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CD45RO expression on bovine T cells: relation to biological function

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SUMMARY

The 180 000 MW isoform of CD45 (CD45RO) has been identified in cattle with a novel monoclonal antibody (mAb) (IL-A116). This has allowed a more precise analysis of T-cell function in relation to CD45 isoform expression. Within the $CD4^+$ and $CD8^+$ T-cell populations, $CD45RO^+$ and $CD45RO^-$ subsets were evident. Most $CD4^+$ and $CD8^+$ T cells that expressed the CD45RO isoform did not express the 220 000 and 205 000 MW isoforms recognized by mAb CC76. In contrast, the $WC1^+$, $CD2^-$, $CD4^-$, $CD8^-$, $\gamma\delta$ T-cell receptor $(TCR)^+$ T cells in bovine peripheral blood mononuclear cells (PBMC) were all $CD45RO^+$. Monocytes and granulocytes were $CD45RO^+$ but B cells were $CD45RO^-$. Sorting experiments with $CD4^+$ T cells from an immunized calf demonstrated that proliferative responses to ovalbumin (OVA) were entirely within the $CD45RO^+$ subset. Following stimulation with concanavalin A (Con A) the $CD45RO^-$ subset of $CD4^+$ T cells produced transcripts for interleukin-2 (IL-2) but not IL-4 or interferon-γ (IFN-γ), while the $CD45RO^+$ subset produced mRNA for IL-2, IL-4 and IFN-γ. Biologically active IL-2 was present in supernatants from both $CD45RO^+$ and $CD45RO^-$, $CD4^+$ T cells, and IFN-γ protein was identified by ELISA in supernatants from the $CD45RO^+$ subset, confirming the production of cytokines implied by polymerase chain reaction (PCR). In contrast, sorting experiments with $CD8^+$ T cells from animals immune to the protozoan parasite *Theileria parva* revealed substantial numbers of cytotoxic T-lymphocyte precursors in both the $CD45RO^+$ and $CD45RO^-$ subsets. Thus it appears that although all antigenically primed $CD4^+$ T cells remain $CD45RO^+$, and expression of this molecule consequently identifies memory cells within PBMC, antigenically primed $CD8^+$ T cells down-regulate CD45RO expression after activation.

INTRODUCTION

The leucocyte common antigen (LCA), CD45, family of molecules is generated by alternative splicing, at the RNA level, of exons 4,5 and 6 (also called A, B and C), which encode amino acids in the amino-terminal extracellular domain of the mature protein. Inclusion or exclusion of products of these three exons results in the synthesis of surface glycoproteins with molecular weights (MW) ranging from about 230 000–180 000. Expression of different isoforms by T-cell subpopulations identifies cells that have different functions, tissue distributions and recirculation pathways. The intracellular domain of CD45 has been shown to have tyrosine phosphatase activity and to be involved in signal transduction by the T-cell receptor (TCR)[1,2] and the variety in extracellular structure is likely to reflect important differences in ligand specificity, which would affect both cell adhesion and T-cell activation. The precise nature of the ligand(s) for CD45 isoforms remains to be established.

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Abbreviations: GAM, goat anti-mouse; PE, phycoerythrin.

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There is general agreement within mammalian systems that resting $CD4^+$ T cells that express high MW CD45 isoforms in peripheral blood taken from immunized animals fail to proliferate in response to specific antigen. These high MW isoforms have been identified in humans and rodents with CD45RA, CD45RB and CD45RC monoclonal antibodies (mAb), the specificity of which has been defined by assessing reactivity with cloned CD45 molecules.[3–5] In sheep and cattle, mAb specific for high MW CD45 isoforms, considered to be most similar to CD45RA and CD45RB, respectively, have also been shown to identify $CD4^+$ T cells from immune animals that fail to respond in proliferation assays.[6,7] Thus, the consensus is that naive $CD4^+$ T cells express a high MW isoform and the reciprocal population that does not stain with mAb to these isoforms contains the memory cells that respond in proliferation assays and provide the majority of B-cell help.

In humans the $CD4^+$ T cells that fail to react with CD45RA or CD45RB mAb are mostly $CD45RO^+$, i.e. express the 180 000 MW isoform that results from the absence of the products of exons A, B and C.[8] However, although the $CD4^+$ T cells that express the 180 000 MW isoform are primarily the reciprocal of the subset that express the high MW isoforms, expression of a high or low MW isoform is not necessarily

mutually exclusive and expression of more than one isoform on a small subpopulation of T cells has been noted.[9] These cells may represent memory cells that have a function distinct from the $CD45RO^+$ cells, which express a single isoform,[9] or cells in the process of changing from one activation state to another.[10–12] Alternatively, $CD4^+$ T cells that express both CD45RO and other high MW isoforms of CD45 may not have been exposed to as many rounds of antigenic stimulation as the $CD4^+$, $CD45R^-$, $CD45RO^+$ population of cells.[13]

Data for $CD8^+$ T cells are not as consistent. Merkenschlager & Beverley[14] showed that sorted human $CD8^+$ T cells that were $CD45RA^-$ contained far more cytotoxic T-lymphocyte precursors (CTLp) effective against Epstein–Barr virus (EBV) than the $CD45RA^+$ subset. Similarly, sorted murine $CD8^+$, $CD45RA^-$ cells contained more CTLp specific for the allogeneic $H\text{-}2K^b$ molecule than the $CD45RA^+$ subset.[15] However, experiments in cattle immune to the protozoan parasite *Theileria parva* showed that $CD8^+$ T cells sorted on the basis of expression of a high MW isoform of CD45 had equivalent numbers of CTLp in both subsets,[7] despite observations with sorted $CD4^+$ T cells being consistent with studies in other mammalian systems.

We have used a new mAb, IL-A116, which recognizes the bovine homologue of CD45RO, that has allowed us to define more precisely isoform expression in relation to functional differences within the $CD4^+$ T-lymphocyte subpopulations, assessed by the ability to proliferate to a recall antigen *in vitro*, an interleukin-2 (IL-2)-like bioassay and cytokine mRNA analysis performed using reverse transcriptase polymerase chain reaction (RT-PCR). Expression of the CD45RO isoform by $CD8^+$ T cells and the relation to CTLp frequency indicated that, unlike $CD4^+$ cells, substantial numbers of $CD8^+$ memory cells were evident within the $CD45RO^-$ subset.

MATERIALS AND METHODS

mAb and other antibodies

The three major bovine T-cell subpopulations in blood were identified with mAb to the CD4 antigen (mAb CC30, CC8 and IL-A12), the CD8 antigen (mAb CC58, CC63 and IL-A105)[16] and the WC1 antigen (mAb CC39 and CC15), a polypeptide of 215 000–300 000 MW present on $\gamma\delta$ TCR^+, $CD2^-$, $CD4^-$, $CD8^-$ T cells in cattle.[17] B cells were identified with mAb CC21, directed against the 145 000 MW antigen called WC3,[16] and mAb IL-A50 or IL-A58 directed against bovine IgM and immunoglobulin light chains, respectively.[18] Monocytes in peripheral blood mononuclear cells (PBMC) were identified with mAb IL-A24[16] and IL-A109 (N. D. MacHugh, unpublished data). The CD25 molecule was identified with mAb IL-A111,[19] and major histocompatibility complex (MHC) class II with mAb CC108 (C. J. Howard, K. R. Parsons & B. V. Jones, unpublished data). Monoclonal antibody CC1 is a bovine CD45 homologue,[20] while mAb CC76[7] recognizes high MW isoforms of bovine CD45 and is most similar to CD45RB in humans but is called $CD45R_{p205/220}$ for accuracy and convenience subsequently in this manuscript. Other murine mAb used as isotype-matched controls were mAb 19 (IgG2a, anti-respiratory syncytial virus) and TRT1 (IgG1, anti-turkey rhinotracheitis virus). Goat anti-mouse (GAM) IgG1 labelled with phycoerythrin (PE), GAM IgG2a–PE, and GAM IgG3–fluorescein isothiocyanate (FITC) (Southern Biotechnology Associates, Birmingham, AL) and biotinylated mAb produced as described previously[21] were used for multicolour staining. Monoclonal antibody IL-A116 (IgG3) identified here as CD45RO was produced from a mouse immunized with bovine PBMC, as described previously.[22]

Immunostaining and flow cytometry

PBMC were isolated from *Bos taurus* and *B. indicus* cattle by density centrifugation. Two-colour immunofluorescence staining was assayed as described elsewhere.[23] Three colour staining was used to analyse expression of $CD45R_{p205/220}$ (mAb CC76) and CD45RO (IL-A116) on T cells. The procedure essentially followed that previously reported[23] involving the use of two mAb of different isotypes, isotype-specific secondary mAb and a biotin-coupled third mAb. Binding of the third mAb was detected with streptavidin–Cy-chrome (Pharmingen, San Diego, CA). Fluorescence was assayed on a FACScan (Becton Dickinson, Mountain View, CA) and analysed using FACScan Research Software. The procedure for cell sorting was essentially that of Howard *et al.*[23]

Biochemical characterization

PBMC surface proteins were labelled with ^{125}I using lactoperoxidase. Sequential precipitation involved preclearing with mAb CC1 (CD45) followed by precipitation with a second mAb. Precipitated molecules were examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography[21].

Assessment of proliferative response to ovalbumin (OVA)

PBMC from an animal immunized 2 years previously with OVA were stained with CD4 and CD45RO mAb, sorted and used in proliferation assays.[23] Proliferative responses of sorted cell populations to concanavalin A (Con A; 5 μg/ml) was also measured after 3 days of culture.

Measurement of T. parva*-specific CTLp in sorted bovine $CD8^+$ T-cell populations*

Parasite-specific CTLp were quantified in $CD8^+$ T-cell populations derived from an animal immunized by infection and treatment.[24] A bulk $CD8^+$ T-cell fraction was prepared from PBMC by complement lysis of $CD4^+$ and $WC1^+$ T cells, B cells and monocytes after staining with mAb IL-A12, CC15, IL-A50, IL-A58 and IL-A109, as described elsewhere.[25] After overnight culture, enriched $CD8^+$ T cells were sorted into $CD8^+$, $CD45RO^+$ and $CD8^+$, $CD45RO^-$ populations on the flow cytometer after staining. The frequency of *T. parva*-specific CTLp in these populations was then determined by limiting dilution analysis, as described previously.[26]

IL-2-like (TCGF) bio-assay

Supernatants from PBMC, irradiated PBMC and sorted cells, that had been cultured with Con A (5 μg/ml) in the presence of irradiated autologous PBMC, were harvested after 36 h. The supernatants were assayed for IL-2-like activity, as described previously by Collins *et al.*[27]

Cytokine mRNA analysis

Isolation of $polyA^+$ mRNA was carried out from a maximum of 5×10^5 cells (whole PBMC, irradiated PBMC or sorted cells) using a MicroFast Track™ mRNA isolation kit (Invitrogen

Corporation, San Diego, CA) following 27 hr of culture in the presence of Con A, as described above. Isolated mRNAs were stored as an ethanol precipitate at −70° until needed, then resuspended in 8 μl of diethylpyrocarbonate-treated double distilled water (DDW), to which was added 2 μl of oligo(dT)$_{15}$ primer (200 μg/ml; Promega Corporation, Madison, WI). This was mixed, heated to 65° for 3 min then allowed to cool to room temperature over a period of 30 min. Following microfugation to bring down any condensation, first strand synthesis was carried out in a 50-μl final volume containing 1 μl of a 25 mM mix of all four deoxynucleotide triphosphates; 10 μl of 5× RT buffer (250 mM Tris–HCl, pH 8·3, 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, 2·5 mM spermidine); 2 μl of RNasin (40000 U/ml; Promega); 25 μl of DDW; and 2 μl of AMV RTase (500 U/ml; Promega). The reverse transcription mixture was incubated at 42° for 1 hr. The primers used for amplification during PCR were IL-2, TGGCACCTACTTCAAGCTCTACG (sense), CTTAAAGGATCCATATTTATCATC (antisense); IL-4, CATTGTCAGTGCAAATAGAG (sense), TCAGCTTCAACACTTGGAG (antisense); IFN-γ, ATGAAATATACAAGCTATTTC (sense), TTACGTTGATGCTCTCCG (antisense) and CD45, GATCCTGCAATTTAGATGAACAGCAGG (sense), AGTCACCTCTCTTCCAGTTGCTTTTTC (antisense). The CD45 primers were based on the human sequence and spanned the bases 1903–1929 and 2490–2516;[28] primers for IL-2, IL-4 and IFN-γ were based on published bovine sequences obtained from the EMBL/GenBank databases (accession numbers M12791, M77120 and M29867 respectively). To 2·5 μl of RT mix the following components were added: 5 μl deoxynucleotide triphosphate mix (10 mM); 5 μl 10× PCR buffer (600 mM Tris-HCl, pH 10, 150 mM $(NH4)_2SO_4$, 20 mM $MgCl_2$); 4 μl of each sense and antisense primer (10 pmol/μl); 29 μl of DDW; and 0·5 μl of Taq polymerase (5 U/ml; Boehringer, Mannheim, Germany). The reaction mixture was overlayed with mineral oil (Sigma, Poole, Dorset, UK) and temperature cycling was initiated with each cycle as follows: (1) 94° for 1 min; (2) 47° for 1 min; (3) 72° for 1 min. Amplified product was detected by running a total of 12 μl of the final PCR reaction mix on a 1% agarose gel at 100 V for 1 hr and visualized by ethidium bromide staining.

Detection and measurement of bovine IFN-γ by ELISA

IFN-γ was measured by ELISA in supernatants from 10^6 whole PBMC or 10^6 sorted cells incubated with 5 μg/ml Con A in the presence of 10^5 irradiated autologous PBMC with 5 μg/ml Con A for 24 hr. Microtitre plates (Falcon 3912; Becton Dickinson) were coated overnight with anti-bovine IFN-γ mAb EC3 (5 μg/ml in coating buffer; 15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide). Plates were washed three times with ELISA buffer (135 mM sodium chloride, 75 mM disodium hydrogen phosphate, 3 mM potassium chloride, 1·5 mM potassium dihydrogen phosphate, 0·05% Tween-20) and remaining sites on the plates were blocked with 1% OVA in coating buffer for 30 min at room temperature. Diluted samples or standards, recombinant bovine IFN-γ, were added and left to incubate for 90 min at room temperature. Monoclonal antibody 3F10 (5 μg/ml in ELISA buffer) was added for 90 min followed by goat anti-mouse IgG2b-conjugated to horseradish peroxidase (Southern Biotechnology Associates Inc.) for 60 min before the addition of O-phenylenediamine (BDH, Poole, Dorset, UK). The colour development was stopped by the addition of 2 N hydrochloric acid and absorbance measured on a Multiskan plate reader at 405 nm. IFN-γ levels were calculated by reference to recombinant bovine IFN-γ standards.

RESULTS

mAb IL-A116 immunoprecipitates bovine CD45RO

Analysis of immunoprecipitates obtained with mAb IL-A116 from surface iodinated PBMC by SDS–PAGE run under reducing conditions revealed a single molecule of 180 000 MW. The apparent MW of this protein was distinct from the two polypeptides of 220 000 and 205 000 MW precipitated by mAb CC76 (CD45R$_{p205/220}$), but identical in size to the additional polypeptide (180 000 MW) precipitated by the non-restricted CD45 mAb CC1 (Fig. 1a). Sequential precipitation showed that the CD45 mAb CC1 effectively removed the 180 000 MW molecule recognized by IL-A116 (Fig. 1b). This mAb thus recognizes an isoform of the bovine CD45 that has the same MW as human CD45RO.

CD45RO expression identifies subsets of CD4$^+$ and CD8$^+$ cells

Two-colour immunofluorescence staining of PBMC identified subsets of the CD4$^+$ and CD8$^+$ T cells that were either CD45RO$^+$ or CD45RO$^-$, as recognized by IL-A116 (Fig. 2). The WC1$^+$ γδ TCR$^+$ T cells identified with mAb CC15 were uniformly CD45RO$^+$, as were the monocytes in PBMC identified with mAb IL-A24. B cells identified in PBMC with mAb CC21 were CD45RO$^-$ (Fig. 2). Granulocytes, identified in lysed blood by their high side scatter were CD45RO$^+$ (data not shown).

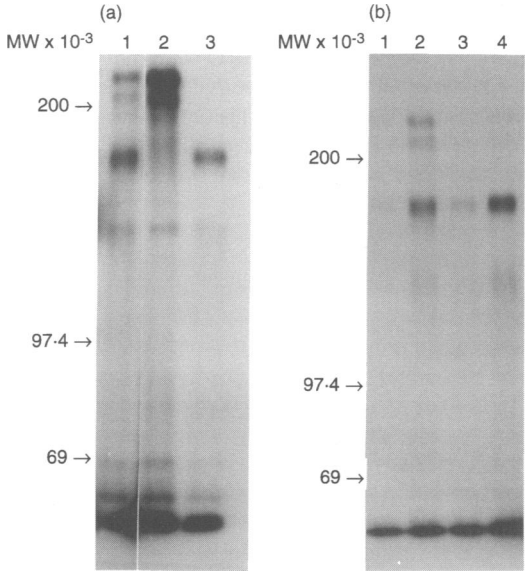

Figure 1. (a) Lanes 1–3 contain precipitates obtained with mAb CC1 (CD45), CC76 (CD45R$_{p205/220}$) and IL-A116 (CD45RO), respectively. (b) Preclearing of surface-iodinated PBMC lysate with CC1. Lane 1, precleared with CC1 followed by CC1 precipitation; lane 2, precipitation with CC1; lane 3, precleared with CC1 followed by IL-A116 precipitation; lane 4, precipitation with IL-A116.

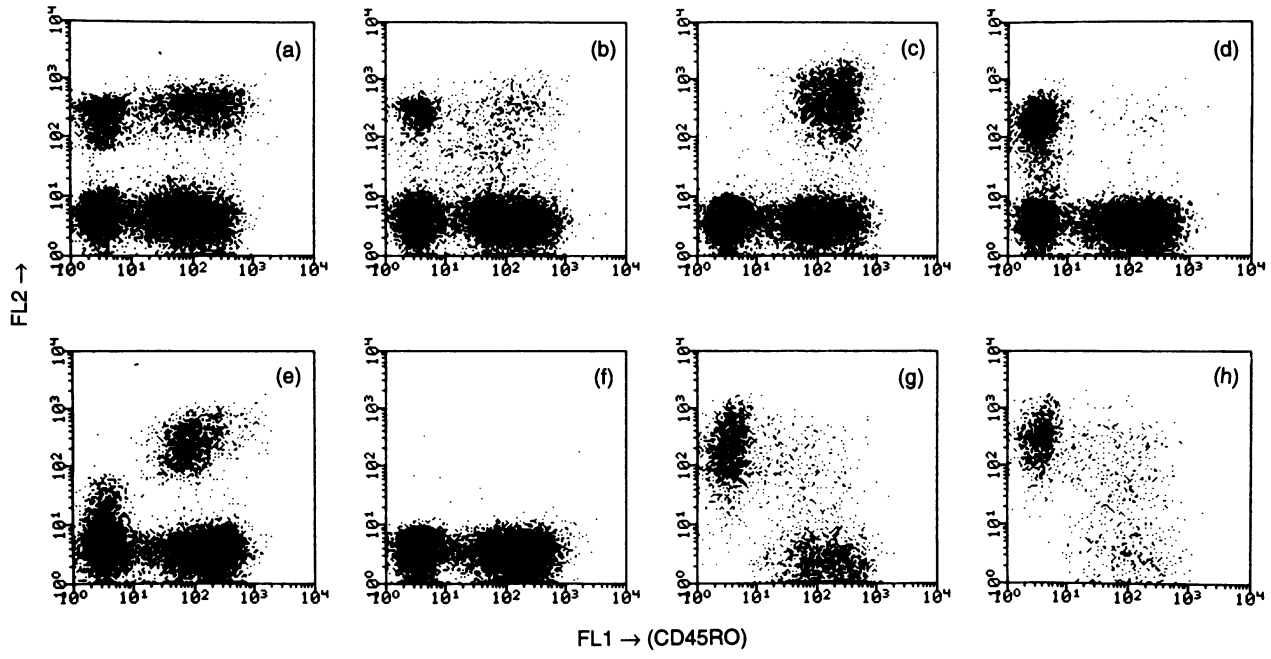

Figure 2. Two- and three-colour immunofluorescence staining of PBMC. (a–f) Two-colour. FL1 = staining with CD45RO (IL-A116). FL2 = staining with (a) CD4 (CC30); (b) CD8 (CC58); (c) WC1 ($\gamma\delta$ T cells, CC15); (d) WC3 (B cells, CC21); (e) monocyte marker (IL-A24); (f) anti-Turkey rhinotracheitis virus (TRT-1). (g, h) Three-colour. FL1 = staining with CD45RO (IL-A116). FL2 = staining with CD45R (CC76). G-cells gated electronically on $CD4^+$ population. H-cells gated electronically on $CD8^+$ population.

Specific proliferative responses *in vitro* are restricted to the $CD45RO^+$ subset of $CD4^+$ T cells

The proliferative responses of PBMC and sorted $CD4^+$, $CD45RO^+$ or $CD4^+$, $CD45RO^-$ T cells from an animal immunized with OVA, using autologous irradiated PBMC as antigen-presenting cells, are shown in Table 1. The $CD45RO^-$ subset failed to proliferate, while intensive proliferation was evident in the $CD45RO^+$ subset. The proliferative responses of the $CD4^+$, $CD45RO^+$ or $CD45RO^-$ cell populations to Con A after 3 days of culture revealed that both populations proliferated equally well within this time, giving mean c.p.m. of 60 000 and 70 000, respectively, compared with background counts of 1000 c.p.m. in control cultures not containing Con A.

$CD4^+$, $CD45RO^+$, but not $CD4^+$, $CD45RO^-$ T cells produce IL-4 and IFN-γ mRNA transcripts

$CD4^+$ T cells were sorted into $CD45RO^+$ and $CD45RO^-$ subpopulations and 10^6 cells stimulated for 27 hr with Con A in the presence of 10^5 irradiated autologous PBMC and analysed for the presence of cytokine and CD45 mRNA transcripts. Amplification of cDNA for a cytoplasmic portion of CD45 was used as a positive control to ensure that cellular mRNA was efficiently extracted and transcribed from the different cell sources. The results revealed that IL-2, IL-4 and IFN-γ mRNA transcripts could be detected from Con A-activated whole PBMC (Fig. 3). However, although both $CD45RO^+$ and $CD45RO^-$ $CD4^+$ T cells produced IL-2 mRNA, only $CD45RO^+$ cells produced detectable amounts of IL-4 and

Table 1. Proliferation induced by OVA in PBMC and sorted $CD4^+$, $CD45RO^+$ and $CD4^+$, $CD45RO^-$ T cells

	C.p.m.* incorporated by indicated cell population				
	PBMC	PBMC†	Stained PBMC	Sorted $CD4^+$, $CD45RO^+$	Sorted $CD4^+$, $CD45RO^-$
Cells alone	2080	100	2680	100	84
Cells + OVA	148 123	124	141 345	2625	186
Cells + PBMC†	3755	—	577	170	83
Cells + PBMC† + OVA	133 640	—	99 280	154 445	866

* Mean of triplicate samples.

† Irradiated (2000 rads) whole PBMC (2×10^4/well); 10^5 PBMC, stained PBMC or sorted cells per well.

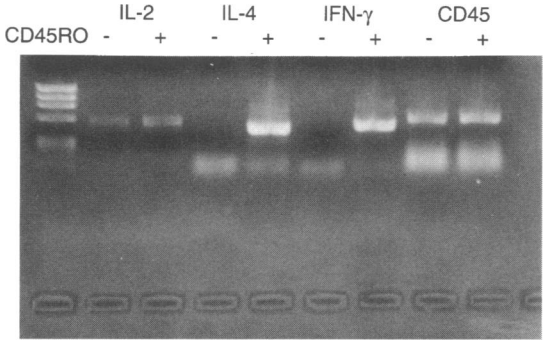

Figure 3. Detection of cytokine mRNA by PCR. Sorted CD45RO$^+$ (IL-A116$^+$) and CD45RO$^-$, CD4$^+$ T cells assayed for IL-2, IL-4, IFN-γ or CD45 mRNA. PCR product for IL-2 and CD45 (non-restricted portion of molecule) evident in CD45RO$^+$ and CD45RO$^-$ cells. PCR product for IL-4 and IFN-γ only evident in CD45RO$^+$ cells. Assessed after 40 cycles.

IFN-γ mRNA transcripts (Fig. 3). To allay fears that we were not detecting low message levels in the CD4$^+$, CD45RO$^-$ population, the number of thermal cycling rounds was increased from 30 to 60 in some experiments but still failed to reveal any mRNA transcripts for IL-4 and IFN-γ.

CD4$^+$, CD45RO$^-$ and CD4$^+$, CD45RO$^+$ T cells produce IL-2

Bovine lymphoblasts proliferated in the presence of recombinant human (rh)IL-2. Conditioned medium from either sorted CD4$^+$, CD45RO$^+$ or CD45RO$^-$ T cells that had been cultured in the presence of Con A induced the proliferation of lymphoblasts. However, the magnitude of the responses were different. The proliferative responses of lymphoblasts to conditioned medium from CD45RO$^-$, CD4$^+$ T cells were greater than those from CD45RO$^+$, CD4$^+$ T cells or whole PBMC (Fig. 4a). No significant proliferation was detected with conditioned medium obtained from irradiated PBMC.

The addition of mAb IL-A111 (CD25), an inhibitor of IL-2-induced proliferation,[19] completely abrogated the proliferative response of lymphoblasts to rhIL-2 at concentrations of 3 U/ml or less, and greatly reduced the proliferation of blasts in the presence of 10 U/ml rhIL-2. The addition of an isotype-matched (non-specific) mAb, TRT-1, had no effect on the rhIL-2-induced proliferation (data not shown). Addition of mAb IL-A111 abolished all proliferation of lymphoblasts induced by the supernatants from CD45RO$^-$, CD4$^+$ T cells. In contrast, the proliferation induced by the supernatants from the CD4$^+$, CD45RO$^+$ T cells, although reduced, was not completely inhibited (Fig. 4b).

Table 2. Measurement of IFN-γ by ELISA

Cells	IFN-γ (ng/ml)*
PBMC	37 ± 3·9
PBMC†	2 ± 0·7
CD4$^+$ CD45R$_{P205/220}$$^+$	2 ± 0·3
CD4$^+$ CD45R$_{P205/220}$$^-$	29 ± 4·4

Culture supernatants from 10^5 PBMC, irradiated PBMC and sorted CD4$^+$ T-cell subpopulations incubated with 10^4 irradiated autologous PBMC and stimulated with Con A were assayed after 24 hr.

* Mean of duplicate samples from duplicate experiments ± SD.

† Irradiated whole PBMC.

CD45RO$^+$, CD4$^+$ cells produce IFN-γ

CD4$^+$ T cells were stained with mAb CC76 (CD45R$_{p205/220}$) and the CD45R$_{p205/220}$$^+$ and CD45R$_{p205/220}$$^-$ populations sorted, the latter population being CD45RO$^+$ cells that had not been incubated with a CD45R mAb (see below). The CD45R$_{p205/220}$$^-$ subset was the predominant producer of IFN-γ protein. Irradiated PBMC and CD4$^+$, CD45R$_{p205/220}$$^+$ T cells did not produce detectable amounts of IFN-γ (< 2·5 ng/ml; Table 2).

Specific CTLp are present in CD45RO$^+$ and CD45RO$^-$ subsets of the CD8$^+$ T-cell population

In an assay representative of several experiments, limiting

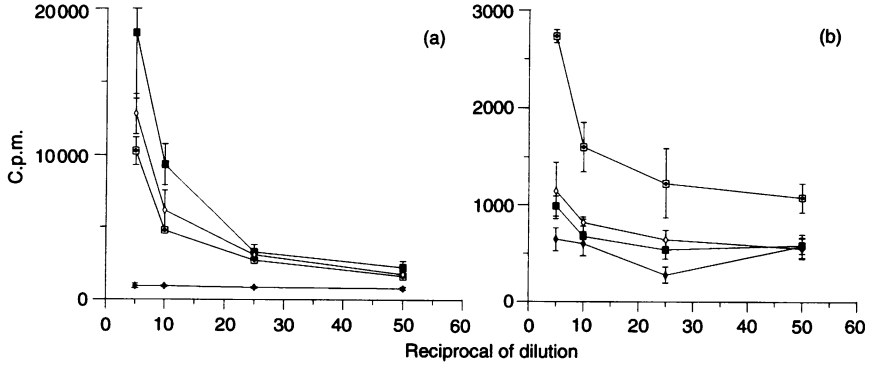

Figure 4. (a) Proliferative responses of 4-day Con A blasts to conditioned medium obtained from CD4$^+$, CD45RO$^+$ (open squares) or CD4$^+$, CD45RO$^-$ (filled squares) or whole PBMC (open diamonds) or irradiated PBMC (filled diamonds; mean of triplicates ± SD). (b) Proliferative responses of Con A blasts to conditioned medium in the presence of mAb IL-A111 (anti-CD25).

Table 3. CTLp frequency in sorted $CD8^+$ T-cell subsets

Sample	CTLp
Whole PBMC	1:2351
$CD8^+$, $CD45RO^+$	1:4890
$CD8^+$, $CD45RO^-$	1:6012

Frequencies of precursor CTL within $CD8^+$ subpopulations identified by mAb IL-A116 (CD45RO) as assayed on *T. parva*-infected lymphoblasts.

dilution analysis revealed the frequency of *T. parva*-specific CTLp in unsorted CTLp to be 1:2351. A similar CTLp was evident in both the $CD45RO^+$ and $CD45RO^-$ subsets of the $CD8^+$ population (Table 3).

Most $CD4^+$ and $CD8^+$ T cells express either $CD45R_{p205/220}$ or CD45RO isoforms

The expression of $CD45R_{p205/220}$ and CD45RO on leucocytes in the circulation of adult cattle was assessed by three-colour flow cytometry. Most $CD4^+$ and $CD8^+$ T cells that were $CD45RO^+$ did not express the $CD45R_{p205/220}$ isoform and vice versa. However, about 10% of $CD4^+$ and 13–20% of $CD8^+$ T cells expressed both (Table 4). Thus, all of the $CD45R_{p205/220}^-$ cells tested for IFN-γ production (above) were $CD45RO^+$ cells that only expressed the 180 000 MW molecule. $WC3^+$ B cells were $CD45R_{p205/220}^+$, $CD45RO^-$, and monocytes and macrophages were $CD45R_{p205/220}^-$, $CD45RO^+$ (data not shown). $WC1^+$ $\gamma\delta$ T cells were all $CD45RO^+$, $CD45R_{p205/220}^-$.

The proportion of the $CD4^+$ and $CD8^+$ T-cell population that was $CD45RO^+$ was higher in adults than calves (Table 4), indicating an increase in the $CD45RO^+$ cells with age. In both calves and adult cattle a higher proportion of $CD4^+$ T cells was $CD45RO^+$ compared to $CD8^+$ T cells (Table 4).

DISCUSSION

The cellular distribution of the CD45RO isoform in cattle has been identified using a novel mAb, IL-A116. The $CD4^+$ T-cell proliferative recall response to OVA was entirely within the $CD45RO^+$ subset. This agrees with previous studies,[7,23] using either dendritic cells (afferent lymph veiled cells) or irradiated PBMC as antigen-presenting cells, in which CD4 proliferative recall responses to soluble antigen were shown to be entirely within the $CD4^+$, $CD45R_{p205/220}^-$ subset.

Transcripts for IL-2 were detected in both $CD4^+$, $CD45RO^+$ and $CD45RO^-$ cells and there was little quantitative differences in the levels secreted. Our data demonstrated that IL-4 and IFN-γ production resides in the $CD4^+$, $CD45RO^+$ population of cells. This is similar to findings in humans.[29–31] The antagonistic nature of IL-4 and IFN-γ suggests that functional heterogeneity exists within the $CD4^+$, $CD45RO^+$ T-cell phenotype, as seen in rodent systems, and it has been proposed that there are functionally distinct subsets within the human $CD4^+$, $CD45RO^+$ population.[9] It was suggested that the human $CD45RA^-$, $CD45RB^+$ memory cells could be regarded as T-helper type-1 (Th1)-like, secreting IL-2 and IFN-γ, while the $CD45RA^-$, $CD45RB^-$ memory cells are Th2-like cells, secreting IL-4.[9] However, Salmon *et al.*[13] recently proposed that CD45 isoform expression is regulated by the level of antigenic stimulation. In this model naive $CD4^+$ T cells exposed to subsequent rounds of antigenic stimulation lost expression of the CD45RB isoform, while expression of the CD45RO isoform increased. This progressive change was associated with a decrease in IL-2 synthesis, increased susceptibility to apoptosis and a change from IFN-γ to IL-4 synthesis. In cattle expression of high or low MW isoforms was not necessarily mutually exclusive and 10% of $CD4^+$ cells were $CD45RO^+$, $CD45R_{p205/220}^+$, which is consistent with observations in humans.

In marked contrast to our findings with $CD4^+$ T cells we have not established a link between CD45RO or $CD45R_{p205/220}$ isoform expression and the number of CTLp cells within the $CD8^+$ population in the blood of animals immune to the protozoan parasite *T. parva*. Naive animals contain < 1 CTLp in 10^5 cells and the important conclusion is that the high number of CTLp in the $CD8^+$, $CD45RO^-$ subset must be the progeny of $CD8^+$ cells derived after stimulation with specific antigen. These observations contrast with studies in humans and mice, in which CTLp were greatly enriched in the $CD45RO^+$ or $CD45RA^-$ populations.[14,15] The use of different antigen-presenting cells may affect the responses of the $CD8^+$ T cells. We used irradiated autologous parasitized T cells. Irradiated autologous EBV-transformed lymphoblastoid cell lines, presumably B cells, were used to induce CTLp activation in isolated human $CD8^+$ T cells.

The more dynamic model of T-cell memory that has been put forward to explain a number of observations that are inconsistent with the earlier static model[32] could resolve the apparently contradictory observations with the $CD4^+$ and $CD8^+$ populations made here. The conclusion that in mammals memory $CD4^+$ T cells are within the $CD45RO^+$ population is supported by the observations that unprimed T cells in cord blood are $CD45RO^-$, but recall responses are almost exclusively within the $CD45RO^+$ subset, which increases with age.[29,33,34] In humans unprimed $CD45RA^+$, $CD45RO^-$ cells become $CD45RA^-$, $CD45RO^+$ on activation.[35] This conversion was thought to be unidirectional but cylical regulation of CD45RA expression has been reported on

Table 4. Three-colour immunofluorescence staining of PBMC from animals aged 2–5 months or 2–5 years

Calf group		$IL\text{-}A116^+$ $CC76^-$	$IL\text{-}A116^-$ $CC76^+$	$IL\text{-}A116^+$ $CC76^+$
2–5 years	$CD4^+$	49 ± 13	40 ± 13	10 ± 1
2–5 months	$CD4^+$	30 ± 5	60 ± 3	10 ± 2
2–5 years	$CD8^+$	21 ± 3	58 ± 8	20 ± 4
2–5 months	$CD8^+$	18 ± 7	69 ± 6	13 ± 3

PBMC stained with mAb to CD4 or CD8 and IL-A116 (CD45RO) together with CC76 ($CD45R_{p205/220}$). Percentage of cells within the $CD4^+$ or $CD8^+$ population that stained with IL-A116 and/or CC76 are shown ± SD. $n = 3$ for each group.

cell lines.[36] Furthermore, re-expression of CD45RC was evident in nude rats reconstituted with $CD45RC^-$ cells.[10,11] The shorter lifespan, in humans, of $CD45RO^+$ T cells compared to $CD45RO^-$ cells and the proposal that maintenance of T-cell memory requires the presence of antigen[37,38] together with the observation that the $CD45RO^+$ cells are larger than the $CD45RO^-$ cells and express higher levels of certain molecules associated with activation[29] has been interpreted as indicating that T-cell memory resides in a continuously proliferating primed population.[32] Thus, although naive T cells are $CD45RO^-$ and become $CD45RO^+$ after exposure to antigen, expression of CD45RO may represent a state of activation and some progeny may revert to a $CD45RO^-$ state. However, our failure to detect recall antigen responses in sorted $CD4^+$ $CD45R_{p205/220}{}^+$ cells, even when dendritic cells were used in the system,[7] implies a very low frequency of reversion to a $CD4^+$, $CD45RO^-$ phenotype. In contrast, the high frequency of CTLp in the $CD8^+$, $CD45RO^-$ subset may represent the progeny of cells that have been stimulated by specific antigen and have reverted to an activation level equivalent to that of naive T cells. There are few $CD45RO^+$ cells within the $CD8^+$ population in PBMC from cattle compared to the $CD4^+$ population; a similar observation has been made in humans.[32] Apoptosis and removal of blasts or changing back to a non-activated state could be important for the maintenance of homeostasis[32] and this may be especially necessary for $CD8^+$ lymphocytes because of the cytolytic potential of the cells.

There is a third major T-cell population in the peripheral circulation of neonatal ruminants, $CD2^-$, $CD8^-$, $CD4^-$, that expresses the WC1 molecule and a $\gamma\delta$ TCR.[17] These cells are uniformly $CD45RO^+$ and their proportionate contribution to the T-cell pool decreases with age. It is most unlikely that all of the cells within this population in neonatal ruminants are memory cells. These $WC1^+$ cells uniformly express the IL-2 receptor (CD25) at a low intensity,[39] indicating that they are activated and further supporting the view that CD45RO expression relates to an activation state for T cells.

We conclude that CD45RO expression relates to functional memory within the resting $CD4^+$ population, identifying cells that are capable of proliferating in response to soluble antigen and rapidly produce mRNA encoding IL-4 and IFN-γ. However, no such correlation is evident for the $CD8^+$ or $WC1^+$ populations. The general homology evident within mammals with respect to expression and function of surface molecules and lymphocyte subpopulations makes it unlikely that this difference between the $CD4^+$ and other T cells is specific for cattle, and although memory and CD45RO expression is associated for $CD4^+$ cells the principle appears not to be generally applicable to $CD8^+$ or $WC1^+$ $\gamma\delta$ T cells.

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