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A new epitope on sheep CD45R molecule detected by a monoclonal antibody

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Abstract

This paper describes the production and characterization of a monoclonal antibody (mAb), Co-46D5, which recognizes a new epitope on the isoform of the homologous sheep leukocyte common antigen (LCA) or CD45. This mAb was submitted to the 3rd workshop on ruminant leukocyte antigens and was assigned to a cluster reactive with B- and T-cells subsets. Co-46D recognizes a 220 kDa molecule on peripheral blood mononuclear cells (PBMC) and spleen cells but not on thymocytes. Flow cytometry (FCM) analysis shows that Co-46D5 reacted with 30% of PBMC and 50% of spleen cells and more than 95% of cells freshly isolated from lymphoid follicles of the ileal Peyer's patches (IPP) of young lambs. By immunohistochemistry, the antigen was detected mainly on B-cell areas of lymph nodes and spleen. It was also found on a subpopulation of medullar thymocytes. Based on these results, we assume that Co-46D5 recognizes a new epitope on the largest isoform of the sheep CD45 receptor, probably on the homologous to the human CD45RA isoform.

Keywords: CD45; Ovine; Monoclonal antibody

Résumé

Cet article décrit la production et la caractérisation de l'anticorps monoclonal Co-46D5, qui reconnaît un nouvel epitope dans l'isoforme de l'homologue ovin du LCA (leukogyte Common Antigen) ou CD45 humain. Cet anticorps a été présenté au '3rd workshop on

Abbreviations: FCB: Flow cytometric buffer, FCM: Flow cytometry, IPP: Ilea Peyer patches, LCA: Leukocyte Common Antigen, MFI: Mean fluorescence intensity, PBMC: Peripheral blood mononuclear cells, PBS: Phosphate buffer saline, PTP: Protein tyrosinase phosphatase.

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ruminant leukocyte antigens' et il a été classé dans un groupe d'anticorps qui réagissent avec des souspopulations de cellules B et T. Co-46D5 reconnaît une molécule de 220 kDa sur les cellules mononuclées du sang péripherique (PBMC) et sur les cellules de rate, mais pas sur les thymocites. L'analyse par cytométrie en flux (FCM) montre que Co-46D5 a réagit avec 30% des PBMC, 50% de cellules de rate et plus de 95% des lymphocytes frais isolés des follicules lymphoïdes des plaques de Peyer illeales (IPP) d'agneau. Par l'analyse inmunohistochimique, l'antigène a été détecté dans les régions riches en cellules B des ganglions lymphatiques et la rate, ainsi que dans une subpopulation de thymocytes médullaires. Ces résultats nous permettent d'assumer que le Co-46D5 reconnaît un nouvel épitope dans l'isoforme majeure du CD45 ovin et probablément dans l'homologue ovin de l'isoforme human CD45RA.

1. Introduction

The CD45 molecule (LCA or T200) is a major transmembrane glycoprotein belonging to the protein tyrosine phosphatase (PTP) family. The human CD45 family included at least nine possible isoforms, eight detected at the mRNA level. The alternative splicing of three exons 4, 5 and 6, generates the different isoforms with mw ranging from 180 to 220 kDa. The encoded peptide segments are designated A, B and C respectively. In humans, five CD45 cDNA of the ninth possible has been isolated; the ABC isoform, that contain all three alternatively used exons; the AB and the BC isoforms, with two of the three exons; the B isoform, with only one exon and the O isoform, with lack of all three alternatively used exons [1].

The structure of CD45 is consistent with a role of receptor. It consists of a heavily glycosylated external domain, a single transmembrane region and a large highly conserved intracytoplasmic domain. The extracellular region varies in length, depending on which combination of exons are spliced into the CD45 mRNA. The cytoplasmic portion of the CD45 molecule, which does not vary between isoforms, contains PTP activity. CD45 is a positive growth regulator required for antigen-stimulated proliferation of both T and B lymphocytes. CD45 plays an essential role in antigen-induced lymphocyte activation and thymic development as it has been demonstrated on CD45-deficient cell lines and knockout mice [2].

While CD45 is abundantly found on the surface of cells from the haematopoietic lineage, the expression of various CD45 specific isoforms is restricted according to cell-lineage and the state of activation of the cell. Various isoforms are selectively expressed by functionally distinct subpopulations of immune effector cells. Monoclonal antibodies to CD45 epitopes have been used to distinguish activated from resting T lymphocytes or memory from virgin T-cells, as well as to recognize distinct functional subpopulations. $CD45RA^{+}$ (220 kDa isoform) lymphocytes are currently considered to be naive or immature. They exhibit low expression of adhesion molecules, and often demonstrate low proliferation and activation in response to exogenous cytokines and mitogens. In

contrast, CD45RO $^{+}$ cells (180 kDa isoform) are refereed to as the memory or mature subset and express high levels of adhesion molecules [1].

In humans, most B lymphocytes and leukaemic B-cell lines predominantly express the 220 kDa isoform (CD45RA) and lack the lowest mw 180 isoform (CD45RO), whereas thymocytes and many leukaemic T-cell lines primary express CD45RO and lack CD45RA [3]. In sheep, CD45 (LCA) is precipitated as an 220, 210 and 190 kDa isoform from leukocytes, also CD45R isoform is present on peripheral B-cells as an isoform of 220 kDa peripheral B-cells, and as two isoforms of 210 and 190 kDa from thymocytes, while cells from IPP express two forms of 220 and 190 kDa [4].

Many mAb to human CD45 isoforms has been characterized by flow cytometric analysis using the mouse cell lines that express the individual human CD45 isofrom, CD45 (LCA) mAb bind to all isoforms, CD45RA mAb bind to the ABC and AB isoforms, CD 45RB bind to ABC, AB, BC and B isoforms and CD45RO mAb bind only to the 180 kDa isoform [5].

Specificity of sheep CD45 mAb is obtained by flow cytometric, immunohistological and biochemical analysis. CD45 cDNA from ruminant has not been isolated for the moment, so it is difficult, if not impossible, to distinguish CD45RA from RB isoform and generally mAbs to these isoforms are labeled as CD45R. MAbs to CD45 or LCA molecule were described [6, 7], and anti-CD45R mAbs have been generated [4]. Moreover, bovine anti-CD45 and anti-CD45RO mAbs cross-reactive with sheep have been described [8, 9].

This report describes the characterization of a mAb generated against sheep PBMC. Its cellular expression in sheep cell suspensions and lymphoid tissues suggests that the molecule recognized by mAb Co-46D5 is present on B leucocytes and on a T-lymphocyte subset. Evidence is given to support that this mAb recognizes the homologue of the CD45RA antigen of human.

2. Materials and methods

2.1. Animals

Merinos ewes were used unless specified. Spleen and lymph nodes were obtained from adult animals, thymus and IPP tissue from 1- to 3-month-old lambs.

2.2. Preparation of cell suspensions

Sheep whole blood was collected into EDTA–K_3 anticoagulant vacuum tubes (Becton Dickinson Vacutainer Systems, Meylan, France). PBMC were isolated by Ficoll–Hypaque gradient (density 1080) and used for immunoprecipitation, immunoblotting or FCM. Ovine peripheral blood monocytes were collected from PBMN after 2 h culture on plastic, washed and recultured overnight with PBS.

Thymus, IPP, lymph nodes and spleen were obtained from lambs at the slaughterhouse. Thymocytes, IPP cells and splenocytes were isolated by gentle perfusion of the thymus and spleen with PBS.

2.3. Production of mAbs

MAb Co-46D5 was produced using previously described immunization and cell fusion procedures [10]. Briefly, female Balb/c mice were immunized with sheep PBMC isolated as described above. Spleen cells from immune mice were fused with Sp2/0 myeloma cells. Hybridoma supernantants were tested for activity by an enzyme-linked immunosorbent assay (ELISA) with coated PBMC as antigen. Antibody-producing hybridomas reacting positively were cloned at least twice by limiting dilution. Immunoglobulin classes and subclasses were determined in a solid-phase ELISA using rabbit antisera specific for mouse heavy and light chains and a peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) (Sigma, USA). mAb Co-46D5 was of the IgG_1 subclass.

2.4. Antibodies used in this study

Other monoclonal antibodies used in this study were: anti-bovine CD5 (CC-17) and anti-ovine CD21 (VPM-30), kindly provided by Serotec, Kidlington; mAbs which recognize ovine T-cell subsets: anti-CD4 (17-D) and anti-CD8 (7C8), were obtained from (EACC, Salisbury); an and anti-CD45RO [8] (IL-A150), was kindly provided by Dr. J. Naessens, International Livestock Research Institute, Nairobi. An polyclonal anti-CD3 and an anti-Igs antibody used as T- and B-cell-marker controls, were purchased from Dakopatts, Glostrup.

2.5. Molecular weight analysis

2.5.1. Immunoblotting

Immunoblotting was performed with lysates from sheep PBMC, splenocytes and thymocytes. Proteins were separated by SDS–PAGE (5–15% gradient gels under reducing conditions). After transfer of proteins to nitrocellulose membranes, free sites were blocked with 3% BSA and incubated overnight with 1:2 diluted mAb, with gentle shaking. After washing, membranes were incubated with peroxidase-conjugated goat anti-mouse Ig for 1 h and developed with chloronaphtol (Sigma). The anti bovine CD45RO antibody, mAb IL-A150, was used as a positive control.

2.5.2. Flow cytometry

Cells preparations were washed with PBS and resuspended at 10^6 cells/ml in PBS containing 0.1% bovine serum albumin (BSA) and 0.1% NaN_3 (flow cytometry buffer, FCB). Cells were incubated on ice for 30 min, with appropriately diluted mAb, or an irrelevant mAb as negative control. After three

washing in FCB, cells were incubated on ice for 30 min with 50 μl of fluorescein isothyocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma), diluted at 1/500 in FCB, per sample. Cells were washed three times in FCB and fixed with 1% paraformaldehyde in PBS. Cells were analyzed using FACScan analyzer (Becton–Dickinson, San José) equipped with the CellQuest™ software. Ten thousand events were collected for each sample. Cells were identified and gated by their characteristic forward and side scatter, fluorescence intensity was acquired on a log scale. Results were scored as the percentage of stained cells within the gated population as well as the mean of fluorescence intensity (channel units).

Dual staining was performed by a combination of the method describe above. After the first labelling step, cells were blocked by incubation with 50 μl of goat Ig (Sigma) (2 mg/ml) for 10 min. After washing three times with FCB, the cells were incubated with the second mAb (anti CD4, CD8, CD5 and CD21), followed by 50 μl of phycoerytrin (PE)-labelled sheep anti-mouse Ig (Sigma) diluted at 1/50, per sample. An isotype control Ig was used to verify specificity and complete blocking. Results were presented as contour plots, in which contour lines show the distribution of events according to their green and red fluorescence intensities.

2.6. Immunoperoxidase staining of tissue sections

Formalin-fixed and paraffin-embedded tissue sections of lymph nodes, spleen and thymus were incubated overnight (O/N) with Co-46D5 (diluted 1:10 in PBS, containing 10% normal goats serum). Biotinylated goat anti-mouse IgG (Dakopatts), diluted at 1:20 in PBS, containing 10% normal goat serum, was applied for 30 min at room temperature. Afterwards, tissue sections were incubated with prediluted ABC (avidin–biotin–peroxidase complex, Vector, Burlingame, CA) in PBS for 1 h at room temperature. Immunoreactive cells were stained with 0.5% 3,3′-diaminobenzidine tetrahydrochloride diluted at 1:10 in 0.05 M Tris, containing 0.3% H_2O_2. Slides were counter-stained with Mayer's haematoxilin.

3. Results

3.1. Cellular distribution of the antigen recognized by Co-46D5

Results of flow cytometry analysis of sheep PBMC, thymocytes and spleen cells stained with mAbs Co-46D5 and IL-A150 (a CD45RO control antibody) are shown in Fig. 1(a) and (b), respectively. A comparison between the fluorescence intensity of cells within the gated population of PBMC, granulocytes, thymocytes and splenocytes for Co-46D5 and Il-A150 was shown in Table 1. In the one-color immunofluorescent staining, Co-46D5 reacted with PBMC (28%, MFI 101.0), granulocytes (92%, MFI 20.5), splenocytes (51%, MFI 37.5) and was negative on erythrocytes and platelets (data not shown). Co-46D5 bound to a small

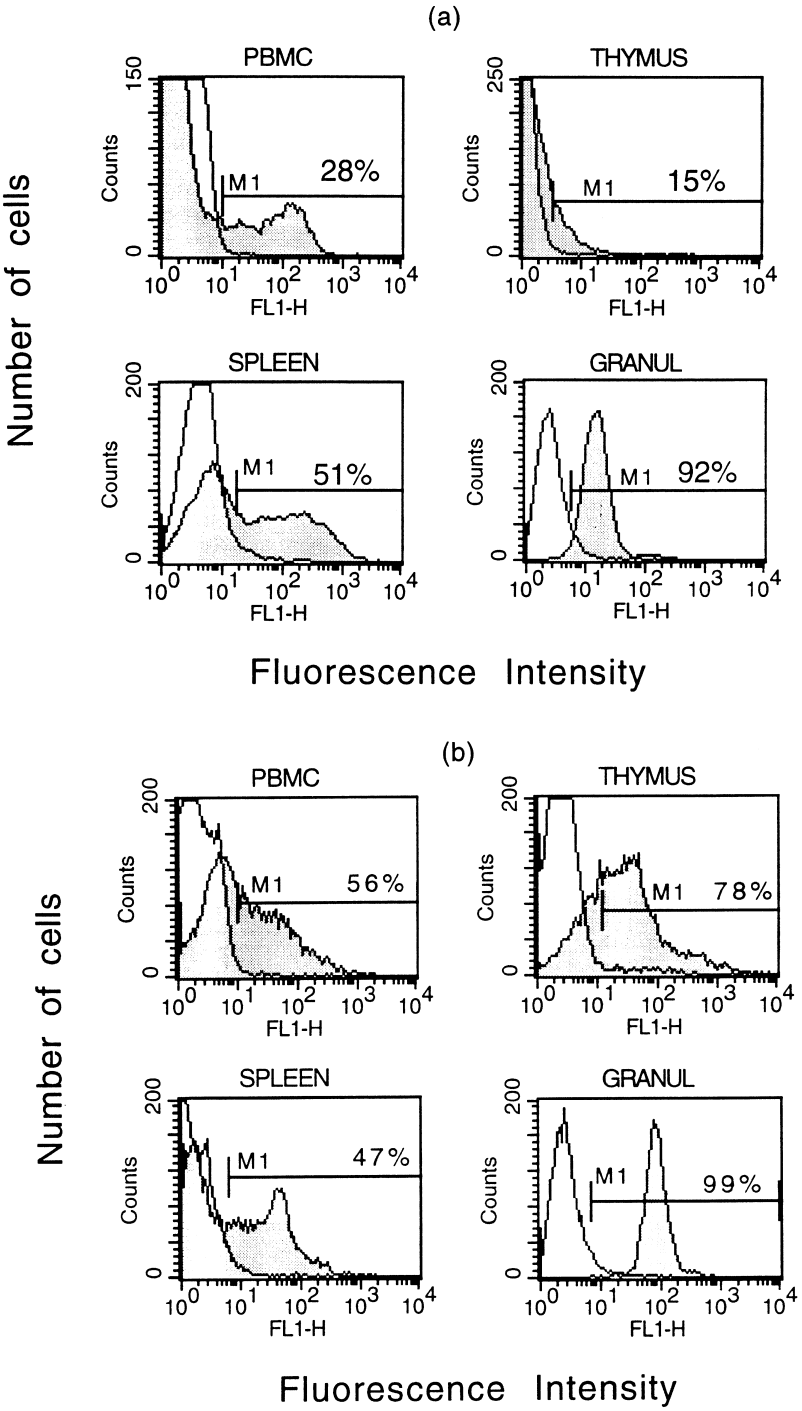

Fig. 1.(a, b)—(*caption on facing page*).

Table 1
Flow cytometric analysis of the antigens recognized by mAbs Co-46D5 and IL-A150, showing a different mean fluorescence intensity (MFI)[a]

Cells	Background	mAB	
		Co-46D5	Il-A150
PBMC	2.9	101.0	57.0
Granulocytes	3.2	20.5	89.5
Thymocytes	1.6	4.1	98.3
Splenocytes	2.2	37.5	48.6

[a]Mean fluorescence intensity (channel units) of cells within the gated population.

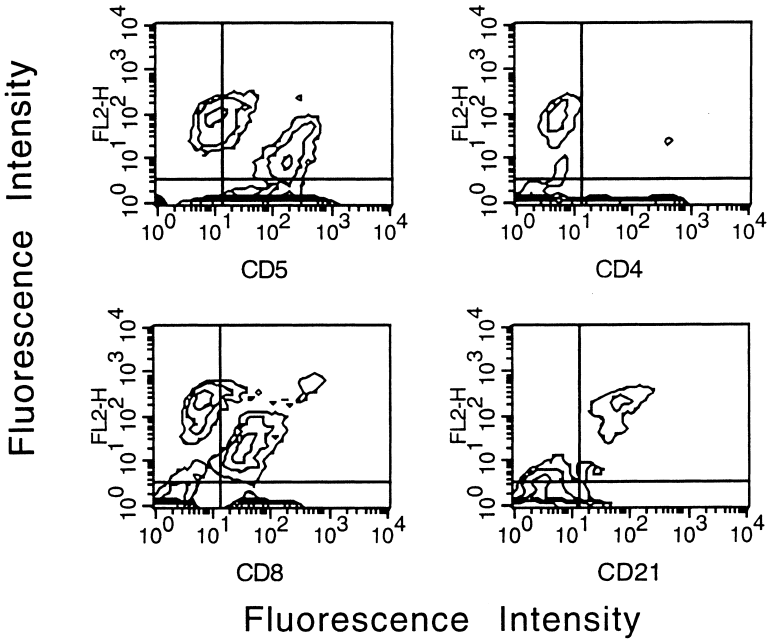

Fig. 2. Contour plots of two-color flow cytometric analysis of the antigen recognized by Co-46D5 (vertical axis) on sheep peripheral blood monucleated cells in combination with anti-CD5 (a), anti-CD4 (b), anti-CD8 (c) and anti-CD21 (d) (horizontal axis). Results are shown as contour plots of the cell population.

Fig. 1. Flow cytometric analysis of the antigen recognized by mAb Co46D5 (a) and IL-A150 (b) on sheep leukocytes from PBMC and lymphoid tissues. Histograms represent fluorescence intensity vs. number of cells. Data were scored as the percentage of fluorescence-positive cells within the gated population over the total. Clear profiles correspond to background staining with an irrelevant mAb. PBMC, peripheral blood mononucleated cells. GRANUL, granulocytes. THYMUS, cell suspension from thymus. SPLEEN, cell suspension from spleen.

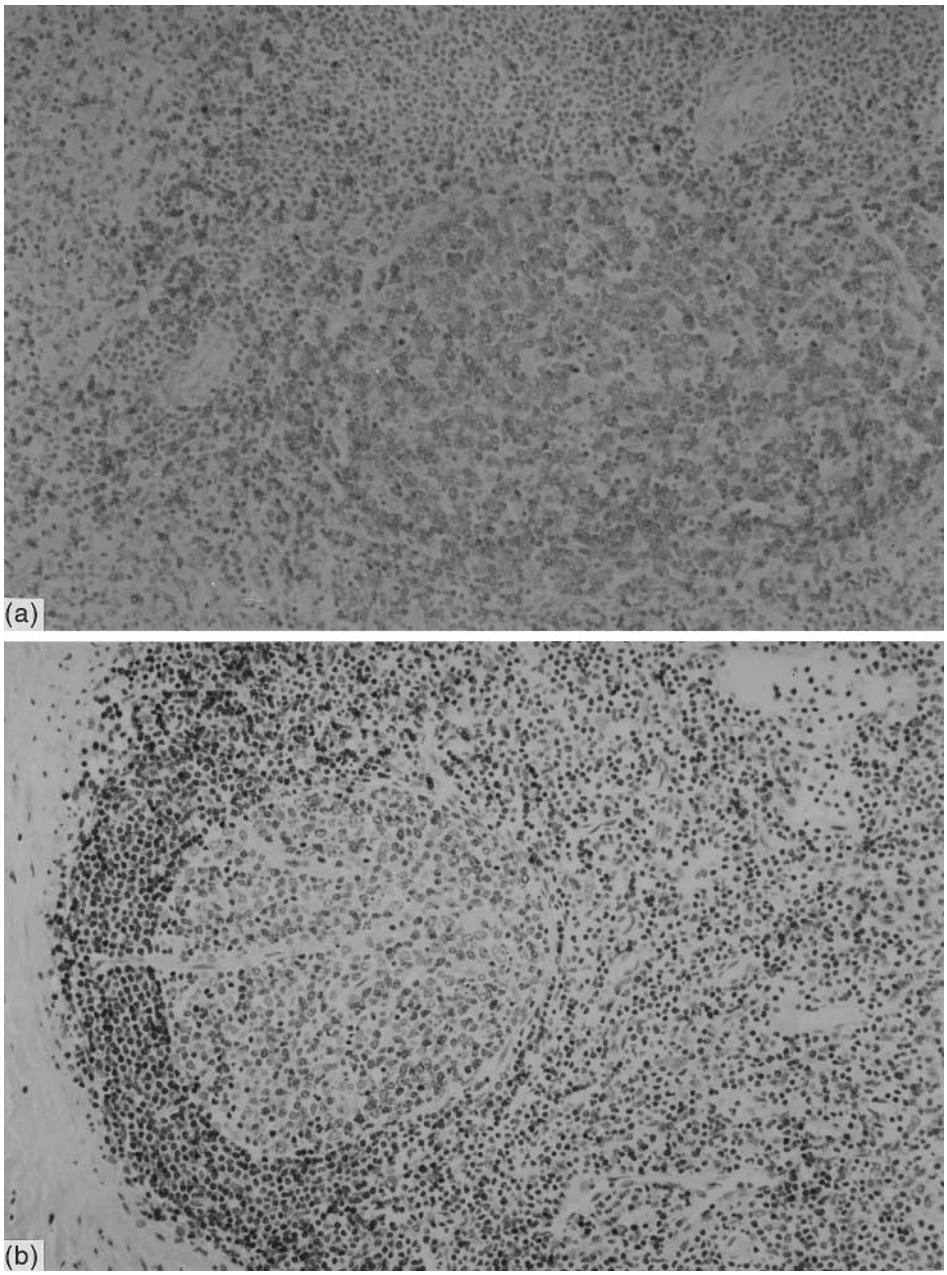

Fig. 3. Paraffin-embedded sections of ovine spleen (a), lymph node (b) and thymus (c) stained with mAb Co-46D5 by the immunoperoxidase method, 40 and 20×.

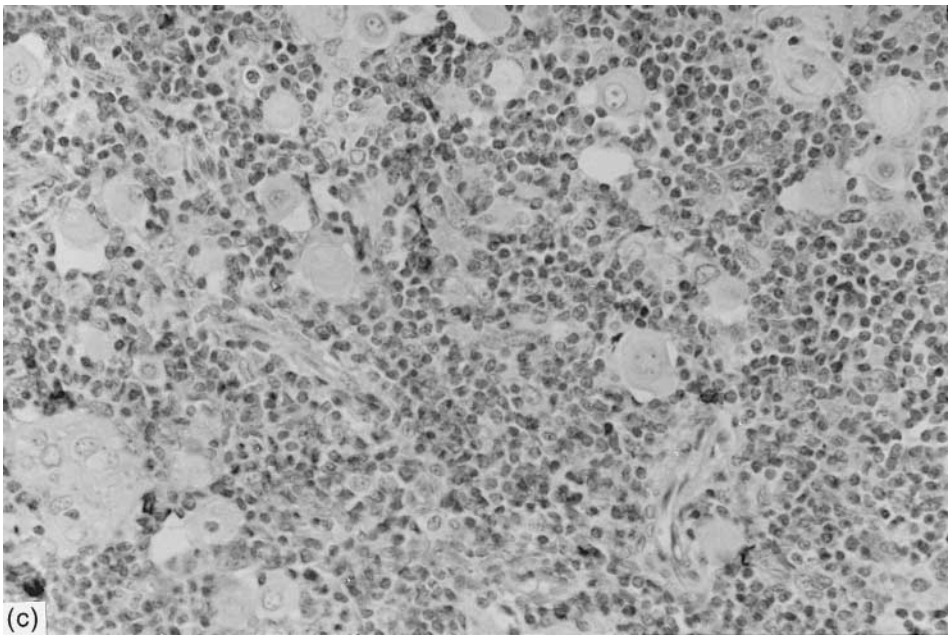

Fig. 3. (c)—(*continued*)

subpopulation of thymocytes (15% MFI 4.1) and stained 35% of peripheral blood monocytes and more than 95% of cells from lymphoid follicles of IPP (data not shown). IL-A150 bound to PBMC, (56% MFI 57.0) granulocytes, (99% MFI 89.5) splenocytes (47% MFI 48.6) and thymocytes (78% MFI 98.3).

The reactivity of mAb Co-46D5 was further investigated using two-color immunofluorescence (Fig. 2). All $CD21^+$ cells (B lymphocytes) were found among the positive Co-$46D5^+$ population. A small proportion of Co-$46D5^+$ cells were also positive for CD5, but showed reduced expression of CD5 compared to other $CD5^+$ cells. Two-color analysis of Co-46D5 against T-cell subsets demonstrated that all Co-$46D5^+$ cells were negative for CD4, but a proportion expressed CD8.

3.2. Molecular weight analysis

No material could be immunoprecipitated with mAb Co-46D5 either from PBMC nor from thymocyte or splenocytes cell lysates. Western-blot analysis of PBMC and spleen cells lysates after SDS–PAGE showed that the antigen recognized by mAb Co-46D5 ran as a single band at 220 kDa mw under reducing conditions (Fig. 4A and B). With thymus lysates, no bands were evidenced by Co-46D5. (Fig. 4C). IL-A150 presents a band at 180 kDa in the PBMC, splenocytes and thymocytes. Fig. 4(D–F).

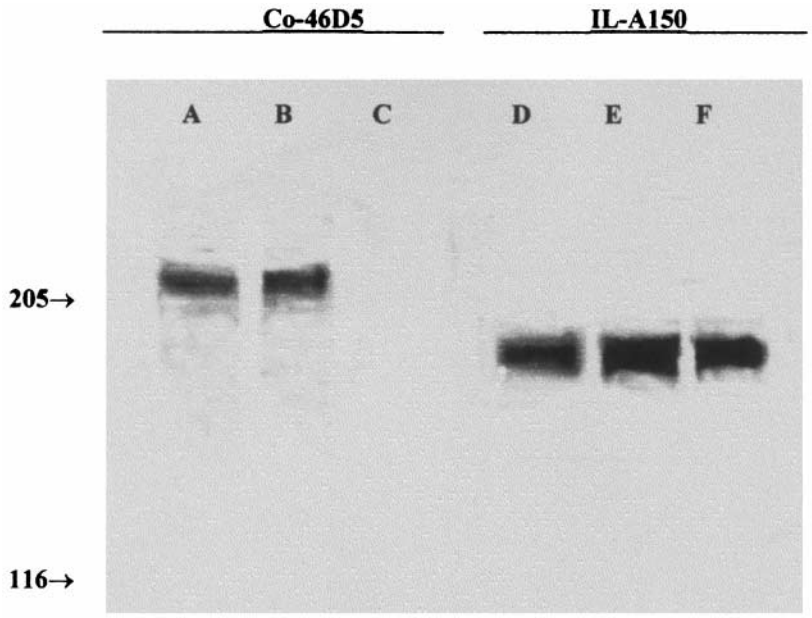

Fig. 4. Western blot analysis of sheep LCA with the monoclonal antibodies Co-46D5 and IL-A150 in lysates of PBMC (a and d), splenocytes (b and e) and thymocytes (c and f).

3.3. Immunohistological analysis of sheep lymphoid tissues

Sheep lymphoid tissue sections were stained with mAbs using the ABC technique. In adults, mAb Co-46D5 stained all lymphocytes within the B-cell follicles as well as scattered cells in interfollicular areas in lymph nodes and in spleen. Within the thymus, mAb Co-46D5 stained mainly cells in the medulla (Fig. 3).

4. Discussion

This report describes the characterization of a mAb, Co-46D5, raised against sheep PBMC. Based on biochemical, histochemical and flow cytometric analysis, the antigen recognized by mAb Co-46D5 appears to be homologous to the largest isoform of human CD45, probably CD45RA.

FCM showed that Co-46D5 reacted with: $CD21^{+}$ cells, cells from lymphoid follicules of IPP, a group of peripheral monocytes, very few T-cells and with a very low intensity with granulocytes. Co-46D5 immunohistological analysis stained all lymphocytes within the B-cell follicle and cells in the medulla of the thymus. These data support that Co-46D5 recognized an molecule mainly present on B lymphocytes and a very reduce group of T lymphocytes, confirming previous

data obtained in the III Ruminant Workshop where Co-46D5 was included in a separate group for B-cells an a T-cell subset [11]. Biochemical data showed that Co-46D5 reacted, with an epitope on a 220 kDa molecule on spleen cells being not detected either on PBMC nor on thymocytes.

Assembling biochemical and tissular expression, we conclude that Co-46D5 detected an epitope on the sheep homologue to human CD45RA or RB isoform. Co-46D5 were predominantly expressed on tissues with high expression of B-cells and it was almost undetected on thymocytes, its molecular weight of around 220 kDa was coincident with the highest isoform CD45RA. CD45RO isoform was discarded since it was mainly present on thymocytes and its molecular weigh was of 180 kDa. Other data like a low density (MFI 20.5) on granulocytes confirm Co-46D5 like specific for the CD45RA or RB isoform.

Human CD45RA or CD45RB mAbs are charactized by cells expressing the suitable isoform, so designate Co-46D5, to CD45RA or RB isoform is difficult, if not impossible, using only FCM, histological and biochemical analysis. Nevertheless in humans, some differences in cells distribution of RA and RB isoform has been described, i.e. monocytes expressed high amounts of CD45RO and CD45RB isoforms and small but readily detectable amounts of CD45RA, also CD45RA were expressed with similar density on bright and dim CD8+ cells [12]. Co-46D5 present weak expression on peripheral monocytes and CD8+ cells, these data will support that our mAbs recognized the sheep homologue to the CD45RA isoform.

Comparison of immunohistological results between 20–96 (a CD45R mAb) [4, 11] and Co-46D5 showed that the cellular distribution of their antigens was similar but staining with Co-46D5 was more intense than with 20–96 [11]. Flow cytometry analysis using two-color immunofluorescence also established differences between both the two mAbs. Co-46D5 recognized an epitope only expressed on B-cells and a CD4−, CD8+, CD5+ lymphocyte subset, while mAb 20–96 recognized an epitope expressed on B-cells and a $CD4^-$, $CD8^-$ and $CD5^+$ lymphocyte subset. Moreover, mAb 20–96 did not react with monocytes [11] and Co-46D5 reacted with a subset of peripheral blood monocytes (35%), recognition of monocytes have been confirmed in the 3rd workshop on ruminant leukocyte antigen [13] where Co-46D5 detected a 68% of peripheral blood monocytes from sheep. These observations would imply that the epitope recognized by Co-46D5 is different from the epitope recognized by mAb 20–96.

We conclude that Co-46D5 recognizes a new epitope on the sheep CD45R molecule, probably the RA isoform. Co-46D5 could be used as a reagent to study changes in the expression of CD45 molecule, such as decreased levels of CD45 in certain leukemias, lymphomas, and other diseases [14]. Provisional data obtained in our laboratory shown a statistically different expression of Co-46D5 on PBMN from sheep vaccinated and not vaccinated against paratuberculosis [15]. Moreover, since this mAb works on paraffin-embedded tissue sections, it can be used as an antigenic marker with which to identify B lymphocytes and leukaemic B-cell lines by immunohistochemistry.

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