Immunology 1997 **91** 58–64

TCR $\gamma\delta^+$ cells are prominent in normal bovine skin and express a diverse repertoire of antigen receptors

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SUMMARY

More than 80% of T cells in bovine skin localized in the superficial 0·5 mm of the dermis. Only 3% occurred within the epidermis or made contact with the *stratum basale* while the remainder occupied deeper dermal sites. The $\gamma\delta$ T-cell receptor (TCR) was expressed by 44% of T cells in skin and 39% and 35% expressed, respectively, the CD4 and CD8 markers. Some cells co-expressed CD8 and the $\gamma\delta$ TCR. A highly diverse repertoire of $\gamma\delta$ TCR was expressed in skin due mainly to the usage of multiple Vδ segments and to extensive sequence variation at the junctions of both TCRγ and TCRδ chains. However, a single receptor isotype was used. Transcripts encoding several new components of the bovine $\gamma\delta$ TCR were identified, including three new Vγ segments, the Cγ5 region and 13 new functional Vδ segments. Taken together with earlier findings, these results emphasize that ruminant $\gamma\delta$ T cells express exceptionally diverse antigen receptors and suggest they may have a more elaborate recognitive capacity than do their counterparts in other species.

INTRODUCTION

Several properties of ruminant $\gamma\delta$ T cells distinguish them from their counterparts in other species. In calves and lambs, $\gamma\delta$ T cells may comprise up to 30–50% of circulating T cells in the first few weeks after birth.[1,2] Circulating sheep $\gamma\delta$ T cells are derived from a thymus-dependent pathway of differentiation and are almost totally and permanently depleted by removing the thymus about half-way through fetal gestation.[3,4] A majority of ruminant $\gamma\delta$ T cells specifically express a unique accessory molecule, a member of the scavenger receptor family termed WC1, that is encoded by a large and complex family of genes.[1,5,6] Finally, ruminant $\gamma\delta$ T cells express a diverse repertoire of T-cell receptors (TCR) encoded by at least five different Cγ segments, around 15–20 different Vγ genes and perhaps as many as 40–50 different Vδ gene segments.[7,8]

In mice, $\gamma\delta$ T cells localizing in different types of mucosal surfaces express a distinctive and usually restricted repertoire of antigen receptors. The populations of $\gamma\delta$ T cells that colonize different epithelia arise at distinct stages of fetal ontogeny and reflect ordered rearrangement of V-genes at the TCRγ and TCRδ loci.[9] T cells expressing different combinations of Vγ-Jγ-Cγ and Vδ-Dδ-Jδ genes appear in the periphery of fetal lambs at different stages during ontogeny.[10] This could also reflect an ordered programme of gene rearrangement in the thymus, although this possibility has not been examined directly. A further question that then arises is whether or not specific subsets of ruminant $\gamma\delta$ T cells with a restricted TCR repertoire either localize or recirculate preferentially to particular types of body surfaces. As a first approach to clarifying this question, we have assessed the frequency and TCR diversity of $\gamma\delta$ T cells in bovine skin, a tissue where these cells localize prominently.[1,11] Our results show that in normal bovine skin, $\gamma\delta$ T cells account for at least 44% of resident T cells. The $\gamma\delta$ T cells express an extremely diverse TCR variable-region repertoire but have a restricted usage of one receptor isotype.

Received 25 November 1996; revised 17 January 1997; accepted 19 January 1997.

TCR V-region sequences reported here are available from the GenBank database under the following accession numbers: Vγ U73186–U73188; Vδ U73380–U73393.

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MATERIALS AND METHODS

Collection of bovine skin

Pieces of skin approximately 15 × 10 cm were collected from the hides of yearling Brown Swiss cattle after slaughter at an abattoir. Skin was removed from a site corresponding to the lateral surface of the neck, placed promptly on ice and transported to the laboratory. Small pieces of skin from each animal were embedded in OCT compound (Tissue-Tek, Miles Inc., Elkhardt, IN), frozen on dry ice and stored at −70°. Serial sections of 8 μm thickness were cut from each sample using a cryotome.

Enumeration of T cells in skin

Duplicate skin sections were stained with a cross-reactive polyclonal antibody to identify CD3 (Dako Diagnostics, Glostrup, Denmark) and monoclonal antibodies (mAb)

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specific for bovine CD4 (clone CC30 IgG1, Serotec, Dottikon, Switzerland), CD8 (clone CC63 IgG2a, Serotec) and the $\gamma\delta$ TCR (clone 86D IgG1[11]) using routine immunoperoxidase procedures.[11] The numbers of positive cells in the epidermis and underlying dermis were counted using an ocular graticule in a light microscope at $\times 125$ magnification. Four areas, each measuring 0·9 mm wide × 1·8 mm deep, were counted for each animal. The graticule subdivided this area into 0·18-mm grids. The average number of T cells in different layers of the skin was then calculated.

Confocal microscopy

Frozen skin sections were reacted with mAb specific for bovine CD4 and CD8 as indicated above. Primary labelling was revealed by reacting the sections with class-specific secondary reagents conjugated to fluorescein isothiocyanate (FITC) or Texas red (Southern Biotechnology Associates Inc., Birmingham, AL) to identify CD4 and CD8, respectively. After blocking binding sites by incubating the sections with normal mouse serum, biotinylated-mAb86D was applied to label the $\gamma\delta$ TCR. Binding of mAb86D was detected with streptavidin–allophyocyanin (APC) conjugate (Molecular Probes Europe, Leiden, The Netherlands). Cover slips were applied over Vectashield™ mounting medium (Vector Laboratories Inc., Burlingame, CA) and the sections examined on a confocal microscope (BioRad MRC 1024). Fluorochromes were excited by krypton/argon laser all lines and emission measured through filters of 522 band pass (bp) (FITC), 605 bp (Texas red) and 685 bp (APC).

Isolation of skin cells

One skin sample was clipped and shaved of hair, washed with detergent and rinsed thoroughly. Strips of epidermis and attached dermis about 0·5 mm deep were removed using a Schink BA706 dermatome (Aesculap, Tuttlingen, Germany). The strips were cut into small pieces, placed in Dulbecco's modified Eagle's medium containing 2 mg/ml collagenase CLS II (Cooper Biomedical Co., Malvern, PA) and 0·5 mg/ml DNAse (Sigma Chemical Co., St. Louis, MO) and digested with agitation for 3·5 hr at 37°. Cells in the digest supernatant were filtered through nylon mesh (40 μm pore size) to remove large clumps and washed three times by centrifugation and resuspension in phosphate-buffered saline (PBS). A pellet containing about $1{\cdot}5 \times 10^7$ cells was used for RNA extraction.

Complementary DNA cloning and sequencing

The methods used to prepare RNA, to amplify expressed Vγ and Vδ transcripts using the anchored polymerase chain reaction (PCR) and to clone and sequence cDNA followed described procedures[10] except that a new primer containing a *Pvu*II restriction site was designed to anneal to known bovine Cγ regions (5′ GTCAGCCAGCTGAACTTCATGTATGTG). The Cδ priming sequence used previously in sheep is conserved in cows (5′GTAGAACTCCTTCACCAAACAAGCGA-CGTTTGTC). Amplified Vγ and Vδ cDNA was digested with *Sac*II–*Pvu*II or *Sac*II–*Hinc*II, respectively, and ligated into appropriate Bluescript plasmid (Stratagene, La Jolla, CA). A total of 16 Vγ and 22 Vδ cDNA clones generated from the skin of a single yearling animal were sequenced.

RESULTS

Frequency and location of T cells

For the purpose of comparing the relative frequency of T-cell subsets, the skin was divided into four regions and positive cells in each were counted. The regions comprised: epidermis, including cells making contact with the *stratum basale*; the first 0·5 mm of dermis; a 0·5–1·0 mm dermal layer; and a 1·0–1·8 mm dermal layer. A representative illustration showing the structure of bovine skin and the distribution of CD3$^+$ cells is shown in Fig. 1(a) and the numbers of different T-cell subsets occurring in the various strata are given in Table 1.

The majority of T cells (82·6%) localized within the first 0·5 mm of the superficial papillary layer of the dermis. Only 3·1% were either within or contacting the epidermis and 14·3% were distributed in the deeper reticular layer or dense collagen layer of the dermis. Very few T cells (2·6%) localized deeper than 1·0 mm from the epidermis. If the CD3 marker is taken to label all T cells, then 39·5% of them expressed CD4, 34·6% expressed CD8 and 44·1% expressed the $\gamma\delta$ TCR detected by mAb 86D (Table 1).

For all regions of the skin, the sum of cells detected by staining for T-subset markers exceeded the number detected with CD3 (range 103·4% to 130·1%, see Table 1). This was especially noticeable in the epidermis and superficial dermis and suggested that some T cells co-expressed subset markers. This possibility was assessed by staining skin sections using three-colour fluorescence to identify each marker and examining them by confocal microscopy. In each animal ($n=6$), some

Table 1. Frequency of T cells in different regions of bovine skin

	CD3$^+$		CD4$^+$	CD8$^+$	TCR$\gamma\delta^+$	Sum of subsets	Sum of subsets as % of CD3$^+$
Epidermis	6·3 ± 2·8	3·1%	1·0 ± 0·9	4·2 ± 1·6	3·0 ± 1·3	8·2	130·1%
Dermis							
0–0·5 mm	166·0 ± 27·4	82·6%	67·2 ± 23·7	53·2 ± 15·8	78·5 ± 8·9	198·9	119·8%
0·5–1·0 mm	23·5 ± 16·2	11·7%	8·8 ± 7·4	9·8 ± 8·2	5·7 ± 3·4	24·3	103·4%
1·0–1·8 mm	5·2 ± 3·8	2·6%	2·5 ± 2·5	2·3 ± 3·1	1·3 ± 1·7	6·1	117·3%
Total	201·0 (100·0%)	100·0%	79·5 (39·5%)	69·5 (34·6%)	88·5 (44·1%)	237·5	118·1%

Data indicate the number of T cells positive for each marker in skin sections derived from six animals (mean ± SD). Areas of skin measuring 0·9 mm × 1·8 mm were scored in quadruplicate for each animal.

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Figure 1. (a) Immunoperoxidase-stained section of bovine skin showing the distribution of CD3$^+$ cells. Most T cells localized in the superficial dermis although some can be seen contacting the stratum basale of the epidermis. Scale bar = 0·5 mm. (b) and (c) Confocal micrographs of skin sections stained by three-colour immunofluorescence to detect CD4$^+$ (green), CD8$^+$ (red) and TCR$\gamma\delta^+$ (blue) T cells. (b) shows a region of superficial dermis and the dotted line indicates the base of the stratum basale of the epidermis. (c) shows a field about 0·25 mm within the dermis. Some cells had a purple-pink colour indicating co-expression of CD8 and the $\gamma\delta$TCR (large open arrows) while rare cells were yellow suggesting co-expression of CD4 and CD8 (small solid arrow). Note the irregular morphology of many skin T cells. Scale bars = 10 μm.

$\gamma\delta$ T cells co-expressed CD8 (Fig. 1b,c) although no attempt was made to enumerate them. Also, rare cells seemed to co-express CD4 and CD8 (Fig. 1b).

Vγ–Jγ gene expression

Only three different Vγ gene segments were represented among the 16 Vγ cDNA clones sequenced (Fig. 2a). Although none of these Vγ genes have been detected before in cattle, they had all been cloned previously from sheep lymphocytes. The convention of naming new bovine V-genes according to their identity to known sheep homologues[12] was therefore followed. At a nucleotide level, the Vγ3.1 and Vγ3.2 segments had 94% identity to each other and 90% and 93% identity, respectively, to sheep Vγ3. The third Vγ segment found among bovine skin cDNA clones has 88% nucleotide identity to the recently identified sheep Vγ7 gene (unpublished data) and this sequence is therefore named bovine Vγ7. The bovine Vγ3 and Vγ7 families share only 60% nucleotide identity. The segments Vγ3.1, Vγ3.2 and Vγ7 were expressed in eight, two and six cDNA clones, respectively.

In 15 out of 16 cDNA clones, the Vγ segments were functionally rearranged to one of two alleles of a new bovine Jγ segment, Jγ5. In 11 clones, the sequence included a stretch identical to sheep Jγ5 while in four clones there had been a coding change resulting in a single amino acid substitution (Fig. 2b). The consensus cDNA sequence of bovine Jγ5 suggests that the gene segment is about three codons longer at the 5′ end than is sheep Jγ5. The remaining TCRγ cDNA clone contained another new Jγ segment that was about 75% identical to bovine Jγ1.1 and Jγ1.2.[12] However, the apparent deletion of two nucleotides within the coding sequence produced a frameshift so that this transcript may have arisen from a bovine Jγ pseudogene.

Vδ – Jδ gene expression

The 22 cDNA clones generated from the skin of a single animal contained 20 different Vδ DNA sequences that were classified into 14 Vδ segments. Sequences that differed by three or fewer nucleotides were considered to represent the same Vδ segment. The Vδ segments had from approximately 80% to 97% DNA sequence identity with each other and all belonged to the large ruminant Vδ1 family. None of the Vδ segments in the skin-derived clones was identical to any of the 12 known bovine sequences.[12] The 14 new Vδ segments were therefore numbered consecutively from Vδ1.13 (Fig. 3a). No individual Vδ segment predominated among the 22 clones. Three segments were represented in three independent clones (Vδ1.15, Vδ1.20,Vδ1.23), two of them occurred independently twice (Vδ1.13, Vδ1.17) while the remaining nine Vδ segments were expressed in a single cDNA clone. The V-segment in one clone (Vδ1.26) contained a stop codon and this may therefore represent a bovine Vδ pseudogene.

(a)

```
                                                                      -14
BVγ3.1                                 TTCTCTTTGAGCACACAGCCCAGCAGACAGG  ATG TCA CCA TTG GAA GCA TTC ACA TTT TTC TCC
BVγ3.2                                                                  --- --- --- C-- --- --- --- --- --- --A ---
BVγ7      ACTTCCTTCCAACAGACCTTGCCTTTTTACCTC--CCCT-GGAGTCAGT-T-GT---GA-  --- G-- TTC C-- --- --G G-- CTC --C -C- ---

                  -1   1                                                                          20
BVγ3.1    TTC TGG ACT TTT GGA CAT GGG TTA TCA AAA GTG GAG CAG GCC CAG ATC TCC CTT TCC ACA GAA GCA AAG AAA AGT ATT GAC
BVγ3.2    --- --- G-- -C- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A--
BVγ7      C-- --- T-- --- --C -T- --- CA- -T- -CT T-- -T- --A A-T G-A G-A --A G-- A-T GG- AC- AG- G-A --G --- --A ATT
                                                                40
BVγ3.1    ATA CAT TGC AAG ATA GAG AGC ACA AAT TTT GAA TCA GAC ACT GTT CAC TGG TAC CGG CAG AAA TTG AAT CAG GTT TTG GAG
BVγ3.2    --- --- --- --- --- --- --- --- --- --- --- --- --A -T- --- T-- --- --- --- --- --- AA- --- --- -C- --- ---
BVγ7      --G TC- --- --- G-G TTC TCT -AG G-C --- AGC AA- --T TAC A-C --- --- --- --- --A --- CCA G-- --A -G- --A --A
                                      60                             67A
BVγ3.1    CAT CTG GCT TAT GTG ACC TCA ATC ACA ACT GCA GCT CGA AAA CAA GTA GAT GGG AAG AAC AAA ATT GAG GCA AGA AAA GAT
BVγ3.2    --- --- -T- --- --- --- --- -C- --- --- --- --- --- --T --- --- --- --A --- --- --- --- --- --- --- --- ---
BVγ7      --G --A CT- -T- --C TTA GAT GC- C-T G-G CTA AAC GAC TT- GG- -GG ... AA- --A --- --G C-- --- --C --- --- ---
                  80                                                                              100             104
BVγ3.1    GCT CGA ATG TTC ACT TCG ACC TTG ACG GTA AAT TTC ATA GAA AAA GAA GAT GTG GGC ATT TAC TAC TGT GCT GGC TGG GGA
BVγ3.2    --- --- --- --- --- --- --- C-T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- AGT
BVγ7      AAA -CC TCT -C- --- --T --- --- -AA A-- -G- --- T-- --G --- --- --- -A- -C- -CA --- --- --- --C --- --- TTG
```

(b)

```
BJγ5      AGT TCA ACC TGG ATC AAG GTA TTT GGT GAA GGA ACT AAG CTC GTA GTA ATA CCT CCT (11/16)
          --- --- --- --- --G --- --- --- --- --- --- --- --- --- --- --- --- --- --- (4/16)
```

Figure 2. Nucleotide sequences of (a) the bovine Vγ3.1, Vγ3.2 and Vγ7 regions and (b) two allelic variants of the Jγ5 region expressed by lymphocytes isolated from bovine skin.

(a)

```
                                                                                -20
BVδ1.13      TGAACTCTCAGCTTGAGGCACAACTAAGCACATTTGCGCAGGGAAATCCATGCCTC  ATG CCG CTT TCC AGT CTG CTC TGG GTG TTC CTG GCC
BVδ1.14   CAC--------------------G---------------N-------------------  --- --- --- --- --- --- --- --- --- --- --- ---
BVδ1.15     -----G---------------G---------T-----TT------------------  --- -T- --C --- --- --- --- --- -C- --- --- ---
BVδ1.16                         G-G---------------TCG----G-T----------  --- --A --C --- --- --- --- --- --- --- --- --T
BVδ1.17    / -----C--------------G---------------TCG-----------------  --- --- --C --- --- --- --- --- --T --- --- -T-
BVδ1.19                   A--AT-----.....--------A---A--G------C-----  --- --- --C --- --C --- -A- --- --- --- --- ---
BVδ1.20   / T--------------------G----G-----G----T------G------------  --- --- --C --- --- --- --- --- --- --- --- ---
BVδ1.21    /  -------------------G----G----------TCG----G------------  --- --A --C --T --- --- --- --- --- --- --- ---
BVδ1.22  CCAC----------A---------G----G----------AT------------------  --- --- --C --- G-G --- --- --- --- --- --- ---
BVδ1.23                                        / T-------------------  --- --- --C --- --- -C- --- --- --- --- A-- ---
BVδ1.24  CCAC---------G----------G---------------CAA----G---------T--  --- --- --C --- --- --- --- --- --- --A -G- ---
BVδ1.25    //  CGTCACGCTCAG------G---------------TCG-----------C-----  --- --- --C -GG --C --- --- --- --- C-- --- ---
BVδ1.26                                  -C------AA-----G------------  --- -T- --C --- --- --- --- --- --- --- --- ---
                                     -1   1
BVδ1.13  TTC ACC TTC TCT GGA CCT GGT GTG GCC CAG AAA GTC ACT CAA GAC CAG TCA GAT GTA TCC AGC CAT GTG GGG CAG TCA GTC
BVδ1.14  --- --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- C-- --- A-- --- --- --A --- --- --- --- ---
BVδ1.15  A-- G-- --- --- -C- T-- --- --- --- --- --- --T --- --- --- --- C-- --- A-- --- --- --C A-- --- --- --- ---
BVδ1.16  G-- G-- --- --- -C- TG- --- --- --- --- --- --T --- --- --- --- C-- --- A-- --- --- --A --- -A- --- --- ---
BVδ1.17  G-T G-- --- --- -C- T-- --- --- --- --- --G --T --- --- --- --- C-- -C- A-- --- -C- --A --- --- --- --- ---
BVδ1.18                        / --- --- --- --- --- --T --- --- --- -G- C-- --- A-- --- --- --A --- --- --- --- ---
BVδ1.19  --T --- --- --- --- T-- --- --- --- --- --- --T --- --- --- --- C-- -TC A-- AT- --T --A --- --- A-- GT- -C-
BVδ1.20  A-- --- --- --- --- T-- --- --- --- --- --- --T -T- --- --- --- C-- T-C T-- A-- --T --A A-A --- --A --T ---
BVδ1.21  A-- --- --- --- --- T-- --- --- --- --- --- --T --- --- --- --- C-- T-C --- A-- --T --A A-A --- --A --T ---
BVδ1.22  G-- --- --- --- --- T-C AC- --- --- --- C-- --A --- --- -T- --- A-- -CC --- C-- --T --A --- --- --- A-- ---
BVδ1.23  A-- G-- --- --- --- T-- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --A --- --- --- --- ---
BVδ1.24  --- --- --- --- --- T-- --- --- T-- --- --- --T --- --- -G- --- --- A-- --- --- --- --A --- --- A-- --- ---
BVδ1.25  --- --- --- --- --- T-- --- --- --G --- --T --T -T- --- --- --- C-- A-C AC- --- G-T --- --- --A --- --- ---
BVδ1.26  A-- --- --- --- --- T-- --- --- --T --- --- --T --- --- --- --- --- --- --- --- --- --A --- --- --- --- ---
         20                                          30A 30B                             40
BVδ1.13  ACC CTG AAC TGT CGG TAT GAA ACA AGT TGG ACC ... ... GCT TAC TAC CTT TAC TGG TAC AAG CAA CTT CCC AGT GGA CAG
BVδ1.14  --- --- --- --- --- --- --- --- --- --- -G- ... ... --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BVδ1.15  --- --- --T --- -AA --- --- --- --- --- -G- ... ... A-- --- --- --- -TT --- --- --- --- --- --- --- --- ---
BVδ1.16  --- --- --T --- -A- --- --- --- --- --- CG- ... ... TA- --- --- --- -TT --- --- --- --- --- --- --- --- ---
BVδ1.17  --- --- --T --- --- --- --- --- --- --- -GT ... ... TA- --- A-- --- -TT --- --- --- --- --- --- --- --- ---
BVδ1.18  --- --- --T --- -A- --- --- --- --- --- CG- ... ... TA- --- --- --- -TT --- --- --- --- --- --T --- --- ---
BVδ1.19  --- --T --- --- -A- --- --- --- --- A-- -A- GTG CAT -TC --- -G- A-- -TT --- --- --- --G --- --- --- --- ---
BVδ1.20  -TT --- --A --- -A- --- --- TT- --- CAC -GT -G- T-C A-G C-- --- T-- -TT --- --- --- --G --- --- --- --- G--
BVδ1.21  -TT --- --- --- T-- --- --- GT- --- --- --T -G- T-C A-G C-- --- --- --T --- --- --- --G --- --- --- --- G--
BVδ1.22  --- --- --T --- --A --- --- GT- --- --- --- AC- G-A TAC --- --- A-- -TT --- --T --A --G --- --- --G --- G--
BVδ1.23  --- --- --- --- --- --- --- --- --- -T- -GT ... ... A-- --- CG- --- --- --- --- --- --- --- --- --- --- ---
BVδ1.24  --- --- --T --- --- --- --- --- --- --- GG- ... ... A-- --- --- --- --- --- --- --- --- --- --- --- --- ---
BVδ1.25  --- --- --- --- --- --- -G- --- -CC --- -G- ... ... TA- --- --- A-G -TT --- --- --- -TG --- GGG -A- --- G--
BVδ1.26  --T --- --T --- --- --- --- --- -A- --A -AT ... ... --- --- --- --- -T- --- --- --- --- --- --- --- --- ---
                                                                     60
BVδ1.13  ATG ACT TAC GTT ATT CGT CAG GGT TCA GAA GTG ACA AAT GCA AGG AAA GAC CGC TAC TCT GTA AAC TTT AAG AAA GCA GAT
BVδ1.14  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BVδ1.15  --- --- --- C-- --- -AA --- TA- --- -G- TAT GGT --- --- --- --T -G- --- --T --C --- --- --C C-- --- --- ---
BVδ1.16  --- --- --- C-- --- -AA --- TA- --- --- TAC -GT --- --- --- --T -G- --- --T --- --- --- --C C-- --- --- ---
BVδ1.17  --- --- --- C-- --- -AA --- TA- --- --- CAC GGC --- --- --- --C -GT --- --T --- --- --- --- C-- --- --- ---
BVδ1.18  --T --- --- C-- --- -AA --- TA- --- -G- TAC -GT --- --- --- --T -G- --- --T --- --- --- --C C-- --- --- ---
BVδ1.19  --- --- --- C-C --C --- --- TA- --- --- -AT GGG --- -A- --- G-C -G- --- --- --- --- --- --C C-- --- --- CG-
BVδ1.20  --- --- -T- C-- --- --- --- -AA --T CTT -GC C-- --- --- --- --T -G- --- --- --- --- --- C-- C-- --- --- --G
BVδ1.21  --- --- -T- C-- --- --- --- -AA --T TCT -GC C-- --- --- --- --C -G- --- --- --- --- --- --- C-- --- --- --G
BVδ1.22  --- --- -T- C-- --- --- --- TA- --- --- -AC GGT --- --- --- --C -G- --- --- --- --- --- --C C-- --- --- ---
BVδ1.23  --- --- --- --- --- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BVδ1.24  --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- G-- --- --- --- --- --- --- --- --- --- --- ---
BVδ1.25  --- -T- --- C-- --- A-- --- -C- --T TCT -GC CAG --- --- --- --T -G- --- --- --- --- --T --- C-- --- --T CG-
BVδ1.26  --- --- --T --- --- --- --A --A --- TT- --C --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- ---
                                         80                                                      94
BVδ1.13  AAA TCC ATC AGC CTC ACC ATT TCA GCC TTA AAA CTG GAA GAC TCT GCA AAG TAC TTC TGT GCT CTC AGT
BVδ1.14  --- --- --- --- --- --- --- --- --- --- C-- --- --- C-- --- --- --- --- --- --- --- --- ---
BVδ1.15  --- --- --- --- --- -T- --- --- T-- --- C-G --- --- --T --- --- --- --T --- --- --- --- T-C
BVδ1.16  --- --- --- --- --- -T- --- --- T-- --- C-G --- --- --T --- --- --- --- --- --- --- --- TC-
BVδ1.17  --- --- --- --- --- -T- --- --- T-- --- C-G --- --- --T --- --- --- --- --- --- --- --- T--
BVδ1.18  --- --- --- --- --- -T- --- --- T-- --- C-C --- --- --T --- --- --- --- --- --- A-- --- TC-
BVδ1.19  --- --- --- --- --T --T --- --- TT- --- --- -CA --- C-T --- --- --- --T --- --- --- --- T-G
BVδ1.20  --C --- --- --- --- --- --- --- --- --- C-G --- --- --- --- --- --- --- --T --- --A G-- CAG
BVδ1.21  --C --- --- --- --- --- --- --- --- --- C-G --- --- --- --- --- --- --- --T --- CG- -AA GTG
BVδ1.22  --- --- --- --- --- -T- --- --- --- --- C-G --T --- --- --- --- --- --- --- --- --- --T T--
BVδ1.23  --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- CA-
BVδ1.24  --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- C--
BVδ1.25  --- --- --- --- --- --- --- A-- -A- --- C-- --- --- --T --- --- G-- --T --- --- --- --- TGG
BVδ1.26  --- --- --A --- --- --- --- --- --T --- C-- --- C-- --- --- --- --- --- --- --- --- --- T--
```

(b)

```
BJδ2     TCC TGG GAC ACC CGA CAG ATA TTT TTT GGA GCT GGC ACC AAA CTC TTC GTG GAG CCC
BJδ3                 --G -CC --- -A- A-A --- --C AAA --A -A- T-T --G AA- --- --A --A (allele)
```

Figure 3. Nucleotide sequences of (a) the bovine Vδ1·13–Vδ1·26 regions and (b) the Jδ2 region and a probable allelic variant of Jδ3.[12] A stop codon (TGA) at residue 29 of the Vδ1·26 segment is underlined.

Three Jδ segments were identified in the 22 clones. Bovine Jδ1 and Jδ3[12] occurred in 13 and eight clones, respectively. Two closely related Jδ3 sequences were found and these probably represent alleles. The final clone contained a new Jδ segment almost identical to sheep Jδ2[10] and it is therefore called bovine Jδ2 (Fig. 3b).

Junctional diversity of TCR γδ chains

Fifteen TCRγ and all TCRδ clones contained functionally rearranged junctions. The junctions between the predicted boundaries of Vγ and Jγ segments were short, never exceeding three amino acids (Fig. 4a). By contrast, the junctional regions of TCRδ clones were more variable and ranged from two to 21 amino acids, probably reflecting the absence or participation of either single or multiple Dδ elements in joint formation (Fig. 4b). Nucleotides appeared to have been removed from the ends of the rearranged gene segments in some clones and this was reflected in the protein sequences. However, because genomic sequences are not yet known, it is not possible to determine the precise contribution made by endonuclease removal and N-region addition of nucleotides during junction

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```
(a)
BVγ3.1   CAGWG   YR    STWIKVFGEGTKLVVIPP (BJγ5)
         ----E   SQ       ---------------
         ----A          S------------------
         -----   --     ------------------
         -----   -     ----M--------------
         ----L   G      ------------------
         ----D         -------------------
         ----L   G      ------------------
BVγ3.2   ----T   Y     -------------------
BVγ7     ----L  LAL    -------------------
         ----S              -M--------------
         ----L   LY    ----M--------------
         ----L   QN    ----M--------------
         ----F   GY    -------------------
         ----L   LY    -------------------

(b)
BVδ1.13 CALS     GYSVHWGPGFRSPTPI         TDKLIFGKGTRLIVEP (BJδ1)
        ----        LLLVGTVGYE            ----------------
BVδ1.14 ----           PQE                ----------------
BVδ1.15 ---C         HWGPSVRGYE           ----------------
        ---W      EGSVRWRDLRLYKVLD        -PEK-----NY-N--- (BJδ3)
        ---W         XLATETTIQN             P-------Y-N--- (BJδ3)
BVδ1.16 ----          ADDLRRWDT           ----------------
BVδ1.17 ---C             SG           SWD-RQIF--A--K-F--- (BJδ2)
        ---C         MLPGGTRVGYN            P-------Y-N---
BVδ1.18 -T--         EQRWVLRTTSN            P-------Y-N---
BVδ1.19 ---W   EGPAYDWGRGTLLVGYYNVQY        P-------Y-N---
BVδ1.20 --VQ           AAYPDTE            ----------------
        --VR        VVGWTGIYAWDTE         ----------------
        --VR        EHXVPWGDGVPTYE        ----------------
BVδ1.21 -RQV       QRRYRGYTTYGVGGYE       ----------------
BVδ1.22 ---C        TALDWGTSVRGRS           ---------------
BVδ1.23 ---L          GTAWGNPLE           ----------------
        ---H       GVQRTGWDGVLRGYE        ----------------
        ---H          TALDWGFWIY            P-------Y-N---
BVδ1.24 ---R         GDLRWGTRTGN            P-------Y-N---
BVδ1.25 ---W     AIQRGLGWDLRAFTTYGSY        P-------Y-N---
BVδ1.26 ---C         ELVEFRGNVGYA            ---------------
```

Figure 4. Amino acid sequences of (a) Vγ–Jγ junctions and (b) Vδ–Dδ–Jδ junctions expressed by lymphocytes isolated from bovine skin. The terminal amino acids of each V-region and the beginning of the J-regions were predicted from consensus alignment of sequences. The nucleotide sequences of bovine Jδ1 and Jδ3 have been reported previously.[12] The cDNA sequences of two Jγ5 alleles, Jδ2 and a probable allelic variant of Jδ3 are described in this paper (see Figs 2 and 3).

formation. Two TCRγ clones contained identical cDNA junctional sequences, suggesting that the clones may have originated from the same RNA template. All other clones contained highly variable junctional sequences (Fig. 4a,b).

Constant region gene usage

The Cγ primer used during PCR annealed about 200 nucleotides downstream of the Jγ–Cγ splice junction and a truncated Cγ sequence was therefore included in each TCRγ clone. The Cγ region in all clones was identical but in 14 of them the sequence was shortened to 55 nucleotides (Fig. 5). At DNA level, the Cγ sequence had 71–76% identity to the three known bovine Cγ regions but 96% identity to sheep Cγ5.[7] This is therefore the first identification of bovine Cγ5. The difference between the lengths of cloned Cγ5 regions probably reflects an allelic polymorphism. For instance, a single nucleotide change at the region where the 14 short clones ended (CAGTTG to CAGCTG) introduces a *Pvu*II restriction site which would have resulted in cleavage at this point during cloning. All TCRδ clones contained a short Cδ segment identical to the known bovine Cδ sequence.[7]

DISCUSSION

The number of TCRγδ$^+$ cells detected in these experiments provides a minimal estimate of their frequency because mAb 86D does not recognize all forms of the bovine γδTCR.[11] Because no existing antibody is specific for the bovine αβTCR and the CD8 molecule was co-expressed on some γδ T cells, the relative numbers of the two T-cell lineages in normal bovine skin cannot be deduced accurately although they seem to be about equally represented. Assuming that each T cell was counted only once in serial sections [the thickness of sections (8 μm) equates with the average diameter of a lymphocyte], the animals examined contained around $3{\cdot}1 \times 10^5$ T cells/cm^2 of skin surface area. The body surface area of cattle ranges from around 1 m^2 in a young calf up to 6 m^2 for a full-grown mature animal.[13] Depending on their age and size, cattle would therefore contain 3×10^9–2×10^{10} T cells in their skin. More than 85% of these localize in a superficial zone comprising the epidermis and, more particularly, the underlying 0·5 mm of dermis.

This pattern of localization differs to the situation in mice where most T cells in skin express the γδ TCR and occur within the epidermis.[9] This population of T cells had earlier been dubbed dendritic epidermal cells (DEC) because of their characteristic morphology.[14,15] When examined by confocal microscopy, the morphology of many T cells in bovine skin was also distinctive from that typically seen in lymph nodes and spleen. Skin-resident T cells were usually irregular in shape and frequently had a flattened outline with wavy cytoplasmic projections. However, this type of morphology was not limited to γδ T cells. It also occurred in CD4$^+$ and CD8$^+$ cells and probably reflects deformation of the plasma membrane as T lymphocytes insinuate themselves between the cellular and extracellular matrices of the skin. All T cells are rather rare in normal human skin and the few γδ T cells present tend to localize in the dermis rather than epidermis.[16,17]

The contrast between the TCR repertoire of γδ T cells in

```
        1                                  10                                      20
        D   R   R   L   D   G   D   L   F   P   K   P   T   I   F   F   P   S   V   E   E   V   K
BCγ5   GAT AGA AGG CTT GAT GGA GAC TTA TTT CCC AAG CCC ACT ATA TTT TTT CCT TCA GTT GAG GAA GTA AAA
                                                                              *  (14/16)
                                    30                                  40
        L   H   R   A   G   T   H   L   C   L   L   Q   N   F   F   P   D   A   I   K   I   Q   W
BCγ5   CTC CAC AGG GCT GGA ACA CAT CTT TGC CTT CTT CAG AAT TTT TTC CCT GAT GCT ATT AAG ATA CAA TGG

                    50                                  60
        K   E   K   N   V   N   T   I   L   E   S   Y   Q   G   N   I   I   K   T   N   D
BCγ5   AAA GAA AAG AAT GTC AAT ACA ATT CTG GAA TCT TAT CAG GGA AAT ATC ATC AAG ACT AAT GAC  (2/16)
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Figure 5. Nucleotide and translated amino acid sequences of the first 67 codons of the bovine Cγ5 segment numbered from the presumed Jγ–Cγ splice site. The nucleotide position corresponding to the cloning site in 14 out of 16 clones is indicated (*) and the region covering a probable allelic variation in bovine Cγ sequences is underlined (see text for details). The Cγ primer used for anchored PCR annealed immediately downstream of codon 67.

bovine skin and the repertoire of corresponding cells in mice and humans is probably the most striking result to emerge from the present work. The repertoire of murine DEC is nearly monoclonal – they express TCR made up of two variable region genes, Vγ3 and Vδ1, and the junctions in each chain are predominantly canonical.[18] The repertoire of γδ T cells in human skin is more diverse, but only slightly so.[19] The situation in cattle is very different. Although only three Vγ genes were used, an extensive pool of Vδ genes was expressed and the junctions in each chain were so variable that the idiotypic repertoire in this population of cells must be extremely large. However, we cannot exclude the possibility that the minor subset of γδ T cells in bovine epidermis may have a more restricted repertoire and that these cells were poorly represented in the samples we prepared.

The repertoire in bovine skin was restricted at the level of receptor isotype, since only one of the four identified bovine Cγ segments was expressed. This may indicate that the different Cγ segments of ruminants, which have distinctive and unusual structures,[7] are specialized for different effector functions at distinctive sites, in a way not unlike the immunoglobulin heavy chains. However, whether or not this is the case and just how it might be manifest remains unknown since no effector role has yet been ascribed to any TCR constant region.

The ruminant Vδ1 family has unusual properties. The 22 cDNA clones examined in the present work contained 20 different DNA sequences that we classified into 14 Vδ1 family members and none of these were identical to 12 published bovine Vδ1 sequences. Sometimes the differences between sequences were small, from one to three nucleotides. We have made similar observations in our analysis of sheep Vδ sequences.[10] Taken at face value, the isolation of so many distinctive V-segments from this number of clones implies that the Vδ1 gene pool is unusually large. However, other factors may contribute to Vδ1 sequence diversity. Polyallelism would account for some sequence variation within an individual animal and may explain the non-overlapping nature of the repertoire in a breed of cattle different from the one we have examined.[12] Small sequence differences may also be generated during PCR. However, the frequency of nucleotide misincorporation under the conditions we used is around 1 in 900 bases[20] so this would not account for much of the sequence diversity.

Most of the variability in the bovine Vδ1 sequences was clustered to two regions between codons 29 and 32, and codons 52 and 56, respectively. This suggests that the V-regions have been selected at sites corresponding quite closely to antibody complementary determining regions (CDR), a feature noted earlier in sheep Vδ segments isolated from blood lymphocytes.[10] It remains an open question as to whether, how, and over what time frame selection may have been exerted but there are two broad possibilities, which are not mutually exclusive. The variability at these sites could reflect the accumulation of a large number of different germ-line sequences over an evolutionary time scale or it might result from somatic mutation of Vδ1 genes. By analogy with other antigen receptor V-genes, in either case the clustering of variability is likely to reflect a selective process that is intrinsic to the mechanism of antigen recognition.[21] This type of molecular evidence argues that ruminant γδ T cells have a better developed capacity to recognize diverse ligands than do similar cells in other species. A more detailed study of ruminant γδ T cells may therefore lead to unexpected insights into the functional evolution of T-cell recognition.

ACKNOWLEDGMENTS

We thank Rhodri Ceredig and Thomas Göbel for constructive comments on the manuscript, Birgit Kugelberg for tissue sectioning and Mark Dessing and Bea Pfeiffer for assistance with confocal microscopy and photography. The Basel Institute for Immunology was founded and is supported by F. Hoffman–La Roche Co. Ltd, Basel, Switzerland.

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