Vet. Res. 34 (2003) 791–801

DOI: 10.1051/vetres:2003033

Original article

Cross-species reactivity of seven monoclonal antibodies with equine lymphocytes by flow cytometry

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(Received 9 July 2002; accepted 12 June 2003)

Abstract – The recognition of equine lymphocyte antigens by monoclonal antibodies (mAbs) directed against human CD11a, CD18, CD21, CD23, CD29 and DR, as well as mouse CD23 was studied by flow cytometry. Unlike anti-CD11a, -CD21, -CD23 and DR mAbs, anti-CD18 and CD29 mAbs labelled the same percentage of horse peripheral blood lymphocytes (PBL) as human PBL. Double-staining with anti-horse immunoglobulin antibodies showed that anti-CD21 and -CD23 mAbs are mainly bound to peripheral blood B lymphocytes. The seven mAbs were also tested on the lymph node and thymus cells. The molecular targets of anti-CD11a, CD18 and CD29 mAbs were confirmed by immunoprecipitation of the membrane proteins. Our results suggest that anti-CD18, -CD29 and -DR mAbs recognise similarly expressed molecular homologues on equine cells, but that anti-CD11a, -CD21 and -CD23 mAbs recognise either different molecules or homologues that are expressed at different levels on horse cells.

horse / lymphocyte / leukocyte antigen / monoclonal antibody / cross-reaction

1. INTRODUCTION

The use of monoclonal antibodies to identify and quantify lymphocytes bearing specific antigens has become a routine procedure in human medicine, but such immunophenotypic analysis is still relatively uncommon in veterinary practice. Some monoclonal antibodies to animal lymphocyte antigens have been developed for research purposes, and cross-reactivities between different species can provide useful reagents. Several lymphocyte antigens have been characterised on equine leukocytes at the two International Equine Leukocyte Antigen Workshops in 1991 [10] and in 1995 [12], where the specificities of CD2, CD3, CD4, CD5, CD8, CD11a/CD18, CD13 and CD44, together with MHC class I and II molecules and immunoglobulin isotypes were established. Further reports describe the specificities to equine CD19 [27], CD28 [6] and FcγII and III [2]. Equine CD23 and TCR have

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been characterised by gene cloning [19, 20, 24], but no monoclonal antibodies are available for them.

To extend the range of available monoclonal reagents to horse leukocyte antigens without recourse to the expensive and time-consuming production of new monoclonal antibodies, several authors have reported useful inter-species cross reactivities [1, 5, 9, 17, 26, 28]. We extended these observations using seven monoclonal antibodies directed to human or mouse B cell antigens, or human integrins or MHC class II molecules. Horse peripheral blood lymphocytes were labelled with these antibodies, and analysed by flow cytometry. These mAbs recognised equine antigens, which were examined for expression on equine peripheral blood B cells, thymocytes and lymph node cells. The biochemical nature of the molecules recognised by anti-integrin mAbs was examined by immunoprecipitation.

2. MATERIALS AND METHODS

2.1. Equine peripheral blood mononuclear cells, thymus and lymph node cells

Venous blood was collected onto dextrose citrate in vacutainer tubes (Becton Dickinson, Le Pont-de-Claix, France) from 11 horses (8 males, 3 females; aged 3–16 years) from a local riding school or presenting to the Lyon veterinary school for treatment. Samples from animals with abnormal immunological or haematological parameters were excluded. Peripheral blood mononuclear cells (PBMC) were prepared by centrifuging over Ficoll (Eurobio, Les Ulis, France) at 600 *g* for 30 min. Cells were re-suspended in phosphate buffered saline (PBS) and centrifuged twice through PBS (180 *g*, 15 min to remove platelets and 450 *g*, 10 min wash) before resuspension and counting.

Thymocytes were recovered from the thymuses of young (< 2 year-old) horses in the Lyon-Corbas (France) slaughterhouse. Macroscopically normal lymph nodes (one tracheo-bronchial lymph node, one liver hilum lymph node and one mediastinal lymph node) were collected from three different horses. Lymphocytes were prepared from these organs by teasing through sterile mesh, filtration through gauze and washing 2× in RPMI-1640 culture medium (Gibco BRL, Cergy-Pontoise, France) by centrifuging at 300 *g* for 10 min. All operations were performed at 4 °C.

2.2. Antibodies

The specificities, origins and nature of the antibodies studied are listed in Table I. Positive controls consisted of mAbs raised against equine antigens and one anti-human CD18 mAb, known to cross-react with the equine homologue [28]. Negative controls included FITC or PE-conjugated, or unconjugated murine IgG1 mAbs directed towards Keyhole limpet haemocyanin (KLH) and an FITC-labelled anti-dinitrophenyl rat IgG2a mAb. Where no isotype control antibody was at our disposal, control cells were incubated with the second-step reagent alone for indirect immunofluorescence reactions. The monoclonal antibody EqT12 recognises equine prothymocytes, and equine B lymphocytes were identified using 100 µL of fluorescein- or biotin-labelled goat $(Fab')_2$ fragments specific for horse $(Fab')_2$ fragments diluted at 1/2000.

2.3. Immunofluorescent staining and flow cytometric analysis

Cell suspensions were adjusted to a concentration of 10^7 cells/mL in PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBA). Aliquots of 5×10^5 cells were incubated with primary antibody at 4 °C in the dark for 30 min, then washed three times in cold PBA. The cells treated with labelled antibodies (see Tab. I)

Table I. Monoclonal antibodies used in this study.

Target antigen	Clone	Host / isotype	Forms	Origin	Use
Horse CD4	CVS4	Ms IgG1	uc	Serotec / Dr Lunn	pct
Horse CD4	HB61A	Ms IgG1	uc	VMRD	pct
Horse CD5	CVS5	Ms IgG1	uc	Serotec / Dr Lunn	pct
Horse CD8	CVS8	Ms IgG1	uc	Dr Lunn	pct
Horse CD8	CVS21	Ms IgG2a	uc	Serotec	pct
Horse CD8α	73 / 6.9.1	Ms IgG3	uc	VMRD	pct
Horse CD8β	HT14A	Ms IgG1	uc	VMRD	pct
Horse EqT12	EqT12	Ms IgG1	uc	VMRD	pct^T
Human CD18	MHM23	Ms IgG1	uc, FITC	Dako	pct
KLH	X-40	Ms IgG1	uc, FITC, PE	BD	nct
DNP	LO-DNP-16	Rat IgG2a	FITC	Caltag	nct
Human CD11a	25.3	Ms IgG1	uc	Beckman-Coulter	tes
Human CD18	25P6	Ms IgG1	uc	Biosys	tes
Human CD21	BL13	Ms IgG1	FITC	Beckman-Coulter	tes
Human CD23	9P25	Ms IgG1	FITC	Beckman-Coulter	tes
Mouse CD23	B3B4	Rat IgG2a	FITC	Pharmingen	tes
Human CD29	4B4	Ms IgG1	FITC, PE	Beckman-Coulter	tes
Human DR	CR3/43	Ms IgG1	uc	Dako	tes

KLH: keyhole limpet haemocyanin, DNP: dinitrophenyl (artificial compound), Ms: mouse, uc: unconjugated, FITC: fluorescein isothiocyanate, PE: phycoerythrin; nct: negative control, tes: tested in this study, pct: positive control, ^{T}: used only on thymocytes.

were then fixed in 1% formaldehyde in PBA, and those treated with unconjugated antibodies were incubated with 400 ng in 20 µL of FITC-conjugated goat $(Fab')_2$ anti-mouse IgG (Fab specific) adsorbed with horse serum proteins (SIGMA, L'Isle d'Abeau-Chesnes, France) before washing (2×) and fixation as above. Biotin-labelled anti-horse Ig were detected using 20 µL of phycoerythrin (PE)-conjugated streptavidin (Becton Dickinson) at a 1/100 dilution. Samples for two-colour fluorescence analysis were simultaneously treated with the FITC- and PE- or biotin-conjugated antibodies.

Labelled cells were analysed on a FACScan cytometer using Lysis II software (Becton Dickinson). Peripheral blood lymphocytes were identified according to their forward scatter and side scatter parameters: the lymphocytes have a smaller size and granularity than monocytes [21]; this was confirmed by back-gating with the anti-CD5 and sIg Abs. Data from 15 000 events from the global population were recorded for each reagent. In general, the positivity threshold was defined so that ≤ 1% control cells were positive ("non specific binding"). The percentage of lymphocytes positive for a reaction with each horse, human or mouse-reactive antibody was the difference between the percentage of cells over the threshold and that of unspecific binding. However, for some mAbs that stained a sub-population of lymph node cells with a weak intensity, the

limit was placed between the first and the second cytometry peak.

2.4. Immunoprecipitation and SDS-PAGE

For each immunoprecipitation, magnetic beads covalently linked with anti-mouse antibodies (Dynal, Compiègne, France) were washed, coated with mAbs for 30 min at 4 °C and washed again, as indicated by the manufacturer.

PBMC or thymocytes (50×10^6/mL) were lysed in a 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1% (v/v) Triton X-100 and Complete™ protease inhibitors (Roche Molecular Diagnostics, Meylan, France) for 30 min at 4 °C. After centrifugation at 15 000 *g* for 10 min, lysates were incubated for 2 h with 2 μg mAb that had previously been coated onto magnetic beads. The beads were washed once in 10 mM Tris-HCl, 150 mM NaCl, Complete™ and twice with the same buffer without Complete™. They were then boiled for 3 min in a denaturing buffer (62.5 mM Tris HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 5% β-mercapto-ethanol and 10% glycerol). The samples were then coloured with 0.002% pyronin G less than 2 h before analysis by SDS-PAGE (polyacrylamide gel electrophoresis) through 10% acrylamide. The gels were stained with silver nitrate as described by Merril et al. [13].

3. RESULTS

Single staining showed that the seven studied mAbs labelled horse peripheral blood lymphocytes, as well as thymus and lymph node cells (Tab. II).

The cells were double stained with anti-horse surface Ig (sIg) antibody to determine the expression of certain mAb targets on horse peripheral blood B-lymphocytes. Immunoprecipitations were carried out to define the biochemical nature of some antigens.

3.1. Anti-CD21 and CD23 mAbs

The anti-CD21 mAb labelled 13.2% horse PBL, and the anti-human and anti-mouse CD23 mAbs stained 20.4% and 21% of these cells, respectively (Figs. 1a–1c and Tab. II). The mAbs of both specificities revealed two subsets with various antigen densities (Figs. 1a–1c).

mAb BL13 (anti-human CD21) mainly stained sIg expressing lymphocytes but most of these cells were $BL13^-$ (Fig. 2a and Tab. III).

The molecules recognised by 9P25 and B3B4 (anti-human and anti-mouse CD23 mAbs) were mostly expressed by B lymphocytes, more precisely by the majority of these cells (76.6–90.6%). They were less restricted to sIg^+ lymphocytes than the antigen recognised by BL13 (Fig. 2b and Tab. III).

Whereas less than 13.7% horse thymus cells were labelled by the anti-CD21 mAb, 13.8 to 49.5% expressed the antigens recognised by the anti-CD23 mAbs (Tab. II).

The anti-CD21 mAb labelled 18.7 to 59.7% lymph node cells (Fig. 3c), and anti-CD23 mAbs stained between 16.1 and 45.8%. The percentages always followed the variations in sIg^+ cells, without being strictly proportional.

3.2. Anti-human integrin mAbs

Except in one blood sample and one lymph node, the anti-human CD18 mAb 25P6 recognised more than 85% equine lymphocytes (Tab. II). Staining profiles (Fig. 3a) were similar to those obtained with MHM23 (data not shown). Immunoprecipitation and SDS-PAGE were performed under reducing conditions. Silver nitrate staining of the gel revealed four protein bands with relative molecular masses of 185, 145, 100 and 29 kDa (Fig. 4, lane C), that were absent from proteins immunoprecipitated with the isotype-matched control mAb (lane B).

Table II. Flow cytometry analysis of horse lymphocytes immunostained with anti-horse, anti-human or anti-mouse leukocyte antigen mAbs.

Target antigen (clone–fluorochrome)	% positive PBL ± S.D. (No.)	% positive thymocytes	% positive lymph node cells ± S.D. (No.)
Horse sIg	29.5 ± 10.9	9.3; 5.5	43.5 ± 12.8
(polyclonal)	(11)		(3)
Horse CD4	49.6 ± 9.0	N.D.	N.D.
(CVS4)	(4)		
Horse CD4	56.25 ± 7.6	56.7; 77.5	44.3 ± 10.4
(HB61A)	(8)		(3)
Horse CD5	79.0; 79.9	86.4; 93.4	66.2 ± 19.3
(CVS5)	(2)		(3)
Horse CD8	25.9; 24.2	N.D.	N.D.
(CVS8)	(2)		
Horse CD8	22.7 ± 1.5	N.D.	N.D.
(CVS21)	(3)		
Horse CD8α	21.1 ± 5.9	47.4; 72.1	13.3 ± 4.0
(73 / 6.9.1)	(5)		(3)
Horse CD8β	21.0 ± 5.1	57.2; 76.2	14.7 ± 4.9
(HT14A)	(8)		(3)
Horse prothymocytes	N.D.	15.4; 10.6	N.D.
(EqT12)			
Human /horse CD18	95.5 ± 1.7	95.3; 94.8	84.2 ± 10.2
(MHM23-FITC or uc)	(5)		(3)
Human CD11a	14.7; 13.6	7.0; 9.2	15.0 ± 3.8
(25.3)	(2)		(3)
Human CD18	83.4 ± 13.1	87.0; 88.2	77.7 ± 13.1
(25P6)	(3)		(3)
Human CD21	13.2 ± 4.8	13.7; 1.4	37.8 ± 20.7
(BL13 – FITC)	(10)		(3)
Human CD23	20.4 ± 7.7	48.2; 13.8	33.5 ± 15.5
(9P25 – FITC)	(4)		(3)
Mouse CD23	21.0 ± 12.8	49.5; 14.1	33.1 ± 14.2
(B3B4 – FITC)	(4)		(3)
Human CD29	38.6 ± 20.8	41.8; 44.3	64.2 ± 8.0
(4B4 – FITC or PE)	(7)		(3)
Human DR	85.6 ; 85.9	38.1; 26.5	67.0 ± 22.9
(CR3/43)	(2)		(3)

No.: number of samples, N.D.: not determined.

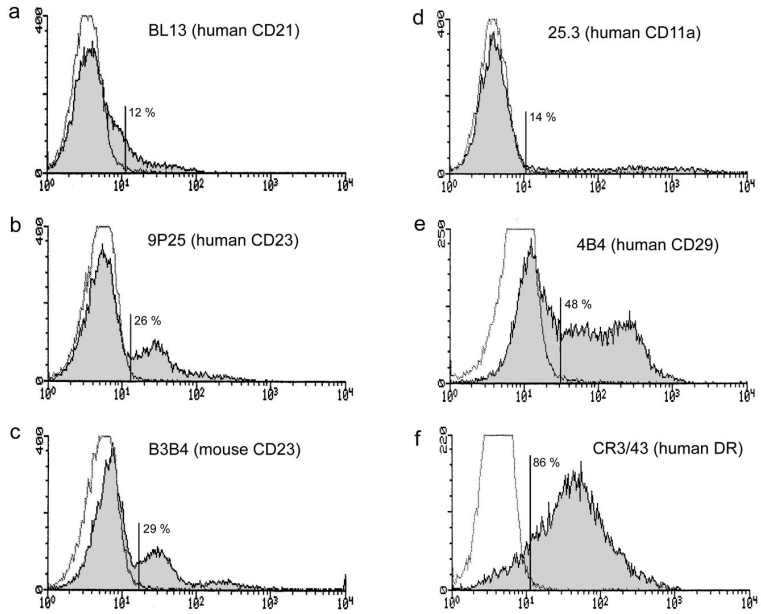

Figure 1. Flow cytometry profiles of horse PBL single stained with various mAbs: (a) FITC-conjugated anti-human CD21 (BL13), (b) FITC-conjugated anti-human CD23 (9P25), (c) FITC-conjugated anti-mouse CD23 (B3B4), (d) anti-human CD11a (25.3), (e) anti-human CD29 (4B4), (f) anti-human DR (CR3/43). Dotted lines represent labelling with a mouse anti-KLH IgG1 (X-40 clone, a–b and d–f) or with a rat anti-DNP IgG2a (LO-DNP-16 clone, c).

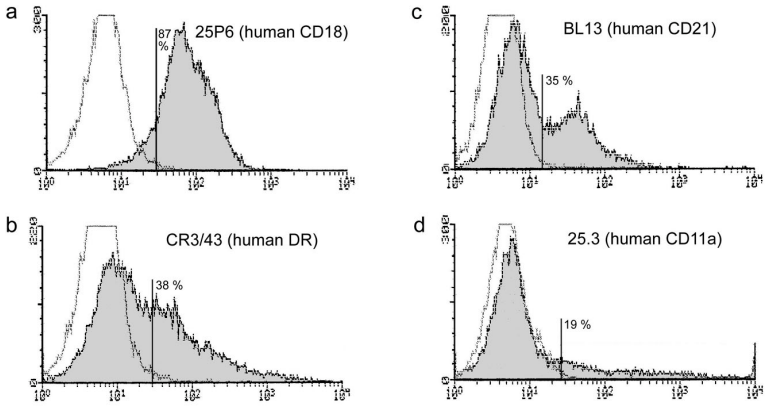

Figure 2. Flow cytometry profiles of horse thymus (a–b) or lymph node (c–d) cells single stained with various mAbs: (a) anti-human CD18 (25P6), (b) anti-human DR (CR3/43), (c) FITC-conjugated anti-human CD21 (BL13), (d) anti-human CD11a (25.3). Dotted lines represent labelling with a mouse anti-KLH IgG1 (X-40).

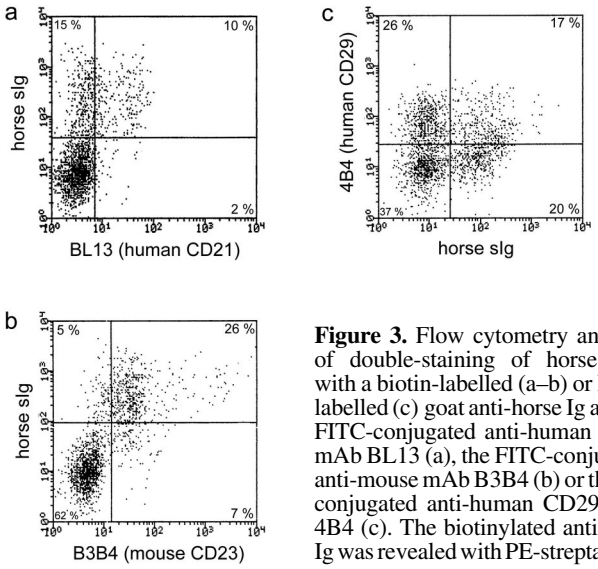

Figure 3. Flow cytometry analyses of double-staining of horse PBL with a biotin-labelled (a–b) or FITC-labelled (c) goat anti-horse Ig and the FITC-conjugated anti-human CD21 mAb BL13 (a), the FITC-conjugated anti-mouse mAb B3B4 (b) or the PE-conjugated anti-human CD29 mAb 4B4 (c). The biotinylated anti-horse Ig was revealed with PE-streptavidin.

The anti-human CD11a mAb 25.3 bound to about 14% PBL (Fig. 1d), 8% thymocytes and 15% lymph node cells (Fig. 3d) in horses (Tab. II). It immunoprecipitated seven polypeptides of 185, 145, 120, 85, 38, 32 and 29 kDa (Fig. 4, lane D).

The anti-human CD29 antibody 4B4 stained various percentages of blood lymphocytes (Fig. 1e and Tab. II), but in two horses, it labelled sIg^+ and sIg^- PBL equally (Fig. 2c and Tab. III). It stained 41.8 to 44.3% thymus lymphocytes and 55 to 70% lymph node cells (Tab. II). Immunoprecipitation products of thymocyte lysates obtained with 4B4 under reducing conditions migrated as three bands of 250, 130 and 110 kDa (Fig. 5, lane C).

3.3. Anti-human DR mAb CR3/43

In our study, the anti HLA-DR mAb CR3/43 reacted with more than 85% PBL (Fig. 1f), 26.5 to 38.1% thymus cells (Fig. 3b) and 67% lymph node cells (Tab. II).

Table III. Percentage of horse total, sIg^+ and sIg^- PBL labelled with the following mAbs: BL13 (anti-human CD21), 9P25 (anti-human CD23), B3B4 (anti-mouse CD23) and 4B4 (anti-human CD29).

Percentage of	Lymphocyte population			Horse
lymphocytes	Total	sIg^+	sIg^-	
$BL13^+$	12.3; 8.2; 10.2	25.6; 19.5; 36.3	0.9; 3.8; 3.3	A; B; C
$9P25^+$	26.6; 31.3	78.4; 76.6	12.4; 10.3	D; E
$B3B4^+$	28.0; 32.6	90.6; 83.0	10.1; 9.4	D; E
$4B4^+$	42.3; 24.9	44.4; 23.4	41.0; 26.1	D; E

For each lymphocyte population, the results were obtained from horses referenced in the right column, and given in the same order.

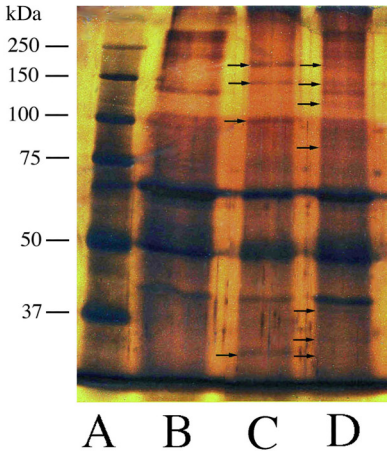

Figure 4. SDS-PAGE analysis of immunoprecipitation from horse PBMC extracts by anti-human leukocyte antigen mAbs. Ten percent acrylamide gels were stained with silver nitrate. Lane (A): relative molecular mass markers, (B): immunoprecipitations with mouse IgG1 isotype control X-40, (C): anti-human CD18 mAb 25P6, (D): anti-human CD11a mAb 25.3.

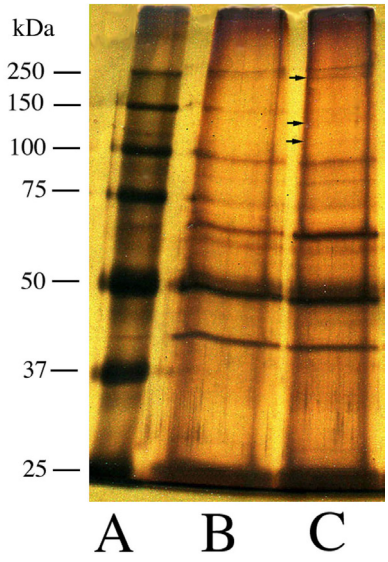

Figure 5. SDS-PAGE analysis of immunoprecipitation of horse thymocyte extracts by an anti-human leukocyte antigen mAb. Lane (A): relative molecular mass markers, (B): immunoprecipitation with mouse IgG1 isotype control X-40, (C): anti-human CD29 mAb 4B4.

4. DISCUSSION

4.1. Anti-CD21 and CD23 mAbs

CD21 (CR2) is the C3d receptor. In human blood, it is expressed at low density on nearly all B cells [15], but CD21 is quite intensively expressed on bovine peripheral blood B lymphocytes [16].

The anti-human CD21 mAb BL13 mainly stained equine blood B lymphocytes (Fig. 2a and Tab. III). Most sIg^+ cells did not, however, express this antigen (Tab. III). Unlike human CD21, the antigen recognised by the anti-human CD21 mAb on horse PBL is only expressed on a B cell sub-population. Horse thymocytes labelled by BL13 may also be B-cells, as described in the human thymus [14]. In lymph nodes (Fig. 3c), the percentage of $BL13^+$ cells was 10% higher than the maximum percentage found among PBL (Tab. II) and followed the variations in sIg^+ cells.

Altogether, these results suggest that the equine antigen recognised by BL13 is a B cell subpopulation marker.

In humans, CD23 (FcεRII) is expressed on activated B cells [4, 15]. Thus it is found on lymph node B cells but not on peripheral blood B lymphocytes.

Anti-human and anti-mouse CD23 mAbs stained most equine blood B lymphocytes (Fig. 2b and Tab. III). Since they mainly stained this cell type, the density of the recognised antigens was not linked to the B/T lymphocyte phenotype.

Anti-CD23 mAbs also stained equine lymph node cells (Tab. II). The proportionality of the percentages of labelled cells and that of sIg^+ cells is in favour of a preferential expression of the recognised antigens on peripheral B lymphocytes in the horse. Since these molecules were expressed by 50% of the cells from the first thymus

(Tab. II), they cannot be thymic B-lymphocyte markers. They may be expressed on immature T-cells.

It is interesting to notice that an anti-human and an anti-mouse CD23 mAb stained the same percentage of lymphocytes in horse blood, thymus and lymph nodes (Tab. II) and labelled the same cell types in peripheral blood (Tab. III). Furthermore, their staining profiles were identical (Figs. 1b and 1c). Both antibodies probably recognised epitopes that are conserved between the human and the equine species for one, and between the murine and the equine species for the other. These epitopes are likely to be different, however, since the mouse anti-human CD23 mAb is unlikely to recognise mouse CD23 too, unless it is an auto-antibody.

4.2. Anti-human integrin mAbs

The leukocyte adhesion molecules LFA-1, Mac-1 (CR3) and p150,95 (CR4) are composed of integrin alpha chains: L (CD11a), M (CD11b) or X (CD11c), respectively, linked to the integrin β2 chain (CD18).

The labelling of equine lymphocytes with the anti-human CD18 mAb 25P6 (Fig. 3a and Tab. II) was similar to that of MHM23 (Tab. II), which recognises both human and equine CD18 [28]. This suggests that 25P6 recognises horse CD18. To ascertain this, immunoprecipitation and SDS-PAGE were performed under reducing conditions. The 29 kDa band produced may come from a degraded integrin chain or from a tetraspan molecule (e.g. CD9). The relative molecular masses of the 185, 145 and 100 kDa bands are, however, very close to those of human CD11a (180 kDa), CD11c (150 kDa) and CD18 (95 kDa), which are co-immunoprecipitated by anti-CD18 mAbs from lymphocyte and monocyte extracts [23]. The 180 and 100 kDa bands are also found after immunoprecipitation of horse PBMC lysates with anti-human CD18 mAb MHM23 or anti-horse CD11a/CD18 mAbs [28]. Thus, 25P6 very probably recognises horse CD18.

Whereas CD11a is expressed on 95–100% human peripheral blood and thymus lymphocytes, the anti-human CD11a mAb 25.3 are bound to less than 15% horse peripheral blood or thymus lymphocytes (Fig. 3d and Tab. II). Among the seven polypeptides immunoprecipitated by 25.3, the 185, 145 and 29 kDa bands were also found in 25P6 immunoprecipitates. However, bands of 120, 85, 38 and 32 kDa were present and the 100 kDa band (which corresponds to CD18) was absent (Fig. 4). Altogether, these results bring us to the conclusion that the anti-human CD11a mAb 25.3 probably does not recognise equine CD11a.

In human leukocytes, CD29 (β1 integrin) is a 130 kDa protein which associates with α1 to 6 integrins (CD49a to f) to form the very late activation (VLA1 to 6) cell adhesion molecules [8]. Various expression percentages have been reported on PBL but Reiter [18] found an average of 55% on both B and T cells.

In our study, the anti-human CD29 mAb 4B4 similarly stained B and T horse PBL (Fig. 2c and Tab. III). Moreover, the percentage of labelled cells from the two thymuses we tested (40%, Tab. II) was not very different from that found by Reiter [18].

The 250 and the 110 kDa equine thymocyte proteins identified in this study differed from human CD49a (210 kDa), CD49d (150 kDa), CD49e (135 kDa) or CD49f (120 kDa) by 10–40 kDa [8]. However, the 130 kDa protein is likely to correspond to CD29, and the 250 and 110 kDa bands may come from horse CD49 proteins, which could have a different molecular mass from that of their human homologues. It is therefore highly probable that 4B4 recognises the equine homologue of human CD29.

4.3. Anti-human DR mAb CR3/43

Unlike human PBL, the majority of adult horse PBL expresses MHC class II molecules

[3, 11]. Since the anti HLA-DR mAb CR3/43 reacted with 85% horse PBL (Fig. 1f and Tab. II), it is likely to recognise its equine homologue. Moreover, cells from the two thymuses tested were stained by CR3/43 (Fig. 3b and Tab. II), which can be correlated with class II molecule expression in the horse thymus [11, 12]. Our results, however, contrasted with those of Jacobsen et al. [9], who found that CR3/43 reacted with 33% horse PBL. This can probably not be explained by age differences because only adult animals were used in that paper as in our study. Hence, this may be due to genetic differences, since the expression of MHC class II molecules on lymphocytes is determined by MHC class II haplotypes [3].

The reason why the mAbs we used reacted on horse lymphocytes may be that they recognised molecules that have been well conserved through evolution. Indeed, the anti-human CD18, CD21, CD29 and DR mAbs had previously been shown to recognise lymphocytes from dogs [7], cats [25] or cattle [22]. The molecules recognised by anti-human CD21 and anti-human or mouse CD23 mAbs on horse lymphocytes remain to be characterised. Double-staining or single-staining on isolated lymphocyte populations will be useful to confirm which lymphocyte populations express these antigens. But overall, the biochemical characteristics of these molecules need to be examined using immunoprecipitation and immunoblotting techniques.

ACKNOWLEDGEMENTS

The authors would like to thank the house veterinary surgeons from the Equine Department of the Lyon Veterinary School and the Corbas slaughterhouse Veterinary Services for blood and tissue samples.

This study was made possible by antibody gifts from researchers: Dr Dominique Rigal (Director of the Lyon Blood Transfusion Centre) and Dr D.P. Lunn (School of Veterinary Medicine, Madison, Wisconsin, USA); and by commercial contributors: the Beckman-Coulter company (Marseille, France).

We finally thank Mrs Elisabeth Granjon for performing blood counts as well as Drs François Guiguen and Yahia Chebloune (National Agronomic Research Institute, Lyon) and Dr Carole Auger for experimental and writing advice.

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