

## Three monoclonal antibodies identifying antigens on all equine T lymphocytes, and two mutually exclusive T-lymphocyte subsets

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### SUMMARY

The aim of this study was to produce monoclonal antibodies (mAb) recognizing equine lymphocyte surface antigens. Fusions were conducted using BALB/c mice hyperimmunized with equine thymocytes. Hybridoma supernatants were screened by flow cytometry and positive hybridomas were cloned twice by limiting dilution. These mAb were then characterized for tissue distribution by immunohistology and flow cytometry, and by precipitation and analysis of the lymphocyte antigens which they recognized. Three mAb (CVS5, CVS4 and CVS8) are described which recognize only T lymphocytes in peripheral blood. Two-colour immunofluorescent studies showed that CVS5 recognized all T lymphocytes and that CVS4 and CVS8 recognized two mutually exclusive subsets of CVS5-positive cells. In the thymus there was a large population of CVS4/CVS8 double-positive cells. Immunohistochemical staining with these mAb was restricted to T-lymphocyte areas. CVS4 and CVS5 precipitated molecules of 58,000 and 69,000 MW, respectively, in both reducing and non-reducing conditions. CVS8 precipitated two molecules of 32,000 and 39,000 MW in reducing conditions, and one molecule of 69,000 MW in non-reducing conditions. This evidence suggests that CVS5, CVS4 and CVS8 recognize the equine homologues of CD5, CD4 and CD8, and that the characteristics of these antigens are similar to those of other species.

### INTRODUCTION

Monoclonal antibodies (mAb) recognizing lymphocyte surface antigens have proven invaluable in immunological research, in that they allow the definition of the immunophenotype of lymphocyte subsets and the biochemical analysis of the antigen which they recognize. A number of such mAb are available for the large domestic species,<sup>1,2</sup> but very few have been produced for use in the horse.

The lymphocyte pool is divided into T and B lymphocytes, and T lymphocytes in man are further divided by the expression of the CD4 or CD8 antigens, which have important roles as accessory and differentiation molecules.<sup>3,4</sup> These lymphocyte surface antigens are best characterized by mAb in man and laboratory rodents,<sup>5</sup> but their homologues have also been defined in several domestic species.<sup>6–11</sup> In the cow, for example, the BoT4 and BoT8 antigens are considered the homologues of CD4 and CD8,<sup>6,8</sup> and their expression in peripheral T lymphocytes and thymocytes has been shown to be similar to that in man.<sup>2</sup>

In the horse few mAb recognizing non-major histocompatibility complex (MHC) lymphocyte surface antigens have been produced. Two reports have described mAb defining T lympho-

cytes, and possibly recognizing an equine homologue of the CD5 antigen.<sup>12,13</sup> The study of equine T-lymphocyte differentiation is of considerable interest in the context of the hereditary condition of severe combined immunodeficiency disease in Arabian foals, which results in the absence of mature circulating T and B lymphocytes.<sup>14</sup> In this condition investigations suggest that the thymic and bone marrow microenvironments are normal and that there is entry of prothymocytes into the thymus.<sup>15,16</sup> However, the further maturation of lymphocytes fails and the normal cortical thymocyte population is not seen. Further investigation of this disease requires the production of additional markers for T-lymphocyte subpopulations.<sup>17</sup> Similarly, the immune response to important equine diseases with a world-wide effect upon the horse industry, such as equine rhinopneumonitis,<sup>18</sup> remain incompletely investigated due to the lack of such reagents.

This report describes three mAb which recognize antigens defining equine T lymphocytes, and two lymphocyte subsets which are mutually exclusive in peripheral blood. The characteristics of these antigens suggest that they may be the equine homologues of CD5, CD4 and CD8.

### MATERIALS AND METHODS

#### *Preparation of cell populations*

Equine mononuclear cells were isolated from peripheral blood collected into EDTA and centrifuged over Ficoll-Hypaque

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**Table 1.** Flow cytometric analyses of immunofluorescent staining with FITC/anti-EqIg, CVS5, CVS4, and CVS8, of isolated PBL and lymphocyte subpopulations

	PBL	T lymphocytes	B lymphocytes	Thymocytes (n = 3)
% FITC/anti-EqIg	37.6 ± 13.0	4.8 ± 1.9	88.4 ± 5.2	2.9 ± 0.6
% CVS5	65.4 ± 14.4	92.6 ± 3.4	8.4 ± 3.0	87.7 ± 6.0
% CVS4	42.0 ± 10.5	68.2 ± 5.0	5.9 ± 3.5	73.0 ± 11.5
% CVS8	9.6 ± 5.0	18.0 ± 6.6	2.3 ± 0.6	71.7 ± 1.1

Mean ± SD, n = 5 unless stated otherwise.

**Table 2.** Expression and co-expression of membrane antigens recognized by CVS5, CVS4 and CVS8 on T lymphocytes (percentage cells staining positively) determined by flow cytometric analyses of immunofluorescent staining

Horse	1 (%)	2 (%)	3 (%)
% CVS4	20	12	27
% CVS8	62	71	60
% mathematical sum of CVS4- and CVS8-positive cells	<u>82</u>	<u>83</u>	<u>87</u>
% Dual staining by CVS4 and CVS8	84	82	86
% CVS5	94	91	88

Results of three experiments in individual horses.

(Nycomed, Oslo, Norway), as described by Bright *et al.*<sup>19</sup> These peripheral blood mononuclear cell (PBMC) preparations were depleted of monocytes by carbonyl iron adherence to yield peripheral blood lymphocytes (PBL) where indicated. The PBL were also separated into T- and B-lymphocyte populations by non-specific panning on uncoated plastic Petri dishes (Optilux-Falcon 1005, Plymouth, Devon, U.K.), as described by Crepaldi *et al.*<sup>20</sup> Equine thymocytes were prepared from foals killed at under 1 year of age. Thymus tissue was teased apart prior to homogenization to yield a single-cell suspension which was processed as for PBMC.

Granulocytes were prepared by treating the red cell and granulocyte pellet, resulting from the Ficoll-Hypaque treatment of blood described above, with distilled water for 30 seconds, followed by addition of 10 times phosphate-buffered saline (PBS) to restore isotonicity. Lysed red cell ghosts and haemoglobin were then removed by several washes in PBS plus 0.4% foetal calf serum (FCS).

#### Production of monoclonal antibodies

BALB/c mice were immunized by intraperitoneal injections of  $1 \times 10^7$  thymocytes three times at 2-monthly intervals. Three days after the final immunization, mice were killed and spleen cells were fused with X63-Ag 8-653 myeloma cells.<sup>21</sup> Hybridomas were assayed for specific antibody production at 14 days by immunofluorescent staining of PBMC and detection by flow cytometry. Selected hybridomas were cloned twice by limiting dilution and expanded to larger volumes. Antibody-containing ascitic fluid was produced in BALB/c mice by conventional methods. Monoclonal antibody isotype was determined using a commercial isotyping kit based on reverse passive red cell agglutination and isotype specific reagents (Serotec, Oxford,

U.K.). Monoclonal antibodies were biotinylated according to a standard protocol,<sup>22</sup> after purification from ascitic fluid by ammonium sulphate precipitation.

#### Immunofluorescent staining

For immunofluorescent staining, all cells were suspended at  $4 \times 10^4$  cells/ml in PBS/0.1% sodium azide plus 0.4% FCS, except for experiments employing avidin-conjugated reagents, where 0.01% bovine serum albumin (BSA) was substituted for the FCS. All incubations were performed at 4°C for periods exceeding 30 min, and cells were washed three times between each incubation step. Direct immunofluorescent staining was performed with a FITC-conjugated polyclonal affinity-purified rabbit antisera to equine immunoglobulins (FITC/anti-EqIg). Indirect immunofluorescence was used for all other single- and two-colour analysis. For single-colour immunofluorescence, and as a screening procedure for the production of the mAb, 50 µl of the cell suspension were incubated with 50 µl of mAb as hybridoma supernatant. Cells were then incubated with a FITC-conjugated F(ab')<sub>2</sub> sheep anti-mouse immunoglobulin reagent (Sigma, Poole, Dorset, U.K.) prior to analysis by flow cytometry using a FACScan analyser (Becton-Dickinson, Mountain View, CA). In all flow cytometric studies 10,000 events were collected and these data were analysed using a gate based on forward and orthogonal light scatter in order to identify only lymphocytes or granulocytes, as required.<sup>23</sup>

For two-colour studies, the single-colour staining protocol was followed, followed by incubation in 1.5% heat-inactivated normal mouse serum (Serotec) to block remaining binding sites on the FITC conjugate. Cells were then incubated with a suitably diluted biotinylated second antibody, and with strepta-

**Table 3.** Expression and co-expression of membrane antigens recognized by CVS5 and FITC/anti-EqIg on PBL (percentage cells staining positively) determined by flow cytometric analyses of immunofluorescent staining

Horse	1	2	3
% CVS5	70	50	71
% FITC/anti-EqIg	34	50	23
% mathematical sum of CVS5- and % FITC/anti-EqIg-positive cells	104	100	94
% Dual staining by CVS5 and FITC/anti-EqIg	98	97	93

Results of three experiments in individual horses.

vidin-conjugated R-phycoerythrin RPE (Serotec) prior to flow cytometric analyses.

#### Immunohistochemistry

Tissues were collected at post mortem from horses of less than 1 year of age, and 8  $\mu$ m cryostat sections of the thymus and mesenteric lymph nodes were preserved and subsequently stained as described by Howard *et al.*<sup>24</sup>

#### Immunoprecipitation and SDS-PAGE analysis

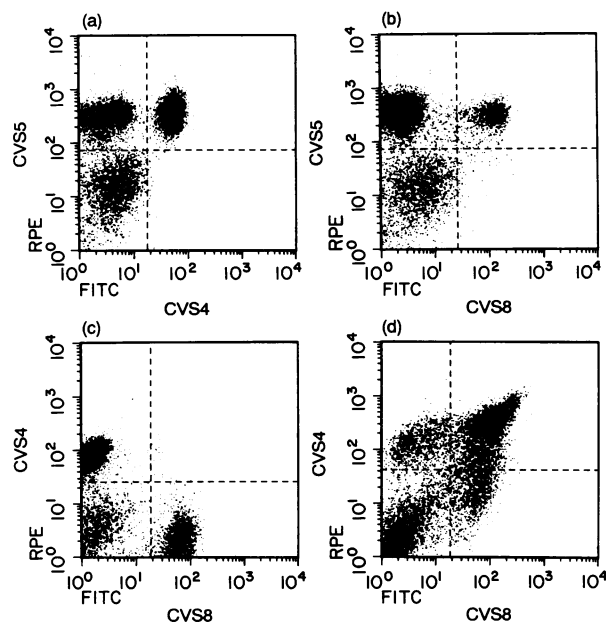
Cell-surface antigens recognized by mAb were analysed by radioiodination, immunoprecipitation and autoradiography. For the purpose of immunoprecipitations, Sepharose beads coupled to rabbit-anti-murine immunoglobulin were prepared. Rabbit anti-mouse serum was affinity purified on mouse immunoglobulin bound to a Sepharose column, and then coupled to cyanogen bromide-activated Sepharose beads (Pharmacia, Poole, Dorset, U.K.)

PBMC were prepared and  $1.5 \times 10^8$  cells were labelled with 1 mCi of  $^{125}$ I (Amersham, Aylesbury, Bucks, U.K.) by the lactoperoxidase-glucose oxidase method.<sup>25</sup> The reaction was stopped by washing in PBS/0.1% sodium azide and cells were then resuspended in 2 ml of lysis buffer (2% NP40, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 20 mM Tris HCl, 1 mM phenol methyl sulphonic acid, pH 8.0). After a 30 min incubation at 4 $^{\circ}$ , the lysate was centrifuged at 10,000 g for 10 min and 100  $\mu$ l aliquots of the supernatant were precleared by addition of 14  $\mu$ l of the anti-mouse Ig-coupled beads and end-over-end rotation at 4 $^{\circ}$  for 1 hr. Beads were then removed by centrifugation and 70  $\mu$ l of mAb supernatant added to the lysate. After a 30-min incubation at 4 $^{\circ}$ , 20  $\mu$ l of the anti-mouse Ig coupled beads were added and incubated for 1 hr with end-over-end rotation. The beads were then washed three times in 1-ml volumes of buffer (0.5% NP40, 10 mM EDTA, 100 mM Tris-HCl, 150 mM NaCl, pH 8.0) and boiled for 2 min in reducing or non-reducing sample buffer (2.3% SDS, 10% glycerol, 0.6 M Tris-HCl, pH 6.8; plus 5% 2-ME for reducing buffer). The supernatants were analysed by SDS-PAGE in 12% gels with standard molecular weight markers (Bio-Rad, Hemel Hempstead, Hertfordshire, U.K.). Gels were dried and autoradiographed using Fuji RX film and intensifying screens at -70 $^{\circ}$ . Exposure times varied from 4 to 7 days.

## RESULTS

### mAb recognizing lymphocyte surface antigens

Two fusions were performed, resulting in the generation of very large numbers of hybridomas. Eight-hundred wells containing

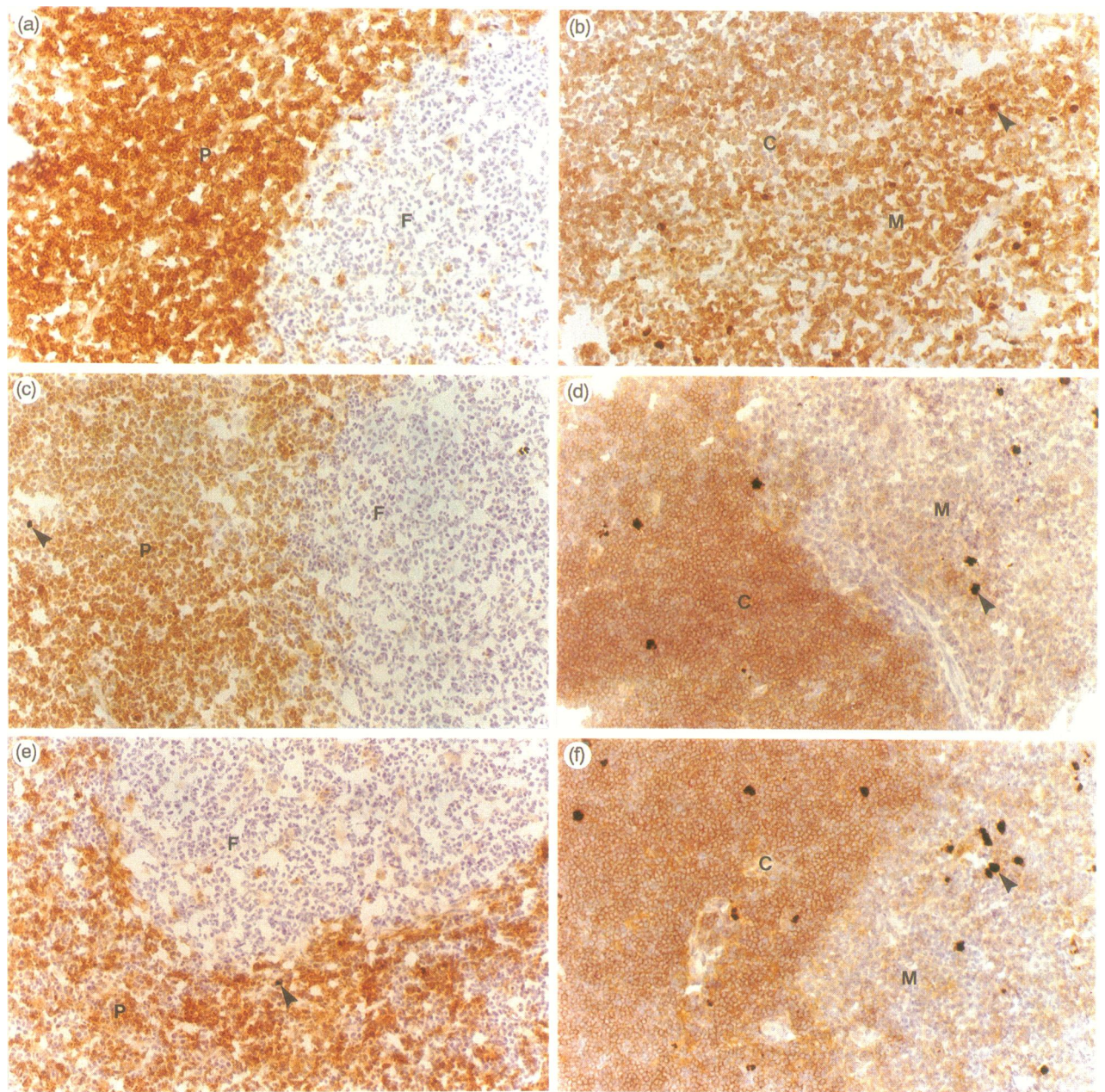


**Figure 1.** Two-colour flow cytometric analyses of immunofluorescent staining of PBL (a, b and c) or thymocytes (d) labelled with CVS5, CVS4 or CVS8. Dotted marker lines were set using an irrelevant negative control antibody.

hybridomas were screened and 26 were selected for cloning, resulting in the production of a panel of eight mAb. Three mAb of isotype IgG1, named CVS4, CVS5, and CVS8, are described in this paper.

### Tissue distribution of antigens determined by flow cytometry

*Distribution of membrane antigens on separated cell populations.* The distribution of staining by the three mAb was studied in separated populations of PBL, T and B lymphocytes, prepared from five adult horses, and in thymocytes prepared from three foals of 7 months of age. The number of lymphocytes bearing surface immunoglobulin in each population was determined by direct immunofluorescent staining using the FITC/anti-EqIg (Table 1). The distribution of staining in PBL, T and B lymphocytes and in thymocytes, for each of the three antibodies as detected by indirect immunofluorescence, is described in Table 1. These results indicate that in populations



**Figure 2.** Immunoperoxidase staining of lymph node (a, c and e) or thymus (b, d and f) with CVS5 (a and b), CVS4 (c and d) or CVS8 (e and f). In thymus sections the cortex (C) and medulla (M) are labelled, and in lymph node sections germinal follicles (F) and the paracortical regions (P) are labelled. In both tissues the darkest cells (arrows) were also stained in negative control sections, and probably contain endogenous peroxidase. Magnification  $\times 200$ .

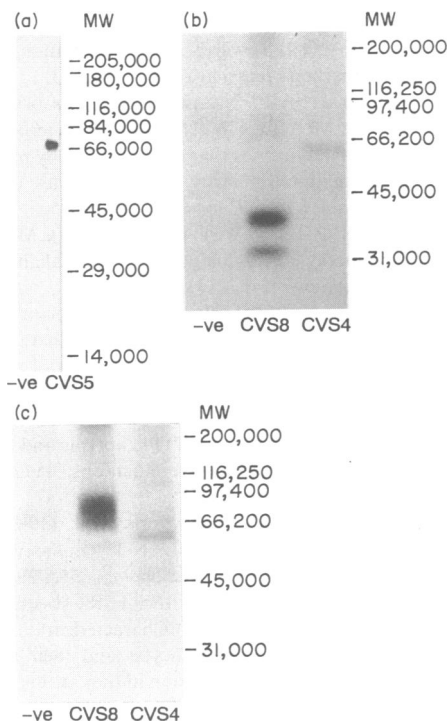
of PBL, CVS5 is a T-lymphocyte marker, and that both CVS4 and CVS8 recognize subpopulations of T lymphocytes.

Almost all thymocytes were negative for surface immunoglobulin, but 88% were positive for CVS5, and antibodies CVS4 and CVS8 both stained approximately 70% (Table 1). None of the mAb were found to recognize granulocytes from the five individual horses that were studied.

*Determination of co-expression of membrane antigens.* The co-expression of antigens recognized by CVS4 and CVS8 was initially investigated by determination of the percentage of T lymphocytes stained by each antibody individually, and the total stained when cells were incubated with both mAb prior to

incubation with the FITC conjugate. The results of three experiments in individual horses are presented in Table 2, and they provide strong evidence that CVS4 and CVS8 recognize mutually exclusive peripheral T-lymphocyte subpopulations.

The co-expression of antigens recognized by CVS5 and the FITC/anti-EqIg conjugate was similarly investigated by first incubating PBL with the latter reagent, and then staining them normally with CVS5. Cells were also stained individually with each reagent. The results are presented in Table 3, and indicate that CVS5 and FITC/anti-EqIg recognize mutually exclusive lymphocyte subsets which in total comprise all or the majority of PBL. This is also supported by the results depicted in Table 1 of



**Figure 3.** Immunoprecipitation of lysates of  $^{125}\text{I}$ -labelled PBMC incubated with the mAb, and then precipitated with anti-murine immunoglobulin coupled to Sepharose. Reduced or non-reduced samples were subject to SDS-12% PAGE and autoradiography. (a) Reduced samples precipitated with an irrelevant negative control mAb (-ve) or CVS5. (b) Reduced samples precipitated with a control mAb (-ve) or with CVS8 or CVS4. (c) Non-reduced samples precipitated with a control mAb (-ve) or with CVS8 or CVS4.

the staining of PBL by these two reagents, and this provides further evidence that CVS5 is a T-lymphocyte marker.

Two-colour immunofluorescent staining was used to determine the co-expression of the antigens recognized by the mAb and FITC/anti-EqIg. For this purpose both CVS5 and CVS4 were biotinylated. The staining of CVS5 was compared with that of both CVS4 and CVS8 in PBL from three adult horses, and in each instance it was found that both CVS4 and CVS8 were present only on cells positive for CVS5 (Fig. 1a, b). The staining of CVS5 was also compared with that of FITC/anti-EqIg in the same horses, and it was found that these reagents recognized two mutually exclusive lymphocyte populations, with less than 5% of cells remaining unstained by either reagent, and with less than 2% of cells staining with both reagents (results not shown). These results again indicated that CVS5 is a T-lymphocyte marker, and that both CVS4 and CVS8 recognize a subpopulation of CVS5-positive lymphocytes. The co-expression of antigens recognized by CVS4 and CVS8 was compared in the PBL of the same three adult horses and in three 7-month-old foals. In each instance positive staining by these mAb was found to be mutually exclusive with less than 0.7% double-positive cells (Fig. 1c), except for one adult with 3.3% double-positive cells.

In the thymocytes of the three foals the pattern of staining of CVS4 and CVS8 was very different (Fig. 1d), with a mean of 63% double-positive cells (standard deviation 12.8) and a mean of 18% double-negative cells (standard deviation 8.8).

These results demonstrate that CVS4 and CVS8 recognize mutually exclusive subsets of CVS5-positive lymphocytes in peripheral blood. However, the sum of CVS4 and CVS8 cells is equal to approximately 80% of the number of CVS5-positive cells in PBL preparations (Table 1) and 90% of CVS5-positive cells in T-lymphocyte preparations (Tables 1 and 2). This indicates the presence of a substantial number of lymphocytes positive for CVS5 staining but negative for CVS4 and CVS8.

#### Tissue distribution of antigens determined by immunohistology

The staining characteristics of each antibody in lymph node and thymus was examined using an indirect immunoperoxidase technique with diaminobenzidine as the substrate and haemalum as the counterstain. It was found that CVS5 stained all cells in the T-lymphocyte dependent areas surrounding the germinal follicles of lymph nodes, and a few cells within the germinal follicles (Fig. 2a). In the thymus CVS5 stained all thymocytes, with slightly more intense staining of cells within the medulla (Fig. 2b). In lymph nodes both CVS4 and CVS8 stained a subpopulation of cells within the paracortex, with CVS4 staining approximately twice as many cells as CVS8 (Fig. 2c, e, respectively). The staining of thymus sections obtained with CVS4 and CVS8 was similar, with both antibodies showing intense staining of almost all cortical cells, and less intense staining of a subpopulation of cells in the medulla (Figs. 2d, f, respectively). This pattern of staining suggests that a large proportion of cortical thymocytes are CVS4/CVS8 double positive.

#### MW of antigens as determined by immunoprecipitation and SDS-PAGE analysis

Immunoprecipitations using CVS5 and analysis under reducing and non-reducing conditions identified a molecule of 69,000 MW (Fig. 3a). The CVS4 antibody identified a 58,000 MW molecule in reducing and non-reducing conditions (Fig. 3b, c). The CVS8 antibody identified two bands of 32,000 MW and 39,000 MW in reducing conditions (Fig. 3b), and a single band of approximately 69,000 MW in non-reducing conditions (Fig. 3c). This indicated that the CVS8-reactive molecule was a disulphide-bonded heterodimer. These immunoprecipitation results were repeated in a total of three experiments.

## DISCUSSION

Lymphocytes recognized by the three mAb described appear to belong almost exclusively to the T-lymphocyte subset, on the basis of immunohistology and immunofluorescent staining of separated lymphocyte populations. The mAb CVS5 recognizes all or most T lymphocytes, and in immunoprecipitation experiments it identifies a 69,000 MW molecule in both reducing and non-reducing conditions. These characteristics are very similar to those of two other mAb defining equine T-lymphocytes described by Crump *et al.*<sup>12</sup> and Wyatt *et al.*<sup>13</sup> It has been postulated that the characteristics of these other reported T-lymphocyte markers indicate that they recognize an equine homologue of the CD5 antigen described in man<sup>26</sup> or the Lyt-1 antigen in mice,<sup>27</sup> and this would also appear to be the case for CVS5.

Single- and two-colour flow cytometry demonstrate that the mAb CVS4 and CVS8 both recognize mutually exclusive

subsets of CVS5-positive equine T lymphocytes. However, in the thymus there as a large CVS4<sup>+</sup>/CVS8<sup>+</sup> double-positive population. These data, combined with immunohistological staining and the biochemical characteristics of the molecule immunoprecipitated by these mAb, suggests that CVS4 and CVS8 recognize the equine homologues of the CD4 and CD8 antigens described in man.<sup>4</sup> These characteristics of the mAb CVS4 and CVS8 are also very similar to the homologues of CD4 and CD8 described in other domestic species, such as the sheep,<sup>10</sup> and cow.<sup>6,8</sup>

It was a consistent finding that the total number of CVS4- and CVS8-positive lymphocytes was less than the number of lymphocytes recognized by CVS5. If these mAb do recognize the CD homologues postulated, this would suggest the presence of a subset (approximately 10%) of CD5-positive, CD4/CD8 double-negative lymphocytes in the equine peripheral circulation. In man CD4<sup>-</sup>CD8<sup>-</sup> T lymphocytes (CD3<sup>+</sup>) comprise approximately 3% of circulating T lymphocytes<sup>28</sup> and express the  $\gamma\delta$  T-cell receptor (TcR). In the cow and sheep this lymphocyte phenotype comprises 5–17% of lymphocytes in young adults, and a frequently much higher proportion in juveniles.<sup>29,30</sup> It is possible that the CVS5-positive, CVS4/CVS8 double-negative lymphocytes may comprise those equine lymphocytes bearing a  $\gamma\delta$  TcR.

Establishing the homology of lymphocyte differentiation antigens in different species relies on the demonstrated conservation of the biochemical and functional characteristics of these antigens through evolution.<sup>27</sup> A more certain indicator of their conservation is comparison of the nucleotide sequence of their respective genomes, such as has been performed in the case of CD5 in man and the Lyt-1 antigen in the mouse.<sup>31</sup> Nevertheless, the biochemical and functional characteristics of lymphocyte antigens recognized by mAb have been used to putatively identify the homologues of CD5 in the cow and sheep,<sup>1,24</sup> and CD4 and CD8 in the cow, sheep, pig, cat and birds.<sup>6, 11</sup>

The evidence provided by this study suggests that the mAb CVS4, CVS5, and CVS8 may recognize equine homologues of the CD4, CD5 and CD8 antigens, and that these equine antigens share many of the characteristics of their counterparts in other species. These mAb are available by application to the authors.

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