEPE	Vδ3.1	DQ275148	Dδ2	Jδ3	13
PBMC 20	ALC	SGGIYEWDT	NPLIFGKGTYLNVEPE	Vδ1.17	U73384	Dδ2, Dδ5	Jδ3	16
PBMC 21	LLV	RWDTQ	TDKLIFGKGTRLIVEPK	Vδ1.28	XM_586316	Dδ5	Jδ1	13
PBMC 22	ALS	DFSGLGYWDT	NPLIFGKGTYLNVEPE	Vδ1.2	D13656	Dδ1, Dδ5	Jδ3	17
PBMC 23	ALR	GADWGPTY	NPLIFGKGTYLNVEPE	Vδ1.7	D16114	Dδ1, Dδ4	Jδ3	15
PBMC 24	AVR	EYNLRAQRGIQN	PLIFGKGTYLNVEPE	Vδ3.1	DQ275148	Dδ2, Dδ5	Jδ3	18

The first column shows the tissue or origin and an identifier of each non-redundant δ chain sequence that was obtained. The CDR3 sequence of the δ chains is given in the next three columns. The names of the V, D, and J segments that are used, and the accession number of the V segments are given in the next columns. V segments are considered to be identical to previously identified V segments (Hein and Dudler, 1997; Ishiguro et al., 1993) if they are >97% identical at nucleotide level. In the column labeled "Genbank accession no. V segment" the accession number is given of previously identified V segments (Hein and Dudler, 1997; Ishiguro et al., 1993) (starting with U or D), of V segments that were predicted based on the bovine genome (starting with XM), or of V segments identified in this paper (starting with EF). If a sequence shares less than 97% identity to a known V segment, but more than 75% to other members of a V family, it is considered a new member of that family. These sequences are numbered Vδ1.27–Vδ1.37 and Vd3.2. An "x" indicates that the obtained sequence was too short on the 5′ side to determine the V segment. CDR3 length was counted between the second cysteine and the FGXG motif. If the CDR3 sequence contained at least six nucleotides of a Dδ segment, this was considered usage of that Dδ segment.

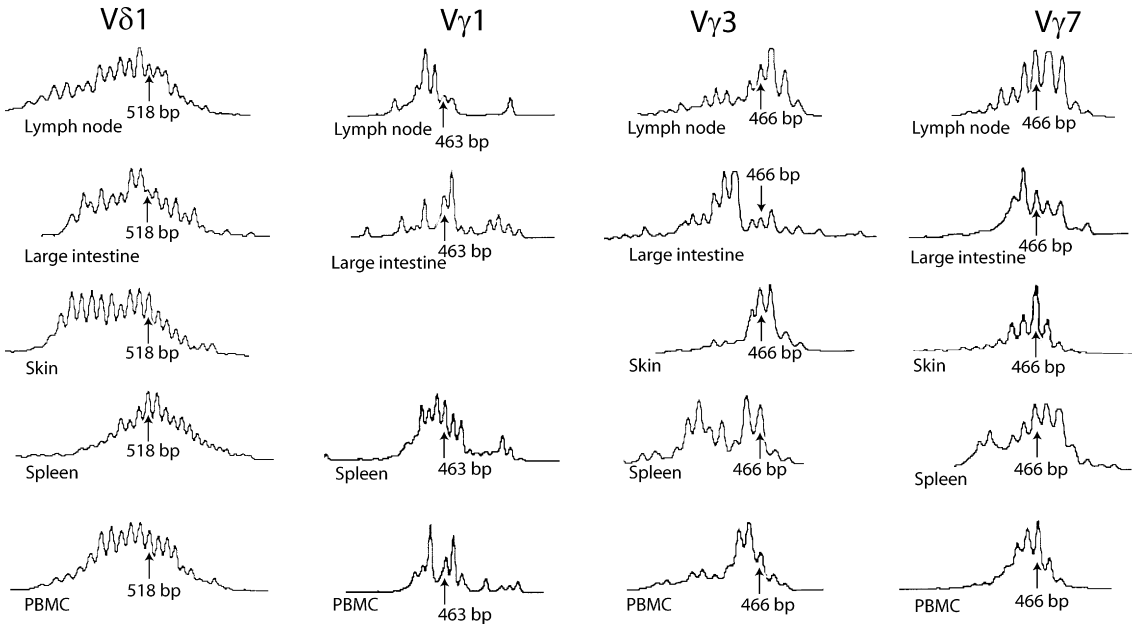

Fig. 1. Spectratyping of Vδ1, Vγ1, Vγ3, and Vγ7 in different tissues. FAM-labeled PCR products were generated from different tissues using an unlabeled primer that specificly amplifies selected V segment families, in combination with a FAM-labeled primer in the C segment. PCR products were analyzed on a 310 ABI Prism Genetic Analyzer. For each family of V segments the plots are aligned according to fragment length. For the Vδ1 PCR product, a fragment length of 518 bp corresponds to a CDR3 length of 21 amino acids.

(A)

Segment	5' nonamer	5' 12 bp spacer	5' heptamer	Dδ segment	3' heptamer	3' 23 bp spacer	3' nonamer
Dδ1	GGTTTTTGT	aaagctctgtag	CACTGTG	**GTTGGACTGGGGG**	CACAGTA	ttacaaacctcaaagagacctct	ACAGAAACT
Dδ2	GGTTTTTGT	aaagctctgcag	CACTGTG	**GGTGGGATTTACGAG**	CACATAG	ctacaatacccaaagagacctgt	ACAGAAATT
Dδ3	GGTTTTTGT	aaagctctgtgg	TACTGTG	**GGTGGGGTACTAC**	CACCGCA	atacaaacatcacacagacctgt	ACAGGAACT
Dδ4	GGTTATTGT	aaagctgtgctt	CACTGTG	**ACAACGTAC**	CACGGAG	gttgaagtgaattaaatccttgt	TCAAAAACC
Dδ5	GGTTTTTGT	aaaggactgtag	CATTGTG	**GGTGGGATACG**	CACGGTG	atacaaaacccacagagacctgt	ACAAAAACT

(B)

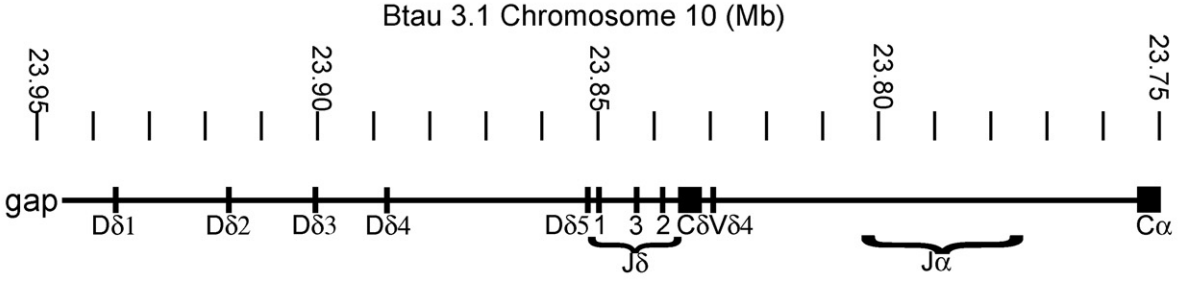

Fig. 2. Genomic location and sequence of Dδ segments. The genomic sequences of the five Dδ segments (A) and their location on chromosome 10 of *Bos taurus* (B). The position of the three Jδ segments, Cδ, Vδ4 and Cα are also indicated. Individual Jα segments are not shown because it is currently unknown how many Jα segments are present in the bovine genome. However, the J segments of the published bovine TCR α chains are located in the indicated region.

reading frames and, in some δ chains, were interspersed with extensive stretches of N nucleotides.

4. Discussion

TCR δ chain sequencing and spectratyping showed that many different Vδ1 family members are expressed in six different bovine tissues, and that a wide variety of δ chain CDR3 lengths is present without any apparent bias towards a certain V segment or CDR3 length. This finding is consistent with previous data generated from bovine PBMC and skin, and we show here that the same applies to bovine small and large intestine, spleen, and lymph node. In contrast, γδ T cells in murine skin and in small and large intestine of humans and mice have been reported to express only one Vδ segment and to be oligoclonal (Asarnow et al., 1988; Chowers et al., 1994; Holtmeier et al., 1995).

In cattle, there is evidence for specific γ chain usage in skin (Hein and Dudler, 1997; Van Rhijn et al., 2007) and it has been suggested that this also holds for other tissues. Spectratyping was performed on the three most widely expressed bovine Vγ

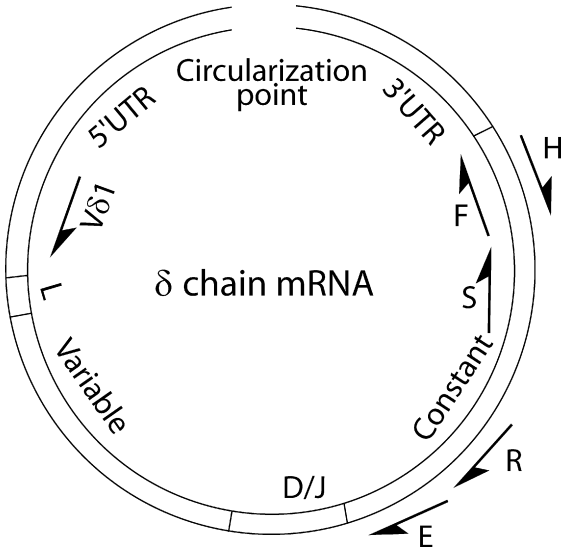

Fig. 3. Location of primers for δ chain analysis. Shown is an example of circularized full length cDNA of a δ chain transcript and the location of the primers used. Note that also parts of transcripts, for example with incomplete or lacking UTRs and incomplete V segments can be circularized and amplified.

segments for comparison between tissues and showed that there is less variation in CDR3 length among γ chains than δ chains. For αβ T cells, skewed CDR3 length and limited V segment usage is often interpreted as a sign of antigen selection. If this is applied to the γδ TCR, our findings may suggest that antigenic selection for certain γ chains has taken place and that δ chain is less important for antigen specificity. However, there are several lines of evidence that lead to the conclusion that antigen specificity of the γδ TCR is mainly determined by the δ chain: (1) The co-crystal of the murine G8 γδ TCR and the non-classical MHC molecule T22 shows that antigen specificity is mainly determined by the CDR3 loop of the δ chain because it forms the main contact area between the two molecules (Adams et al., 2005). (2) Vδ and Vγ usage does not strictly correlate with T22 antigen specificity (Shin et al., 2005), but a CDR3 consensus sequence of the δ chain, but not the γ chain, does. (3) Synthetic peptides mimicking the δ chain CDR3 show the same antigen specificity as the whole γδ TCR (Xu et al., 2007).

So, paradoxically, it seems that γ chain usage is restricted in a tissue-specific manner, but on the other hand, δ chains, which are more likely to determine the antigen specificity of γδ TCR are not. One way to look at this is to acknowledge that limited γ chain repertoire does not necessarily reflect antigenic selection but may be caused by other processes, like for example by waves of Vγ segment expression during ontogeny and concomitant availability of space in the tissues for thymic emigrants, or the coupling of Vγ segment expression to the expression of known adhesion molecules or chemokine receptors. This is possible if the γ chain is indeed not the main determinant of the γδ TCR specificity. The fact that the δ chain repertoire that we describe is extremely diverse may simply mean that there has not been enough antigenic challenge to skew it.

We have shown here that bovine δ chains can use up to four D segments, which fully explains the wide CDR3 length variation that was observed. In bovine γδ T cells, the CDR3 length variation and the theoretical number of combinations of V segments, 0–4 D segments, and J segments is much higher than in human and murine γδ T cells. It is currently unknown how diverse the bovine αβ TCR repertoire is, but it is possible that αβ T cells and γδ T cells show opposite characteristics in γδ high and γδ low species in terms of complexity and actual numbers of T cells.

References

Adams, E.J., Chien, Y.H., Garcia, K.C., 2005. Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22. Science 308, 227–231.

Antonacci, R., Lanave, C., Del Faro, L., Vaccarelli, G., Ciccarese, S., Massari, S., 2005. Artiodactyl emergence is accompanied by the birth of an extensive pool of diverse germline TRDV1 genes. Immunogenetics 57, 254–266.

Asarnow, D.M., Kuziel, W.A., Bonyhadi, M., Tigelaar, R.E., Tucker, P.W., Allison, J.P., 1988. Limited diversity of gamma delta antigen receptor genes of Thy-1+ dendritic epidermal cells. Cell 55, 837–847.

Blumerman, S.L., Herzig, C.T., Rogers, A.N., Telfer, J.C., Baldwin, C.L., 2006. Differential TCR gene usage between WC1 (−) and WC1 (+) ruminant gammadelta T cell subpopulations including those responding to bacterial antigen. Immunogenetics 58, 680–692.

Chowers, Y., Holtmeier, W., Harwood, J., Morzycka-Wroblewska, E., Kagnoff, M.F., 1994. The V delta 1 T cell receptor repertoire in human small intestine and colon. J. Exp. Med. 180, 183–190.

Hedges, J.F., Cockrell, D., Jackiw, L., Meissner, N., Jutila, M.A., 2003. Differential mRNA expression in circulating gammadelta T lymphocyte subsets defines unique tissue-specific functions. J. Leukoc. Biol. 73, 306–314.

Hein, W.R., Dudler, L., 1997. TCR gamma delta+ cells are prominent in normal bovine skin and express a diverse repertoire of antigen receptors. Immunology 91, 58–64.

Hein, W.R., Mackay, C.R., 1991. Prominence of gamma delta T cells in the ruminant immune system. Immunol. Today 12, 30–34.

Herzig, C., Blumerman, S., Lefranc, M.P., Baldwin, C., 2006a. Bovine T cell receptor gamma variable and constant genes: combinatorial usage by circulating gammadelta T cells. Immunogenetics 58, 138–151.

Herzig, C.T., Blumerman, S.L., Baldwin, C.L., 2006b. Identification of three new bovine T-cell receptor delta variable gene subgroups expressed by peripheral blood T cells. Immunogenetics 58, 746–757.

Holtmeier, W., Chowers, Y., Lumeng, A., Morzycka-Wroblewska, E., Kagnoff, M.F., 1995. The delta T cell receptor repertoire in human colon and peripheral blood is oligoclonal irrespective of V region usage. J. Clin. Invest. 96, 1108–1117.

Ishiguro, N., Aida, Y., Shinagawa, T., Shinagawa, M., 1993. Molecular structures of cattle T-cell receptor gamma and delta chains predominantly expressed on peripheral blood lymphocytes. Immunogenetics 38, 437–443.

Itohara, S., Farr, A.G., Lafaille, J.J., Bonneville, M., Takagaki, Y., Haas, W., Tonegawa, S., 1990. Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. Nature 343, 754–757.

Machugh, N.D., Mburu, J.K., Carol, M.J., Wyatt, C.R., Orden, J.A., Davis, W.C., 1997. Identification of two distinct subsets of bovine gamma delta T cells with unique cell surface phenotype and tissue distribution. Immunology 92, 340–345.

Massari, S., Antonacci, R., Lanave, C., Ciccarese, S., 2000. Genomic organization of sheep TRDJ segments and their expression in the delta-chain repertoire in thymus. Immunogenetics 52, 1–8.

O'Keeffe, M.A., Metcalfe, S.A., Cunningham, C.P., Walker, I.D., 1999. Sheep CD4(+) alphabeta T cells express novel members of the T19 multigene family. Immunogenetics 49, 45–55.

Shin, S., El-Diwany, R., Schaffert, S., Adams, E.J., Garcia, K.C., Pereira, P., Chien, Y.H., 2005. Antigen recognition determinants of gammadelta T cell receptors. Science 308, 252–255.

Uematsu, Y., 1991. A novel and rapid cloning method for the T-cell receptor variable region sequences. Immunogenetics 34, 174–178.

Van Rhijn, I., Rutten, V.P.M.G., Charleston, B., Smits, M., van Eden, W., Koets, A.P., 2007. Massive, sustained gammadelta T cell migration from the bovine skin. Mol. Immunol., in press.

Wilson, E., Aydintug, M.K., Jutila, M.A., 1999. A circulating bovine gamma delta T cell subset, which is found in large numbers in the spleen, accumulates inefficiently in an artificial site of inflammation: correlation with lack of expression of E-selectin ligands and L-selectin. J. Immunol. 162, 4914–4919.

Wilson, S.B., Kent, S.C., Patton, K.T., Orban, T., Jackson, R.A., Exley, M., Porcelli, S., Schatz, D.A., Atkinson, M.A., Balk, S.P., Strominger, J.L., Hafler, D.A., 1998. Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. Nature 391, 177–181.

Xu, C., Zhang, H., Hu, H., He, H., Wang, Z., Xu, Y., Chen, H., Cao, W., Zhang, S., Cui, L., Ba, D., He, W., 2007. Gammadelta T cells recognize tumor cells via CDR3delta region. Mol. Immunol. 44, 302–310.