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Identification of a bovine surface antigen uniquely expressed on $CD4^-CD8^-$ T cell receptor γ/δ^+ T lymphocytes*

In this study, two monoclonal antibodies, IL-A29 and CC15, are described that identify a novel bovine cell surface marker of 215/300 kDa. The antibodies reacted with a discrete population of resting lymphocytes in peripheral blood which, in young animals, constituted about 25% of the mononuclear cells. Thymus, lymph nodes and spleen contained < 5% positive cells. These cells were negative for surface Ig, a monocyte/granulocyte marker, and the T lymphocyte antigens CD2, CD6, CD4 and CD8. Immunohistological analyses revealed the presence of IL-A29/CC15-positive lymphocytes in the thymic medulla, in the outer cortex of lymph nodes, in the marginal zones of the spleen, in the dermal and epidermal layers of the skin and in the lamina propria of the gut. The IL-A29/CC15$^+$ cells in unfractionated blood mononuclear cells responded in autologous and allogeneic mixed lymphocyte cultures, and when purified they responded to concanavalin A in the presence of recombinant interleukin 2. These observations suggested this population of cells belonged to the T cell lineage. In order to unambiguously define their lineage, cDNA clones encoding bovine T cell receptor (TcR) and CD3 proteins were isolated. Northern blot analyses of IL-A29/CC15$^+$ cell populations and of established cell lines of various lineages demonstrated that they expressed TcR δ and CD3 γ, δ and ε mRNA; TcR α was not expressed, whereas only a truncated form of TcR β mRNA was present. These results indicate that the IL-A29 and CC15 antibodies define a unique population of $CD4^-CD8^-$, γ/δ T cells.

1 Introduction

Studies of murine and human T lymphocytes have demonstrated two distinct populations expressing different TcR. One population expresses the "conventional" α/β heterodimeric TcR [1–5], which is known to recognize foreign antigen presented in the context of class I or class II MHC molecules. The other population expresses a heterodimeric TcR made up of γ and δ chains [6–9]. Although cloned populations of the γ/δ T cells have been shown to have cytotoxic activity, usually of a non-MHC-restricted nature [6, 10, 11], the precise function of this cell population is not known. Both types of TcR are non-covalently associated on the cell surface with several invariant polypeptides, collectively known as CD3, which are believed to be involved in transmembrane signaling [5, 12].

A prominent feature of human γ/δ T cells is that they are present in larger numbers in fetuses and neonates than in adults. The expression of other T lymphocyte differentiation antigens on γ/δ T cells varies. While a substantial proportion of them have the $CD2^+CD5^+CD4^-CD8^-$ phenotype, populations which do not express CD2 or which are positive for CD8 or CD4 have also been described [13–16]. They are all positive for CD3 and they express only low levels of the determinant recognized by mAb WT31 which reacts strongly with α/β T cells [16]. So far, no unique markers have been identified on $CD4^-CD8^-$ γ/δ cells. At present the only definitive means of positively identifying the γ/δ T cells is by using mAb or DNA probes specific for the TcR γ/δ itself.

Efforts to produce mAb against bovine T lymphocyte differentiation antigens have yielded reagents specific for molecules which are the homologues of CD2, CD4, CD5, CD6 and CD8 in man [17–21]. However, it has not so far been possible to generate antibody reagents specific for CD3 or the associated TcR.

Herein, we describe the phenotypic and functional characteristics of a discrete subpopulation of bovine T lymphocytes identified by two mAb specific for a 215/300-kDa cell surface antigen. Northern blot analyses with CD3 and TcR cDNA probes isolated in this study demonstrated that the 215/300-kDa antigen represents a specific marker for bovine $CD4^-CD8^-$ γ/δ T cells in cattle.

[I 8094]

* This is ILRAD publication No. 810.

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2 Materials and methods

2.1 Animals and cell populations

The cattle used in this study comprised female or castrated male Friesians (*Bos taurus*) and Borans (*Bos indicus*) aged between 1 week and 9 years. Inbred BALB/c mice were used for production of mAb. PBMC were isolated from

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venous blood by density gradient centrifugation. Highly enriched populations of erythrocytes, platelets, polymorphonuclear leukocytes and monocytes were obtained from peripheral blood as described previously [18, 22]. Suspensions of cells were prepared from samples of LN, spleen and thymus, collected immediately following slaughter of healthy animals, as described elsewhere [18]. Blocks of tissue were also collected, frozen in OTC mounting medium and stored in liquid nitrogen.

2.2 mAb

The production and preliminary characterization of the two mAb discussed herein, IL-A29 (IgG_1) and CC15 (IgG_{2a}), have been reported previously [23, 24].

2.3 Staining of cell suspension and tissue sections

Suspensions of lymphoid cells were stained by indirect immunofluorescence and analyzed on a fluorescence-activated cell sorter (FACStar plus, Becton Dickinson, Sunnyvale, CA) [18, 25]. Purification of specific populations of lymphocytes for preparation of RNA and for functional studies was also achieved by immunofluorescence staining and FCM. Cryostat sections of bovine lymphoid tissues were stained by the indirect immunoperoxidase technique.

The following mAb, in addition to those described herein, were used: CH128A (IgG_1) and IL-A43 (IgG_2) are specific for bovine CD2 [19]; IL-A57 (IgG_2) is specific for an antigen expressed on mature bovine T cells [20], believed to be the homologue of CD6 in man; IL-A11 (IgG_2) and CC30 (IgG_1) are specific for the bovine homologue of CD4 [17], and IL-A67 (IgG_1) and IL-A17 (IgG_1) are specific for the homologues of CD5 and CD8, respectively [18, 21]; IL-A30 (IgG_1) is specific for bovine IgM [26], and IL-A24 (IgG_1) reacts with an antigen restricted to bovine monocytes and granulocytes [27].

2.4 Cell lines

Fibroblast cell lines were established in culture from biopsy samples of bovine skin. Cloned cell lines transformed by the protozoan parasite *Theileria parva* were established from purified populations of lymphocytes, as described previously [28]. Briefly, lymphocyte populations expressing IgM, CD2 or the antigen recognized by mAb IL-A29 were purified to > 98% purity by FCM and exposed to suspensions of *T. parva* sporozoites for 1–2 h. They were then distributed at LD into 96-well microtiter plates containing bovine fibroblasts as a feeder layer. Infected cells growing at clonal frequency were selected and expanded in the absence of a feeder layer. Such infected cells can be maintained indefinitely in culture. Both fibroblasts and parasitized cell lines were grown in RPMI 1640 medium containing 20 mM Hepes buffer, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-ME and 50 μg/ml gentamycin.

2.5 Immunoprecipitation

PBMC, lymphocytes from an autologous MLC and cloned cell lines infected with *T. parva* were surface-labeled with ^{125}I by the lactoperoxidase technique. Antigens reactive with the mAb were precipitated from cell lysates with protein A-Sepharose and precipitates analyzed by SDS-PAGE in 5% polyacrylamide gels run under reducing or nonreducing conditions. Labeled antigens were visualized on dried gels by autoradiography.

2.6 Preparation of cDNA libraries from bovine PBMC

RNA was prepared from freshly isolated bovine PBMC by the acid guanidinium thiocyanate phenol chloroform method [29]. Poly(A)$^+$ RNA was purified over oligo-dT cellulose, and converted into cDNA using the BRL cDNA synthesis kit according to the manufacturer's instructions (BRL, Gaithersburg, MD). The cDNA was Eco RI methylated, and Eco RI linkers were ligated and cleaved by Eco RI digestion. The cDNA was size-fractionated and separated from free linkers by centrifugation on a 5%–20% potassium acetate gradient at 300 000 × g for 2 h. Fifty nanograms of pooled cDNA (> 800 bp) was ligated into 1 μg of Eco RI-digested, phosphate-treated λgt10 or gt11 arms. In both cases, 5×10^4–10^5 primary recombinant phages were obtained after infection of the *E. coli* NM514 strain.

2.7 Isolation of cDNA clones encoding bovine CD3 and TcR proteins

Plaque lifts were performed before amplification of the primary libraries and the nitrocellulose filters were sequentially hybridized under low-stringency conditions with human TcR α (pY14, [2]), TcR β (pJR216, C. Hall and C. Terhorst, unpublished), TcR δ (pO-240, [30]), CD3 γ (pJ6T3γ, [31]), CD3 δ (pPGBC9, [32]) and CD3 ε (pJ4, [33]). cDNA probes were labeled by random oligo priming. Five to 25 primary positive clones were identified with each probe. Four clones of each cDNA species were plaque purified, their insert lengths were determined and the longest inserts were subcloned in pUC19 and subjected to double-stranded sequencing from both ends using the dideoxy method.

2.8 RNA isolation and Northern blot analysis

Total cellular RNA was prepared as described above. Samples of 10 μg RNA were analyzed by standard Northern blotting, under high stringency conditions, with bovine CD3 and TcR probes labeled as described above. Washes were performed at 60 °C in 0.2 × SSC. After exposure, blots were stripped by boiling in 0.1% SDS and reprobed. A bovine class I MHC cDNA clone served as a positive control.

2.9 Functional assays

Allogeneic and autologous MLC and cultures stimulated with Con A or PWM were established in 96-well or 24-well plates as described previously [24, 34]. Intact PBMC were

used both as stimulator cells and as responder cells in the allogeneic MLC, whereas the autologous MLC was established with monocyte-depleted PBMC [24] as responder cells and intact PBMC as stimulator cells. Proliferation in the cultures was evaluated by measuring incorporation of ^{125}I iododeoxyuridine. This was done after 3 days of culture with mitogens and after 5 days in the case of MLC.

3 Results

3.1 Cells recognized by mAb IL-A29 and CC15 in peripheral blood

The mAb IL-A29 and CC15 reacted with a discrete population of cells in peripheral blood. These were small cells as indicated by their low forward and side scatter properties, and cytological examination of positive cells purified by FCM showed that they had the morphological characteristics of lymphocytes. Neither of the mAb reacted with granulocytes, monocytes, platelets or erythrocytes isolated from peripheral blood. The percentage of positive cells in PBMC varied between individual animals, but was consistently higher in young animals than in adults. As shown in Table 1, IL-A29 reacted with about 25% of PBMC in calves less than 3 weeks of age, with about 15% of PBMC in animals 3–12 months of age and with only 5% in animals over 3 years of age. When both mAb were tested on the same animals, in most instances they reacted with a similar percentage of cells. However, in some of the young animals, particularly those less than 3 weeks of age, CC15 reacted with a small additional population, always constituted $<5\%$ of the PBMC.

Table 1. Percentages of cells reacting with mAb IL-A29 in bovine lymphoid tissues

Tissue cells	No. of animals	Age	Percentage positive cells[a)]
PBMC	25	1–3 weeks	26.5 (5–54)
	14	3 months	14.4 (9–26)
	17	1 year	15.2 (5–34)
	14	3–9 years	5.0 (2– 9)
LN	6	1–2 years	4.0 (2– 8)
Spleen	4	1–2 years	4.7 (3– 6)
Thymus	7	3 months–2 years	2.4 (1– 4)

a) Mean (range).

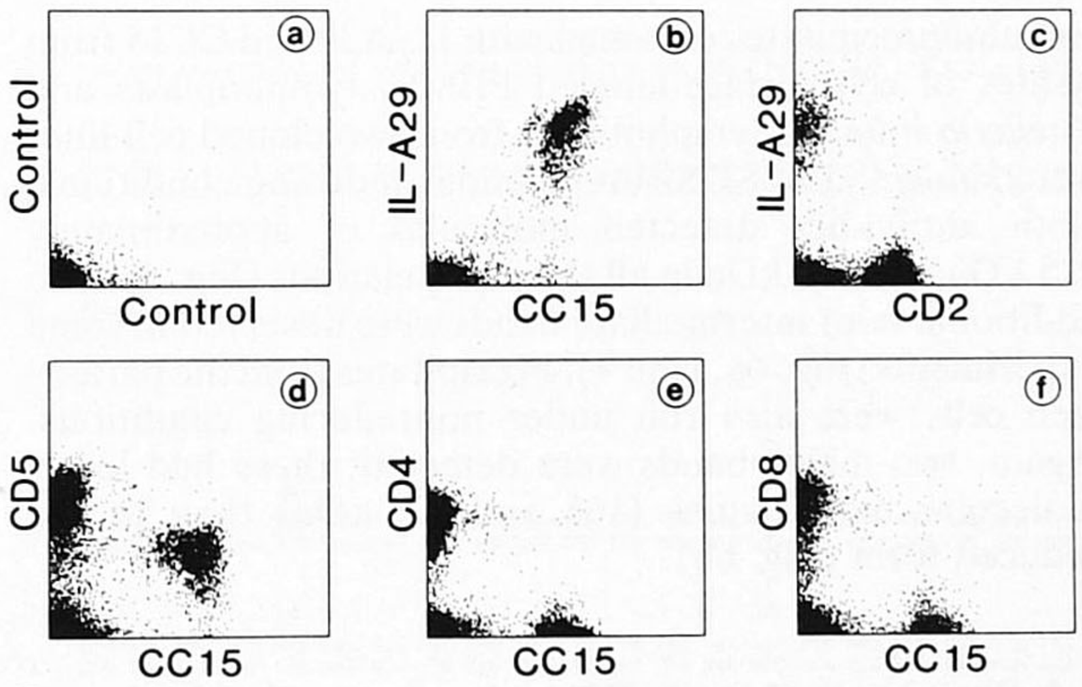

Figure 1. Two-color FCM analyses of bovine PBMC showing red fluorescence (PE) on the vertical axes and green fluorescence (fluorescein) on the horizontal axes. Staining with CC15 and IL-A29 demonstrates a small population of CC15$^+$ IL-A29$^-$ cells (b). The majority of the CC15$^+$ cells express low levels of CD5 (d) but are negative for CD4 (e) and CD8 (f). (c) shows that the IL-A29$^+$ cells are also negative for CD2. mAb IL-A43, IL-A67, CC30 and IL-A17 were used to detect CD2, CD5, CD4 and CD8, respectively. Fluorescence on both axes is displayed on a logarithmic scale.

Examination of PBMC by two-color immunofluorescence showed that the population of cells recognized by mAb IL-A29 and CC15 did not express surface IgM, the monocyte/granulocyte antigen recognized by IL-A24, or the T cell antigens CD2, CD6, CD4 and CD8 (Fig. 1). Bovine PBMC exhibit a bimodal density of expression of the CD5 antigen. The majority of the IL-A29$^+$ and CC15$^+$ cells were within the weak positive population. Two-color staining also confirmed the presence of a small population of CC15$^+$ IL-A29$^-$ cells in some animals (Fig. 1b).

3.2 Tissue distribution of cells recognized by mAb IL-A29 and CC15

About 4% of cells in cell suspensions from LN and spleen reacted with mAb IL-A29 and CC15 (results for IL-A29 shown in Table 1). Positive cells constituted about 2% of thymocyte suspensions (Table 1); two-color staining demonstrated that the phenotype of these cells was similar to that of the positive cells in the blood, *i.e.* they were CD2$^-$CD5$^+$CD4$^-$CD8$^-$ (not shown).

The two mAb stained a small population of cells in sections of thymus; these cells were concentrated mainly in the medulla but a few were also observed scattered through the cortex (Fig. 2). Positive cells in LN were found predominantly in the outer areas of the cortex adjacent to the subcapsular sinuses (Fig. 3). A few cells were also present in the sinuses and in the paracortex but positive cells were rarely seen in the B cell follicles. In the spleen, positive cells

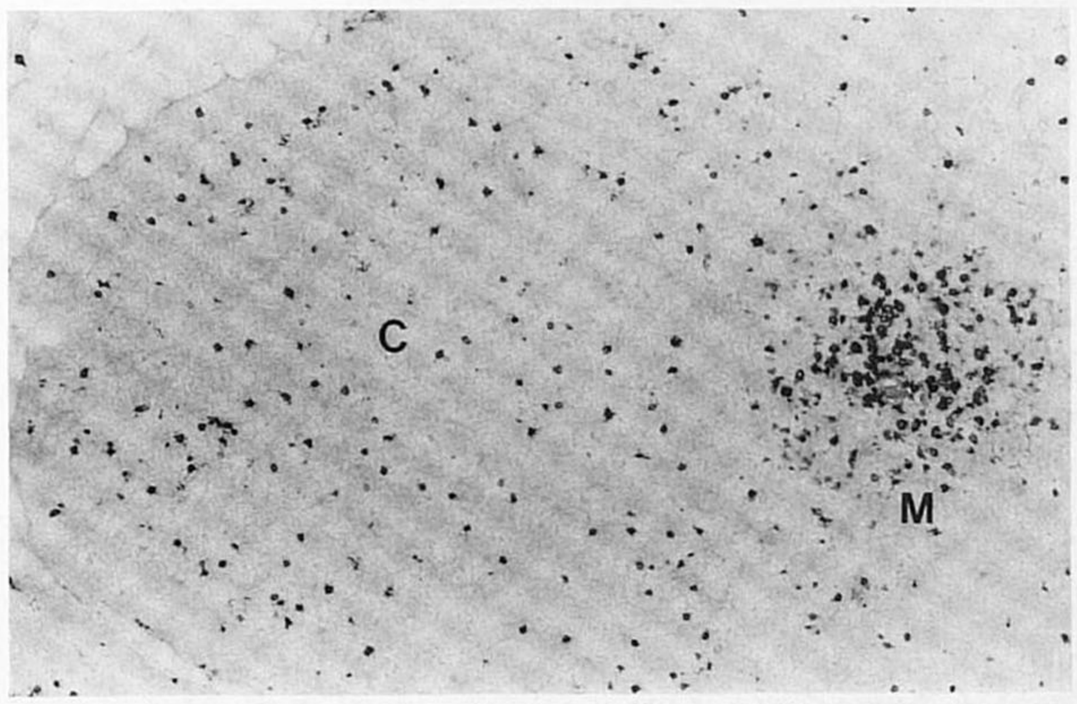

Figure 2. Immunoperoxidase staining of bovine thymus with IL-A29: positive cells are concentrated in the medulla (M) and are also found in smaller numbers scattered through the cortex (C).

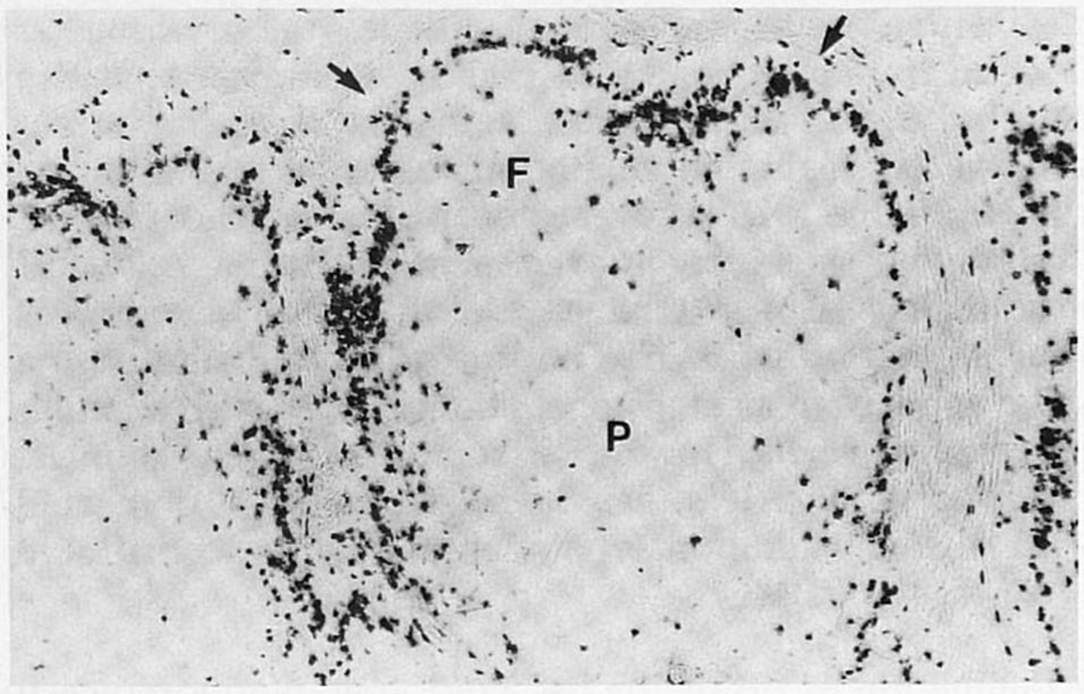

Figure 3. Immunoperoxidase staining of bovine LN with IL-A29: positive cells are concentrated in the peripheral areas of the cortex adjacent to the subcapsular and cortical sinuses. (F) B cell follicle; (P) paracortex; arrows, subcapsular sinus.

were concentrated mainly in the marginal zones (Fig. 4), with a few cells in the red pulp and sometimes in the periarteriolar regions.

Other tissues examined included skin, gastrointestinal tract, lungs, liver, kidney and brain. Populations of cells reactive with IL-A29 and CC15 were prominent in the skin and gastrointestinal tract. The majority of positive cells in the skin were found in the dermis, but a few were also detected in the basal layers of the epidermis (Fig. 5). These cells were larger than the positive cells detected in other tissues and they had a dendritic morphology. In the

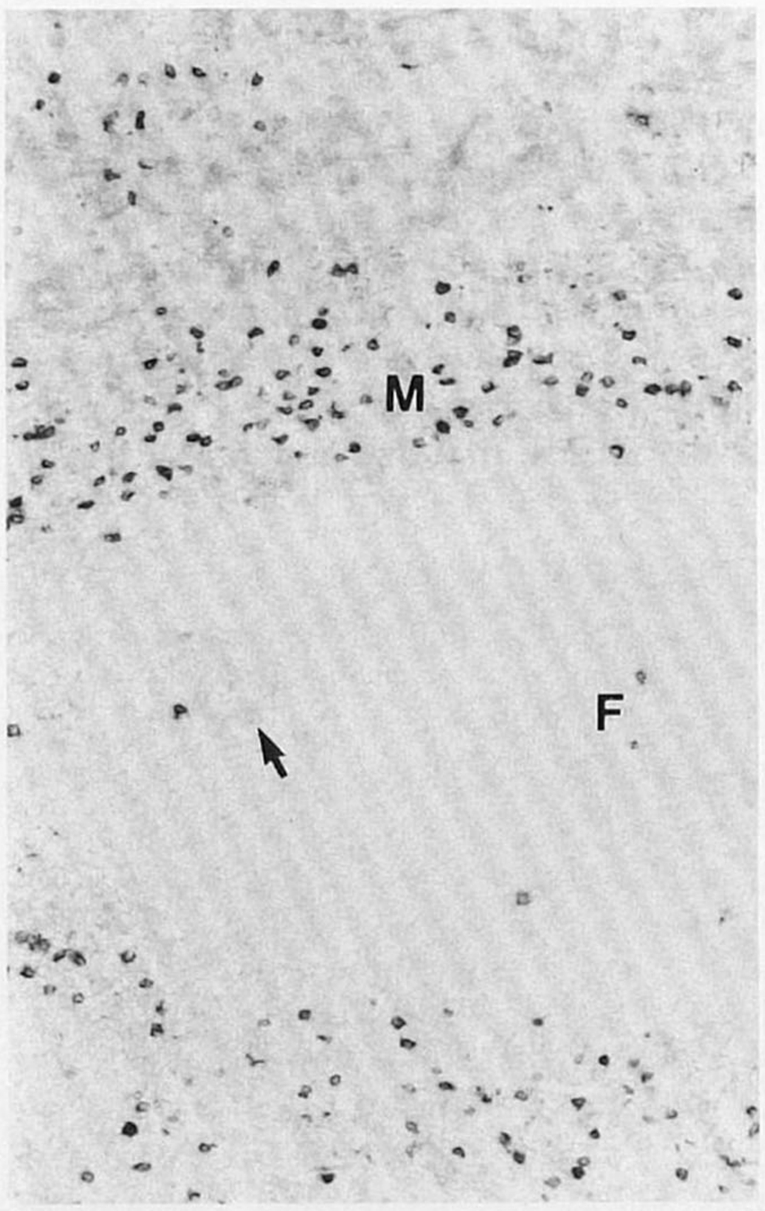

Figure 4. Immunoperoxidase staining of bovine spleen with IL-A29: positive cells are concentrated in the marginal zones (M). There are virtually no positive cells in the periarteriolar region (arrow, central arteriole) or the B cell follicle (F).

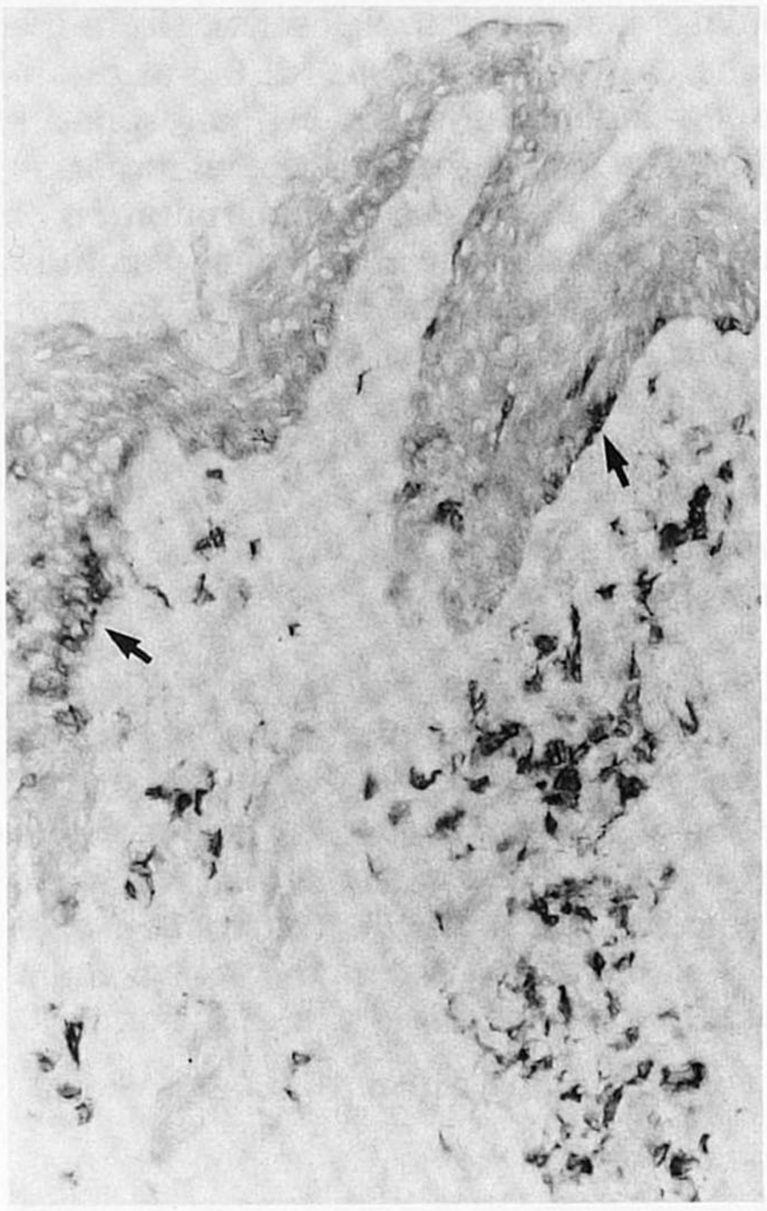

Figure 5. Immunoperoxidase staining of bovine skin with IL-A29. There are numerous positive cells in the dermis. A few are also present in the basal layers of the epidermis (arrows).

intestinal tract, most of the positive cells were found in the lamina propria, although a few cells were also detected within the epithelium. Very few positive cells were observed in the Peyer's patches but always in the interfollicular areas. Other tissues examined contained only occasional positive cells.

3.3 Immunoprecipitation

Immunoprecipitates obtained with IL-A29 and CC15 from lysates of cell surface-labeled PBMC, lymphoblasts and *Theileria*-infected lymphoblasts from two cloned cell lines were subjected to SDS-PAGE under reducing conditions. Both antibodies detected molecules of approximately 215 kDa and 300 kDa in all three populations (Fig. 6). Two additional faint intermediate bands were observed in some experiments (Fig. 6a, lane 4). Precipitates from the parasitized cells were also run under nonreducing conditions. Again, two major bands were detected; these had lower molecular mass values (165 and 275 kDa) than in the reduced form (Fig. 6b).

3.4 Characterization of cDNA clones encoding bovine CD3 and TcR proteins

Cloned cDNA encoding CD3 and TcR proteins were obtained by screening cDNA libraries prepared from bovine PBMC, with human cDNA probes. Homology comparisons of the partial sequences of the bovine CD3 and of complete sequences of the TcR cDNA clones with the available human and mouse sequences established the identity of full-length clones for bovine TcR α (pBTCRa1),

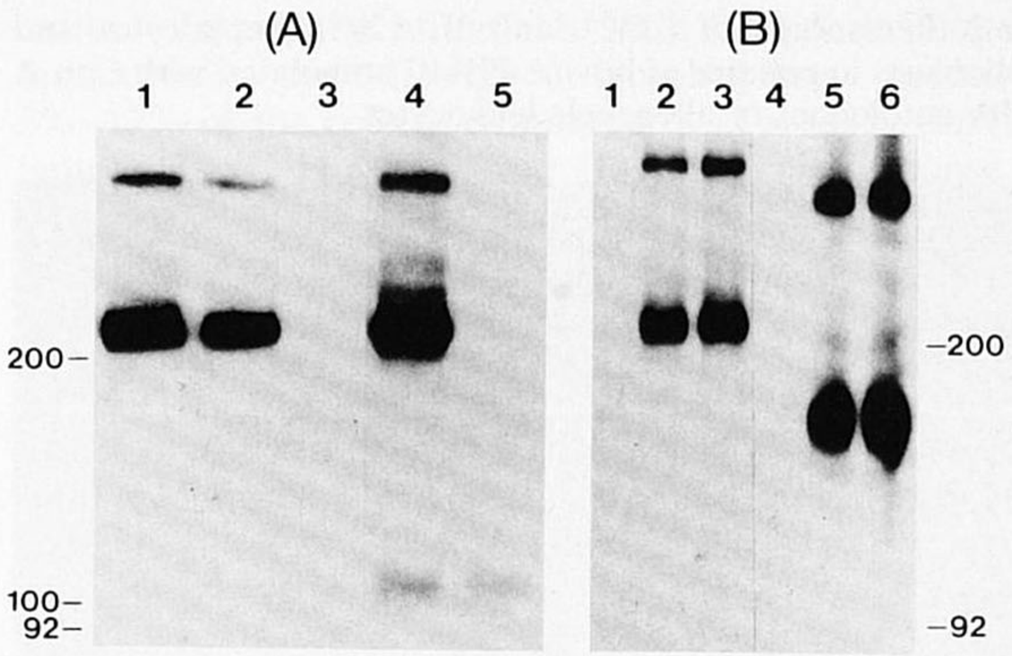

Figure 6. SDS-PAGE of immunoprecipitates obtained with IL-A29 and CC15 from ^{125}I surface-labeled cells (A) lanes 1, 2 and 3 contain immunoprecipitates from PBMC with CC15, IL-A29 and a control IgG_{2a} mAb, respectively, run under reducing conditions. Lanes 4 and 5 contain immunoprecipitates from autologous MLC lymphoblasts with IL-A29 and a control IgG_1 mAb, respectively, run under reducing conditions. (B) Immunoprecipitates from a cloned *Theileria*-infected cell line with CC15 and IL-A29 are shown run under reducing (lanes 2 and 3) and nonreducing (lanes 5 and 6) conditions. Lanes 1 and 4 contain control antibodies.

TcR β (pBTCRb1), CD3 γ (pBCD3c1), CD3 δ (pBCD3d1) and CD3 ε (pBCD3e1). The longest TcR δ clone (pBTCRd1) lacked 300–400 bp at the 5′ end. The 5′ nucleotide sequences and predicted amino acid sequences of the bovine CD3 cDNA are given in Fig. 7. The complete sequences of the TcR clones were determined and will be reported elsewhere (A. Bensaid and S. Dunlap, manuscript in preparation).

CD3γ

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                -24             -20                             -10                  ‖ 1
                Met Asp Met Glu Gln Gly Lys His Leu Ala Gly Leu Ile Leu Ala Val Phe Leu Leu Gln Gly Thr Met Ala‖His Val Lys
1    GAGAACAGAG ATG GAT ATG GAG CAG GGG AAG CAT CTG GCT GGC CTC ATC CTG GCT GTC TTT CTT CTT CAA GGT ACT ATG GCC ‖CAT GTG AAA

                          10                              20                              30
     Glu Val Lys Val Asp Asp Asn Arg Glu Asp Gly Ser Val Ile Leu Ile Cys Val Thr Asn Asp Thr Thr Ile Thr Trp Leu Lys Asp Val
92   GAA GTA AAA GTG GAT GAC AAT CGA GAA GAT GGT TCC GTA ATT CTG ATT TGT GTC ACG AAT GAC ACA ACG ATC ACA TGG CTT AAA GAT GTG

                          40                              50                              60
     Glu Gln Ile Gly Ser Gly Asp Thr Lys Lys Asn Xxx Trp Asn Leu Gly Ser Ser Thr Lys Asp Pro Arg Gly Ile Tyr Lys Cys Glu Gly
182  GAA CAA ATA GGT TCT GGA GAC ACA AAG AAA AAT NCT TGG AAT CTT GGA AGT AGT ACC AAA GAC CCT CGA GGA ATA TAT AAA TGT GAA GGA

                       69
     Ser Asn Asn Gln Ser Lys
272  TCA AAT AAC CAA TCA AAA
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CD3δ

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                -21                             -10                          ‖ 1
                Met Glu His Ser Arg Phe Leu Ser Cys Leu Ile Leu Ala Ala Leu Leu Ser Gln Val Asn Pro‖Arg Ile Leu Lys Val
1    GAATTCCGGGGGAG ATG GAG CAC AGC AGG TTT CTC TCT TGC CTG ATA CTG GCT GCC CTT CTC TCC CAA GTG AAT CCC ‖CGC ATT CTA AAA GTG

                10                              20                              30
     Leu Glu Pro Glu Asp Lys Val Met Leu Glu Cys Asn Ser Ser Ile Thr Leu Leu Gln Gly Thr Glu Gly Gln Glu Val Ser Gly Asn Asn
93   CTG GAA CCT GAG GAC AAA GTA ATG TTG GAA TGC AAT TCC AGC ATC ACA TTG CTA CAA GGA ACA GAA GGA CAA GAG GTG TCA GGC AAT AAT

          38
     Lys Leu Gly
183  AAA CTC GGA
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CD3ε

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                -21                             -10                          ‖ 1
                Met Gln Ser Gly Asn Leu Trp Arg Ala Leu Gly Leu Cys Leu Leu Leu Val Gly Ala Trp Ala‖Gln Asp Ala Asp
1    GTCCAGTGAGGGAAGG ATG CAG TCG GGG AAT CTC TGG CGA GCT CTG GGA CTC TGC CTC TTA TTA GTT GGT GCT TGG GCA ‖CAA GAT GCT GAT

                10                              20                           31
     Glu Gln Lys Pro Tyr Glu Val Ser Ile Ser Gly Asn Thr Val Glu Leu Thr Cys Pro Arg Glu Phe Glu Gly Glu Ile His
92   GAA CAG AAA CCA TAT GAA GTC TCC ATC TCT GGA AAC ACT GTT GAG CTG ACA TGC CCT AGA GAG TTT GAA GGT GAA ATA CAT T
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Figure 7. Nucleotide sequences and predicted amino acid sequences of the 5′ ends of cDNA clones encoding bovine CD3 γ, δ and ε. The predicted N-terminal amino acid sequence of CD3 γ shared 51 out of 92 residues with it's human homologue (for comparison, human and mouse CD3 γ share 54 residues in the same stretch of sequence). The N-terminus of bovine CD3 δ shared 21 out of 40 residues with it's human homologue and 23 out of 40 with it's mouse homologue (human and mouse CD3 δ share 25 residues in the same stretch). The predicted N-terminal amino acid sequence of CD3 ε shared 33 out of 52 residues with it's murine homologue (human and mouse CD3 ε share 24 of their most 5′ 52 residues).

3.5 Northern blot analyses

Radiolabeled CD3 γ, δ and ε, and TcR α, β and δ cDNA were used to probe Northern blots prepared from two different sets of cells. The first set consisted of three sorted populations of resting lymphocytes, purified from PBMC by FCM, and skin fibroblasts, all derived from the same animal; the three populations of lymphocytes were sorted to >97% purity on the basis of expression of surface IgM, the IL-A29 determinant and CD4 or CD8, respectively. The second set of cells consisted of six cloned cell lines infected with *T. parva*, all derived from the same animal; two of these were B cell lines, two were IL-A29$^+$ cell lines, one was a CD4$^+$ T cell line and one was a CD8$^+$ T cell line. Representative results of the Northern blot analyses are shown in Fig. 8.

The CD3 γ and ε probes both identified single species of RNA of approximately 1.3 and 1.7 kb, whereas the CD3 δ probe identified two transcripts of approximately 1.5 and 2.2 kb. Transcripts of CD3 γ, δ and ε were detected in unfractionated PBMC, in purified cells expressing CD4 or CD8 and in purified IL-A29$^+$ cells, but not in purified B cells or fibroblasts. Similar transcripts were detected in IL-A29$^+$, CD4$^+$ and CD8$^+$ cell lines infected with *T. parva* but were not detected in infected B cell lines.

The TcR α probe identified transcripts of about 1.6 kb while the TcR β probe identified two sizes of transcript of approximately 1.2 and 1.5 kb. By analogy with findings in human and mouse [4], it was assumed that the truncated 1.2-kb transcript represented incomplete D-J-C β message. Transcripts of TcR α and both forms of TcR β were detected in RNA from unfractionated PBMC, from purified popu-

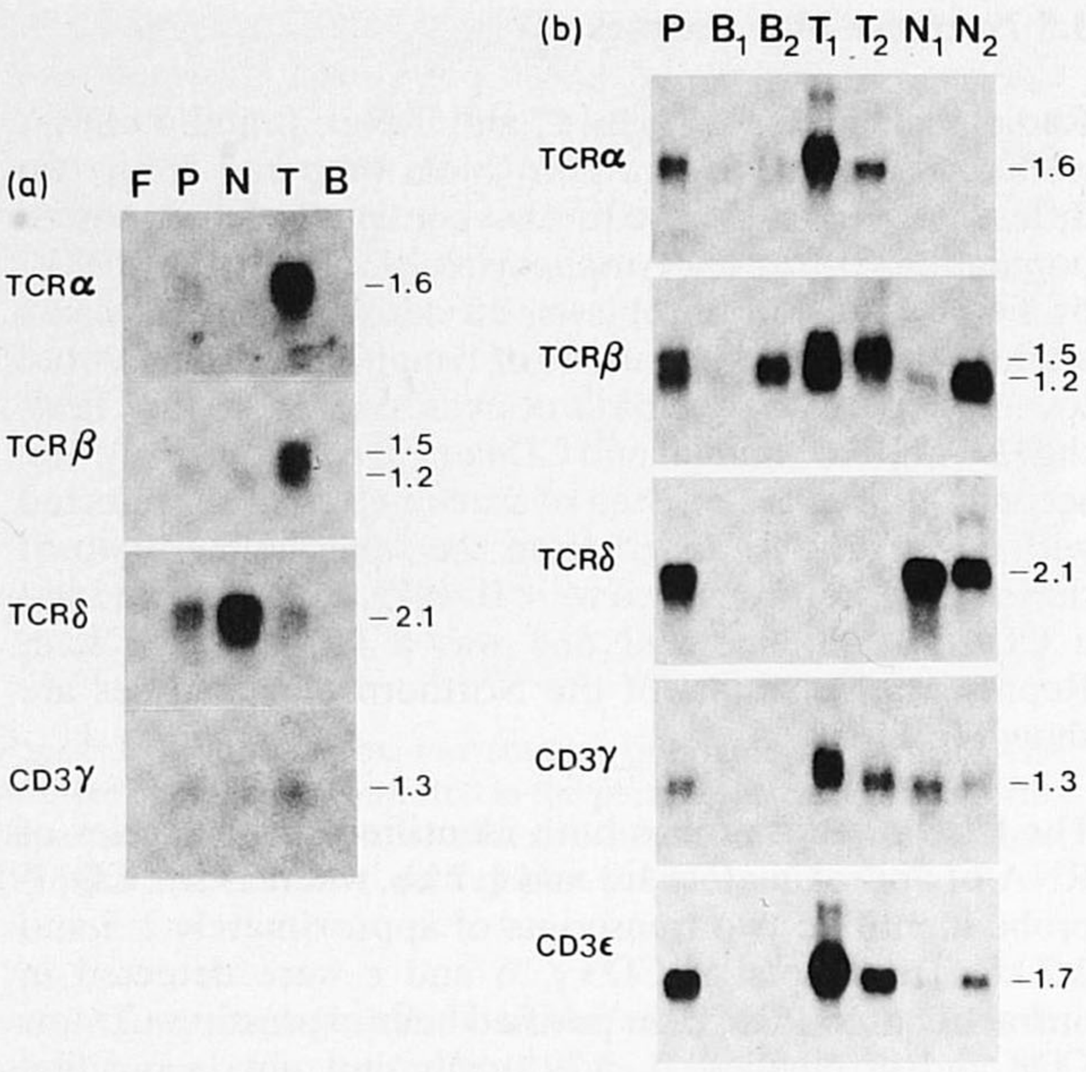

Figure 8. Expression of bovine TcR and CD3 RNA in purified populations of resting lymphocytes and cloned parasitized cell lines. (a) Northern blots of RNA prepared from fibroblasts (F), unfractionated PBMC (P) and lymphocyte populations purified by FCM on the basis of expression of the IL-A29 determinant (N), CD4 or CD8 (T) and surface IgM (B). The filter was probed sequentially with bovine cDNA probes for TcR α, β and δ and CD3 γ. (b) Northern blots of RNA prepared from cloned cell lines infected with *T. parva*. Lanes B_1 and B_2 contain RNA from B cell lines. Lanes T_1 and T_2 contain RNA from $CD4^+$ and $CD8^+$ T cell lines, respectively. Lanes N_1 and N_2 contain RNA from IL-$A29^+$ cell lines. The infected cell lines were derived by infection of the respective purified lymphocyte populations with *T. parva* sporozoites and cloned by LD. The filter was probed sequentially with TcR α, β and δ and CD3 γ and ε. Further probing of the above filters was not possible. However, in separate experiments expression of CD3 ε (sorted populations) and CD3 δ was shown to be similar to that of CD3 γ.

lations of lymphocytes expressing CD4 or CD8 and from cloned $CD4^+$ and $CD8^+$ parasitized cell lines. By contrast, the purified IL-$A29^+$ lymphocytes and the IL-$A29^+$ cloned cell lines were negative for TcR α and contained only the truncated 1.2-kb form of TcR β message. The latter was also detected in the infected B cell lines, as was a trace of TcR α message. No message for TcR α or β was detected in the purified B lymphocytes or in the cultured fibroblasts.

Table 2. Percentage of $CD2^+$ and IL-$A29^+$ lymphocytes and lymphoblasts in cultures of bovine PBMC stimulated with Con A or with autologous or allogeneic leukocytes

Culture	Donor animal	Percentage of cells of each phenotype (blast cells in parenthesis) Input cells $CD2^+$	IL-$A29^+$	Cultured cells[a] $CD2^+$	IL-$A29^+$
Con A	E55	60	8	61 (18)	18 (5)
	E77	55	16	53 (16)	19 (8)
	F304	32	6	61 (24)	24 (10)
Allogeneic MLC	E55	60	8	54 (18)	18 (5)
	E77	55	16	38 (11)	36 (12)
	F304	32	6	50 (19)	18 (4)
Autologous MLC	E76	44	8	27 (8)	57 (20)
	E77	66	6	29 (9)	50 (27)
	E192	79	5	48 (20)	32 (14)
	E223	48	11	31 (11)	44 (12)

a) Con A-stimulated cultures were analyzed on day 3 and MLC on day 5. Blast cells were defined by FACS analysis as lymphocytes displaying greater forward angle scatter than resting blood lymphocytes.

The TcR δ probe detected transcripts of approximately 2.1 kb in purified IL-$A29^+$ lymphocytes and in the IL-$A29^+$ parasitized cell lines. A weak signal was also detected in the purified population of lymphocytes expressing CD4 or CD8, but the $CD4^+$ and $CD8^+$ parasitized cell lines did not contain detectable message for TcR δ. The purified B cells, parasitized B cell lines and cultured fibroblasts were also negative. Reprobing the same blots with a bovine class I MHC cDNA probe demonstrated that the RNA prepared from B cells and fibroblasts did contain comparable levels of RNA.

3.6 Conditions for stimulation of proliferation of IL-$A29^+$ cells

The participation of IL-$A29^+$ cells in various *in vitro* proliferative responses involving T lymphocytes was examined by enumerating IL-$A29^+$ lymphocytes and lymphoblasts in responses of PBMC to Con A and in autologous and allogeneic MLC. As shown in Table 2, there was an increase in the percentage of IL-$A29^+$ cells and the appearance of IL-$A29^+$ lymphoblasts in all three types of

Table 3. Responses of purified populations of $CD2^+$ and IL-$A29^+$ bovine T cells to stimulation with Con A, PWM and bovine rIL 2[a]

	(ng/ml)	$CD2^+$ T cells –	Con A	PWM	IL-$A29^+$ cells –	Con A	PWM
Medium		653	64 562	45 183	465	1 852	447
rIL 2	100	43 840	85 092		15 836	137 413	
	33	43 440	77 473		16 978	119 631	
	11	24 623	85 232		16 224	134 678	
	3.6	14 868	78 773		13 532	129 799	
	1.2	7 686	55 758		13 810	73 113	
	0.4	2 813	57 421		9 111	33 950	

a) The cell populations were purified by FCM to >97% purity. Cultures were conducted in half-area microtiter plates at 1×10^5 cells in 100 μl/well. Proliferation was evaluated by measuring incorporation of ^{125}I iododeoxyuridine after 3 days of culture; the results are expressed as mean cpm of incorporated radioactivity.

culture. In cultures stimulated with Con A or allogeneic leukocytes, the IL-A29$^+$ lymphoblasts constituted 5%–12% of the cells as compared to 11%–24% for CD2$^+$ lymphoblasts. However, the IL-A29$^+$ cell response was particularly marked in the autologous MLC; there was a 4–9-fold increase in the percentage of IL-A29$^+$ cells and, in three of the four autologous MLC examined, the IL-A29$^+$ cells constituted $>40\%$ of the cells and outnumbered CD2$^+$ cells in terms of both total numbers and numbers of blast cells (Table 2). Two-color staining of the IL-A29$^+$ cells in these cultures demonstrated that they were still negative for CD2, CD6, CD4 and CD8 (not shown).

In order to address the question of what signals are required for stimulation of proliferation of the IL-A29$^+$ cells, the proliferative responses of purified populations of IL-A29$^+$ and CD2$^+$ cells to Con A, PWM and bovine rIL 2 were examined. The results of a representative experiment are shown in Table 3. The purified CD2$^+$ T lymphocytes gave potent proliferative responses to PWM, Con A or rIL 2. By contrast, the IL-A29$^+$ cells did not respond to PWM, responded very weakly to Con A and gave only moderate responses to rIL 2. However, addition of rIL 2 to the Con A-stimulated cultures resulted in marked potentiation of the proliferative response, even at low concentrations of rIL 2. These results indicate that IL-A29$^+$ cells express receptors for IL 2 but that they require a second signal for full expression of proliferative activity.

4 Discussion

The results of this study show that the antigen identified by mAb IL-A29 and CC15 is uniquely expressed on a discrete subpopulation of bovine T cells containing mRNA for CD3 and the δ chain of the TcR. These cells are phenotypically distinct from other populations of bovine T lymphocytes, in that they do not express CD2, CD6, CD4 or CD8, either in the thymus or as resting or activated mature cells.

Both mAb immunoprecipitate molecules of 215 kDa and 300 kDa from resting and activated lymphocytes. The two entities are expressed on the surface of cells from cloned cell lines indicating that they are not clonally expressed on different subpopulations. The results of SDS-PAGE of precipitates run under nonreducing conditions demonstrated that the two molecules are not covalently associated on the cell surface; moreover, the enhanced migration of the molecules under nonreducing conditions indicates that they contain disulfide bonds. Further studies will be required to determine whether the two molecules are the products of one or two genes.

The detection of a small but discrete population of IL-A29$^+$ cells in the thymus, together with the reactivity of the cells in functional assays, suggested that they belonged to a T cell lineage. In order to define their lineage, cDNA for bovine CD3 and TcR were cloned from cDNA libraries using human probes. Northern blot analyses with the isolated bovine probes demonstrated that purified IL-A29$^+$ cells and cloned IL-A29$^+$ parsitized cell lines contained transcripts for CD3 γ, δ and ε, and TcR δ but did not contain transcripts for TcR α and contained only a truncated form of TcR β message. By contrast, a population of T lymphocytes purified with a mixture of mAb to CD4 and CD8 contained readily detectable transcripts for CD3 γ, δ and ε, TcR α and full-length TcR β. Preliminary findings with cloned bovine TcR γ cDNA indicate that there are C_γ regions with different levels of homology to a human TcR γ probe (S. Dunlap, unpublished data). The study of TcR γ gene expression in various bovine T cell subsets will have to await further elucidation of the complexity of the TcR γ locus. Nevertheless, the findings of the present study indicate that IL-A29 and CC15 identify a subpopulation of T cells expressing TcR γ/δ.

A similar population of T lymphocytes, expressing a 220-kDa antigen recognized by mAb, has also been identified in sheep [35, 36]. Indeed, the anti-sheep and anti-bovine mAb cross-react between the two species (W. I. Morrison, W. R. Hein and A. Bensaid, unpublished data) and clearly recognize the same surface antigen. Parallel studies of TcR expression by these cells in sheep have yielded similar results to those reported herein [37]. In addition, cell surface expression of a heterodimeric molecule believed to represent the TcR γ/δ has been demonstrated in sheep [38].

The phenotype of the bovine T cells recognized by IL-A29 and CC15 differs somewhat from that reported for human γ/δ T cells. The majority of the human cells have been shown to express CD2; a significant proportion of them also expresses CD8 and a minor population expresses CD4 [13–16]. By contrast, these markers are absent from the bovine IL-A29$^+$ cells at all stages of development. The absence of CD4 and CD8 implies that the TcR on these cells may not interact with conventional class I or class II MHC molecules. However, the detection of a small quantity of TcR δ message in the purified population of bovine cells expressing CD4 or CD8 but not in the CD4$^+$ or CD8$^+$ cloned cell lines indicates that there is an additional population of γ/δ T cells in cattle which are IL-A29$^-$ and express CD4 and/or CD8. These observations suggest that there may be a functional dichotomy among bovine γ/δ T cells. It will be of interest to determine whether these populations utilize differently rearranged TcR γ/δ genes.

A particularly striking observation in the present study was the high representation of IL-A29$^+$ cells in the blood of young animals. In animals less than 3 weeks of age, they constituted about 25% of PBMC and often outnumbered other T lymphocytes. Similar observations have been made in sheep [39]. Hence, if the IL-A29$^+$ cells have a role in immune responses to foreign antigens, it is likely to be in the primary responses of young immunologically naive animals. The results obtained would indicate that ruminants possess larger numbers of γ/δ T cells than other mammalian species so far examined. This is certainly true for γ/δ T cells which do not express CD2, CD4 or CD8. However, Groh and others [16] have recently reported a mean value of 4.9% δ$^+$ T cells in the PBMC of 29 human subjects over 23 years of age; many of these cells expressed CD2 and CD8. This figure is not substantially different from that obtained for IL-A29$^+$ cells in adult cattle. Thus, more detailed information is required before meaningful comparisons between species can be made. A notable feature in both cattle and humans is the marked variation between individuals in the number of positive cells detected; this variation is much greater than that observed for other T lymphocyte populations.

The finding of small numbers of IL-A29$^+$ cells localized mainly in the medulla of the thymus is similar to that reported for human δ^+ cells [16] and indicates that expression of the antigen is restricted to more mature cells. However, the distribution of the IL-A29$^+$ cells in secondary lymphoid tissues differed from that reported for human γ/δ T cells. While the latter are apparently distributed throughout the T-dependent areas in the spleen and LN [16], the bovine IL-A29$^+$ cells are concentrated in the marginal zones of the spleen and in the other cortex adjacent to the subcapsular sinues of the LN. It is of note that these are the sites of entry of cells and antigen into these organs from the blood and afferent lymph, respectively. This suggests that the IL-A29$^+$ cells may participate in the initial events in induction of immune responses. Populations of γ/δ T cells have been detected in the epidermal layers of the skin [40, 41] and in the intestinal epithelium [42, 43] in mice. In cattle, small numbers of IL-A29$^+$ cells were detected within these epithelial sites. In addition, large populations of IL-A29$^+$ cells were found in the dermis and in the lamina propria of the intestine. It is of note that the intra-epithelial γ/δ cells in the intestine of the mouse all express CD8 [42, 43].

Preliminary *in vitro* experiments on the functional properties of the IL-A29$^+$ cells have shown that, in common with other T cell populations, they respond to stimulation with Con A. However, IL 2 was required for optimal responses, suggesting that under physiological conditions the cells require two signals for activation. Blasting IL-A29$^+$ cells were detected in all of the T cell proliferative assays examined, but were particularly numerous in the autologous MLC. Previous studies have shown that this response occurs in cultures conducted in autologous serum or in serum-free medium [34]. Taken together, these observations imply that the IL-A29$^+$ cells react to autologous cell surface determinants in the presence of interleukins generated by other populations of T lymphocytes.

Further definition of the function of the IL-A29$^+$ cells, and of γ/δ T cells in general, is dependent on identification of the molecule(s) with which the TcR reacts on the surface of APC. As already indicated, the absence of CD4 and CD8 from the IL-A29$^+$ cells would argue against them recognizing conventional class I or class II molecules. This is consistent with recent evidence that murine γ/δ T cells recognize antigen presented on the non-polymorphic Qa class I-like molecules [44]. The fidelity with which the molecule identified by IL-A29 and CC15 is expressed on a discrete population of γ/δ T cells implies that the molecule performs an important function unique to these cells. This may be analogous to the function fulfilled by CD4 and CD8 on α/β T cells. Further molecular characterization of the molecule and it's putative ligand should, therefore, provide valuable information on the function of γ/δ T lymphocytes.

We wish to thank Drs. C. Hall and C. Terhorst for providing the pJR216 cDNA probe, Dr. M. S. Krangel for the pPGBC9 probe, Dr. T. W. Mak for the pY14 probe, Dr. M. J. Crumpton for the pJ6T3γ probe and Immunex Corporation, Seattle, WA, for providing bovine recombinant IL 2. We are also grateful to Mr. Elias Awino for technical assistance.

Received November 3, 1989.

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