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Report of the Second Equine Leucocyte Antigen Workshop, Squaw Valley, California, July 1995

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Abbreviations: BSA, bovine serum albumen; CD, human cluster of differentiation antigen; CTL, cytotoxic T lymphocyte; EDTA, ethylene diamine tetracetate; ELAW I, First Equine Leucocyte Antigen Workshop; ELAW II, Second Equine Leucocyte Antigen Workshop; EqCD, equine orthologue of a human cluster of differentiation antigen; FACS, fluorescence activated cell staining; FCS, fetal calf serum; Ig, immunoglobulin; kDa, kilodalton; LAK, lymphokine-activated killer; mAb, monoclonal antibody; MHC, major histocompatibility complex; PHA, phytohemagglutinin; NaN_3, sodium azide; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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1. Introduction

The Second International Workshop on Equine Leucocyte Antigens (ELAW II) was held from July 15 to 16, 1995 in Squaw Valley, CA. One hundred and thirteen monoclonal antibody (mAb) reagents to leucocyte antigens and immunoglobulins were evaluated by investigators from eight laboratories. ELAW II extended characterization of 48 previously reported anti-leucocyte reagents and introduced 29 new anti-leucocyte reagents and 34 anti-immunoglobulin reagents. The clusters recognized by ELAW II are EqCD2 (formerly EqWC3), EqCD3, EqCD5, EqCD8, EqCD11a/18, EqCD13, EqCD44, EqMC I, EqMHC II, EqWC1, EqWC2 and EqWC4. In addition ELAW II evaluated mAbs to equine immunoglobulin isotypes and sub-isotypes and identified antibodies recognizing IgGa, IgGb, IgGc, IgG(T), IgA, IgM and light chain.

1.1. Objectives of the workshop

The aim of ELAW II was to continue the studies of equine leucocyte cell surface antigens undertaken by the First International Workshop on Equine Leucocyte Antigens (ELAW I), which was held in July 1991 in Cambridge, UK (Kydd et al., 1994). In addition ELAW II included studies of mAbs recognizing equine immunoglobulins. Eight laboratories from North America, Europe and Asia participated actively in ELAW II and additional investigators in the field of equine immunology were invited as observers to the final ELAW II meeting in order to promote further communication and collaboration in this field.

The three principle objectives of the ELAW II workshop were: (1) to determine the specificity of individual mAbs and to assign them to cluster designations when possible; (2) to achieve consensus on the rules for naming equine leucocyte and immunoglobulin molecules; (3) to promote collaboration among equine investigators studying equine immunity and to support their efforts through definition and provision of well-characterized reagents.

1.2. Nomenclature

The nomenclature used for naming equine leucocyte surface molecules follows that adopted by ELAW I (Kydd et al., 1994). The criteria used for assignment of specificities by ELAW II used a combination of the tissue distribution of the antigen together with functional data in some instances, and either the molecular weight or the genetic sequence of the antigenic molecule. Studies of the homology of equine leucocyte antigens with the antigens of other species would be furthered by the availability the genetic sequence of the equine antigens, but currently this is available only for the EqCD2, EqCD44, EqMHC I and EqMHC II molecules (Tavernor et al., 1993, 1994; Barbis et al., 1994; Szalai et al., 1994a,b).

1.3. Participating laboratories and organization of the workshop

A total of 10 laboratories submitted antibodies to ELAW II (Table 1) although only eight laboratories conducted an analysis of the panel. The work of ELAW II began in

Table 1
ELAW II participants

Participants	Laboratory	Laboratory code
Christopher Stokes, Philip R. Widders	School of Veterinary Science, Langford House, Langford, Bristol, UK	BVS
Jefferey L. Stott, Peter F. Moore, Myra Blanchard-Channell	School of Veterinary Medicine, Agricultural Experiment Station, Department of Veterinary Microbiology and Immunology, University of California, Davis, CA 95616-8738, USA	CAL
D.F. Antczak, Jessica Baker, Karen Cannizzo	James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA	COR
Mark A. Holmes, Linda Butler, Neeraj Agerwal, Abhineet Sheoran	Department of Clinical Veterinary Medicine, Cambridge University, Madingley Road, Cambridge, CB3 OES, UK	CVS
D. Paul Lunn, Brian R. Schram, Karen E. Vagnoni	School of Veterinary Medicine, University of Wisconsin, 2015 Linden Drive West Madison, WI 53706, USA	CVS
Cristopher K. Grant	Custom Monoclonals, 813 Harbor Blvd., West Sacramento, CA 95691, USA	GRA
Takeo Sugiura, Takashi Kondo	Equine Research Institute, Epizootic Research Station (Tochigi Branch), 1400-4, Shiba, Kokubunji-machi, Shimotsuga-gun, Tochigi 329-04, Japan	JRA
Smain Bendali-Ahcene, J.C. Monier	Laboratoire D'Immunologie, Universite Claude Bernard, U.E.R.-Faculte de Medecine Alexis Carrel, Rue Guillaume Paradin, 69372 Lyon Cedex 08, France	LYO
Julia H. Kydd, Duncan Hannant, Terry O'Neil	Animal Health Trust, P.O. Box 5, Newmarket, Suffolk CB8 7DW, UK	MAC
William Davis, Katherine M. Byrne, Mary Jo Hamilton	Departments of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040, USA	WSU

Table 2
Antibodies submitted to ELAW II

Workshop number	Source of mAb	Name of mAb	First (1991) workshop number
1	CVS	neg ctrl	
2	GRA	IgA9-6A	
3	GRA	CAG8-7C	
4	CVS	CVS3	
5	CVS	CVS44	
6	JRA	F12-6C10	
7	CVS	CVS48	
8	CVS	CVS36	
9	CVS	CVS43	
10	CVS	CVS37	
11	CVS	CVS38	
12	GRA	CM6E	
13	CVS	CVS1	41
14	GRA	IgA5-3A	
15	BVS	BVS2	
16	CVS	CVS47	
17	CVS	CVS41	
18	CVS	CVS2	
19	BVS	BVS1	
20	JRA	F14-10G12	
21	JRA	F6-5G2	
22	CVS	CVS49	
23	GRA	CM7	
24	CVS	CVS42	
25	CVS	CVS50	
26	JRA	F13-6D6	
27	CVS	CVS46	
28	GRA	FIG1-7A	
29	CVS	CVS45	
30	CVS	CVS40	
31	JRA	F13-7G12	
32	CVS	CVS51	
33	CVS	CVS39	
34	WSU	E47B	23
35	COR	CZ1.2	47
36	COR	CZ1.7	37
37	CVS	CVS22	
38	CVS	CVS4 dupl.[a]	41
39	LYO	1A8E9	
40	COR	CZ5.1	35
41	WSU	E18A	22
42	COR	CZ4.6	57
43	COR	CZ11	48
44	LYO	13B11F10	
45	COR	CZ3.1	45
46	CVS	CVS4	1
47	WSU	HB7A	
48	LYO	4H11G10	
49	CVS	CVS13	76

Table 2 (continued)

Workshop number	Source of mAb	Name of mAb	First (1991) workshop number
50	COR	CZ2.2	36
51	CAL	F13C.3(D9)	
52	COR	CZ4.1	40
53	LYO	13G4B8	
54	CVS	CVS19	32
55	LYO	7H3H11	
56	CVS	CVS7	62
57	COR	CZ6	54
58	COR	CZ1.4	63
59	WSU	DH16A	73
60	WSU	PG173A	
61	COR	CZ1.1	6
62	COR	CZ5.5	50
63	COR	CZ5.15	69
64	WSU	HT8B	64
65	COR	CZ1.6	14
66	CVS	CVS35	
67	CVS	CVS23	
68	CVS	CVS6	55
69	WSU	HB84A	52
70	JRA	LB5C7	
71	JRA	TH2A10	
72	CVS	CVS18	61
73	COR	CZ2.1	65
74	CAL	F13A.3(B8)	
75	LYO	12F10E11	
76	WSU	PT25A	
77	COR	CZ3.2	8
78	WSU	HT6A	70
79	JRA	LB1D8	
80	JRA	LB3C3	
81	CVS	CVS5	67
82	JRA	TH1G4	
83	LYO	5B9C2	
84	CVS	CVS8	49
85	COR	CZ5.2	58
86	CVS	CVS24	
87	WSU	BAQ150A	
88	JRA	LB3F3	
89	CVS	CVS11	30
90	COR	CZ1.5	26
91	COR	CZ4.7	27
92	CVS	CVS21	
93	COR	CZ4.4	24
94	CAL	F18E.2	
95	CAL	F6B.3	
96	COR	CZ1.3	44
97	CAL	F18P.2	
98	CAL	F6G.3(G12)	
99	CVS	CVS20	

Table 2 (continued)

Workshop number	Source of mAb	Name of mAb	First (1991) workshop number
100	COR	CZ4.3	42
101	CAL	F18H.2	
102	COR	CZ3.3	33
103	MAC	MAC288	79
104	MAC	MAC292	81
105	MAC	MAC291	84
106	MAC	MAC290	85
107	MAC	MAC293	86
108	CVS	CVS53	
109	CVS	CVS52	
110	WSU	IFA6	7
111	WSU	HB88a	78
112	WSU	HB86a	21
113	WSU	HB65a	29

[a]A duplicate of antibody Workshop number 38 (CVS4) was included for the purposes of analysis.

the summer of 1994 with the distribution of a panel of 113 mAbs to the eight participating laboratories. The panel included a negative control and a duplicate control antibody, 77 anti-leucocyte antibodies and 34 anti-immunoglobulin antibodies. A list of these reagents, their submitting laboratory, ELAW II workshop number, and when appropriate their workshop number from ELAW I is presented in Table 2. In December of 1994 an initial clustering of this antibody panel was performed on the basis of results submitted by participating laboratories of reactivity of antibodies with leucocyte subpopulations and cell lines as determined by flow cytometry. In addition the anti-immunoglobulin reagents were partially characterized in terms of their reactivity with purified equine immunoglobulin isotypes by two laboratories. The results of these initial analyses were immediately distributed to all participating laboratories. Results of further studies of the reagents were collated in June 1995 and antibodies were assigned to provisional clusters (Table 3) prior to final assignment of specificities at the ELAW II meeting in July 1995, at Squaw Valley, CA.

2. Methods and assays

2.1. Flow cytometry

Equine leucocyte populations were isolated from the peripheral blood of adult horses using either erythrocyte lysis or density-gradient centrifugation using Ficoll-Hypaque (Carter, 1990). Single and two color flow cytometric analyses (FACS) using the ELAW II panel antibodies were performed as described in ELAW I (Kydd et al., 1994). For single color analyses, cells were suspended in 'labeling buffer' (typically PBS contain-

Table 3
Results of initial antibody clustering

Specificity	Workshop number	Species/isotype
Leucocyte reagents		
EqCD2 (EqWC3)	103	rat/G2a
	110	mur/G1
	111	mur/G1
EqCD3	98	mur/G1
EqCD4	38	mur/G1
	46	mur/G1
EqCD5	51	mur/G1
	71	mur/G2b
	81	mur/G1
EqCD8	84	mur/G1
	92	mur/G2a
	97	mur/G2a
	101	mur/G1
EqCD11a/18	45	mur/G1
	65	mur/G1
	77	mur/G2a
EqCD13	54	mur/G1
EqCD44	53	mur/G1
	63	mur/G1
	72	mur/G1
	75	mur/G1
EqMHC I	37	mur/G1
	57	mur/G1
	105	rat/G2b
EqMHC II	43	mur/G1
	74	mur/G1
	99	mur/G1
EqWC1	49	mur/G1
	61	mur/G1
	80	mur/G1
	89	mur/G1
EqWC2	35	mur/G1
	36	mur/G1
	58	mur/M
	59	mur/M
	68	mur/G1
	90	mur/G1
	96	mur/G1
EqWC4	112	mur/G1
	113	mur/G2a
B cell markers	67	mur/G1
	73	mur/G1
	104	rat/M
Macrophage markers	50	mur/G1
	102	mur/M
Individual antibodies	34	mur/M
	41	mur/G2a
	44	mur/M
	47	mur/G2a

Table 3 (continued)

Specificity	Workshop number	Species/isotype
	48	mur/M
	56	mur/G1
	60	mur/M
	62	mur/M
	64	mur/M
	66	mur/
	69	mur/G1
	76	mur/G3
	78	mur/G3
	79	mur/G2b
	82	mur/M
	83	mur/M
	85	mur/G1
	86	mur/G1
	87	mur/G3
	88	mur/M
	91	mur/G1
	94	mur/G2b
	95	mur/G1
	100	mur/G1
	106	rat/G2a
	107	rat/M
Immunoglobulin reagents		
IgGa	7	mur/G1
	16	mur/G1
	22	mur/G2a
	24	mur/G1
	27	mur/G1
	29	mur/G1
IgGb	4	mur/M
	5	mur/G2a
	6	mur/G2a
	13	mur/G2b
	18	mur/G1
	25	mur/G1
	33	mur/G1
IgGc	20	mur/G1
	108	mur/G1
	109	mur/G1
IgG(T)	11	mur/G1
	21	mur/G1
	30	mur/G1
	32	mur/G1
IgA	15	mur/
	19	mur/
IgM	10	mur/G1
	12	mur/G2a
	23	mur/G1
	26	mur/G1
	31	mur/G1

Table 3 (continued)

Specificity	Workshop number	Species/isotype
Pan-IgG	17	mur/G2b
Pan-Ig	8	mur/G1
Negative antibodies		
	1	mur/G2a
	2	mur/G1
	3	mur/G1
	9	mur/G1
	14	mur/G2b
	28	mur/G1
	39	mur/M
	40	mur/G1
	42	mur/G1
	52	mur/G1
	55	mur/M
	70	mur/M
	93	mur/M

ing 0.5% BSA plus 0.1% NaN_3) and incubated with the workshop antibodies as first stage reagents followed by commercial fluoresceinated second stage antibodies to murine or rat Ig. All incubations were performed at 4°C for periods exceeding 30 min and cells were washed three times between each incubation step. For two color analyses, the single color staining protocol was followed, and subsequently cells were incubated with 1.5% murine serum, followed by a biotinylated monoclonal antibody recognizing either EqCD5 (CVS5: WS81) or EqCD8 (CVS8: WS84) (Lunn et al., 1991) and finally streptavidin-conjugated phycoerythrin. Stained cells were either analyzed fresh or fixed by incubation in buffer containing 2% paraformaldehyde. Flow cytometric analyses were performed using a FACScan analyzer (Becton-Dickinson, San Jose, CA), or a Coulter-Epics I or II flow cytometer (Coulter, Hieleah, FL). Lymphocytes and granulocytes were differentiated during FACS analysis using forward and orthogonal light scatter properties (Sharpe, 1988).

Additional single color FACS analyses of fetal kidney cells, thymocytes and an equine lymphoblastoid line EqT8888 (Hormanski et al., 1992) were performed with the entire ELAW II panel. Finally FACS analyses of COS cells transfected with recombinant equine CD2 (Tavernor et al., 1994) and stained with EqCD2 antibodies was also performed.

2.2. Immunohistochemistry

Conventional immunohistological techniques were used to study the distribution of antigens identified by the workshop antibodies on a variety of frozen tissues using conventional techniques (Lunn et al., 1991). Tissues examined included lymph nodes, spleen and thymus.

2.3. Immunoprecipitation

The molecular weights of antigens identified by workshop antibodies were determined by immunoprecipitation using mAbs as supernatants and anti-murine antisera linked to agarose beads. Immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography of radioidonated cell surface proteins (Lunn et al., 1991) or chemiluminescent detection of biotinylated cell surface proteins (Blanchard-Channell et al., 1994). In some instances precipitated antigens which were heavily glycosylated were treated with endoglycosidase F in order to cleave glycosyl residues. This was performed by completing the immunoprecipitation in the normal manner and then treating a 14 μl aliquot of the agarose beads with the attached precipitated antigen with 2 μl of 10% 2-mercaptoethanol and 4 μl of 0.1% SDS. This preparation was boiled for 1 min, and then placed on ice prior to addition of 10 μl (0.5 U) of endoglycosidase F (Boehringer Mannheim) and incubation at 37° for 14 h. The preparation was then subjected to SDS-PAGE analysis in the normal manner. The molecular weights of equine immunoglobulins identified by antibodies was also determined by SDS-PAGE of affinity purified proteins and Western blotting of whole serum as described in Section 4.2.

2.4. ELISA

Reactivity of anti-immunoglobulin reagents was tested using ELISAs with purified equine immunoglobulin isotypes. The pure equine immunoglobulin isotypes were prepared both by separation based on physicochemical properties (i.e., gel filtration, ion exchange chromatography, staphylococcal protein affinity and electrophoresis) and by monoclonal antibody affinity chromatography. The mAbs were tested against IgGa, IgGb, IgGc, IgG(T), IgA and IgM. A detailed description of all immunoglobulin analyses is given below in Section 4.2.

2.5. Functional studies

Individual workshop participants have previously performed functional studies of ELAW II antibodies in cytotoxic T lymphocyte (CTL) assays and in lymphokine-activated killer (LAK) assays and these are cited where appropriate. Functional studies conducted during ELAW II were limited to the examination of the effect of including mAbs with mitogen-treated lymphocytes in culture and observing subsequent changes to the uptake of tritiated thymidine in comparison to PBS and monoclonal antibody negative controls. For these assays mAbs as supernatants were dialyzed into RPMI (Flow Labs., Hemel Hempstead, Herts.) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 u/ml penicillin, 50 μg/ml streptomycin and 0.001 mM 2-mercaptoethanol. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation as described above and incubated with increasing amounts of mAbs in replicates of three wells of a 96 well U bottomed microtitre plate. Phytohaemagglutinin (PHA, Sigma, Poole, Dorset) was added at a predetermined sub-optimal concentration of 1 mg/ml. Plates were incubated at 37°C in 5% CO_2 for 48 h. Each well was pulsed with 0.5 μCi methyl ^{3}H thymidine (Amersham International, Little Chalfont, Bucks.)

for 18 h then harvested onto filter paper and β emissions counted. Controls included addition of either dialysed medium or 0.1% sodium azide in medium or EqT6 (anti-equine thymocyte mouse ascites, VMRD, Pullman, WA) at 2.5 or 5 μg/ml in medium. EqT6 was chosen as control reagent due to its commercial availability. The two concentrations of ascites were chosen to discount the possible immunosuppressive effects of excess protein and to reflect the estimated specific antibody concentration present in tissue culture supernatants. Additional control mAbs MAC288 (WS103) (anti-EqCD2) and MAC291 (anti-equine MHC class I) were included in both assays and gave identical results. Two assays were performed, using PBMC from two Welsh Mountain pony mares, with the full range of control reagents in each. In both assays, addition of increasing volumes of the control reagents resulted in minor inhibition of PHA-induced lymphoproliferation. A subset of the ELAW II panel of mAbs were tested, including: WS51, WS71, WS81 (EqCD5); WS92, WS97, WS101 (EqCD8); WS49, WS61, WS80, WS89 (EqWC1); WS35, WS59, WS68, WS90, WS96 (EqWC2); WS63, WS72 (EqCD44); WS103 (EqCD2); WS37, WS57 (EqMHC class I); WS38 (EqCD4); WS45, WS77, WS95 (EqCD11a/18).

2.6. Statistics and clustering

Statistical analysis was performed to provide an initial guide to the clusters of antibodies likely to be identifying the same antigen or cell populations. This analysis was performed only on FACS results from mAbs submitted as anti-leucocyte reagents using the blind coded panel. A preliminary cluster analysis was applied to the initial data using software written by Dr. Steve Cobbold for the Canine Leukocyte Antigen Workshop (Cobbold and Metcalfe, 1994). The results were coded as a value between 0 and 100% together with a letter to indicate the contributors' interpretation of that value: N to indicate negative, A to indicate an accurate positive, and C to indicate a complex result. The software uses the contributors' confidence indications to weight certain results during the analysis using the LTDB3 workshop method (Spiegelhalter and Gilks, 1987) of calculating distances and results in the production of a dendrogram showing the distance between each antibody and its closest matching antibody or cluster. A final statistical analysis was performed just before the workshop meeting using data collected from the preliminary analysis together with all data submitted subsequently. This analysis was performed on complete target cell datasets and those that fell outside the consensus grouping were excluded. These exclusions were performed in an attempt to reduce the bias caused by multiple submissions of peripheral blood data and the noise resulting from the inclusion of data sets with high backgrounds.

3. Results of leucocyte antigen cluster

The results of the provisional clustering of antibodies performed prior to the ELAW II meeting are summarized in Table 3, which also describes the isotype and species of origin of the specific antibodies as provided by the submitting laboratory. These clusters are discussed in detail below and the results of final clustering are presented in Table 4,

Table 4
Summary of results of ELAW I and ELAW II

Specificity	ELAW II workshop number	First[a] workshop number	Name of mAb	Lab. of origin	Species/ isotype[b]	Molecular weight[c] (kDa)
Leucocyte reagents						
EqCD2 (EqWC3)	103	79	MAC288	MAC	rat/G2a	Tf+
	110	7	IFA6	WSU	mur/G1	Tf+
	111	78	HB88a	WSU	mur/G1	Tf+
EqCD3	98		F6G.3(G12)	CAL	mur/G1	20
EqCD4	38 and 46	41	CVS4	CVS	mur/G1	58
		72	HB61A	WSU	mur/G1	56
EqCD5	51		F13C.3(D9)	CAL	mur/G1	69
	71		TH2A10	JAP	mur/G2b	69
	81	67	CVS5	CVS	mur/G1	69
		3	EqT3	WSU	mur/G1	65–69
		28	HB80A	WSU	mur/G1	69
		39	HB19A	WSU	mur/G1	69
		43	RVC1	RVC[d]	mur/n.d.	67
		53	HT23A	WSU	mur/G1	69
EqCD8	84	49	CVS8	CVS	mur/G1	32,39
	92		CVS21	CVS	mur/G2a	32,39
	97		F18P.2	CAL	mur/G2a	32,39
	101		F18H.2	CAL	mur/G1	32,39
		12	HT14A	WSU	mur/G1	32,39
		74	HB20A	WSU	mur/G1	30–35
EqCD11a/18	45	45	CZ3.1	COR	mur/G1	100,180
	77	8	CZ3.2	COR	mur/G2a	100,180
		18	CVS9	CVS	mur/G1	100,180
		38	H20A	WSU	mur/G1	100,180
EqCD13	54	32	CVS19	CVS	mur/G1	140–150
EqCD44	63	69	CZ5.15	COR	mur/G1	Tf+
	72	61	CVS18	CVS	mur/G1	76 (100) Tf+
		15	BAT31A	WSU	mur/G1	Tf+
EqMHC I	37		CVS22	CVS	mur/G1	45,12
	57	54	CZ6	COR	mur/G1	44
	105	84	MAC291	MAC	rat/G2b	45,12
EqMHC II	43	48	CZ11	COR	mur/G1	34
	74		F13A.3(B8)	CAL	mur/G1	33
	99		CVS20	CVS	mur/G1	34
		71	CVS10	CVS	mur/G1	34
EqWC1	89	30	CVS11	CVS	mur/G1	22
		11	CVS14	CVS	mur/M	22
		31	CVS12	CVS	mur/G2A	22
		34	HB32A	WSU	mur/n.d.	22
EqWC2	68	55	CVS6	CVS	mur/G1	172
	90	26	CZ1.5	COR	mur/G1	180
	96	44	CZ1.3	COR	mur/G1	160
		82	MAC289	MAC	rat/G2a	145
EqWC4	112	21	HB86a	WSU	mur/G1	46
	113	29	HB65a	WSU	mur/G2a	46
B cells	73	65	CZ2.1	COR	mur/G1	85
	104	81	MAC292	MAC	rat/M	240

Table 4 (continued)

Specificity	ELAW II workshop number	First[a] workshop number	Name of mAb	Lab. of origin	Species/ isotype[b]	Molecular weight[c] (kDa)
Macrophages	50	36	CZ2.2	COR	mur/G1	unknown
	102	33	CZ3.3	COR	mur/M	unknown
Immunoglobulin reagents						
IgGa	7		CVS48	CVS	mur/G1	
	16		CVS47	CVS	mur/G1	
	22		CVS49	CVS	mur/G2a	
	24		CVS42	CVS	mur/G1	
	27		CVS46	CVS	mur/G1	
	29		CVS45	CVS	mur/G1	
IgGb	4		CVS3	CVS	mur/M	
	5		CVS44	CVS	mur/G2a	
	6		F12-6C10	JRA	mur/G2a	
	13	41	CVS1	CVS	mur/G2b	
	18		CVS2	CVS	mur/G1	
	25		CVS50	CVS	mur/G1	
	33		CVS39	CVS	mur/G1	
IgGc	20		F14-10G12	JRA	mur/G1	
	108		CVS53	CVS	mur/G1	
	109		CVS52	CVS	mur/G1	
IgG(T)	11		CVS38	CVS	mur/G1	
	21		F6-5G2	JRA	mur/G1	
	30		CVS40	CVS	mur/G1	
	32		CVS51	CVS	mur/G1	
IgA	15		BVS2	BVS	mur/n.d.	
	19		BVS1	BVS	mur/n.d.	
IgM	10		CVS37	CVS	mur/G1	
	12		CM6E	GRA	mur/G2a	
	23		CM7	GRA	mur/G1	
	26		F13-6D6	JRA	mur/G1	
	31		F13-7G12	JRA	mur/G1	
Pan-IgG	17		CVS41	CVS	mur/G2b	
Pan-Ig	8		CVS36	CVS	mur/G1	

[a] Kydd et al. (1994).
[b] Species of origin of antibodies: mur, murine.
[c] Molecular weights as determined by SDS-PAGE analysis under reducing conditions. In some instances specificity was determined by recognition of transfected COS cell lines: Tf+.
[d] RVC: originated from Royal Veterinary College, Royal Street, Camden, London, NW 10TY, UK.

which also lists antibodies with firm cluster assignments from ELAW I which were not re-submitted to ELAW II.

3.1. EqCD2 (EqWC3)

Three mAbs recognize the equine antigen previously defined as EqWC3: WS103, WS110, and WS111. This antigen is an orthologue of the human CD2 T cell marker on

Table 5
Flow cytometric analysis of peripheral blood leucocyte populations, thymocytes, the EqT8888 cell line and fetal kidney cells labeled with mAbs listed in provisional clusters

mAb	Percentage of positive (labeled) cells[a]										
	PBL			Granulocytes			Thymocytes			EqT8888	Fetal kidney
	Mean	Range	*n*	Mean	Range	*n*	Mean	Range	*n*		
EqCD2											
WS103	67	29–100	13	2	0–7	5	60	53–72	4	1	1
WS110	85	82–88	2	n.d.			n.d.			n.d.	n.d.
WS111	88	85–91	2	n.d.			n.d.			n.d.	n.d.
EqCD3											
WS98	75	54–92	13	2	0–7	5	79	58–93	4	3	5
EqCD4											
WS38	57	42–70	13	4	0–14	5	67	51–80	4	5	1
WS46[b]	58	41–71	13	5	0–17	5	70	54–83	4	7	1
EqCD5											
WS51	77	55–88	13	6	0–19	5	92	90–94	4	0	1
WS71	79	53–93	13	7	0–28	5	94	92–96	4	16	1
WS81	77	48–92	13	11	0–34	5	93	92–97	4	14	1
EqCD8											
WS 84	24	9–56	13	4	0–16	4	78	69–96	4	0	1
WS 92	15	4–27	13	5	0–14	5	66	53–96	4	4	n.d.
WS 97	14	8–32	13	2	0–7	5	63	54–71	4	2	1
WS 101	18	0–35	13	2	0–6	5	67	59–77	4	4	0
EqCD11a / 18											
WS 45	25	2–68	12	95	88–99	6	95	93–97	3	3	2
WS 65	86	55–99	13	38	0–99	6	97	95–98	4	0	5
WS 77	80	52–99	13	78	61–96	6	98	97–98	4	0	1
EqCD13											
54	5	0–17	13	66	34–86	6	14	0–35	4	13	1
EqCD44											
WS 53	92	70–100	13	95	84–99	6	98	97–99	4	99	99
WS 63	77	28–100	12	71	23–99	6	92	80–98	4	15	93
WS 72	92	58–99	13	90	68–99	6	97	96–98	4	86	99
WS 75	95	87–100	13	87	55–99	6	98	96–99	4	99	99
EqMHC I											
37	94	75–100	13	86	74–99	6	97	97–98	4	99	99
57	95	72–100	13	71	38–98	6	89	76–94	4	99	97
105	92	49–100	13	43	6–99	6	88	66–98	4	99	97
EqMHC II											
43	70	24–99	13	6	0–21	6	64	45–79	4	97	1
74	45	9–84	13	8	0–24	6	56	51–61	4	99	1
99	77	55–99	13	4	0–13	6	70	49–82	4	99	6

Table 5 (continued)

mAb	Percentage of positive (labeled) cells[a]										
	PBL			Granulocytes			Thymocytes			EqT8888	Fetal kidney
	Mean	Range	*n*	Mean	Range	*n*	Mean	Range	*n*		
EqWC1											
49	51	21–78	13	33	0–85	6	49	37–59	4	0	2
61	47	19–69	12	54	27–82	6	39	29–48	4	0	1
80	53	34–78	13	44	16–85	6	49	35–59	4	0	1
89	58	37–88	13	56	29–86	6	51	37–62	4	0	0
EqWC2											
35	89	67–100	13	70	52–90	6	89	79–98	4	40	3
36	80	37–99	13	75	60–88	5	89	77–98	4	70	7
58	44	12–69	13	78	31–99	6	7	0–14	4	32	2
59	60	34–78	13	90	67–99	6	43	33–51	4	60	3
68	82	64–97	13	85	69–99	3	97	95–99	4	70	2
90	82	62–99	13	84	65–98	6	97	94–99	4	87	4
96	78	54–95	13	15	0–36	6	89	71–99	4	76	1
EqWC4											
112	12	12–13	2	n.d.			n.d.			n.d.	n.d.
113	11	11–12	2	n.d.			n.d.			n.d.	n.d.
B cells											
67	15	3–32	12	4	0–12	5	4	2–6	4	13	2
73	13	1–56	13	7	0–23	5	1	0–3	4	1	3
104	20	9–66	13	6	0–30	6	13	3–25	4	6	3
Macro											
50	10	0–28	13	2	0–7	6	1	0–2	4	14	0
102	3	0–23	12	1	0–4	5	0	0–1	3	1	1
Individual mAbs											
34	70	31–91	13	16	8–23	5	31	15–48	4	6	1
41	7	1–24	13	17	4–34	6	0	0–1	4	1	1
44	4	0–15	13	1	0–5	6	0	0–1	4	3	8
47	15	0–43	13	1	0–4	4	37	21–53	4	12	20
48	2	0–11	12	3	0–11	5	1	0–2	4	6	7
56	42	20–57	13	12	1–37	6	33	20–46	4	0	9
60	25	9–55	13	11	0–34	5	33	21–55	4	0	12
62	77	59–97	12	24	20–27	4	28	18–37	4	0	2
64	38	11–87	13	46	16–77	6	51	10–93	4	0	51
66	4	0–14	13	5	0–21	6	2	0–4	4	2	2
69	54	38–70	12	5	0–17	6	85	75–91	4	0	1
76	19	1–43	13	9	0–34	5	80	73–85	4	0	99
78	24	1–52	13	41	11–79	6	38	21–95	4	6	1
79	10	1–28	13	7	0–28	5	1	0–1	4	1	1
82	19	8–28	13	79	39–96	6	30	10–45	4	13	2
83	44	24–72	13	51	18–80	6	24	15–34	4	6	9
85	65	42–88	12	6	0–21	5	98	97–99	4	99	99
86	12	0–34	12	6	0–21	5	34	14–65	4	0	2
87	15	0–55	12	4	0–11	5	18	6–35	4	99	1

Table 5 (continued)

mAb	Percentage of positive (labeled) cells[a]										
	PBL			Granulocytes			Thymocytes			EqT8888	Fetal kidney
	Mean	Range	*n*	Mean	Range	*n*	Mean	Range	*n*		
88	34	10–72	13	17	0–81	6	26	8–40	4	0	4
91	8	0–23	13	8	0–36	6	1	0–1	4	6	7
94	90	62–99	13	94	79–99	6	97	97–98	4	0	99
95	91	71–100	1.	89	79–99	3	97	96–98	4	3	1
100	63	15–90	13	4	0–12	5	97	96–98	4	99	99
106	67	31–97	13	6	0–20	5	26	16–37	4	6	10
107	81	38–94	13	41	7–76	6	24	18–36	4	1	10

[a]Percentage of positive (labeled) cells are reported as the mean and range when more than one determination was made; 0, negative, no labeling detectable above the negative control; n.d., not done.
[b]Duplicate of WS 38 included as an internal control for clustering purposes.

the basis of expression cloning and gene sequencing (Tavernor et al., 1994). On this basis the EqWC3 cluster was renamed EqCD2. While no successful immunoprecipitations have been conducted with the mAbs in this cluster during ELAW II it has been reported that WS111 recognizes a 58 kDa T cell antigen (Tumas et al., 1994), consistent with the molecular weight of CD2 (Barclay et al., 1993). Experiments were conducted during ELAW II to express the EqCD2 molecule in COS cells as previously described (Tavernor et al., 1994). All three mAbs in this cluster recognized the transfected COS cells in FACS analyses. In FACS analysis these antibodies recognized 67–88% of lymphocytes (Table 5), which in two color analysis proved to comprise the great majority of T cells (EqCD5 + ve) (Fig. 1). Using immunohistochemistry EqCD2

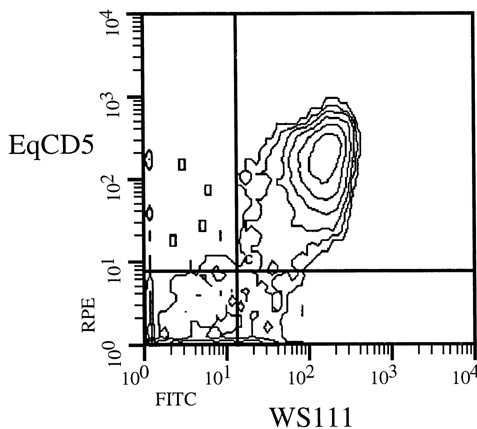

Fig. 1. Two color FACS profiles of lymphocytes from an adult horse stained with WS111 (EqCD2) and the T lymphocyte marker WS81 (EqCD5). Negative marker set with an irrelevant mAb.

antibodies have previously been shown to recognize all cells in T-dependent regions of lymph nodes and the majority of mature medullary thymocytes together with many cortical thymocytes (Kydd et al., 1994). FACS analysis using mAb WS103 confirmed that the majority of thymocytes are recognized (Table 5). The distribution of EqCD2 is similar to human CD2, in contrast to the mouse where CD2 is also present on B cells (Barclay et al., 1993).

The human CD2 T cell antigen binds its ligand, CD58, on cytotoxic targets, antigen presenting cells, or memory T cells and binding of CD2 can mediate T cell activation via its cytoplasmic domain (Barclay et al., 1993). In functional analyses WS103 (the only EqCD2 mAb tested) partially inhibited PHA-induced lymphoproliferation.

3.2. EqCD3

The only mAb that recognized an equine orthologue of the CD3 antigen was WS98, and its characterization has been described elsewhere (Blanchard-Channell et al., 1994). In single color FACS analyses (Table 5) WS98 staining was restricted to lymphocytes and thymocytes, and in two color FACS staining was restricted to EqCD5 + ve lymphocytes (data not shown). In immunohistochemistry WS98 was a very effective T cell marker (Blanchard-Channell et al., 1994; Lunn et al., 1995b). Immunoprecipitation experiments using chemiluminescent detection of biotinlylated cell surface proteins showed that WS98 precipitated a complex of proteins with molecular weights from 18,000 to 42,000. On further dissociation of the precipitated complex, two proteins were identified with molecular weights of 22,000 and 27,000. It is not known which component of the EqCD3 complex WS98 recognizes, however this antibody can induce interleukin-2 receptor expression on T lymphocytes (Blanchard-Channell et al., 1994) which is a feature shared with antibodies specific for the ϵ chain of human and murine CD3.

3.3. EqCD4

Only WS38 recognized EqCD4, and this antibody was also assigned to this cluster in ELAW I (Kydd et al., 1994). The characterization of this antibody has been extensively described elsewhere (Lunn et al., 1991). A duplicate of WS38 was included in the workshop panel (WS46) for the purposes of quality control and statistical analyses. The mAb WS38 is effective in FACS analysis and in immunoprecipitation experiments (precipitates a 58 kDa antigen in reducing and non-reducing conditions), but has generally given a weak result in immunohistology. At ELAW II the Davis laboratory reported that after re-cloning the hybridoma and incorporating a commercial growth supplement in the medium a much higher titer of antibody was achieved in the supernatant and this resulted in much improved performance in immunohistology. In functional analyses WS38 partially inhibited PHA-induced lymphoproliferation as has been reported in other species when a soluble anti-CD4 reagent is used (Parnes, 1989; Band and Chess, 1985).

One additional reagent was submitted by the Tochigi laboratory late in the course of ELAW II that had the characteristics of an EqCD4 reagent. This mAb was not included in the main workshop analysis. Some details of its characteristics are given in Section 5.

3.4. EqCD5

Three antibodies, WS51, WS71 and WS81 were assigned to the EqCD5 cluster on the basis of FACS (Table 5) and immunohistological analyses of tissue distribution, and results of immunoprecipitation experiments. Two of these antibodies have been described elsewhere, WS51 (Blanchard-Channell et al., 1994) and WS81 (Lunn et al., 1991), and one was also included in ELAW I (WS81). Both WS51 and WS81 precipitate a 69 kDa antigen in reducing and non-reducing conditions. The new reagent in this group is WS71 and the Tochigi laboratory which produced this mAb showed that it precipitated a 69 kDa structure in reducing conditions (Fig. 2). No laboratory reported any evidence of EqCD5 expression by B lymphocytes, in contrast to the situation in humans (Kipps, 1989). In functional analyses all three anti-EqCD5 mAbs consistently enhanced PHA-induced lymphoproliferation consistent with a role for the EqCD5 antigen in T cell activation (Lydyard and Mackenzie, 1989).

Blocking experiments using FACS analysis were conducted by both the Cambridge (1 experiment) and the Madison (2 experiments) laboratories. In these experiments two color FACS analysis was performed using biotinylated WS81 as the second color reagent. In every instance labeling with WS81 as a first step completely prevented labeling by WS81 as a second step, and labeling with either WS51 or WS71 as a first step almost entirely blocked labeling. These results indicate that these mAbs are likely to

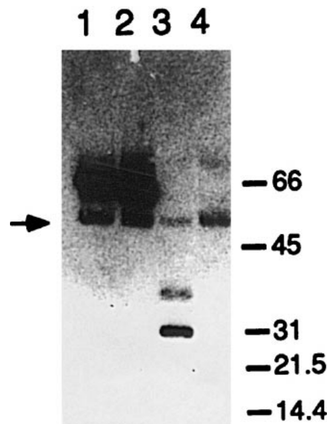

Fig. 2. Autoradiographs of SDS-PAGE analysis of ^{125}Iodine labeled equine lymphocyte surface molecules immunoprecipitated under reducing conditions using the Tochigi mAbs WS71 (TH2A10) in Lane 1 (anti-EqCD5), LB4A1 in Lane 2 and LB3G11 in Lane 3 (see Section 5 for specificity) and a negative control antibody in Lane 4; the arrow identifies a non-specific band in all lanes. Molecular weight markers are shown on the right in kDa.

recognize epitopes on the same antigenic structure, and may possibly recognize the same epitope.

3.5. EqCD8

Four mAbs were assigned to this cluster: WS84, WS92, WS97, and WS101. Of these only WS84 was submitted to ELAW I. All of these antibodies recognized a small subset of lymphocytes on FACS analysis (Table 5) which consisted entirely of T cells on two color FACS (data not shown). All four mAbs precipitated a heterodimer of 32 and 39 kDa as determined by SDS-PAGE analysis in reducing conditions (Fig. 3). Analysis of the WS84 and WS92 immunoprecipitates in non-reducing conditions identified a single band of 68 kDa confirming that the heterodimer is covalently linked. A detailed description of the tissue distribution of the EqCD8 antigen in immunohistology and in two color FACS analysis with the EqCD4 antigen has been presented elsewhere (Lunn et al., 1991).

In functional analyses the addition of soluble WS92, WS97 and WS101 partially inhibited PHA-induced lymphoproliferation. In contrast Lunn et al. (1996) have shown that positive selection of EqCD8 + equine lymphocyte precursors using WS84 attached to agarose beads can lead to increased LAK activity after incubation with recombinant human IL-2 (Lunn et al., 1996). The explanation for these different responses is uncertain, although it has similarly been demonstrated in humans that labeling CD8 with soluble antibody (as opposed to cross-linking CD8 molecules) down-regulates cellular

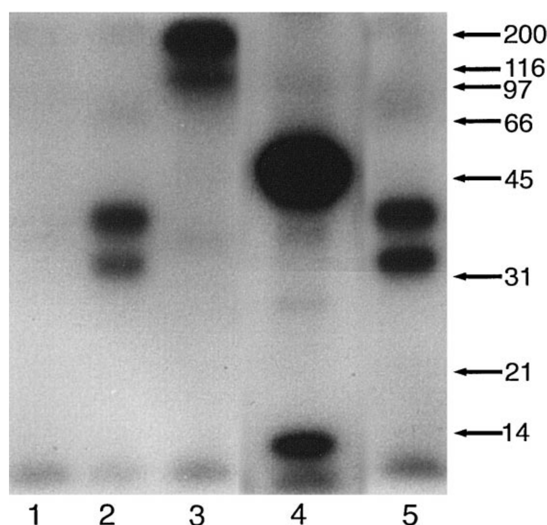

Fig. 3. Autoradiographs of SDS-PAGE analyses of ^{125}Iodine labeled equine lymphocyte surface molecules imunoprecipitated under reducing conditions. Lanes are: (1) negative control mAb; (2) WS84 (anti-EqCD8); (3) WS77 (anti-EqCD11a/18); (4) WS37 (anti-EqMHC I); (5) WS92 (anti-EqCD8). Molecular weight markers are shown on the right in kDa.

activation (Welte et al., 1983). These functional effects of binding EqCD8 receptor with soluble antibody, or cross-linking it with immobilized antibody (as was done in the LAK study) may be a result of blocking or activating the cytoplasmic protein-tyrosine kinase $p56^{ick}$, which is complexed to the α chain of the CD8 molecule in humans (Rudd, 1990).

Blocking experiments using FACS analysis were conducted by the Madison laboratory using PBMC from two different horses. Two color FACS analysis was performed using biotinylated WS84 as the second color reagent. In each instance incubation with WS84 as a first step completely prevented labeling by WS84 as a second step. Labeling with WS97 similarly blocked WS84 labeling, while labeling with WS92 or WS101 did not block labeling and gave a double-positive staining pattern (Fig. 4). This result suggests that WS84 and WS97 are likely to recognize epitopes on the same antigenic structure, and may possibly recognize the same epitope. It is possible that WS84 and WS97 recognize either the α or β chain of the EqCD8 molecule while WS92 and

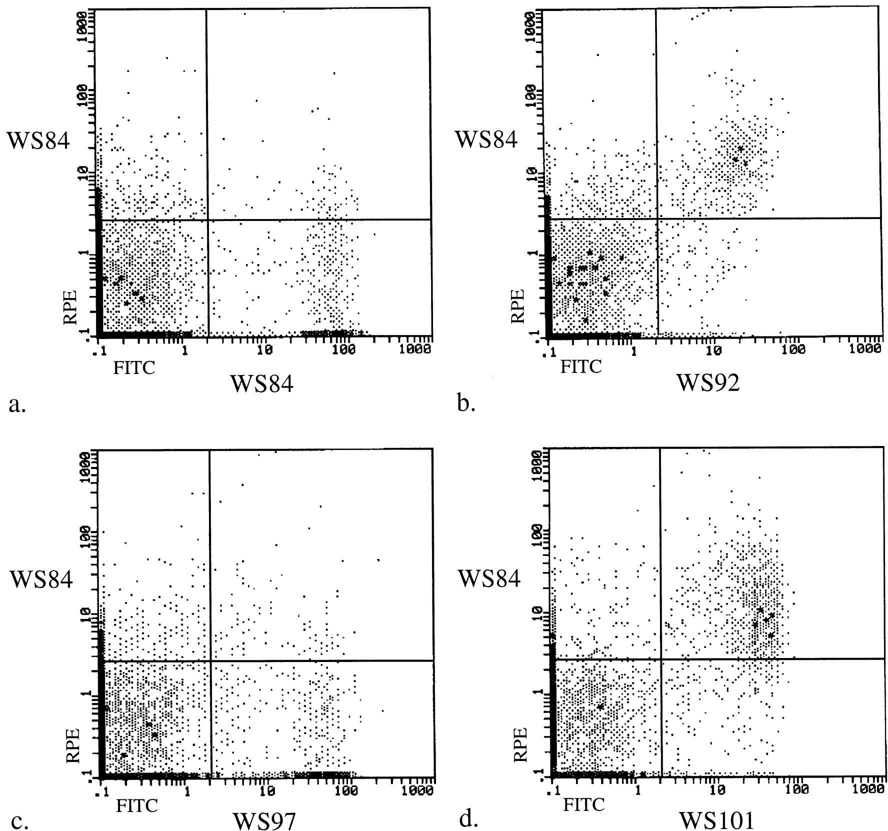

Fig. 4. Two color FACS profiles of lymphocytes from an adult horse stained with WS84 as a second color in each instance, and with: (a) WS84; (b) WS92; (c) WS97; (d) WS101. Panels a and c show evidence that labeling with WS84 as a second color reagent is blocked. Negative markers set with an irrelevant mAb.

WS101 recognize the other chain. Careful immunoprecipitation and absorption experiments will be needed to resolve this issue.

One additional reagent was submitted by the Tochigi laboratory late in the course of ELAW II that had the characteristics of an EqCD8 reagent. This mAb was not included in the main workshop analysis. Some details of its characteristics are given in Section 5.

3.6. EqCD11a / 18

Three mAbs were initially assigned to this cluster on the basis of FACS analysis: WS45, WS65, and WS77. All of these reagents were also assigned to this cluster in ELAW I. Immunoprecipitation data from ELAW I (Kydd et al., 1994) and ELAW II demonstrated that WS45 and WS77 recognized a non-covalently linked heterodimer of 100 and 180 kDa molecular weight (Fig. 3). No successful immunoprecipitations were conducted with WS65, and it was decided to finally assign only WS45 and WS77 to the EqCD11a/18 cluster, together with two antibodies analyzed in ELAW I (Table 4). No clear effect of anti-EqCD11a/18 mAbs on PHA-induced lymphoproliferation could be demonstrated. However in a previous study of equine LAK cells it was shown that an anti-EqCD11a/18 mAb from ELAW I (CVS9, see Table 4) was capable of blocking LAK activity, consistent with the critical role of this antigen in intercellular adhesion events (Lunn et al., 1994b).

Single color FACS analyses of whole blood leucocyte populations using CVS9, an EqCD11a/18 antibody characterized in ELAW I (see Table 4), demonstrated a very consistent pattern of staining when linear side scatter was plotted against log fluorescence 1 (Fig. 5). This result was obtained repeatedly in analysis of whole blood leucocyte populations from a total of eight horses by the Madison laboratory. After comparison of fluorescent staining with both forward and side scatter characteristics

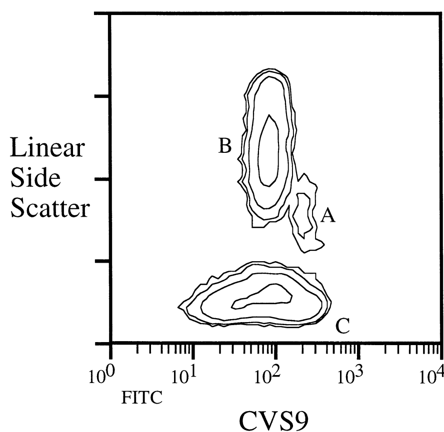

Fig. 5. Flow cytometric staining of whole blood leucocytes with an anti-EqCD11a/18 mAb (CVS9; see Table 4). Linear side scatter is plotted against logarithmic fluorescence 1. Three populations are shown, A, B and C, corresponding to macrophages, granulocytes and lymphocytes respectively, as described in the text.

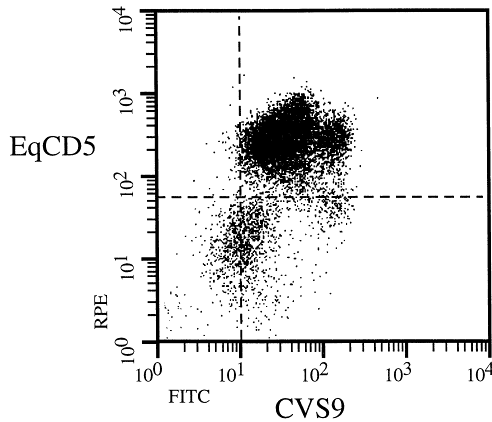

Fig. 6. Two color FACS profiles of lymphocytes from an adult horse stained with an anti-EqCD11a/18 mAb (CVS9; see Table 4) and the T lymphocyte marker WS81 (EqCD5). Negative marker set with an irrelevant mAb.

using Paint-a-Gate™ software (Becton-Dickinson, San Jose, CA) it was apparent that population (A) represented granulocytes, (B) represented macrophages, and (C) represented lymphocytes. This indicates that macrophages show a uniform high intensity of EqCD11a/18 expression, lymphocytes show a wide range of staining intensities and granulocytes show a homogenous intermediate staining pattern. Examination of two color FACS analysis of lymphocytes with CVS9 and the T cell marker WS81 (EqCD5) demonstrates that the range of staining obtained with lymphocytes is due to low intensity staining of B lymphocytes and a sub-population of T cells, and high intensity staining of a sub-population of T cells (Fig. 6). This range of T cell EqCD11a/18 expression is consistent with higher levels of expression on memory compared to naive T cells (Wallace and Beverley, 1990; Dustin and Springer, 1991).

3.7. EqCD13

Only one mAb, WS54, recognized an equine orthologue of CD13 in that it stained only myeloid cells in FACS analyses (Table 4) and immunohistology, and precipitated a 150 kDa molecule in both reducing and non-reducing conditions. A detailed report of the characterization of this antibody has been submitted elsewhere (Lunn et al., manuscript in preparation).

3.8. EqCD44

Four antibodies, WS53, WS63, WS72 and WS75, were assigned to this cluster on the basis of tissue distribution as determined by FACS analysis (Table 5). Of these, both WS63 and WS72 had previously been demonstrated to recognize a cDNA clone of the EqCD44 molecule expressed in COS cells (Tavernor et al., 1993). Unfortunately it was

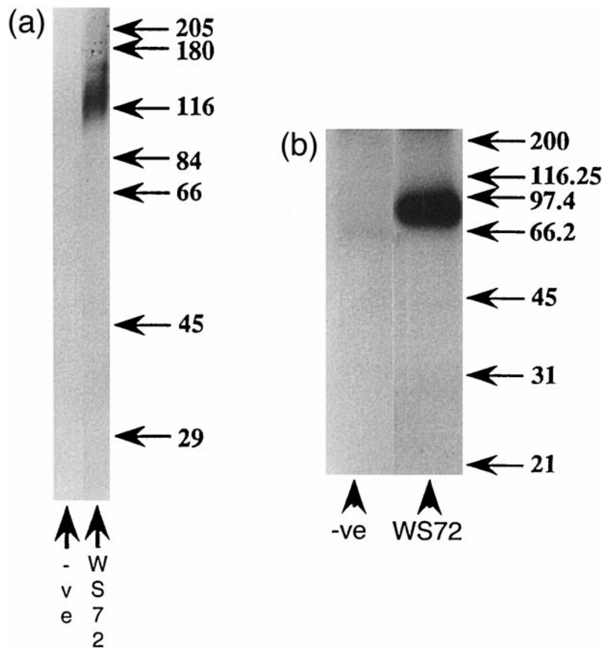

Fig. 7. Autoradiographs of SDS-PAGE analyses of ^{125}Iodine labeled equine lymphocyte surface molecules imunoprecipitated under reducing conditions. Two separate gels are presented. Lanes are: Gel A—(1) no mAb; (2) WS72 (anti-EqCD44). Gel B—(1) no mAb; (2) WS72 (anti-EqCD44) after endoglycosidase F treatment. Molecular weight markers are shown on the right in kDa.

not possible to repeat these expression experiments during ELAW II to confirm the specificity of WS53 and WS75. In immunoprecipitation experiments WS72 precipitated a 'smear' in the 100 kDa position in both reducing and non-reducing conditions which could be resolved to a 76 kDa band after endoglycosidase treatment of the precipitate to remove glycosyl residues (Fig. 7), as is typical of human CD44 (Stoll et al., 1989). The Lyon (France) laboratory performed immunoprecipitations with WS53 and WS75, and observed two bands of 100 kDa and 80 kDa in both reducing and non-reducing conditions. This immunoprecipitation result was confirmed by the Madison laboratory for WS53. This result is not typical of CD44, and because of this, together with the unavailability of the EqCD44-transfected cells for screening, it was decided not to assign WS53 or WS75 to the EqCD44 cluster at this time.

Single color FACS analyses of whole blood leucocyte populations using WS72 as described in Section 3.6 demonstrated a consistent pattern of staining when linear side scatter was plotted against log fluorescence 1 (Fig. 8). Two populations of cells were observed with population (A) representing macrophages and granulocytes, and (B) representing lymphocytes. This result indicates that macrophages and granulocytes express EqCD44 with a uniform high intensity, while lymphocytes show a wide range of staining intensities. In ELAW I it was demonstrated that B cells showed a lower

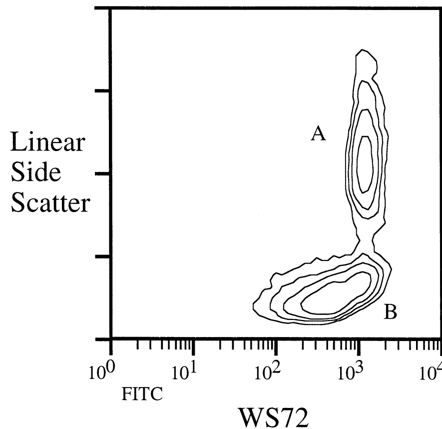

Fig. 8. Flow cytometric staining of whole blood leucocytes with an anti-EqCD44 mAb (WS72). Linear side scatter is plotted against logarithmic fluorescence 1. Two populations are shown, A and B, corresponding to macrophages plus granulocytes, and to lymphocytes respectively, as described in the text.

intensity of EqCD44 expression (Kydd et al., 1994). No clear effect of anti-EqCD44 mAbs on PHA-induced lymphoproliferation could be demonstrated.

3.9. EqMHC I

Three mAbs, WS37, WS57 and WS105 were assigned to this cluster on the basis of tissue distribution (Table 5) and precipitation of a 44–45 kDa α chain, together with the 12 kDa $\beta 2$ microglobulin chain in the case of WS37 and WS105 (Fig. 3). Both WS57 and WS105 have been shown to block MHC I-restricted cytotoxic lymphocytes in vitro (O'Brien et al., 1991), although they had no effect on PHA-induced lymphoproliferation.

3.10. EqMHC II

Three mAbs, WS43, WS74 and WS99 were assigned to this cluster on the basis of tissue distribution (Table 5) and immunoprecipitation data; each of the antibodies precipitated either a 33–34 kDa band or two closely related bands in this region in both reducing and non-reducing conditions. In two color FACS analysis of adult PBMC all three antibodies stain B cells with a high intensity together with a large subset of T cells (data not shown), as is typical of equine MHC II markers (Lunn et al., 1993; Crepaldi et al., 1986).

3.11. EqWC1

Four mAbs WS49, WS61, WS80 and WS89 were provisionally assigned to this cluster on the basis of their staining of a major T lymphocyte subset, all medullary thymocytes, and granulocytes in FACS analyses (Table 5 and data not shown). These characteristics of this workshop cluster were assigned at the previous workshop (Kydd et

al., 1994) and investigated in detail in an associated study (Lunn et al., 1994a). Of the mAbs submitted to ELAW II only WS49 was effective in immunoprecipitations, identifying a 22 kDa molecule in reducing and non-reducing conditions. Given the fact that several other reagents were identified in the previous workshop that were effective in immunoprecipitations and identified a similar structure, only WS49 was finally assigned to this cluster together with three antibodies described in the previous workshop (Table 4). The identity and role of the EqWC1 antigen remains unknown although it may represent a orthologue of Thy-1. However, attempts to verify this theory and identify equine Thy-1 using cross-reactive antibodies from other species for comparison were ineffective. In functional analyses EqWC1 mAbs had no consistent effect on PHA-induced lymphoproliferation and have previously been shown to be incapable of blocking LAK activity (Lunn et al., 1994b).

3.12. EqWC2

Seven mAbs, WS35, WS36, WS58, WS59, WS68, WS90 and WS96 were assigned to this cluster on the basis of staining of all T lymphocytes and granulocytes on FACS analyses (Table 5 and data not shown). Only WS68, WS90, and WS96 were effective in immunoprecipitation experiments, identifying a 160–180 kDa band in reducing and non-reducing conditions, and therefore only these three reagents were firmly assigned to this cluster (Table 4). EqWC2 mAbs had no consistent effect on PHA-induced lymphoproliferation but have previously been shown to be capable of blocking LAK activity (Lunn et al., 1994b) consistent with a role in intercellular interactions. The identity of the EqWC2 antigen remains unknown, although its biochemical characteristics are consistent with either the low molecular weight isoform of CD45 (CD45RO), or a member of the CD49 integrin family. However in the absence of more definitive information the precise identity of this antigen remains undetermined.

3.13. EqWC4

Two mAbs, WS112 and WS113 were placed in cluster EqWC4 on the basis of recognition of a small subset of EqCD5 + ve T cells (Table 5 and Fig. 9). Immunohistochemical studies revealed an abundance of $EqWC4^{+}$ cells in the paracortex of lymph nodes with few cells evident in follicles. Immunoprecipitation and SDS-PAGE analyses under reducing conditions demonstrated the apparent molecular weight of the molecule was 46 kDa. On this evidence it was suggested that the mAbs might recognize the equine orthologue of CD28.

Further studies conducted by the Washington State laboratory using the EqWC4 mAbs showed the molecule is expressed in several breeds of horses including Pony, Arabian, and Thoroughbred. Two color flow cytometric analysis of peripheral blood lymphocytes demonstrated that the mAbs recognize 6.3% (± 3.5) of $EqCD4^{+}$ lymphocytes and a smaller population of $EqCD8^{+}$ lymphocytes (0.5% $\pm$ 2.5). FACS analyses of thymocytes revealed EqW4 is expressed on approximately 1% of these cells. Immunoprecipitation of peripheral blood lymphocytes under reducing conditions confirmed that these mAbs identified a peptide with a molecular weight of 46 kDa (Fig. 10).

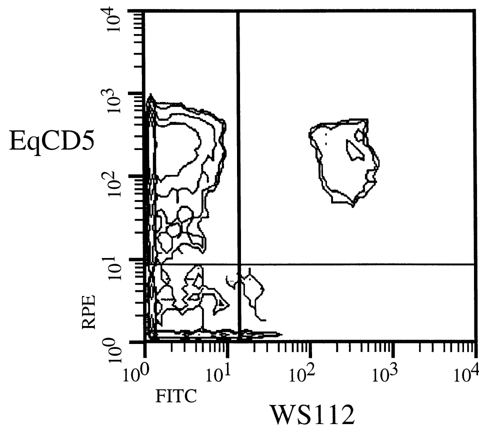

Fig. 9. Two color FACS profiles of lymphocytes from an adult horse stained with WS112 (EqWC4) and the T lymphocyte marker WS81 (EqCD5). Negative marker set with an irrelevant mAb.

Further analysis by Western immunoblot of molecules derived from thymocytes under reducing conditions yielded two bands, one at 46 kDa and 52 kDa. Under non-reducing conditions, a third band of molecular weight 92 kDa was evident suggesting the formation of protein dimers (Fig. 11). Activation of equine lymphocytes with concanavalin A or phytohemagglutinin did not increase the percentage of EqWC4 + cells, indicating that it may not be an activation molecule.

It is still unclear whether EqWC4 is the orthologue of CD28. Although the biochemical characteristics of this equine antigen are similar to that of CD28, the number of

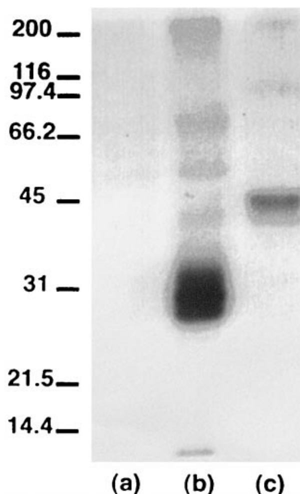

Fig. 10. Autoradiographs of SDS-PAGE analyses of ^{131}Iodine labeled equine lymphocyte surface molecules imunoprecipitated under reducing conditions. Lanes are: (a) negative mAb; (b) control anti-EqCD8 mAb; (c) WS113 (anti-EqWC4). Molecular weight markers are shown on the left in kDa.

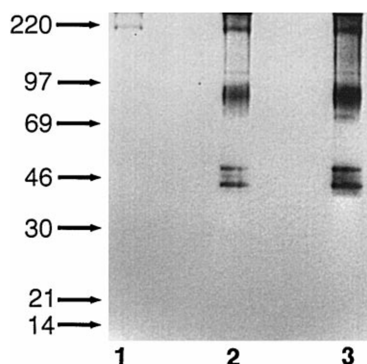

Fig. 11. Western blot analysis of biotinylated cell surface protein analyses in non-reducing conditions using ECL system with control antibody in Lane 1 (HB61A), and anti-EqWC4 antibodies in Lanes 2 and 3 (WS113:HB65A and WS112:HB86A). Molecular weight markers are shown on the left in kDa.

$EqCD4^+$ and $EqCD8^+$ equine lymphocytes expressing WC4 differs considerably from the populations of human lymphocytes expressing CD28. Human CD28 has been reported to be expressed on most T lineage cells and plasma cells, with 95% of $CD4^+$ cells and 50% of $CD8^8$ cells positive for CD28. Human CD28 is also expressed on a large proportion on thymocytes with the highest expression on mature cells. The proportion and phenotype of cells expressing EqWC4 is clearly different from the characterized populations of human and mouse lymphocytes expressing CD28. With this difference in the pattern of expression, it will be necessary to clone and sequence the equine gene to determine whether the observed differences are attributable to a species difference in expression or to the fact that EqWC4 recognizes a new molecule with as yet no identified orthologue in humans or mice.

3.14. B cell reagents

Three reagents, WS67, WS73 and WS104 were clustered on the basis of their expression being restricted to B cells. Two of these reagents, WS73 and WS104, precipitated antigens of 85 and 240 kDa respectively in reducing conditions, while WS67 was ineffective in immunoprecipitations. The WS73 reagent was described in detail in a companion paper to ELAW I (Zhang et al., 1994) and it was tentatively proposed that this reagent may recognize an equine orthologue of the CD19 antigen (95 kDa in humans). However, no additional evidence for this proposal has been accumulated and therefore the identity of the WS73 antigen remains uncertain. The role of the antigen recognized by WS104 is similarly unknown, although its weight and tissue distribution are consistent with the B cell isoform of the CD45 molecule that includes the A, B, and C exon encoded sequences (Barclay et al., 1993). With the information available it was not possible to assign specificities to these reagents beyond their reactivity with B lymphocytes, and therefore the mAbs for which immunoprecipitation data was available, WS73 and WS104, were assigned to a B cell cluster (Table 4).

3.15. Macrophages

Two antibodies were clustered solely on the basis of their recognition of macrophages: WS50 and WS102. Neither of these reagents recognize peripheral blood lymphocytes, granulocytes, thymocytes, or either of the two other cell lines tested (Table 5). However, both reagents recognize macrophages in FACS analysis of peripheral blood as described in detail in ELAW I (Kydd et al., 1994). In the absence of immunoprecipitation data these reagents and the macrophage cluster remain poorly characterized (Table 4). Nevertheless both reagents represent useful macrophage markers.

3.16. Individual anti-leucocyte antibodies

A total of 26 mAbs were classified as 'individual' reagents as it was not possible to cluster them effectively on the basis of tissue distribution. Details of tissue distribution are presented in Table 5. Immunoprecipitation data were available only for WS83, which was reported to precipitate a 70–90 kDa antigen, and for WS107, which precipitated a non-covalently linked dimer of 145 and 240 kDa. The immunohistological evaluation of many of these reagents is presented in ELAW I (Kydd et al., 1994). While this large group of unclustered antibodies represent a frustrating puzzle, they may well contain many of the 'missing pieces' from the equine armamentarium of immunological reagents and therefore warrant further study. One observation that can be made from the FACS data (Table 5) is that the differential expression of these antigens on cell lines suggests that several of the individual lymphocyte markers could be assigned to individual clusters, and this emphasizes the importance of developing additional equine cell lines as a powerful tool for resolving the identify of reagents such as these.

4. Immunoglobulin reagents

4.1. Introduction and overview

During ELAW II a new category of reagents identifying equine immunoglobulins was included. Although surface immunoglobulin has long been recognized as a surface antigen on B cells, these molecules have not traditionally been included in similar workshops in other species. However the relative lack of research on equine immunoglobulins and the knowledge that a number of research groups are now working in this area led the organizers to request the submission of mAbs recognizing equine immunoglobulins to the workshop panel.

Previous studies have revealed the presence of IgA, IgE, IgG, and IgM in equine serum. Although it would be inappropriate to include a full review of the equine immunoglobulin isotypes and sub-isotypes here, the following paragraphs include a concise summary of the state of knowledge prior to the workshop.

4.1.1. IgG

Four IgG sub-isotypes have been described. Three are designated as IgGa, IgGb and IgGc on the basis of their increasing anodal mobility in immunoelectrophoresis (Rockey

et al., 1964; Klinman et al., 1965; Rockey, 1967). However, the separation of IgGa from IgGb has proved very difficult due to the similarity in charge of these two molecules and studies have often been restricted to partial purification resulting in IgGab and IgGc (Rockey, 1967; McGuire et al., 1973). Some confusion exists in early reports about the status of IgG(T) as a sub-isotype of IgG. Some authors have proposed that IgG(T) be classified as a separate Ig isotype, IgT (Dorrington and Rockey, 1968; Montgomery et al., 1969; Montgomery, 1973). Widders et al. (1986) purified IgG(T) free from any contamination with IgG and conducted investigations of the antigenic relationship between IgG and IgG(T) using immunoelectrophoresis and double immunodiffusion. These experiments unequivocally classified IgG(T) as a sub-isotype of IgG. The reports of the molecular weights of the sub-isotypes of IgG show similar weights of approximately 152 kDa for the whole molecules and H and L chain weights ranging from 52.2 kDa to 53.9 kDa and 22.3 kDa to 23.1 kDa, respectively (Montgomery, 1973).

4.1.2. IgM

IgM exists in serum as a single sub-isotype in a pentameric structure with a molecular weight of 990 kDa (Kabat, 1939; McGuire et al., 1973) which after reduction and alkylation separates into five monomeric sub-units each capable of antigen binding, but without precipitating and agglutinating properties (Hill and Cebra, 1965). Although the molecular weight of μ chain has not been reported, the structure of IgM appears to be similar to that of man with a single J chain of molecular weight of 15 kDa associated covalently with each pentamer (Mitchell et al., 1977). To date no sub-isotypes or allotypes of IgM have been demonstrated.

4.1.3. IgA

The existence of equine IgA analogous to that found in man was indicated when immunological cross-reaction was discovered between an immunoglobulin in equine serum and milk with anti-human α chain antiserum (Vaerman et al., 1971). Later secretory IgA and free secretory component were identified and isolated in equine milk and other secretions (McGuire and Crawford, 1972; Pahud and Mach, 1972). Antigenic determinants specific for secretory IgA associated with secretory component were demonstrated by McGuire and Crawford (1972). Equine serum IgA exists predominantly as dimers while monomers, trimers and tetramers also occur. The molecular weight of serum IgA extends from 150 kDa to about 700 kDa with the majority being about 350 kDa suggesting a predominant dimeric form in serum similar to the cow and the pig, but distinct from human serum IgA which exist largely as monomers (Roberts, 1975). The molecular weight range of secretory IgA is similar to that of serum IgA but the majority is larger than 350 kDa, the difference being assumed to be due to the presence of a secretory component of a molecular weight of 80 kDa (McGuire et al., 1973). The molecular weight of IgA heavy chain and the existence of sub-isotypes and allotypes of IgA in the horse have not been reported.

4.1.4. IgE

Matthews et al. (1983) and Suter and Fey (1983) provided preliminary evidence of the presence of equine IgE and described its partial purification and the preparation of

antisera. The existence of IgE in the horse has now been firmly established with recent characterization of its cDNA and deduced amino acid sequence (Navarro et al., 1995).

4.1.5. Light chains

Equine Igs possess κ and λ L chains (Allen et al., 1968) and both have been sequenced (Home et al., 1992; Ford et al., 1994). Over 90% of the L chains in horse serum Igs are λ. The explanation for this is unclear, as the variable λ gene repertoire in the horse is relatively limited (Home et al., 1992) and the κ locus has been shown to be functional and potentially have a similar number of variable region genes (Ford et al., 1994).

4.1.6. Other Immunoglobulins

One additional form of equine Ig has been reported to exist but has never been assigned a conclusive isotype or sub-isotype designation. This Ig has $\gamma 1$ mobility and exists as a salt-dissociable noncovalently linked aggregates, and was first isolated from hyperimmune equine anti-pneumococcal serum (Sandor et al., 1964) and subsequently designated as aggregating immunoglobulin (AI) (Zolla and Goodman, 1968). Studies have shown that aggregating immunoglobulin is closely related to IgG but there has not been any agreement on whether it represents a sub-isotype of IgG or is a distinct isotype. It has been designated by various investigators as $\gamma 1$ component (Helm and Allen, 1970), as an Ig isotype IgB (Montgomery, 1973), and as a sub-isotype of IgG termed IgG(B) (Allen and Johnson, 1972).

4.2. Results of analysis of immunoglobulin reagents

Conventional ELISA techniques using purified equine immunoglobulins were used as the main assay for clustering anti-Ig reagents. Both affinity chromatography using mAbs of known specificity and other physicochemical purification methods were used to produce the purified immunoglobulins. Representative methods used are described in the paper by Sheoran et al. (1997) accompanying this report. The Cambridge laboratory used WS29, WS33, WS109 and WS30 for the affinity purification of IgGa, IgGb, IgGc and IgG(T), respectively, and the Tochigi laboratory used WS20, WS21 and WS31 for the preparation of affinity purified IgGc, IgG(T), and IgM, but used non-workshop antibodies for the other equine Igs. The Madison laboratory used WS15 for the affinity purification of IgA, and salt precipitation followed by gel filtration for the preparation of IgM.

The results of ELISA assays showing the reactivity of the workshop panel against affinity purified equine immunoglobulins are shown in Table 6. A summary of these results is included in Table 7 together with FACS analysis results for the expression of sIg on equine B cells using the methods described in Section 2.1. Peripheral blood mononuclear cell preparations were incubated overnight at 37° to eliminate the effects of Fc receptor binding, and monocytes removed by incubation with iron powder and use of a magnet. It was found that a prolonged incubation at 37° to eliminate Fc receptor

Table 6
Summary of ELISA optical density results from ELAW II anti-Ig mAbs tested against affinity purified equine immunoglobulins

WS#	Tochigi						Cambridge				Wisconsin		mAb name	Cluster	WS#
	IgGa	IgGb	IgGc	IgG(T)	IgM	IgA	IgGa	IgGb	IgGc	IgG(T)	IgA	IgM			
1	0.00	0.03	0.00	0.00	0.01	0.01	0.03	0.01	0.03	0.03	0.70	0.84	neg ctrl	negative	1
2	0.00	0.03	0.00	0.00	0.01	0.00	0.03	0.01	0.02	0.03	0.09	0.31	IgA9-6A	negative	2
3	0.00	0.04	0.00	−0.01	0.00	0.00	0.02	0.01	0.03	0.02	0.00	0.12	CAG8-7C	negative	3
4	−0.01	1.84	0.00	0.00	0.00	0.00	0.03	1.23	0.02	0.02	0.05	0.38	CVS3	IgGb	4
5	0.00	1.35	0.00	−0.01	0.00	0.00	0.04	1.27	0.30	0.05	0.04	0.06	CVS44	IgGb	5
6	−0.01	1.15	0.00	−0.01	0.00	0.00	0.02*	0.85*	0.02*	0.06*	0.00	0.15	F12-6C10	IgGb	6
7	1.21	0.10	0.01	0.01	0.01	0.01	1.87	0.03	0.23	0.13	0.21	0.24	CVS48	IgGa	7
8	1.07	0.75	0.70	0.59	0.71	0.89	2.19	1.51	2.60	2.34	1.87	1.72	CVS36	Pan-Ig	8
9	0.05	0.03	0.00	0.00	0.00	0.00	0.13	0.00	0.05	0.08	0.27	0.07	CVS43	negative	9
10	0.00	0.02	0.00	−0.01	0.04	0.00	0.02	0.01	0.01	0.02	0.07	1.43	CVS37	IgM	10
11	0.03	0.10	0.30	1.60	0.01	0.08	0.04	0.06	0.06	2.62	0.15	0.49	CVS38	IgG(T)	11
12	0.05	0.23	0.34	0.01	1.16	0.32	0.64	0.73	1.96	1.05	0.47	1.56	CM6E	IgM	12
13	0.01	1.12	0.10	0.00	0.01	0.02	0.03	0.46	0.01	0.05	0.02	0.15	CVS1	IgGb	13
14	0.00	0.04	0.00	−0.01	0.00	0.00	0.03	0.01	0.02	0.12	0.00	0.34	IgA5-3A	negative	14
15	0.01	0.04	0.15	0.00	0.01	0.10	0.17	0.09	1.10	0.13	1.10	1.34	BVS2	IgA	15
16	1.18	0.04	−0.01	−0.01	0.00	−0.01	1.87	0.02	0.08	0.12	0.62	0.16	CVS47	IgGa	16
17	1.26	1.08	1.05	0.70	0.90	0.94	1.99	1.25	2.68	2.49	0.66	0.86	CVS41	light chain	17
18	0.00	0.90	0.00	−0.01	0.00	0.00	0.08	1.39	0.63	0.18	0.09	0.90	CVS2	IgGb	18
19	0.02	0.04	0.25	0.00	0.01	1.04	0.22	0.12	1.23	0.11	1.53	1.36	BVS1	IgA	19
20	0.07*	0.03*	1.24*	0.08*	0.10*	0.17*	0.03*	0.02*	0.83*	0.04*	0.01	0.03	F14-10G12	IgGc	20
21	0.01	0.07	0.29	0.98	0.00	0.03	0.04	0.04	0.09	2.66	0.06	0.47	F6-5G2	IgG(T)	21
22	1.78	0.05	0.00	−0.01	0.00	0.00	1.90	0.02	0.08	0.06	0.00	0.21	CVS49	IgGa	22
23	0.02	0.08	0.08	0.00	0.73	0.07	0.38	0.40	1.65	0.66	0.05	0.32	CM7	IgM	23
24	1.15	0.06	0.01	0.00	0.01	0.00	1.66	0.02	0.09	0.05	0.00	0.02	CVS42	IgGa	24
25	0.00	0.07	0.00	−0.01	0.01	0.00	0.02	0.03	0.00	0.03	nd	nd	CVS50	IgGb	25
26	0.00	0.06	0.02	0.00	0.35	0.01	0.22	0.29	1.32	0.43	0.20	1.43	F13-6D6	IgM	26
27	1.21	0.10	0.01	0.00	0.01	0.01	1.78	0.03	0.20	0.06	0.13	0.21	CVS46	IgGa	27
28	0.00	0.03	0.00	−0.01	0.01	0.00	0.03	0.01	0.02	0.05	0.00	0.21	FIGI-7A	negative	28
29	1.10	0.04	0.00	−0.01	0.00	0.00	1.26	0.02	0.04	0.06	0.00	0.02	CVS45	IgGa	29
30	0.00	0.03	0.01	0.68	0.00	0.00	0.03	0.01	0.01	2.37	0.00	0.03	CVS40	IgG(T)	30
31	0.00	0.04	0.01	−0.01	0.52	0.02	0.14	0.16	0.80	0.25	0.45	1.52	F13-7G12	IgM	31
32	0.02	0.11	0.30	0.78	0.01	0.05	0.04	0.05	0.15	2.84	0.04	0.57	CVS51	IgG(T)	32
33	0.00	0.41	0.00	−0.01	0.00	−0.01	0.05	1.30	0.40	0.14	0.00	0.00	CVS39	IgGb	33
108	0.11*	0.29*	1.94*	0.01*	0.04*	0.07*	0.02	0.01	1.95	0.03	nd	nd	CVS53	IgGc	108
109	0.09*	0.27*	0.48*	0.01*	0.02*	0.06*	0.12	0.01	1.17	0.02	nd	nd	CVS52	IgGc	109

Results were received from laboratories in Tochigi, Cambridge and Madison. Results marked with an asterisk (*) were obtained in separate experiments performed following the workshop meeting. The letters 'nd' in a result indicate that an assay was not performed with that antibody.

Table 7
Reactivity of anti-immunoglobulin mAbs with purified immunoglobulins in ELISAs or with lymphocytes (%Ly) and B lymphocytes (%EqCD5-ve) in FACS analyses (after 8 h pre-incubation to allow for Fc receptor turnover)

WS#	IgGa	IgGb	IgGc	IgG(T)	IgA	IgM	%Ly	%EqCD5-ve
Anti-IgGa								
7	+	−	−	−	−	−	3	26
16	+	−	−	−	−	−	3	27
22	+	−	−	−	−	−	7	59
24	+	−	−	−	−	−	3	25
27	+	−	−	−	−	−	3	28
29	+	−	−	−	−	−	3	26
Anti-IgGb								
4	−	+	−	−	−	−	1	4
5	−	+	−	−	−	−	1	4
6	−	+	−	−	−	−	0	3
13	−	+	−	−	−	−	1	4
18	−	+	−	−	−	−	1	3
25	−	+	−	−	−	−	0	0
33	−	+	−	−	−	−	0	0
Anti-IgGc								
20	−	−	+	−	−	−	0	1
108	−	−	+	−	−	−	0	2
109	−	−	+	−	−	−	0	1
Anti-IgG(T)								
11	−	−	−	+	−	−	0	1
21	−	−	−	+	−	−	0	0
30	−	−	−	+	−	−	0	1
32	−	−	−	+	−	−	0	0
Anti-IgA								
15	−	−	−	−	+	−	0	0
19	−	−	−	−	+	−	0	0
Anti-IgM								
10	−	−	−	−	−	+	10	79
12	−	−	−	−	−	+	9	71
23	−	−	−	−	−	+	12	95
26	−	−	−	−	−	+	11	87
31	−	−	−	−	−	+	9	76
Anti-pan-Ig								
8	+	+	+	+	+	+	12	100
17	+	+	+	+	−	−	12	100

binding was a critical step. Table 8 shows the results of a separate experiment in which flow cytometry was performed on PBMC with either no pre-incubation or an 8 h pre-incubation to allow for the turnover of Fc receptors occupied by equine immuno-

Table 8
Results of two colour FACS on PBMC using anti-EqCD5 (WS81) and anti-Ig mAbs with or without an 8 h pre-incubation to allow for Fc receptor turnover

mAb reactivity	%CD5-ve cells with 8 h pre-incubation	%CD5-ve cells with no pre-incubation
Anti IgGa (WS27)	28	79
Anti IgGb (WS13)	4	85
Anti IgGc (WS108)	2	68
Anti IgG(T) (WS30)	1	96
Anti IgA (WS15)	0	4
Anti IgM (WS31)	76	84
Anti pan-Ig (WS8)	100	99

globulins. Results of a single, representative two-color experiment showing the binding of the antibodies to equine lymphocytes (%Ly) and to EqCD5-ve lymphocytes (%EqCD5-ve) are shown. It was observed that without an 8 h incubation step peripheral blood lymphocytes typically expressed several immunoglobulin isotypes.

4.2.1. IgGa

Six mAbs, WS7, WS16, WS22, WS24, WS27 and WS29 showed specificity for equine IgGa and did not cross react with other Ig isotypes. Two different strategies were employed by the Tochigi and Cambridge laboratories to separate IgGa and IgGb. Protein A and G affinity chromatography was used in Cambridge while Sugiura et al. used ion exchange chromatography and starch block electrophoresis. Subsequently both laboratories produced affinity purified IgGa which was used in the ELISA assays described in Table 6. The molecular weight of the IgGa heavy chain in reducing conditions was 50 kDa with a 27 kDa light chain and the whole molecule weighed 178 kDa in non-reducing conditions. The expression of IgGa on the cell surface of equine B cells was a phenomenon detected by all the antibodies in this cluster. The majority of the antibodies stained 3% of total lymphocytes representing 25–28% of CD5-ve lymphocytes in dual staining (Tables 7 and 8).

4.2.2. IgGb

A panel of 7 mAbs, WS4, WS5, WS6, WS13, WS18, WS25 and WS33 showed unique specificity for equine IgGb, which was prepared using affinity chromatography. One further data set was submitted from Madison using WS13 affinity purified protein which has not been included in these results. The molecular weight of the IgGb heavy chain in reducing conditions was 53 kDa with a 27 kDa light chain and the whole molecule weighed 160 kDa in non-reducing conditions. Following overnight incubation no cells expressed sIg when labeled with these antibodies (Tables 7 and 8).

4.2.3. IgGc

The initial panel of mAbs submitted to the workshop contained only a single IgGc specific candidate (WS20). Subsequently two further antibodies were added (WS108 and

109). Unfortunately WS20 produced only negative results prior to the workshop meeting. Following the workshop meeting, further supplies of the mAbs and affinity purified proteins were exchanged by the two laboratories that produced these reagents (Cambridge and Japan). The results of these experiments have been included in Table 6 and show that all three mAbs identify IgGc. The molecular weight of the IgGc heavy chain in reducing conditions was 52 kDa with a 27 kDa light chain and the whole molecule weighed 169 kDa in non-reducing conditions. Following overnight incubation no cells expressed sIg when labeled with these antibodies (Tables 7 and 8). All results were similar to negative control values.

4.2.4. IgG(T)

Four mAbs, WS11, WS21, WS30 and WS32 showed specificity for equine IgG(T) and did not cross react with other Ig isotypes. The molecular weight of the IgG(T) heavy chain in reducing conditions was 58 kDa with a 27 kDa light chain and the whole molecule weighed 188 kDa in non-reducing conditions. Following overnight incubation no cells expressed sIg when labeled with these antibodies (Tables 7 and 8).

4.2.5. IgA

Two mAbs, WS15 and WS19, identifying equine IgA were submitted to the panel. Affinity purified IgA was used for testing by the laboratories in Japan and Madison. The Tochigi IgA was prepared using a mAb not submitted to ELAW II, but described in a companion paper (Sugiura et al., 1997). The Madison IgA affinity column was prepared using WS15 according to a previously described protocol (Lunn et al., 1995a). Briefly, WS15 was purified from ascites by HPLC and coupled to *N*-hydroxy succinimide activated agarose beads (Affi-gel 10, Bio-Rad, Richmond CA). Affinity purified IgA was initially prepared by passing whole equine serum over the column and analyzed for the presence of IgG, IgG(T), IgA and IgM using commercially available radial-immunodiffusion plates (VMRD). This preparation was found to contain IgA with a trace of IgM contamination. Subsequently equine serum was subjected to 50% ammonium sulfate precipitation and the precipitate was dialyzed extensively against PBS. When this

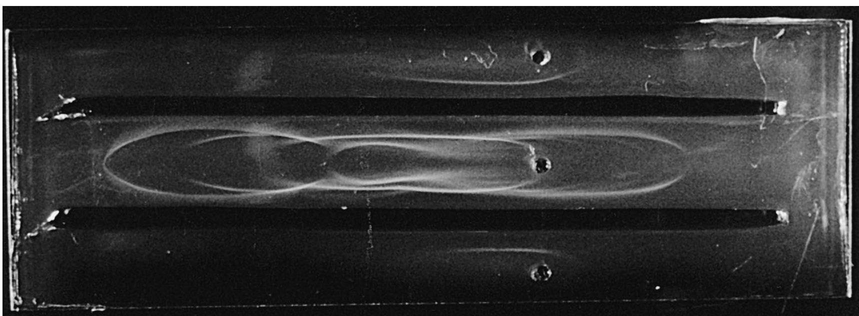

Fig. 12. Immunoelectrophoresis analysis of IgA prepared by affinity chromatography of whole equine serum using mAb WS15. The upper and lower well contained the affinity purified IgA and the middle well contained whole serum. Rabbit anti-equine serum was placed in the trough. The cathode is to the left.

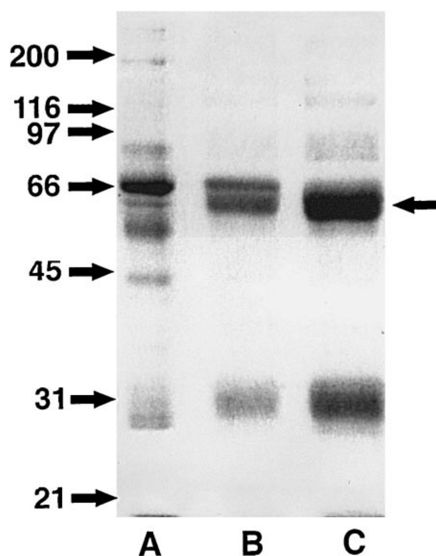

Fig. 13. Results of SDS-12% PAGE analysis in reducing conditions of affinity chromatography purified fractions of normal equine serum prepared with mAb WS15; molecular weight markers are on the left (kDa). Lane A represents whole equine serum. Lane B represents a WS15 affinity extract of whole serum with the heavy chain of IgA visible at 61 kDa, together with some contaminating albumen at 66 kDa. Lane C represents a WS15 affinity extract of partially purified equine immunoglobulins prepared by precipitation of whole serum in 50% ammonium sulfate. A purer preparation of IgA is obtained.

preparation was subjected to affinity chromatography, a pure IgA preparation resulted. This preparation was analysed by immunoelectrophoresis (Fig. 12) and SDS-PAGE (Fig. 13) using previously described methodology (Lunn et al., 1995a). In SDS-PAGE analysis in reducing conditions the molecular weight of the IgA heavy chain was 61 kDa. Following overnight incubation no cells expressed sIg when labeled with these antibodies (Tables 7 and 8).

4.2.6. IgM

Purification of IgM using salt precipitation and gel filtration provided pure preparations for use in ELISAs. Testing revealed that 5 mAbs, WS10, WS12, WS23, WS26, and WS31 bound specifically to equine IgM. Following overnight incubation of PBLs these reagents stained 71–95% of EqCD5-ve cells showing that the majority of B lymphocytes in the circulation express sIgM (Tables 7 and 8 and Fig. 14).

4.2.7. Pan-IgG / pan-Ig

Two mAbs, WS8 and WS17, showed reactivity to more than one equine Ig isotype. One mAb, WS17, principally bound the sub-isotypes of equine IgG and WS8 bound to all Ig isotypes. However both mAbs bound 100% of EqCD5-ve peripheral blood lymphocytes. Following overnight incubation of cells prior to staining all EqCD5-ve lymphocytes were identified by these antibodies (Tables 7 and 8 and Fig. 14).

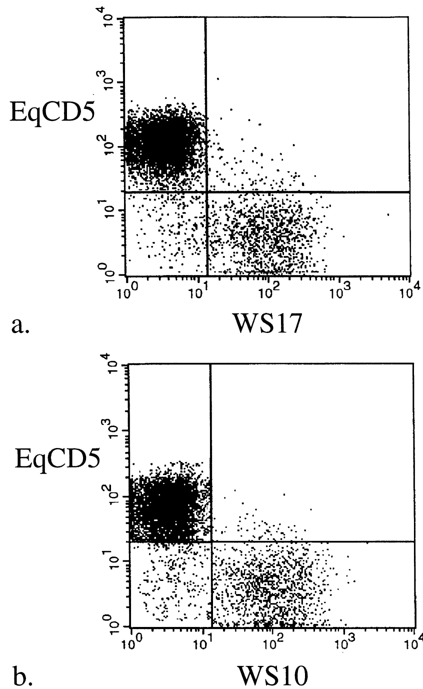

Fig. 14. Two color FACS profiles of lymphocytes from an adult horse stained with EqCD5 (WS81) and (a) anti-pan-Ig and (b) anti-IgM. Distribution of EqCD5 staining is plotted on the vertical axis, and distribution of staining with anti-Ig mAbs is plotted on the horizontal axis. Negative markers were set with irrelevant mAbs.

4.3. Discussion of anti-immunoglobulin reagents

As a result of ELAW II a panel of mAbs recognizing the four IgG sub-isotypes, IgA and IgM was identified, providing valuable reagents for future studies. No evidence concerning the identity of aggregating immunoglobulin was presented during the workshop and no antibodies from the panel were tested against this form of equine Ig.

A considerable amount of FACS data was submitted where the binding of these antibodies to equine leucocytes was examined. A previous report (Lunn et al., 1995a) demonstrated that the majority of B lymphocytes in the horse express the IgG isotype recognized by the mAb CVS1 (WS13) which was found to be specific for IgGb in this workshop. Studies performed using the workshop panel have shown that this may be the result of Fc receptor binding. The previous study assumed that 2–4 h preincubations in equine serum free medium were sufficient to ensure turnover of occupied Fc receptors, however the workshop studies demonstrated that at least 8 h are required for this to occur. Almost no surface expression of IgGb, IgGc, IgG(T) or IgA was shown after overnight incubation in serum free conditions, while approximately 30% of B cells expressed IgGa and 71–95% of B cells expressed IgM (Tables 7 and 8 and Fig. 14). Both WS8 and WS17 identified all B cells although WS17 was principally reactive only

Table 9
Summary of monoclonal antibodies to equine leucocyte antigens not examined in ELAW II

Specificity	Clone	Notes	Citation
CD3	PC3/188A	Cross-reactive antibody raised against synthetic peptide sequences from cytoplasmic region of antigen.	(Jones et al., 1993)
CD4	LB4A1	Described in Section 5 in this report	ELAW II report
CD8	LB3G11	Described in Section 5 in this report	ELAW II report
CD18	MHM23	An anti-human CD18 mAb that recognizes all equine leucocytes on FACS analysis.	(Jacobsen et al., 1993)
CD18	CA16.2G1	An anti-canine CD18 mAb that recognizes all equine leucocytes on FACS analysis.	P.F. Moore[a]
CD41/61	Co.35E4 and Co.2oA1	Characterized by cellular distribution and immunoprecipitation as recognizing equine glycoprotein IIb/IIIa (the homologous human antigen is designated CD41/61) This β3 integrin adhesion receptor is expressed on platelets only.	(Pintado et al., 1995)
CD79a	HM57; M7051, Dako	Cross-reactive antibody raised against synthetic peptide sequences from cytoplasmic region of CD79a, a B-cell marker also known as MB-1 which is part of the B cell antigen receptor complex. CD79a is expressed at the pre-B cell stage and continues to be present on B-cells throughout their differentiation.	(Jones et al., 1993)
MHC I	6 reagents, see citation	A panel of mAbs raised against equine MHC I (4 mAbs) or anti-murine mAbs that cross-react with equine MHC I (2 mAbs)	(Donaldson et al., 1988)
MHC II	F39.2	A rat mAb raised against equine MHC II	(Crepaldi et al., 1986)
MHC II	7 reagents, see citation	A series of rat and murine mAbs recognizing HLA-DR, DQ and DP antigens were shown to recognize class II MHC antigens on equine lymphocytes on FACS analysis.	(Monos et al., 1989)
B cells	B29A	Partially characterized in the First Equine Leucocyte Workshop as mAb WS68, this reagent was subsequently described in a second publication as recognizing a complex B cell surface antigen complex not described in other species.	(Kydd et al., 1994; Tumas et al., 1994)
NK cells	5C6	A mAb that recognizes a functifunction-associated molecule on fish NK cells that is evolutionarily conserved in man, sheep, cattle, and horses.	(Harris et al., 1993)

Table 9 (continued)

Specificity	Clone	Notes	Citation
Macrophages	1.646	Recognizes a cytoplasmic antigen of equine mononuclear phagocytes consisting of two proteins (150 and 30 kDa). This product works on deparaffinised formalin fixed tissues for immunohistological staining.	(Sellon et al., 1993)
Granulocytes and macrophages	DH59B	Partially characterized in the First Equine Leucocyte Workshop as mAb WS25, this reagent was subsequently described in a second publication as recognizing a pan-granulocyte/monocyte 96 kDa antigen	(Kydd et al., 1994; Tumas et al., 1994)

with IgG isotypes in ELISAs against purified Ig (Table 6). Data presented in an accompanying paper (Sheoran et al., 1997) show that WS17 identifies one of the equine light chain molecules.

5. Non-ELAW II antibodies recognizing equine leucocyte antigens

Several reagents capable of recognizing equine leucocyte differentiation antigens were not submitted to either of the equine leucocyte workshops, but nevertheless represent useful reagents. A listing of published reagents known to react or cross-react with equine antigens is given in Table 9 together with citations. In addition further reagents are available which have not been described in the literature but may prove valuable with further characterization and will be briefly described here.

Table 10
Flow cytometric analysis of equine leucocyte populations labeled with Tochigi monoclonal antibodies putatively reactive with EqCD4 (LB4A1) and EqCD8 (LB3G11)

mAb	Percentage of positive (labeled) cells[a]		
	PBL[b]	PBL[c]	Granulocytes[b]
LB3G11	19 (10–30) $n=8$	12 (10–15) $n=4$	1 (0–2) $n=3$
LB4A1	53 (28–62) $n=8$	67 (61–69) $n=4$	1 (0–2) $n=3$

[a]Percentage of positive (labeled) cells are reported with mean and range given when more than one determination was made; 0, negative, no labeling detectable above the negative control.
[b]Data from Tochigi laboratory.
[c]Data from Madison laboratory.

At the ELAW II workshop meeting, Drs. Kondo and Sugiura of the Equine Research Institute, Tochigi, Japan, described two additional reagents from their laboratory that were not submitted to ELAW II. These reagents are both of the murine IgG1 isotype and are named LB4A1 and LB3G11, and putatively recognize EqCD4 and EqCD8 respectively. Single color FACS analyses from the Tochigi and Madison laboratory are presented in Table 10. Results of two color FACS analysis performed by the Tochigi laboratory are presented in Fig. 15 and demonstrate that these reagents recognize two mutually exclusive T cell subsets. After the ELAW II meeting the Madison laboratory repeated the two color FACS analyses comparing these reagents to another EqCD5 reagent (WS81) and confirmed these observations in two separate experiments in different horses. In addition the Madison laboratory demonstrated blocking of binding of the EqCD8 antibody by LB3G11 in two FACS experiments identical to those described in Section 3.5. Subsequently the Tochigi laboratory provided immunoprecipitation data

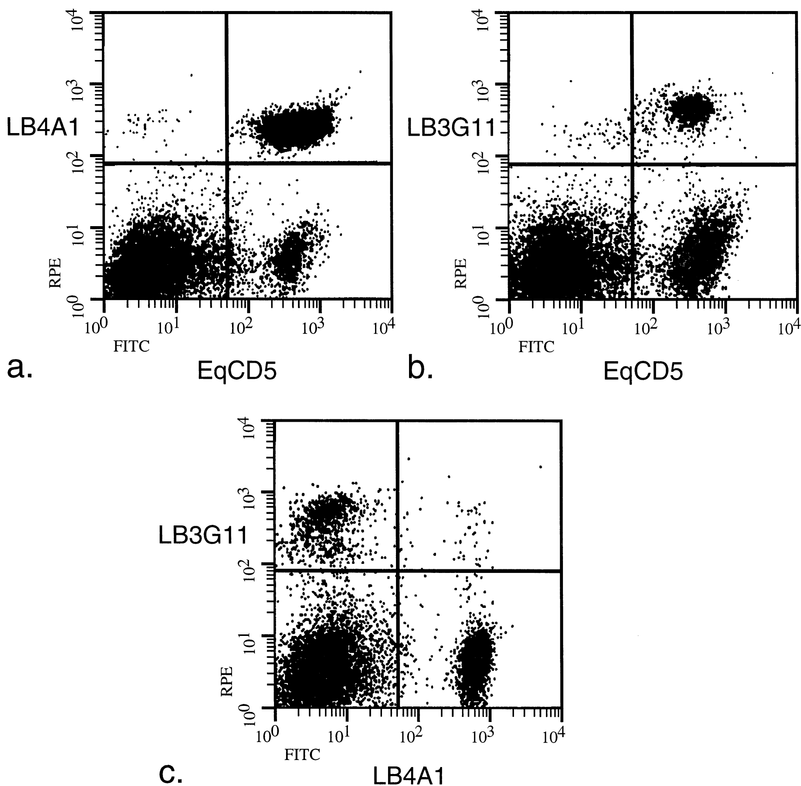

Fig. 15. Two color FACS profiles of lymphocytes from an adult horse stained with WS71 (EqCD5) on the horizontal axis and (a) LB4A1 (anti-EqCD4) and (b) LB3G11 (anti-EqCD8), on the vertical axis. These results demonstrate that both antibodies recognize T cell subsets (EqCD5 + ve). The results of two color FACS staining with both LB4A1 (horizontal axis) and LB3G11 (vertical axis) are shown in c. This result demonstrates that both antibodies stain mutually exclusive subsets. Negative markers were set with irrelevant mAbs.

using these two antibodies, and it is presented in Fig. 2. This data indicates that the putative EqCD4 antibody (LB4A1) precipitates a 58 kDa antigen in reducing conditions, and the putative EqCD8 antibody (LB3G11) precipitates a heterodimer of 32 and 39 kDa in reducing conditions. Overall these data are consistent with the proposed specificities of these mAbs. Formal assignment of these reagents will be made in a further workshop.

During the course of ELAW II the Madison laboratory conducted a series of FACS analyses in six separate horses using two commercially available mAbs (MY4, Coulter; and Tuk 4, Dako) that recognize human CD14 and have been demonstrated to cross-react with these antigens in other species (Jacobsen et al., 1993; B.D. Darien and S. Helfand, Madison, WI, personal communication). No equine leucocyte populations stained with either of these reagents indicating their inability to identify an equine orthologue of CD14.

6. Summary and conclusions

The final assignment of antibody clusters for leucocyte antigens and immunoglobulins, as described in detail in Sections 3 and 4, is summarized in Table 4. Together with other mAbs developed outside of ELAW II (Table 9) this pool of reagents represent a powerful array of tools for the study of equine immunity. The Second Equine Leucocyte Antigen Workshop made considerable advances in pursuing the objectives of establishing the specificities of mAbs and achieving consensus on the nomenclature for equine leucocyte and immunoglobulin molecules. Of equal importance, several productive collaborations were fostered among the participating laboratories and observers. Overall, enormous advances have been made in the past decade since mAbs specific for equine leucocyte antigens and immunoglobulins were first reported.

There remains enormous scope and need for further studies of equine leucocyte antigens and immunoglobulins, both for the purposes of comparative immunology and for the good of the horse. In the future novel techniques will be required to develop reagents for specific target antigens such as the orthologues of the CD25 or CD45 isoforms. In studies of equine immunoglobulins the functional role of the IgG isotypes must be better established, reagents for IgE must be developed, and cloning of the immunoglobulin heavy chain genes will be essential if the complexities of the IgG sub-isotypes are to be elucidated. The tasks still facing the currently small group of equine immunologists throughout the world remain formidable, and will only be tackled successfully in a spirit of collaboration.

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