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Characterization of monoclonal antibodies specific for equine homologues of CD3 and CD5

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SUMMARY

Two monoclonal antibodies (mAb), UC F6G-3 and UC F13C-5, were characterized as being specific for the apparent equine homologues of CD3 and CD5, respectively. Both antibodies exhibited characteristics of pan-T-lymphocyte markers based upon immunohistology and two-colour flow cytometry. UC F6G-3 precipitated a complex of proteins (up to seven) with molecular weights ranging from 18,000 to 42,000, similar to the human and murine CD3 complex. Upon further dissociation of the precipitated complex, two proteins were identified with molecular weights of 22,000 and 27,000. Immobilized UC F6G-3 was effective at inducing interleukin-2 receptor (IL-2R) expression on T lymphocytes, a feature consistent with antibodies specific for the ε chain of human and murine CD3. Three populations of cells in the thymus were distinguishable by UC F6G-3 target antigen density, suggesting increasing stages of T-cell maturation. UC F13C-5 precipitated a 67,000 MW protein, consistent with reported values for CD5 in multiple species. While this antibody exhibited characteristics of a pan-T-cell marker, low numbers of B lymphocytes also expressed the target antigen. Phorbol esters induced variable increases in target antigen density on B lymphocytes. These two antibodies, taken together with the few equine CD markers currently available, represent a substantial resource for further defining the equine immune system in health and disease.

INTRODUCTION

The development of monoclonal antibodies (mAb) specific for leucocyte differentiation antigens has greatly facilitated phenotypic and functional definition of immune systems of multiple species. More specifically, such reagents have been invaluable in better defining leucocyte subpopulations, signal transduction, maturity and traffic. Relative to animal species of veterinary significance, minimal reagents for identification of equine CD antigens have been reported. Unfortunately, most CD antigen-specific antibodies developed for other species exhibit minimal species cross-reactivity. While an early report[1] described multiple mAb with apparent specificities for equine T lymphocytes, characterization did not include precipitation of the target antigens, making them of limited value. Two recent reports have described mAb specific for the equine homologues of CD5, CD4 and CD8.[2,3] While CD5 expression approaches that of a pan-T-cell marker, variable numbers of B lymphocytes may also express the antigen. In this context, antibodies specific for CD3 are invaluable as this protein complex is only expressed in conjunction with the T-cell receptor (TcR). In addition, many antibodies specific for CD3 subunits can induce signal transduction resulting in polyclonal T-cell activation and proliferation;[4–7] the specificity of this activation is well defined and thus is preferable to use of mitogen-induced activation.

This report describes the characterization of two mAb specific for the apparent homologues of CD3 and CD5. CD5 expression on equine B lymphocytes, before and after activation, is described. Application of the CD3-specific antibody to delineation of T-cell maturation in the thymus and induction of T-cell activation are also described.

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MATERIALS AND METHODS

Monoclonal antibody production

BALB/c mice were immunized with 2×10^6 mononuclear leucocytes, intraperitoneally, at 3-week intervals. Monoclonal antibodies were produced as previously described,[8,9] screened by flow cytometry and cloned by limiting dilution. Antibody isotype was determined by flow cytometry using isotype-specific, fluorescein isothiocyanate (FITC) conjugated reagents (Zymed Laboratories Inc., South San Francisco, CA).

Ascitic fluid was purified using Bakerbond ABX mixed function ion-exchange matrix (J.T. Baker Inc., Phillipsburg, NJ). Purified mAb were directly labelled with FITC utilizing Quick Tag kit, following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN).

Additional murine mAb used in the characterization of UC F6G-3 and UC F13C-5 included an irrelevant mAb (IgG1), CVS4 (equine homologue of CD4)[3] and CVS8 (equine homologue of CD8);[3] CVS4 and CVS8 antibodies were generously provided by Drs Lunn and Holmes (Cambridge, U.K.).

Cell and tissue preparation

Equine mononuclear leucocytes (PBML) were isolated from whole blood collected in EDTA using one of two methods; centrifugation of buffy coat over Histopaque 1077 (Sigma, St Louis, MO; 15 min at 500 *g*)[10] or centrifugation in Leukoprep tubes (Becton Dickinson, San Jose, CA; 7 min at 1800 *g*). Cells in organized lymphoid tissues were isolated by gentle dissociation of tissues into phosphate-buffered saline (PBS). In all cases, cells were washed in PBS (without Ca^{2+} or Mg^{2+}) before use. Where applicable, cells were cultured in Dulbecco's modified minimal essential medium and 20% fetal bovine serum (DMEM/FBS) as previously described.[11] Where utilized, recombinant human interleukin-2 (rIL-2; generously provided by Cetus Corp, Emeryville, CA) and leucohaemagglutinin (PHA-L, Sigma) were added at concentrations of 100 U/ml and 1 μg/ml, respectively. Lymphoid tissues for immunohistology were snap frozen in O.C.T. Compound (Miles, Elkhart, IN).[12]

Immunoprecipitation and PAGE

Freshly isolated and cultured (with rIL-2 and PHA-L) cells were used as antigen sources for immunoprecipitation. Leucocytes were labelled with sulpho-NHS-biotin[13] (Pierce, Rockford, IL) with modifications as previously described.[14] Cellular proteins were solubilized with lysis buffer (LB) containing 1·5% w/v Brij 99 and 0·5% Brij 96.[14]

Purified mAb were bound to agarose beads (affigel-10 active ester agarose, Bio Rad, Richmond, CA) per manufacturer's instruction at a concentration of 20 mg protein/1 ml packed volume. Labelled cell lysates were incubated with immunoglobulin-coated beads overnight at 4°. Beads were pelleted and washed repeatedly in LB. The complex precipitated with UC F6G-3 was then incubated with a mild dissociation buffer [50 mM Tris–HCl, pH 7·6/300 mM NaCl/0·2% sodium dodecyl sulphate (SDS)/0·1% Triton X-100/0·2% sodium deoxycholate][7] for 30 min, on ice, followed by a LB wash. Precipitated proteins, removed from the solid support by incubation with sample application buffer (containing 2-mercaptoethanol), were analysed by discontinuous SDS–polyacrylamide gel electrophoresis (PAGE),[15] along with unlabelled and biotinylated molecular weight standards. Proteins were transferred to Immobilon P (Millipore, South San Francisco, CA) and blocked membranes (5% non-fat dry milk in Tris-buffered saline with 0·5% Tween-20) were probed with streptavidin–horseradish peroxidase (Zymed Laboratories Inc.). Membranes were developed with enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) per the manufacturer's instructions and light emission recorded on Hyperfilm-ECl (Amersham).

Immunofluorescent staining and flow cytometric analysis

Cell preparations were analysed by flow cytometry as previously described.[10] Briefly, 1×10^6 cells were blocked with 5% goat serum and stained with mAb using direct FITC conjugates or indirectly with $F(ab')_2$ anti-mouse (anti-Ms) IgG (H + L) [FITC or phycoerythrin (PE)-conjugated; Caltag, South San Francisco, CA]. B lymphocytes were stained directly with FITC-conjugated goat anti-horse IgG (H&L) [FITC–SIg (surface Ig); Jackson Immunoresearch, West Grove, PA]. Two-colour flow cytometric analysis was achieved by indirectly labelling mAb with PE anti-Ms IgG, followed by incubation with the FITC-conjugated antibody of interest. Non-specific binding of Ig to cells was determined by indirect FITC labelling of irrelevant antibody. In all cases, incubations were performed on ice for 30 min. Cells were analysed on a FACSTAR-Plus flow cytometer (Becton Dickinson), using a 5-W argon ion laser. Ten thousand events were collected in list mode, using a live gate set to include only mononuclear cells identified by forward and side light-scatter characteristics.

Immunohistochemistry

Undiluted hybridoma tissue culture supernatants were applied to frozen sections of lymphoid tissue. Antibody binding was detected using biotinylated anti-Ms IgG (Vector Laboratories, Burlingame, CA) followed by streptavidin–horseradish peroxidase (Zymed Laboratories Inc.) and DAB substrate as previously described.[12]

Lymphocyte activation

Monoclonal antibodies were immobilized onto plastic using previously described methodology.[16] Briefly, purified antibody (1 μg/well) was added to wells in 48-well flat-bottomed tissue culture plates (Costar, Van Nuys, CA). After washing, freshly isolated PBML (4×10^5/well) suspended in DMEM/FBS were added to wells. Antibodies utilized included an irrelevant IgG1 mAb (negative control), UC F6G-3 and UC F13C-5. In all cases, the negative control and UC F6G-3 were bound to the plate while UC F13C-5 was used in cell suspensions and considered 'free'. Additional control wells contained cells cultured without antibody, with rIL-2 alone, or with rIL-2 and PHA-L. Cells were harvested at 48, 72, and 96 hr, and analysed by flow cytometry for interleukin-2 receptor (IL-2R) expression after incubation with IL-2/PE (R&D Systems, Minneapolis, MN).

Expression of UC F13C-5 by SIg^+ cells

PBML and node cell suspensions were cultured at 37° for 48 hr in DMEM/FBS, with and without phorbol 12-myristrate 13-acetate (PMA; 5, 10 or 20 ηg/ml, final concentration; Sigma).[17] Cells were labelled for two-colour flow cytometric analysis with UC F13C-5 (PE indirect) versus FITC–SIg.

RESULTS

Monoclonal antibodies

UC F6G-3 and UC F13C-5 reacted to large subpopulations of lymphocytes as determined by flow cytometry. These mAb were pursued due to the likelihood of their recognition of significant and diagnostically useful CD antigens. Both antibodies are of the IgG1 isotype.

Immunoprecipitation of target antigen

Immunoprecipitation techniques were applied to determine the molecular weight of the antigen recognized by

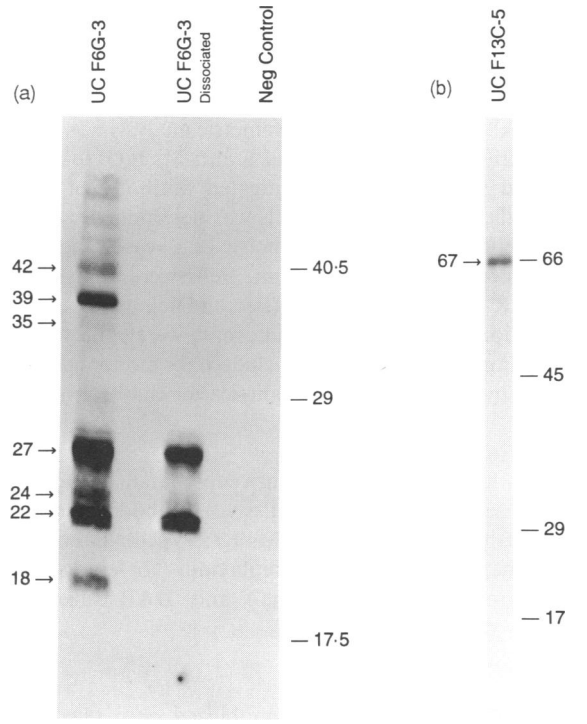

Figure 1. Film of proteins immunoprecipitated by UC F6G-3 (gel a, PHA-L cultured PBML) and UC F13C-5 (gel b, lymph node), separated by SDS–PAGE under reducing conditions (15 and 10% respectively), transferred to Immobilon P membrane and developed with chemiluminescence. Gel (a) also depicts the results of treating precipitated UC F6G-3 antigen with dissociation buffer prior to the addition of SAB, as well as the precipitation of cultured PBML lysate with irrelevant antibody. Molecular weight standards ($\times 10^3$) are indicated on the sides of each gel.

each mAb (Fig. 1). UC F6G-3 precipitated a complex of up to seven proteins, having M_r of *c.* 42,000, 39,000, 35,000, 27,000, 24,000, 22,000 and 18,000 (Fig. 1a). Multiple attempts at further dissociation of the complex consistently precipitated two bands; 27,000 and 22,000 MW (Fig. 1a).

UC F13C-5 precipitated a single protein with an apparent molecular weight of 67,000 (Fig. 1b). Precipitation of antigen from cultured cells often resulted in a second minor band with a M_r of *c.* 60,000 MW (data not shown).

Immunohistology

UC F6G-3 and UC F13C-5 stained cells in the thymus and T-cell domains of lymph nodes (Fig. 2). UC F6G-3 staining in the thymus was most intense in the medulla; the cortical staining was diffuse and generally less intense except for scattered individual cells and small clusters of intensely stained cells (Fig. 2a). UC F13C-5 staining in the thymus was of similar intensity in the cortex and the medulla; randomly scattered individual cells and small clusters of intensely stained cells occurred at equal frequency in both locations (Fig. 2b). UC F6G-3 and UC F13C-5 staining of lymph nodes was identical. Both antibodies stained cells in the paracortical domain and the apical light zone of follicular germinal centres (Fig. 2c).

Flow cytometric analysis

Relative percentages and calculated absolute cell counts (per μl) of PBML labelled with anti-equine SIg, UC F6G-3 and UC F13C-5 are presented in Table 1. UC F13C-5$^+$/SIg$^+$ cells represent a very small percentage of the B cells (1–5%) in the eight animals examined and demonstrate no evidence of age-related variation. The mean of absolute counts of PBML stained with all three markers are significantly different ($P < 0{\cdot}05$, Student's *t*-test) between animals under 1 year and those over 3 years of age; a trend for an increased T : B cell ratio in the older animals was also suggested.

Representative histogram profiles of each antibody as it reacts in peripheral blood and organized lymphoid tissues are provided in Fig. 3. Thymic profiles were dramatically different; UC F13C-5 labelled a large number of cells with a relatively uniform density of expression, whereas UC F6G-3 marked fewer cells distinguished by two distinct densities (31% low density and 12% high density). Cells in lymph nodes express higher densities of both UC F6G-3 and UC F13C-5 when compared to cells in the peripheral blood, while the density of SIg expression in lymph node cells is less than on PBML.

UC F6G-3 and UC F13C-5 produced profiles consistent with two distinct pan-T-cell markers as illustrated by the

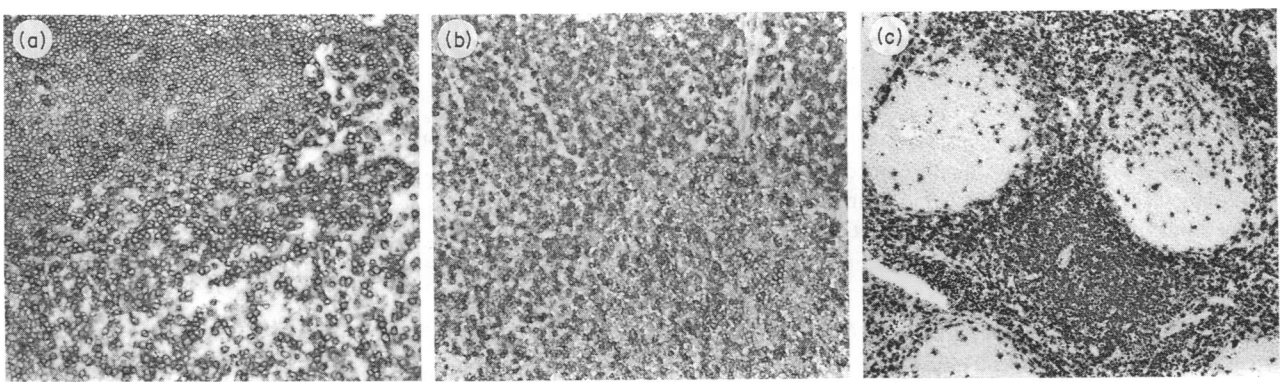

Figure 2. Immunoperoxidase staining of lymphoid organs: (a) UC F6G-3 staining of thymic cortex and medulla (magnification ×130); (b) UC F13C-5 staining of thymic cortex and medulla (magnification ×65); (c) UC F6G-3 staining of mesenteric lymph node (magnification ×65).

Table 1. PBML—% and absolute numbers of cells (per μl) reacting with UC F13C-5, UC F6G-3 and SIg as a function of age

Horse no.	Age (years)	UC F6G-3 %	UC F6G-3 no./μl	UC F13C-5 %	UC F13C-5 no./μl	SIg %	SIg no./μl	$F13C^+$ SIg^+ (%)
1	<1	59	2447	55	2281	28	1161	2
2	<1	45	3315	45	3315	53	3684	2
3	<1	58	3614	53	3360	42	2663	2
4	<1	73	4409	72	4349	30	1812	2
Mean ± SD		3446 ± 810		3326 ± 844		2280 ± 1012		
5	3–5	77	1664	73	1577	11	238	1
6	3–5	60	2761	72	2945	30	1380	2
7	3–5	71	1546	75	1633	15	566	5
8	3–5	72	1757	72	1757	13	317	2
Mean ± SD		1932 ± 559		1978 ± 649		625 ± 522		

percentage of cells stained (Table 2) and representative two-colour analysis with anti-equine SIg, CSV4 and CSV8. Dual staining of these two antibodies with anti-SIg revealed mutually exclusive populations in all tissues (data not shown). All $CSV4^+$ and $CSV8^+$ cells in secondary lymphoid tissues carried antigens recognized by both UC F6G-3 and UC F13C-5. Thymic profiles demonstrate that these markers identify thymocytes in different stages of maturation, with UC F13C-5, CVS4 and CVS8 identifying a population of UC F6G-3-negative cells (Fig. 4d, e and f). In contrast, all thymus-derived $CSV4^+$ and $CSV8^+$ cells expressed the UC F13C-5 target antigen (Fig. 4a and b). UC F6G-3-labelled cells in the thymus ranged from 40 to 58%, while UC F13C-5-labelled cells ranged from 60 to 90%; SIg^+ cells accounted for $\leqslant 5\%$ of all events (Table 2). Two-colour analysis on both PBML and mesenteric node revealed that a percentage of the $CD4^+$ cells carry a greater level of expression of the antigen recognized by UC F13C-5 than do $CD8^+$ cells (illustrated in PBML; Fig. 4c).

Monoclonal antibody-mediated lymphocyte activation

UC F6G-3 and UC F13C-5 were tested for their ability to activate T cells as measured by IL-2R expression (Fig. 5). While UC F13C-5 showed little to no ability to increase IL-2R expression (immobilized or free), immobilized F6G had a profound effect. The effect of unbound UC F6G-3 was not determined. Despite animal variability, trends in relative levels of IL-2R expression, when comparing various cell treatments,

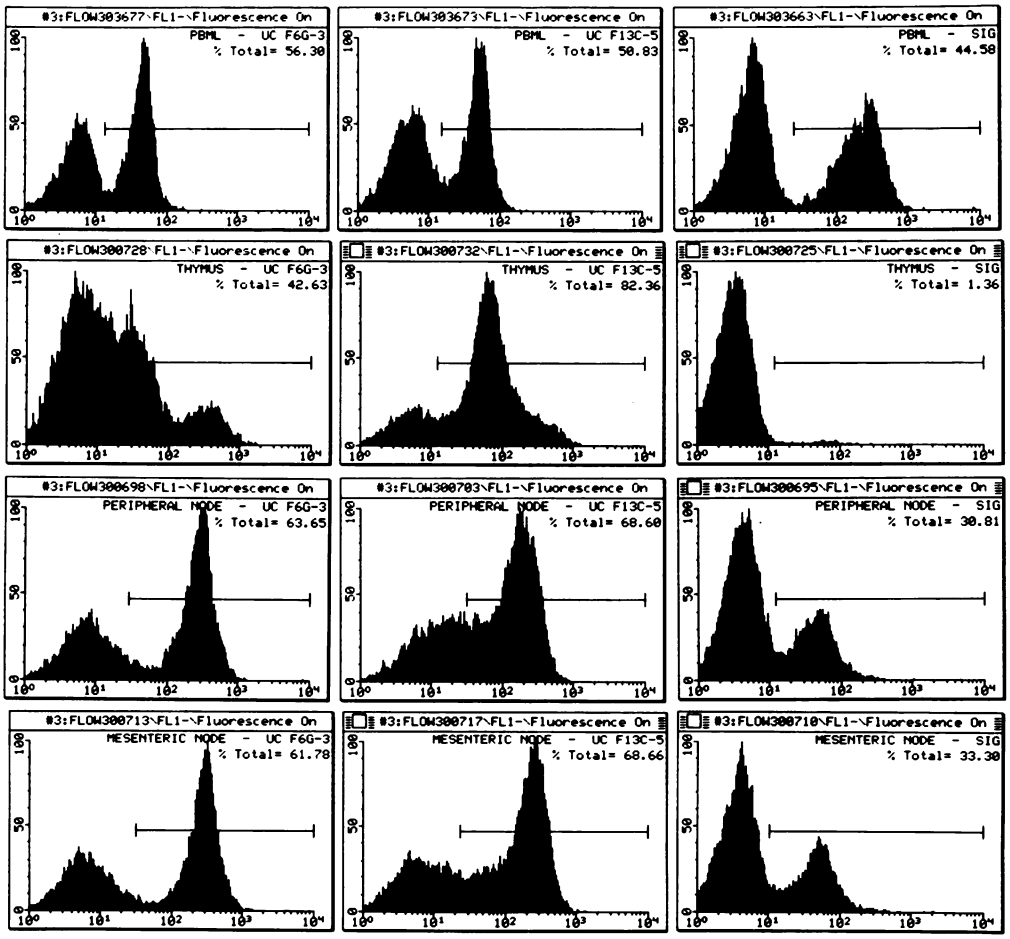

Figure 3. Representative histogram profiles of PBML, thymocytes, peripheral node and mesenteric node labelled with UC F6G-3, UC F13C-5 and anti-equine surface immunoglobulin as determined by flow cytometry.

Table 2. Percentage of lymphocytes derived from organized lymphoid tissues, expressing UC F6G-3, UC F13C-5 and SIg

Tissue	Ab	Foal no. 1 (%)	Foal no. 2 (%)	Foal no. 3 (%)	Foal no. 4 (%)	Foal no. 5. (%)
Axillary node	F6G-3	61	64	60	70	71
	F13C-5	52	47	70	72	69
	SIg	30	33	30	23	32
Mesenteric node	F6G-3	71	52	62	51	59
	F13C-5	69	53	71	53	55
	SIg	11	42	28	43	44
Thymus	F6G-3	58	53	41	53	40
	F13C-5	86	85	90	91	60
	SIg	5	2	1	1	5

were consistent. All animals demonstrated peak levels of expression at 72 hr incubation. Bound UC F6G-3 (Fig. 5E) promoted greater IL-2R expression than did IL-2 + PHA (Fig. 5D). Slight increases in expression were seen when either UC F13C-5 or IL-2 were added to bound UC F6G-3 preparations (Fig. 5F and G) in all but one animal.

Expression of UC F13C-5 on B lymphocytes

Lymphocytes from multiple lymphoid compartments were examined for the presence of SIg^+/UC F13C-5^+ cells before and after culture with PMA. Most cell cultures responded to PMA with slight to pronounced increased proliferation of SIg^+ cells and corresponding drops in the UC F13C-5^+ cells (data not shown). While most cultures (control and PMA treated) never exceeded 10% UC F13C-5^+/SIg^+, a twofold increase in double positives was observed in mesenteric and peripheral node-derived lymphocytes from one animal. The majority of B lymphocytes expressing UC F13C-5 did so at a low density relative to UC F13C-5^+ T lymphocytes.

DISCUSSION

The two antibodies described in this report recognize major T-lymphocyte populations. The monoclonal antibody, UC F6G-3, recognizes the apparent homologue of CD3 . Cells expressing UC F6G-3 and SIg were mutually exclusive and taken together represented the majority of lymphocytes in secondary lymphoid organs; this observation was consistent with immunohistological examination of lymph node wherein

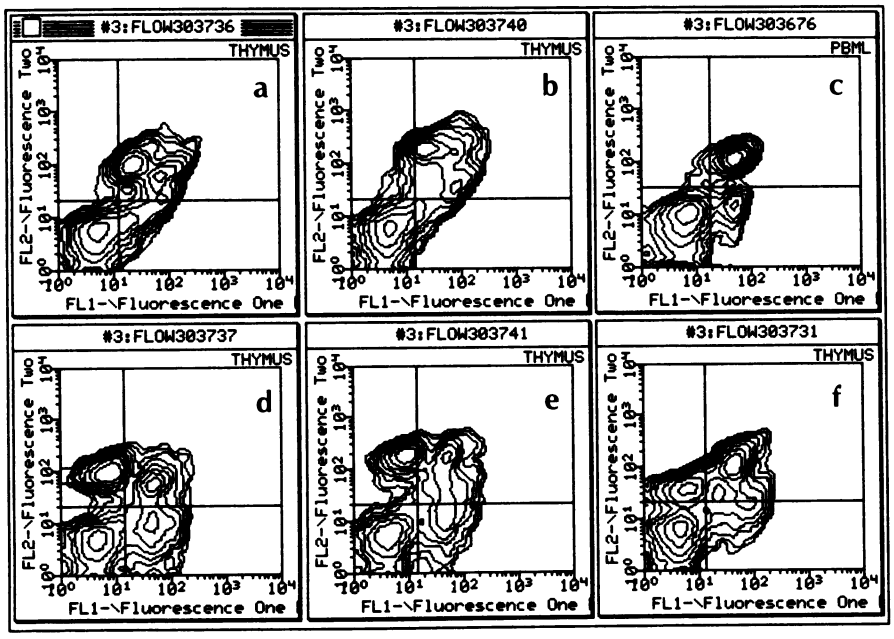

Figure 4. Representative two-colour flow cytometric analysis of UC F13C-5, UC F6G-3, CSV4 and CSV8 in thymus and PBML: (a) CSV4 (PE) versus UC F13C-5 (FITC); (b) CSV8 (PE) versus UC F13C-5 (FITC); (c) CSV4 (PE) versus UC F13C-5 (FITC) in PBML; (d) CSV4 (PE) versus UC F6G-3 (FITC); (e) CSV8 (PE) versus UC F6G-3 (FITC); (f) UC F13C-5 (PE) versus UC F6G-3 (FITC). FL1, FITC; FL2, PE.

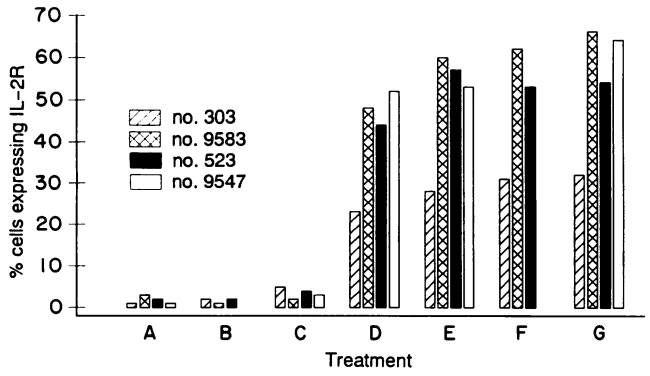

Figure 5. Comparison of the percentage of cells expressing IL-2R after cell cultures were incubated for 72 hr at 37°. Presented are the reactions of four different horses to the following treatments: (A) negative control (DMEM/FBS only); (B) free UC F13C-5; (C) IL-2; (D) IL-2 + PHA-L; (E) bound UC F6G-3; (F) bound UC F6G-3 + UC F13C-5; (G) bound UC F6G-3 + IL-2.

UC F6G-3 stained cells lying within the T-cell zone (paracortical region) while staining only an occasional lymphocyte within the B-cell domain (follicle) (Fig. 2c). Additional evidence supporting recognition of a pan-T-cell antigen is that all CD4- and CD8-expressing lymphocytes derived from secondary lymphoid tissue expressed the UC F6G-3 target antigen. In contrast, a population of UC F6G-3 negative thymocytes expressing CD4 and CD8 were observed (Fig. 4d and e), suggesting that cell-surface TcR-CD3 is expressed subsequent to CD4 and CD8. Cell-surface density of the UC F6G-3 target antigen on thymocytes revealed three distinct T-lymphocyte populations (Fig. 3). Based upon previous studies of CD3 density on human and murine thymocytes,[4,5,7,18] we predict that density increases reflect advancing stages of T-cell maturation. Comparative analysis of target antigen density (UC F6G-3, UC F13C-5 and SIg) on lymphocytes from secondary lymphoid compartments was also of interest in that variation existed between peripheral blood- and lymph node-derived lymphocytes (Fig. 3). Such observations suggest that peripheral blood lymphocytes are not representative of the lymphocyte pool that resides in organized lymphoid tissues, a feature that requires consideration when using peripheral blood-derived lymphocytes as a window for phenotypic and/or functional characterization of an animal's adaptive immune system.

Precipitation of the UC F6G-3 target antigen revealed a minimum of seven proteins with estimated molecular weights ranging from 18,000 to 42,000 (Fig. 1) which is consistent with that previously described for human[4,6,18–20] and murine[5,7,21,22] antibodies specific for the CD3 complex. This complex typically yields the α and β chains of the TcR in addition to four or five proteins that compose the CD3 complex (γ, δ, ε, ξ and sometimes η).[23] With the exception of the ξ chain, the CD3 complex-associated proteins vary considerably in molecular weight between human and murine species. While we speculate that the lowest molecular weight protein (18,000) precipitated by UC F6G-3 represents the ξ chain, unequivocal designation of the equine CD3-associated protein subunits must await further biochemical and genetic characterization. The non-covalent association of the CD3 complex with the TcR permits dissociation of the complete immunoprecipitate with ionic detergents.[5,7,20] Such treatment of the cell-surface complex precipitated by UC F6G-3 yielded two proteins with estimated molecular weights of 22,000 and 27,000 (Fig. 1a). Previous studies utilizing similar detergent conditions to characterize murine and human CD3 ε chain-specific mAb often resulted in the precipitation of two distinct proteins with molecular weights in the range of 20,000 and 28,000.[5,24] This information, taken together with the apparent immunodominant nature of the ε chain relative to the other CD3-associated proteins, favours the specificity of UC F6G-3 for the ε subunit.[5,25]

The primary function of the TcR-associated CD3 complex appears to be that of signal transduction following engagement of the receptor with antigen. Both the δ and ε subunits have phosphorylation sites[21,22] and mAb specific for these proteins can induce T-lymphocyte activation, IL-2 production and proliferation.[4–7,16] Immobilized UC F6G-3 induced substantial T-lymphocyte activation as determined by IL-2R expression (Fig. 5). Previous studies have demonstrated an augmentation of anti-CD3 ε-induced T-lymphocyte (pure cultures of T cells) activation by addition of IL-2 or antibody specific for CD5;[7,16,26,27] augmentation of UC F6G-3-induced lymphocyte activation with either protein was minimal (Fig. 5F and G). Because the mononuclear cells used in our assay were not pure populations of T lymphocytes, we speculate that sufficient secondary or accessory activation signals were provided by the non-T-contaminating cells to obtain maximum stimulation.

Two pan-T-lymphocyte-specific mAb with apparent specificities for equine CD5 have previously been described.[2,3] UC F13C-5 also recognizes the apparent homologue of CD5 with the molecular weight of the target antigen (67,000) being consistent with previous estimations.[2,3] UC F13C-5 primarily recognizes a target antigen expressed on T lymphocytes, as few double positives (UC F13C-5$^+$/SIg$^+$) were identified (Table 1). Immunohistology also supported a pan-T designation as the majority of lymphocytes in the paracortex stained while only a few cells stained within the follicles. While flow cytometric and immunohistologic data generated from secondary lymphoid tissues were almost identical for UC F13C-5 and UC F6G-3, two-colour flow cytometry in the thymus demonstrated both single and double positives (Fig. 4f). Furthermore, unlike UC F6G-3, UC F13C-5 target antigen was expressed at a relatively constant density throughout the thymus (Fig. 3), stained a greater percentage of thymocytes (Fig. 3) and stained most of the CD4- and CD8-expressing thymocytes (Fig. 4a and b). Thus it would appear that the target antigen of UC F13C-5 is expressed earlier during T-lymphocyte maturation, and at a constant cell-surface density, as compared to that of UC F6G-3.

Expression of CD5 on one or more subsets of murine and human B lymphocytes has previously been described and its possible immunoregulatory role recently reviewed.[28] Previous studies have reported minimal expression of CD5 on peripheral blood-derived B lymphocytes.[2,3] Few CD5$^+$ B lymphocytes were identified in this study, regardless of tissue origin or animal age (Fig. 3; Table 1); a profile more consistent with murine species than human (reviewed in ref. 29). Previous studies have demonstrated phorbol ester-induced induction of CD5 on normal resting B lymphocytes[17] although their relationship to CD5-expressing B cells *in vivo* is unknown.

PMA induced minimal to extensive expression of CD5 on equine B lymphocytes; the underlying basis of this animal variability is unknown. Variation of lymphocyte phenotype within a given sample may influence the outcome, as IL-4 has been demonstrated to inhibit B-cell activation-associated CD5 expression.[30]

Establishment of the mAb specific for equine CD antigens described in this report, and others (equine CD4, CD5 and CD8),[2,3] will facilitate multiple immunological studies in this species. Development and characterization of UC F6G-3, with an apparent specificity for CD3, is especially important as the only pan-T marker to date has been for CD5, the latter being a CD antigen variably expressed on B lymphocytes. Application of the putative markers for CD3 and CD5 have demonstrated animal age to be an important variable (Table 1), emphasizing the need for establishment of baseline lymphocyte subpopulation values.

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