The immunobiology of ovine γδ T cells

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This thesis is submitted as part of the course requirements for the degree of Doctor of Philosophy at the University of Edinburgh.

1995

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To my nana;

Missed so dearly and never forgotten.

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Declaration:

The experiments and composition of this thesis are, unless otherwise stated, my own work. No part of this work has been or is being submitted for any other degree, diploma or other qualification.

Brett T. Lund (August, 1995)

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ABSTRACT OF THESIS

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γδ T cells have been found in every species so far examined yet no function has been attributed to this population of lymphocytes. WC1 is a cell surface glycoprotein expressed exclusively by γδ T cells in ruminants. The function of this protein is unknown. The γδ TCR molecule is similar to the TCR expressed on αβ T cells. αβ T cells recognise foreign antigen which is presented on the surface of antigen presenting cells in association with MHC class I or MHC class II molecules. It has been a general assumption that γδ T cells, because of the similarity of the antigen receptor molecules, may recognise antigen, and therefore function in a similar manner to αβ T cells. This however has not yet been shown to be the case.

In this thesis, the expression of the WC1 protein on ovine γδ T cells was analysed. A large number of monoclonal antibodies exist which recognise epitopes of this molecule in the bovine species. Their reactivity with ovine WC1 protien was examined, both at a cellular level and protein level. The results in this thesis, along with those published by others during the course of the thesis, identified WC1 as a protein complex with great diversity at the genetic level, and possibly at the post-transciptional or post-translational level. Work in this thesis also confirmed that, with respect to the expression of WC1 proteins, γδ T cell immunochemistry in the ovine species is similar in some, but not all, respects to that seen in the bovine species.

γδ T cells are more prevelant in the ovine species than in either the human or murine species. This may imply that sheep have a greater requirement for γδ T cells than either the human or murine species. The *in vitro* activation and proliferative response of ovine γδ T cells, following *in vitro* culture with mitogen or antigen, was examined and compared to the responses of ovine αβ T cells. The responses by γδ T cells to both antigen and mitogen were different to the responses by αβ T cells. All γδ T cells expressed markers of activation within 8hrs of culture with mitogen, in contrast, it took 24hrs for all αβ T cells to express the same markers. In addition, all γδ T cells expressed markers of activation following culture with antigen whereas there was little change in the expression of these markers on αβ T cells. Purified γδ T cells proliferated following culture with mitogen but did not proliferate in response to culture with antigen. In addition, purified γδ T cells did not proliferate following culture with allogeneic lymphocytes. These result implied that the function of γδ T cells within the immune system may be markedly different to the function of αβ T cells.

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List of contents:

List of abbreviations:

APC	antigen presenting cells
ARAM	antigen recognition activation motif
bp	base pair
b-NMS	biotinylated normal mouse sera
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid (to ribonucleic acid)
Ci	curie
CD	cluster of differentiation
CDR	complementarity determining region
CIP	calf intestinal alkaline phosphatase
CNBr	Cyanogen bromide
ConA	concanavalin A
CPM	counts per minute
CTL	cytotoxic T lymphocyte
DAB	diamino benzimidine
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunoadsorbent assay
fHBSS	calcium and magnesium free Hank's balanced salt solution
FACScan	fluorescence activated cell scanner
FCS	fetal calf serum
FDA	fluorescein di-acetate
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	gravity
GALT	gut-associated lymphoid tissue
^{3}H-thy	tritiated thymidine
HEPES	4-2(hydroxyethyl)-1-piperazineethanesulfonic acid
HBSS	Hank's balanced salt solution
HLA	histocompatability leucocyte antigen
HSA	heat stable antigen
hsp	heat shock protein
iIEL	intestinal intra-epithelial lymphocyte
IFNγ	gamma interferon
Ig	immunoglobulin
IL	interleukin
IL-2R	interleukin-2 receptor
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LPL	lamina propria lymphocytes
LMP	low molecular mass proteins

mRNA	messenger RNA
MACS	magnetic activated cell sorter
M.tb.	*Mycobacterium tuberculosis*
MHC	major histocompatability complex
MLR	mixed lymphocyte reaction
MW	molecular weight
NBT	nitroblue tetrazolium
ND	not determined
NK	natural killer cells
NMS	normal mouse serum
OD	optical density
OVA	ovalbumin
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSF	phosphate buffered saline supplemented with 1% fetal calf serum
PCR	polymerase chain reaction
PLC	phospholipase C
PMSF	phenylmenthyl sulphonyl fluoride
PTK	protein-tyrosine kinases
PPD	purified protein derivative
RNA	ribonucleic acid
SA-PE	streptavidin-phycoerythrin
scid	severe combined immunodeficient mice
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDW	sterile distilled water
SI	stimulation index
sIg	surface immunoglobulin
SPF	specific pathogen free
SRCR	scavenger receptor cysteine rich
SSC	side scatter
TAP	transport associated with antigen processing
TCR	T cell receptor (for antigen)
TEMED	N,N,N',N'-tetramenthyl-1,2-diaminoethylene
TNF-α	tumour necrosis factor-alpha
Tris	tris(hydroxymenthyl)aminomethane
UV	ultraviolet
WC	workshop cluster

CHAPTER 1

Introduction

1.1 Cells of the immune system.

The cells of the immune system are made up of non-specific and specific elements. The non-specific components include killer cells (e.g natural killer (NK) cells and lymphokine activated killer (LAK) cells), granulocytes, macrophages, dendritic cells and other phagocytic and/or antigen presenting cells which may be both circulating or resident in tissue. The specific cells of the immune system are the lymphocytes. In all species so far examined, the lymphocyte population can be divided into subsets, T cells and B cells. The majority of T cells are produced in the thymus and express a specialised T cell receptor for processed antigen, they can be divided both phenotypically and functionally into subsets. B cells are produced in the bone marrow (or Bursa of Fabricus in birds) and recognise antigen via surface immunoglobulins.

Before discussing the populations of lymphocytes, it is worth defining some of the molecules used to describe the different populations. In order to characterise and co-ordinate the reactivity of the increasing number of monoclonal antibodies, a series of Leucocyte Differentiation Antigen Workshops were organised (Bernard et al., 1984; Reinherz et al., 1986; McMichael, 1987; Knapp,1990). Monoclonal antibodies which demonstrated identical patterns of cellular reactivity and immunoprecipitated the same molecular weight components as visualised by SDS-PAGE were grouped into clusters of differentiation (CD) and assigned a number.

CD3 was one of the first group of human T cell surface antigens identified by monoclonal antibodies and the CD3 complex is intimately associated with the TCR on the surface of all T cells (Clevers et al., 1988). CD3 is involved in signalling from the TCR and is comprised of three CD3 chains (γ, δ and ε) and two further transmembrane polypeptides (ζ, and η in a proportion of receptors). The surface proteins CD4 and CD8 are transmembrane glycoproteins which are expressed on mutually exclusive subpopulations of mature T lymphocytes (Maddon et al., 1985; Maddon et al., 1987; Parnes, 1989). The expression of CD4 and CD8 on cells is tightly correlated with the recognition of antigen in association with MHC class II and class I molecules respectively. CD4 is a nonpolymorphic glycoprotein of Mr 55kDa. It interacts with a nonpolymorphic determinant of MHC class II molecules on APC thereby increasing the avidity of the interaction of T cell and APC (Doyle and Strominger, 1987). CD4 has also been shown to associate with the TCR on cells involved in immune responses (Janeway, 1992). CD8 is a 70kDa heterodimer of an α and β chain, though this protein can be found on populations of lymphocytes as an α chain homodimer (e.g. intraepithelial lymphocytes). CD8 directly binds MHC class I molecules thereby contributing to the cytotoxic activity of CD8-positive T cells (Norment et al., 1988; Salter et al., 1989). A physical association between CD8 and the TCR has also been demonstrated (Janeway, 1992).

CD22 is a lineage specific B lymphocyte molecule expressed in the cytoplasm of all B lymphocytes including B cell progenitors. Surface expression is associated with B cell maturity and antigenic responsiveness.

1.1.1 T lymphocytes.

Once exported from the thymus, T cells migrate to T cell specific areas of the spleen, lymph nodes and other lymphoid organs. Many T cells continue in a migratory lifestyle, recirculating out of lymphoid tissues into the lymphatics, then via the thoracic duct to the blood, and again into lymphoid tissues through specialised sinuses or venules. A smaller proportion exits into nonlymphoid tissues and eventually makes its way to lymphoid tissues via afferent lymphatics.

1

This continual migration is believed to allow exposure, of a large proportion of the total pool of T cells, to antigen which may be quite locally distributed. Under normal circumstances, the great majority of the lymphocytes in a healthy individual are quiescent but following exposure to antigen T cells are activated, divide and become effector cells. Activation of T cells occurs primarily in the lymph nodes which drain the site of the infection and this leads to an expansion of all antigen specific cell populations. Each daughter T cell has an identical antigen specificity to that of its parent cell.

T cells play an important role in the function and regulation of the immune system. Once activated they directly or indirectly kill invading bacteria or parasites, infected or damaged cells, tumour cells and inopportunately, the bodies own cells. T cells regulate other T cells by cell-to-cell contact or through the release of soluble factors such as cytokines. T cells also regulate B cell function and are essential for expansion, differentiation and Ig class switching.

In all species so far examined, mature T cells can be divided into three subsets based on the expression of the proteins CD4 and CD8. CD4-positive cells are predominantly regulatory "helper cells" and react with foreign antigens associated with MHC class II molecules. These antigens are usually peptides derived from exogenous proteins. CD4-positive cells do not express CD8. CD8-positive T cells are mostly cytotoxic cells and react with antigens associated with MHC class I molecules. These antigens are usually peptides derived from endogenous molecules. CD8-positive cells do not express CD4. CD4-positive T cells and CD8-positive T cells use the $\alpha\beta$ dimer as their TCR. The third group of cells do not express CD4 or CD8 and do not use the $\alpha\beta$ dimer as their T cell receptor. Instead these cells express a $\gamma\delta$ TCR dimer. $\gamma\delta$ T cells have not yet been assigned any distinct function, nor have any presentational elements been identified which are specific for $\gamma\delta$ T cells.

1.1.1.1 Helper T cells.

Helper T lymphocytes are generally the CD4-positive population though some CD8-positive T cells and some $\gamma\delta$ T cells can carry out helper functions. The functional distinction of helper cells is in the array of cytokines that they produce as a consequence of antigen-stimulated activation. Cytokines act by binding to high-affinity receptors expressed on target cells and by inducing biochemical signals within those cells that profoundly affect their behaviour.

CD4-positive T cells do not constitute a homogeneous class of cells and cytokine biology has identified two subsets designated T_{H1} and T_{H2} helper T cells. When stimulated by receptor engagement, CD4-positive T cells produce IL-2, but little or no IL-4 or IFNγ (Swain et al., 1991). These pre-helper T cells have the potential to develop into either a population of T_{H1} helper cells or a population of T_{H2} helper cells. Much work has attempted to dissect the necessary steps of development, this is reviewed by Paul and Seder (1994). There is evidence that the ligand presented to CD4-positive T cells may itself regulate the T_{H1} or T_{H2} pattern of the responding cells (Murray et al., 1989) and this may be dependent on the density of the ligand presented. The cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 are secreted by T_{H2} cells and act on B cells. IL-4 acts as a costimulant of B cell growth and in the murine species also controls Ig class switching from IgM to IgG1 (IgG4 in humans), and IgM to IgE. IL-13 has a similar activity to that of IL-4 (Punnonen et al., 1993). IL-5 is important in the development of stimulated B cells into antibody secreting cells (Hamaoka and Ono, 1986) and IL-6 and IL-10 promote Ig secretion. T_{H1} cells produce the cytokines IL-2, IFNγ and TNFβ. These cells cause T cell mediated inflammation and

2

are well suited to induce enhanced microbicidal activity in macrophages and are therefore of particular value in intracellular infections. IL-10 and IL-4, secreted by T_{H2} cells, oppose the effects of IFNγ and thus these cells may play a role in the control of self-inflicted injury due to the effects of T_{H1} cells (Rapoport et al., 1993; Kuhn, 1993).

1.1.1.2 Cytotoxic T cells.

CTL play a major role in protection and recovery from viral, bacterial and parasitic infections. CTL also mediate allograft rejection and are implicated in certain autoimmune diseases and tumour immunity (Moller, 1983, 1988). Classically, CTL were described as those cells which recognise antigen in the context of MHC class I molecules. This occurs in association with the CD8 protein expressed on the surface of the target cell (Podack et al., 1991). MHC class II and CD4 restricted cytotoxicity does occur however, as do cytotoxic $\gamma\delta$ T cells. The first signal in cytotoxicity is antigen recognition by the TCR. The CTL then become activated and carry out their effector functions. A co-stimulatory signal for CD8 cells is not known though IL-1 is thought to be the co-stimulatory signal for CD4 restricted cytotoxicity.

Cytolysis is known to be unidirectional with the effector T cell spared the lethal cytotoxic hit delivered to the target cell (Fishelon and Berke, 1978; Zagury et al., 1979; Mueller and Tschopp, 1994). Two mechanisms for cytolysis have been identified, one based on the secretion of lytic proteins and one which depends on cell-surface ligand-receptor interaction (Squier and Cohen, 1994). The first mechanism involves perforin and serine esterases which are contained within cytoplasmic granules in CTL (Lu et al., 1992). Perforin has been demonstrated to form pores in cell membranes analogous to the complement proteins (Joag et al., 1989). The second mechanism involves the expression of the cell-surface protein Fas on the target cell and the ligand for Fas on CTL (Walsh et al., 1994; Lowin et al., 1994)

1.1.1.3 $\gamma\delta$ T cells.

$\gamma\delta$ T cells were not noticed by cellular immunologists in innumerable studies of humoral and cell mediated immune responses. It was the discovery of rearranged genes other than Ig and $\alpha\beta$ TCR rather than an observation of a new function which led to their discovery. The immunology of $\gamma\delta$ T cells has since progressed in a reverse direction to that of $\alpha\beta$ T cells. From discovery of the γ and δ genes, to the discovery of the TCR and discovery of a new class of lymphocyte. A definitive function for $\gamma\delta$ T cells has yet to be identified.

In 1984, monoclonal antibodies were raised against T cell clones and the subsequent identification of the TCR for antigen was possible. These monoclonal antibodies immunoprecipitated protein heterodimers of rearranged α and β gene transcripts (Allison et al., 1982; Meuer et al., 1983; Acuto et al., 1983). cDNA's encoding the TCR β subunit were first isolated by subtractive hybridisation and differential screening (Hedrick et al., 1984; Yanagi et al., 1984) and subsequently, candidate cDNA's encoding α genes were cloned. One of these genes did not have the consensus site for asparagine linked glycosylation and this cDNA rearrangement was termed the γ gene (Saito et al., 1984). The first evidence that a second heterodimer existed followed analysis of cells with monoclonal antibodies raised against the $\alpha\beta$ TCR molecule and monoclonal antibodies raised against the CD3 proteins (Brenner et al., 1986; Bank et al., 1986; Weiss et al., 1986; Littman et al., 1987). It was shown that there was a small percentage of peripheral blood CD3-positive T cells which did not express the $\alpha\beta$ TCR. These

3

cells were shown to express a CD3-associated heterodimer of a γ-chain with an unidentified chain. Antibodies raised against synthesised γ-chain peptides subsequently revealed the cell surface expression of a second heterodimer, the γδ TCR (Brenner et al., 1986). The δ gene was subsequently found located within the TCR γ locus and cloned (Chien et al., 1987). The products of the γ and δ genes were shown to be expressed as cell surface heterodimers and cells bearing this receptor represent a different class of T cells (Bank et al., 1986; Lanier et al., 1987)

There are few phenotypical and morphological differences between αβ and γδ T cells. In all species examined, γδ T cells do not generally express the proteins CD4 and CD8 (Groh et al., 1989; Hayward et al., 1989;. Miyawaki et al., 1990; Braakman et al., 1991; Inghirami et al., 1990; Faure et al., 1989; Cron et al., 1989; Mackay et al., 1988; Evans, 1993). In addition, in ruminants, γδ T cells do not express the protein CD2 (Mackay et al., 1988a; Evans, 1993) but uniquely express a protein called WC1 (previously referred to as T19). The WC1 molecule is the only known unique surface protein expressed on γδ T cells (MacKay et al., 1989; Clevers et al., 1990).

1.1.2 B cells.

B cells are responsible for antibody formation; each B cell produces an antibody with a single specificity (Awdeh et al., 1970). Antibodies are the antigen recognition molecules of the B cell and largely recognise conformational epitopes of regions of complex molecules such as glycoproteins (Warner, 1974). Antibody may be a secreted form or membrane-bound. The B cell receptor for antigen is the membrane bound form of antibody. Activation of B cells in peripheral lymphoid organs leads to the secretion of antibody by the plasma cell progeny of these B cells. The antibodies are identical or closely related to the cell surface receptors present on the B cell progenitors that were triggered (Tonegawa et al., 1981; Leder, 1982). Each type of antibody plays a different role in the body (Spiegelberg, 1974; Davies et al., 1990). IgA molecules are distributed in the serum and in secretory fluids from the salivary glands and gastrointestinal system (Fisher et al., 1979). IgG proteins are the most abundant antibodies in the serum, while IgD molecules are found predominantly on the surface of B lymphocytes (Spiegelberg, 1974). IgE antibodies are mediators of allergic responses and of immune reactions to parasitic infections (Ishizada and Ishizada, 1975). IgM proteins occur in two forms, as a receptor on the surface of B cells (Spiegelberg, 1974) or a secreted form in the serum, which is a pentameric structure linked by an additional polypeptide called the J chain.

Antibodies consist of four polypeptide chains or multiples thereof. There are two mostly unglycosylated light chains and two glycosylated heavy chains (Edelman, 1970; Porter, 1973). In the mouse, light chains fall into two groups (κ and λ) while the heavy chains can be divided into five classes (α, γ, δ, ε and μ). The genes encoding the heavy and light chain of antibodies are made up from segments which have to be rearranged before the genes can be transcribed and translated (Hanjo et al., 1989). The H gene is made from four distinct clusters of gene segments encoding the variable (V), diversity (D), joining (J) and constant (C) regions. Light chains are made from V J and C region segments. Light chains associate with any of the heavy chains to produce IgA(α), IgG(γ), IgD(δ), IgE(ε) and IgM(μ) antibodies. The heavy chain used is indicated in parenthesis. The amino terminal domains of the two chains are variable from antibody to antibody and are the antigen binding site. The more carboxyterminal constant (C) domains are involved in functions of the antibody which are unrelated to their antigen-binding specificities. Many of the effector functions of antibodies are mediated by the binding of C

4

domains to complement proteins or to Fc receptors expressed on other cells (Reid and Porter, 1981). Different classes of antibodies bind to different types of Fc receptor. Binding of antibody to Fc receptors can induce antibody-dependent cellular cytotoxicity (ADCC), antigen uptake for processing and presentation to T cells by antigen presenting cells, and immunoglobulin class switching. ADCC is carried out by killer cells and is IgG dependent although other forms of antibody can synergise with IgG to improve the ADCC responses.

1.2 Ontogeny of the immune system.

The immune system is built up from a small number of embryonic stem cells. These cells persist throughout the life of an individual and replenish the immune system. Such cells have been used experimentally as transplantable cells to regenerate parts of the immune system (Ikuta et al., 1992). The most primitive known progenitor of lymphoid cells is the pluripotent hematopoietic stem cell (HSC). HSC express little or no surface markers which are characteristic of committed blood cell lineages. However, in the human and mouse, these cells have been shown to include in their progeny, $\gamma\delta$ T cells, $\alpha\beta$ T cells and precursors of inflammatory cells (Spangrude and Weissman, 1988; Ikuta et al., 1990). In addition, HSC have the capacity to self-renew and have been shown to protect lethally irradiated hosts following transplantation by the early and sustained reconstitution of all blood cell types, including stem cells (Spangrude et al., 1988a).

During the ontogeny of the immune system, B cells progenitors are the first lymphocyte committed precursors to be seen (Kantor and Herzenberg, 1993). These B-lineage precursors are found in the embryonic part of the placenta and in embryonic blood at 10-14 days of gestation. At this time, they then enter the stromal compartments of the bone marrow where B cell differentiation takes place. At the same time, HSC committed to differentiate along T cell lineages migrate from the bone marrow to the cortical regions of the thymus. The thymic stroma contains elements of both ectodermal and endodermal origin which are important during the thymic differentiation of thymocytes (Goldstein and MacKay, 1969; von Gaudecker, 1986). The HSC seed the thymus and in a short time begin to express Thy-1 and CD5, characteristic of mouse thymocytes (Ezine et al., 1987; Spangrude et al., 1988b, 1989). The process by which stem cells develop into immunocompetent, exportable T lymphocytes is an elaborate one which takes about three weeks (Shortman, 1992).

1.3 Thymic development of T cells.

The first major developmental event in T cell production occurs when HSC seed the thymus. Under the influence of chemoattractants such as β2-microglobulin (Dunon et al., 1990; Zijlstra et al., 1990; Koller et al., 1990) and using homing receptors such as CD44 (Pgp-1) (Rothenberg 1992; O'Neill, 1989) the HSC localise at the thymic corticomedullary junction (Penit and Vasseur, 1988). HSC then begin to express the thymic T-cell markers Thy-1 and CD44 (Nikolic-Zugic, 1991). The cells are initially CD8-negative, TCR-negative, CD3-negative and express moderate levels of CD4 (Wu et al., 1991a). These then become CD4-negative and express high levels of HSA (Adkins et al., 1987; Rothenberg, 1992). Subsequent transient expression of CD25 has been reported (Shimonkevitz et al., 1987). By 15 to 20 weeks of gestation, T cell precursors expressing $\alpha\beta$ and $\gamma\delta$ TCR are present in the thymus (Wilson et al., 1992) and these cells are now committed to their T cell lineage (Petrie et al., 1990). $\alpha\beta$ and $\gamma\delta$ T cells develop from the same precursor cell though rearrangements and surface expression of the γ and δ genes precede those of the α and β genes (Raulet et al., 1985; Pardoll et al., 1987; Itohara et al., 1989).

5

Using γδ TCR transgenic mice it was suggested that αβ and γδ T cell lineages segregate prior to the expression of either TCR on the cell surface (Bonneville et al., 1989; Ishida et al., 1990). It was also shown that γδ T cells developed normally in αβ T cell deficient mice (Mombaerts et al., 1992) and that normal αβ T cell development occurs without γδ T cells (Itohara et al., 1993). Furthermore, nonproductive rearrangements of γ genes are commonly found in αβ T cells (Heilig and Tonegawa, 1987). It is widely known that a variety of different cytokines intervene as paracrine and autocrine growth and differentiation factors in intrathymic T-cell differentiation (Barcena et al., 1991; Gutierrez-Ramos et al., 1991)

1.3.1 T cell receptor variable and constant genes.

The TCR loci, termed α, β, γ and δ, consist of variable (V), joined (J), diverse (D - in the case of the β and δ loci) and constant (C) regions. These regions undergo somatic rearrangements and generate functional genes. The rearrangements are mediated by processes similar to those that control the rearrangement of immunoglobulin genes. This comprises both looping out and deletion of gene segments (Fujimoto and Yamagishi, 1987; Okazaki et al., 1987; Lai et al., 1987) and inversion (Malissen et al., 1986).

In the human and murine species the TCR γ and δ loci contain a limited number of germline V and C segments. This is in contrast to the TCR α and β loci. In humans, 14 Vγ genes (of which only 7 or 8 are functional) belonging to four families have been identified along with 6 Vδ genes (Moretta et al., 1991). The murine γ locus encodes 7 Vγ genes belonging to 5 families whilst the δ locus encodes 8 individual Vδ genes (Raulet, 1989). In the human species , some 700 V region recombinations are possible for the γδ heterodimer, whilst some 2500 αβ TCR combinations are thought possible (Davis and Bjorkman, 1988).

Despite the limited number of these germline V and C segments there is extensive junctional diversity. The variability in the junctional region is due to the imprecision in the joining processes and the addition of short stretches of nucleotides, called N regions, for both TCR γ and δ genes. N-nucleotide insertion in V-J joints is controlled by the expression of the terminal deoxynucleotidyl transferase gene (Alt and Baltimore, 1992; Komori et al., 1993; Gilfillan et al., 1993). Because of the N regions, the number of different junctional recombinations for the human γδ heterodimer are higher than those of the αβ heterodimer (Brenner et al., 1988; Davis and Bjorkman, 1988). This limited V region repertoire of the γ and δ loci appears not to be a consensus of all species. Hein and Dudler (1993) have shown that in the ovine species, γδ T cells express a broad repertoire of receptors. There is greater diversity in the V and C region gene segments of both the γ and δ loci. There are 28 Vδ genes belonging to 4 families and 10 Vγ genes belonging to 6 families. In addition the ovine γ loci encodes 5 constant genes and the δ loci one. Specific splicing of each of the Cγ genes to distinct sets of rearranged Vγ segments increases the diversity of the ovine γδ TCR in comparison to other species.

1.3.2 γδ T cell receptor development.

There are numerous nomenclatures describing the variable gene usage of the γδ T cell subsets. These are discussed by Raulet (1989). In this introduction, the V region nomenclature used will be that followed by Heilig and Tonegawa (1986).

In the mouse, γδ T cells consist of multiple lineages of cells which differ from each other by

parameters such as time of appearance in ontogeny, anatomical location, TCR repertoire and thymus dependence (Haas et al., 1993). The first virgin T cells seen in the thymus are CD4 negative and CD8 negative cells expressing the TCR rearrangement Vγ5Vδ1. This is followed by a second wave of cells expressing the Vγ6Vδ1 TCR (Havran and Allison, 1988; Ikuta et al., 1992; Ito et al., 1989). These two γδ T cell subsets are unusual in that their TCR show virtually no diversity (Lafaille et al., 1989). The appearance from the thymus of Vγ5 and Vγ6 T cells slows down and ends in late fetal life. Vγ4, Vγ7 and TCR αβ rearrangements then appear in succession and these rearrangements continue to be expressed in adult life (Ikuta et al., 1992; Elliott et al., 1988; Korman et al., 1988; Takagaki et al., 1989). It was reported, in the mouse, that the precursors of all γδ T cells exported from the thymus must express both HSA and Thy-1 (Zorbas and Scollay, 1993). The mechanisms that control the sequential appearance and disappearance of Vγ regions are still unclear. In the adult, Vγ5 expressing cells home to the epidermis (Asarnow et al., 1988; Havran and Allison, 1990). Cells found in the skin express only Vγ5Vδ1 sequences whereas Vγ5 expressing cells in the intestinal epithelium have a broad receptor diversity (Tonegawa et al., 1989). Presumably the monomorphic TCR expression in the skin and epithelium arose through an evolutionary pressure and these cells will have only one specificity. Vγ6 expressing cells are found in the mucosal epithelia of the female reproductive tract and the tongue (Itohara et al., 1990; Tonegawa et al., 1989). These cells again have no TCR diversity and in human oral epithelium it has been shown that there is preferential expression of the Vδ1 sequence (Pepin et al., 1993). Cells expressing the Vγ4 rearrangement are found in the blood, spleen and lymph node of adults and have a high degree of TCR diversity.

The molecular mechanisms underlying the appearance of distinct γδ T cell subsets are obscure. It was recently shown that an intracellular mechanism acting at the level of DNA rearrangement in the thymus, rather than a cellular selection mechanism, plays a key role in the differential generation of γδ T cell subsets (Itohara et al., 1993).

1.3.3 αβ T cell receptor development.

The principle pathway for development of T cells that will express αβ TCR complexes is again in the thymus. Firstly, the TCR β chain is expressed in disulphide linkage with the development protein gp33 and in association with CD3 (Groettrup et al., 1993). Cross-linking of this induces calcium mobilisation (Groettrup et al., 1992; Groettrup and von Boehmer, 1993) and is responsible for promoting the development of thymocytes. This occurs to avoid the accumulation of thymocytes with either no, or a nonproductive TCR β chain rearrangement (Mombaerts et al., 1992; Mallick et al., 1993). Following this, further TCR β chain rearrangements are suppressed ensuring the expression of only one TCR β allele. The TCR β chain may also enhance TCR α chain rearrangements. Once the signal has been delivered, TCR α chain rearrangements are accelerated and gp33 expression is terminated. The next stage of development is different in the human and mouse species. Human cells differentiate into CD4hi, CD8-negative, CD3-negative (Kraft et al., 1993) while mice cells differentiate into CD8hi, CD4-negative, CD3-negative (Guidos et al., 1989, 1989a). This leads to the expression of both co-receptors and gives rise to double positive cells: CD8-positive, CD4-positive blast cells. These cells express low levels of the αβ TCR-CD3 complex and are the starting point for most of the reported TCR based selection within the thymus. The blast cells either progress into a small cell progeny and die off or follow a two-stage differentiation into CD4-positive (CD8-negative) TCRhi or CD8-positive (CD4-negative) TCRhi virgin T cells (Guidos et al., 1990).

7

1.4 T cell selection in the thymus.

Antigen recognition by the TCR molecule occurs in association with MHC class I or MHC class II molecules. Antigen recognition and MHC presentation is discussed later (section 1.8). The TCR is predicted to have a similar structure to immunoglobulin molecules and is orientated to interact with peptide fragments and the MHC molecules which hold them (Hong et al., 1992; Kaye and Hendrick, 1988; Malissen et al., 1988). The antigen binding sites of the TCR has three variable domains termed complementarity determining regions (CDR). CDR1 and CDR2 are made up from the variable regions of the genome and CDR3 is formed from the V(D)J segments (Jorgensen et al., 1992). The CDR3 region is involved in the recognition of the peptide fragment (Hong et al., 1992) whilst the CDR1 and CDR2 regions make contact with the α-helices of the MHC molecules (Clavarie et al., 1989; Davis and Bjorkman, 1988; Bjorkman et al., 1987).

Because of the nature of antigen receptor formation there will be receptors which are specific for either self-MHC α–helices which are in association with foreign antigenic peptides or self-MHC α–helices which are in association with self peptides or no peptide at all. There has to be therefore, some form of negative selection which prevents self-specific lymphocytes from becoming auto-aggressive and also positive selection by internal ligands, so avoiding the accumulation of redundant lymphocytes.

A major function of the thymus is the selection of cells that co-recognise peptides which are held in association with self-MHC α–helices only. Cells that survive and are found in the periphery of an individual have undergone positive and negative selection. Positive selection leaves behind cells which recognise, but are not stimulated by self-MHC. Negative selection removes those cells with receptors which strongly recognise either self-MHC alone or self-MHC plus self antigens. Negative selection occurs predominantly by a clonal abortion process (Kappler et al., 1987). It is not known whether positive selection or negative selection occurs first (Guidos et al., 1990; Borgula et al., 1991).

Most reports in the literature describe positive and negative selection of αβ T cells. It has however been suggested that γδ T cells are subject to positive and negative selection in a manner analogous to the αβ T cell population (Matis et al., 1991; Wells et al., 1993). Some reports contradict this and have demonstrated that γδ T cells can develop normally in the absence of MHC class II molecules (Bigby et al., 1993). It has been proposed that heat shock proteins or endogenous ligands, such as viral sequences, may play a role in the positive selection of γδ T cells though the more restrictive rearrangements of the γδ TCR implied a less stringent selection process (Lafaille et al., 1990; Sim and Augustin, 1990). It was also reported recently (Dent et al., 1993) that self reactive γδ T cells undergo programmed cell death following negative selection.

1.4.1 Positive selection.

Numerous models have described positive selection (Teh et al., 1988; Kisielow et al., 1988; Scott et al., 1989) which was recently reviewed by Bevan (1994). The nature of the MHC ligands inducing positive selection is unknown though the recognition of a peptide is thought to be essential for positive selection. The thymus is thought to be able to generate virtually all the peptides that could possibly fit into the groove of a given MHC. This may occur at sufficiently low concentrations to allow for positive selection, without causing active responses leading to negative selection (Kourilsky et al., 1989). An alternative view is that the peptides are needed simply to stabilise the MHC molecules on the cell surface and that the ligands required for

positive selection are generally different to those inducing T cell activation.

It is believed that T cells which express a TCR with intermediate avidity for either the MHC class I or MHC class II peptide complexes are positively selected. Following positive selection via the TCR, the selected cells further develop into functionally mature cells. T cells which bind MHC class I peptide complexes make a second contact between the CD8 molecule and the MHC class I molecules. T cells which bind MHC class II peptide complexes make a second contact between the CD4 molecule and the MHC class II molecule. The second contact induces a decrease in the cell surface expression of unengaged CD4 or CD8 molecules (Robey et al., 1991; Borgula et al., 1991).

Associated with the positive selection via the TCR is a shut-down of the recombinase associated genes, *RAG1* and *RAG2*, which terminate the cells' TCR rearrangements. The TCR α-chain product, in contrast to the TCR β-chain, does not induce feedback regulation of further TCR α-chain rearrangements and two different TCR α-chain transcripts have been observed in the same cell at both the RNA and protein level (Casanova et al., 1991; Borgula et al., 1992).

1.4.2 Negative selection.

Negative selection is the process by which a TCR-antigen interaction results in the death of the T cell expressing the given TCR molecule (reviewed by Nossal, 1994). If the avidity between the MHC peptide complex on the thymic stroma and the TCR on the T-cell is sufficiently high, the thymocyte will undergo apoptosis and is deleted from the lymphocyte repertoire of that individual (Jenkinson et al., 1989; MacDonald and Lees, 1990; Ucker et al., 1992). The death of a self-reactive lymphocyte is termed clonal deletion. The majority of clonal deletion of thymocytes occurs when the thymocyte has a CD4-positive, CD8-positive phenotype (Spain and Berg, 1992). T cells can also be silenced by persistent stimulation with an antigen without undergoing apoptosis. This is termed clonal anergy (Nossal and Pike, 1980).

1.5 The immunobiology of γδ T cells.
1.5.1 Extrathymic origin of γδ T cells.

It is known that not all γδ T cells are generated in the thymus. Populations of murine γδ T cells have been shown to be generated extrathymically in the gut (Guy-Grand et al., 1991, 1991a; Rocha et al., 1991), the liver (Abo et al., 1991) and the lungs (Sim and Augustin, 1990: Sim and Augustin, 1991; Augustin et al., 1989). Human di George syndrome patients (van Dongen et al., 1990) and thymoectomised chickens (Bucy et al., 1991) have also been shown to generate populations of γδ T cells. In contrast, thymoectomised lambs do not generate any populations of γδ T cell populations (Hein et al., 1990a).

1.5.1.1 Intraepithelial γδ T cells.

The populations of iIEL in all species examined comprise T cells bearing γδ and αβ TCR. Most iIEL expressing a γδ TCR are extrathymically derived and it has been shown that tolerance, functional anergy and deletion occur in this environment (Barrett et al., 1993). γδ T cells are a major population of iIEL in humans (Deusch et al., 1991), mice (Allison and Havran 1991; Taguchi et al., 1991) and sheep (Gyorffy et al., 1992). γδ iIEL are found primarily in the small intestine interspersed between the villous epithelial cells and are unique to all other γδ T cells in

numerous ways. Firstly, murine γδ iIEL preferentially use Vγ7 variable region and express extensive junctional diversity. This is in contrast to all other γδ T cells localised at epithelial sites within this species (Robijn et al., 1993; Bonneville et al., 1988; Asarnow et al., 1989; Takagaki et al., 1989a). Secondly, γδ iIEL express CD8 αα homodimers (Bandeira et al., 1991; Boneville et al., 1988). The functional significance of the lack of CD8β chain expression is unclear but CD8α-chain expression is sufficient for signal transduction (Dembic et al., 1987). CD8 αα homodimers can also be expressed by activated γδ T cells from peripheral tissues (Cron et al., 1989) and lectin stimulated thymocytes (MacDonald et al., 1988). Expression of the CD8 αα homodimers may be indicative of an activated γδ T cell lineage within the gastrointestinal microenvironment (Guy-Grand et al., 1991). Alternatively, expression of the CD8 αα homodimers may imply that γδ iIEL have the potential to recognise foreign peptide presented by MHC class I or MHC class I-like molecules such as TL or CD1 antigens. MHC class I, TL antigens or CD1 antigens are all expressed by gut epithelial cells and are implied to have functional roles as presentational elements in antigen presentation to γδ T cells (Taguchi et al., 1991; Hershberg et al., 1990; Bleicher et al., 1990; Wu et al., 1991; Eghesady et al., 1992). Thirdly, few murine γδ IEL express CD5 and only half of the cells express Thy-1 (Ledbetter et al., 1980; Lefrancois, 1991).

It was recently shown that in microbe-deprived mice, the level of γδ iIEL remained constant whilst the αβ iIEL level decreased with a loss in cytolytic activity also observed (Kawaguchi et al., 1993). It is therefore believed that there is no requirement for the intestinal flora for the presence of γδ T cells.

1.5.2   γδ T cell function

There is, as yet, no clear delineation of the function of γδ T cells although γδ T cells have been attributed a great number of functions. These functions however have all been identified with γδ T cells clones or with transgenic animals devoid of certain subsets of cells. This obviously reduces the significance of any results found, due simply to the artificial circumstances and environments under which the cells were treated and responses analysed.

From *in vivo* studies, γδ T cells are believed to play a large part in the immunosurveillance system and in primary immune response to several infectious organisms. Increases in γδ T cell number were observed in the peripheral blood during numerous bacterial and parasitic infections of both humans and mice (Janis et al., 1989; Eichelberger et al., 1991; Carding et al., 1990; Ohga et al., 1990; Alaibac et al.,1993; van der Heyde et al., 1993; Takada et al., 1993). During the course of diseases such as leprosy (Modlin et al, 1989; Falini et al., 1989), Graves disease (Roura-Mir et al., 1993), tonsillitis (Kawaguchi, 1993), HIV infected patients with AIDS (Autran et al., 1989; De Paoli et al., 1991) and multiple sclerosis (Hvas et al., 1993) an increased number of tissue resident and circulatory γδ T cells were also observed. Additionally, γδ T cells have been isolated from the synovial fluid of patients with rheumatoid arthritis (Holoshitz et al., 1989) and from the lungs of patients with sarcoidosis (Balbi et al., 1990) which suggested a role for these cells in the pathogenesis of autoimmune diseases.

In addition to increases in the γδ T cell population as a whole, individual sub-populations of γδ T cells with single variable chains are known to respond to certain infections. A preferential expansion of the Vδ2 subset was observed following Brucella meltensis infection (Bertotto et al., 1993) and similarly during the acute stage of Epstein-Barr Virus the number of circulating Vδ2

expressing γδ T cells was also greater. Little of the evidence however suggested that the expansion in the circulation, or accumulation of γδ T cells in the infected tissues, was due to recognition of microbial antigens. Responses were thought to be directed toward a host derived stimuli. In some cases, no detectable *in vitro* response by γδ T cells from an infected individual, was observed to antigens from the infectious agents, (Bertotto et al., 1993; Eichelberger et al., 1991).

1.5.3 Ligand recognition by γδ T cells.

A diverse array of antigens have been identified as ligands for γδ T cells in both the murine and human species. This heterogeneous group of ligands includes classical and non-classical MHC molecules (Matis et al., 1987, 1989; Bluestone et al., 1988; Bonneville et al., 1989a), mycobacterial heat shock proteins (Born et al., 1990, 1990a; Russo et al., 1993), superantigens such as staphylococcal enterotoxins (Rust et al., 1990) and self antigens including CD1 (Porcelli et al., 1989), cell surface immunoglobulins (Wright et al., 1989) and autologous stress proteins (Born et al., 1990; Ramanujam et al., 1990). None of these antigens however appear to be representative targets for γδ T cells as a whole or their defined subsets. The exceptions, which are described below, are the recognition of *Mycobacterium tuberculosis* antigen by human peripheral blood γδ T cells, the recognition of heat shock proteins by murine peripheral blood γδ T cells and the recognition of keratinocyte antigens by skin γδ T cells in the murine species.

In human peripheral blood 50-70% of γδ T cells express a Vγ9/Vδ2 heterodimeric structure whereas Vγ9/Vδ2 γδ T cells represent only a minor population in cord blood (Parker et al., 1990; Faure et al., 1990). The majority of human peripheral blood Vγ9/Vδ2 γδ T cells respond to *Mycobacterium tuberculosis* lysates and stimulation of human cord blood with *Mycobacterium tuberculosis* lysates selectively expanded the Vγ9/Vδ2 γδ T cell population. Pfeffer and colleagues (1990, 1992) reported that the moiety which stimulated the human Vγ9/Vδ2 γδ T cell population was a lectin-binding, protease resistant ligand of 1-3kDa which exhibited the hallmarks of a superantigen for γδ T cells.

Murine γδ T cells expressing Vγ1/Vδ6 were shown to recognise both mycobacterial hsp 65 and murine hsp 60 (Born et al., 1990, 1990a; Happ et al., 1989). The cross reactivity of Vγ1/Vδ6 γδ T cells with eukaryotic and prokaryotic heat shock proteins occurs because of the high degree of conservation between heat shock proteins (Kaufmann, 1990). Furthermore, murine Vγ1/Vδ6 γδ T cells hybridomas recognised a 16 amino acid peptide corresponding to residues 180-196 of mycobacterial hsp 65 (O'Brien et al., 1989, 1991) and γδ T cells responded to this peptide *in vivo*. In the murine species, γδ T cells which recognise heat shock proteins are proposed to have a developmental role (Ferrick and Gemmell-Hori, 1992) and a role in bacterial immunity and autoimmunity (Reardon et al., 1992). In the human species, γδ T cells which recognise heat shock proteins are proposed to have an autoaggressive role in muscle disease (Hohlfeld and Engel, 1992)

Invariant, murine γδ T cells expressing Vγ3/Vδ1 reside in intimate contact with keratinocytes and recognised self antigens on stressed neighbouring cells in a non-MHC restricted fashion (Havran et al., 1991). The keratinocyte antigen recognised is a low molecular weight moiety which can be acid eluted from stressed keratinocytes (Havran and Boismenu, 1994).

1.5.4 Effector functions of γδ T cells.
1.5.4.1 Secretion of cytokines by γδ T cells.

The secretion of cytokines is an integral part of the function of αβ helper T cells following antigenic stimulation. It has been shown in humans and mice that γδ T cells, like αβ T cells, can be stimulated to secrete many lymphokines. As the antigen specificity and restriction elements of γδ T cells are unknown, non-specific stimuli were used to stimulate the γδ T cells. In contrast, many γδ T cell clones in cattle could not be stimulated to secrete the major cytokines IL-2, IL-4 and γ-IFN (W.I. Morrison, personal communication). In the human and mouse species IL-2 is secreted by γδ T cells following stimulation though the levels of IL-2 produced are considerably lower than the levels of IL-2 produced by αβ T cells (Bender and Kabelitz, 1990; Patel et al., 1989; Warren et al., 1989). The cytokines IL-3, IL-4, IL-5, IFNγ, TNF-α, TNF-β and GM-CSF are also produced by γδ T cells in response to a variety artificial stimuli (Biassoni et al., 1991; Patel et al., 1989; Krangel et al., 1990; Carding et al., 1990a; Carrel et al., 1991). Different combinations of lymphokines were shown to be produced by different γδ T cell clones though it is not clear if there are any analogies to the T_{H1} and T_{H2} subsets of the αβ T cell lineage (Bluestone et al., 1991; Krangel et al., 1990; Morita et al., 1991; Spits et al., 1989; Raziuddin et al.,1992; Taguchi et al., 1991). In the absence of details of the physiological roles or antigenic stimuli of γδ T cells, it is difficult to speculate on the function of the produced cytokines.

1.5.4.2 Cytotoxic γδ T cells.

γδ T cells have been shown to lyse target cells in a manner analogous to CD8-positive T cells, NK cells and LAK cells in humans (Koide et al., 1989; Spits et al., 1989), mice (Goodman and Lefrancois, 1988; Klein, 1986) and sheep (MacKay et al., 1988a). Those γδ T cells which express a disulphide-linked form of the T cell receptor (Cγ1) are more highly cytotoxic than those expressing the non-disulphide linked form (Cγ2) (Christmas 1989; Dastot et al., 1990). It was also suggested that the cytotoxicity of human γδ T cells is associated with surface phenotype. CD8-positive γδ T cell clones and CD4-negative, CD8-negative γδ T cell clones mediated cytotoxic function whilst CD4-positive γδ T cell clones did not (Morita et al., 1991). The expression of CD8 by some cytotoxic γδ T cell clones may suggest that these cells recognise cells in a MHC class I restricted manner. This however, has not been shown although γδ T cell mediated lysis via non-polymorphic class Ib molecules has been reported (Porcelli et al., 1989). In addition, γδ T cells are known to localise at epithelial sites where MHC class Ib molecules are known to be expressed (Vroom et al., 1991; Eghesady et al., 1992)

Indirect evidence of a cytotoxic function of γδ T cells was found in the murine species were electron dense granules were located in the cytoplasm of murine γδ T cells (Koizumi et al., 1991). It is known for CD8-positive cytotoxic T cells that perforin is stored in cytoplasmic electron dense granules which are visible by electron microscopy (Kraehenbuehl and Tschopp, 1990). In addition, both human and murine γδ T cells express mediators of cytotoxicity such as perforin and serine esterases 1 and 2 (Nakata et al., 1990; Koizumi et al., 1991). In contrast, in the ovine species, electron dense granules were not observed in γδ T cells in the ovine species (Evans, 1993).

1.6 The immune response.

Immune activation of lymphocytes occurs in the periarteriolar lymphoid sheaths of the spleen and the paracortex of the lymph node where antigen specific cells are selectively sequestrated.

The spleen is the locus of immune responses to most blood borne antigens whilst the different lymph nodes and the Peyer's patches are the loci of the immune responses to antigen taken up into the afferent lymph.

The number of T cells specific for the foreign peptides of a given pathogen is very low in an unprimed individual. Dendritic cells appear to provide a mechanism which ensures that these few specific T cells and antigen meet (Steinman, 1991). Dendritic cells express both MHC class I and class II molecules and the turnover of these molecules is very low. In addition, MHC class Ib molecules and MHC class I-like molecules such as CD1, which have been shown to play a role in antigen presentation, are expressed by dendritic cells (Steinman, 1991). Dendritic cells migrate to lymph nodes, spleen and mucosal sites where they strongly adhere. Quiescent, recirculating T cells "scan" the MHC surfaces of the dendritic cells and once an interaction of the correct avidity occurs, the antigen specific T cells are activated, divide, and become effector cells.

In the lymph node, primary B cell follicles contain mostly resting B cells, while T cells are predominantly found in the T cell domains of the paracortex (Gutman and Weissman, 1972). When stimulated, CD4-positive T cells move from the paracortex of the lymph node to the interface between it and the B cell dominated primary follicle (Gutman and Weissman, 1972; Rouse et al., 1982). These activated CD4-positive T cells are thought to stimulate antigen specific, follicular B cells. A few days after antigenic challenge, proliferating B cells can also be detected in the periarteriolar lymphoid sheath of the spleen and are subsequently found in primary lymphocyte follicles. In the lymph node, large germinal centres are prominent for about two weeks and contain: i) follicular dendritic cells which effectively store and present antigen, ii) activated T helper cells, iii) proliferating B cells and their non-dividing progeny. After several days the expanded populations are released into the circulation and carry out effector functions.

1.7 Memory cells.

Following the clearance of antigen at the end of a primary response wide scale T cell elimination is known to occur (Rocha and von Boehmer, 1991; Moskophidis et al., 1993) and most effector B cells (plasma cells) die within 1-2 weeks (Ho et al., 1986). A proportion of antigen specific cells however remain and become long-lived memory cells. Memory T cells are derived from effector T cells (Moskophidis et al., 1993) and the resulting memory cells have identical TCR affinities to the effector cells. Memory T cells (CD45R^{hi}, L-selectin^{hi}, CD44^{lo}) have a distinct surface phenotype compared to naive cells (CD45R^{lo}, L-selectin^{lo}, CD44^{hi}) (Fowell et al., 1991; Swain et al., 1991; Budd et al., 1987) though memory T cells have been reported to revert to a naive phenotypic status (Bell and Sparshott, 1990). Memory T cells also express a number of other markers lacking on naive cells (Shimizu et al., 1990; Mackay, 1993) and differ in their pattern of cytokine release (Mossmann and Coffman, 1989). It is believed that maintenance of immunological memory requires the persistence of antigen and the incomplete elimination of viral infections. Dendritic cells and sequestered antigen are retained in the germinal centres of a lymph node for months to years (Mandel et al., 1980; Tew et al., 1990). It has also been seen in some viral infections that viruses can avoid complete elimination (Ahmed, 1992; Oehen et al., 1992; Gray, 1993).

The generation of memory B cells occurs by affinity maturation in the germinal centres and is associated with somatic hypermutation and immunoglobulin class switching (MacLennan, 1994; Berek et al., 1991; Rajewsky, 1992; Leanderson et al., 1992; Nossal, 1992). Memory and naive B cells, unlike the equivalent T cells, are believed to originate from two different precursor cell

13

sub-populations distinguished by different levels of HSA (a phosphoinositol-glycolipid linked glycoprotein) (Linton et al., 1989; Yin and Vitetta, 1992). HSA-positive cells elicit strong primary responses whereas HSA[lo] or HSA-negative cells function well as precursors of memory cells. Memory B cells express higher levels of CD44 than naive B cells and they also differ in the isotype of immunoglobulin expressed on the cell surface.

1.8 Antigen processing and presentation.

The majority of T cell clones do not recognise native antigen but instead recognise a peptide derived from proteolytic cleavage of the native antigen (Unanue, 1984; Townsend and Bodmer, 1989). Two major intracellular routes of antigen processing and presentation have been identified. One route generates peptides from endogenously synthesised proteins which are presented in association with MHC class I molecules (Townsend and Bodmer, 1989; Morrison et al., 1988). The second route generates peptides from exogenously acquired protein material which are presented in association with MHC class II molecules (Unanue and Allen, 1987)

1.8.1 MHC molecules.

Immunologists first became aware of the MHC loci, which encode the molecules involved in antigen presentation, as a result of intraspecies tissue grafting experiments (Counce et al., 1956). MHC class I molecules have one integral membrane subunit, a 45kDa heavy chain, non-covalently associated with the 12kDa molecule β2 microglobulin which is encoded outside the MHC (Bjorkman and Parham, 1990). MHC class I molecules are expressed on all nucleated cells of the body although expression varies and is typically highest on hematopoietic cells. MHC class II molecules are heterodimers of two transmembrane proteins, an α subunit of 33kDa and an β subunit of 29kDa. MHC class II molecules are constitutively expressed on B cells, macrophages, monocytes, dendritic cells and endothelium. Under the influence of the cytokine γ-interferon, however, MHC class II expression can be induced on diverse cell types.

The X-ray crystallographic structure of MHC class I molecules (Bjorkman et al., 1987; Madden et al., 1992) and MHC class II molecules (Brown et al., 1993) have been determined. MHC class I and MHC class II molecules have a peptide groove consisting of a floor of eight β strands of amino acids which support walls of α helices. It has been proposed, due to sequence homology, that the MHC molecule peptide-binding regions may have evolved from the peptide-binding domain of hsp70 chaperones (Flajnik et al., 1991; Rippmann et al., 1991). For both classes of MHC molecules, the diverse peptide binding capacity arises from hydrogen bonds formed between conserved residues of the MHC molecule and backbone or terminal groups of the associated peptides.

1.8.2 MHC class I antigen processing and presentation.

MHC class I molecules generally present peptides which are generated by degradation of cytosolic protein. Townsend and colleagues (1985) showed that CD8-positive cytotoxic T cells required MHC class I molecules and cytoplasmic or nuclear viral proteins for their activity. In addition, cytoplasmic and nuclear proteins were seen to be the principle source of peptides eluted from purified MHC class I molecules (Hunt et al., 1992a). The processing of endogenously derived proteins to the peptides associated with MHC class I molecules occurs in the cytosol (Morrison et al., 1986; Moore et al., 1988; Townsend et al., 1985, 1986). This process involves a

14

multicatalytic, ATP-dependant protease assembly called the 26S proteasome (reviewed by Goldberg and Rock, 1992). The first step in the hydrolysis of most endogenous protein involves the covalent conjugation of the protein to ubiquitin (Rechsteiner, 1987; Hough et al., 1987; Finley and Chau, 1991). This is the rate limiting step and requires ATP. Once associated with ubiquitin, the polypeptide undergoes rapid hydrolysis by the ATP-dependant 26S proteasome.

An essential component of the 26S protease assembly is the 20S proteasome called CF-3 (Tanaka et al., 1988). Complexes referred to as low molecular mass proteins (LMP), which are similar in composition and size to the 20S proteosome, have been mapped to the MHC locus (Monaco and McDevitt, 1982; Brown et al., 1991; Martinez and Monaco, 1991). It is suggested that LMP are specialised types of 20S proteasomes (Goldberg and Rock, 1992).

Monaco (1992), identified genes encoding TAP-1 and TAP-2 proteins (TAP=transport associated with antigen processing) which are responsible for the ATP-dependent transport of short MHC class I binding peptides from the cytoplasm to the endoplasmic reticulum and then onto MHC molecules (Shepherd et al., 1993; Neefjes et al., 1993). It was shown that the peptide was a prerequisite for stable expression of the MHC class I heavy chain and β2-microglobulin complex and that empty MHC class I heavy chains are not transported out of the E.R. hence reducing the likelihood of exogenous peptide acquisition and presentation. The retention of empty MHC class I heavy chains is due to the binding of calnexin (p88, Ip90) (Degen et al., 1992). Calnexin is dissociated when β2-microglobulin and peptide have become associated with the heavy chain to form a stable MHC molecule. To date, no physical association has been found between the proteasomes or LMP and the TAP proteins.

MHC class I molecules bind antigenic peptides, using topographically clustered residues at the very ends of their binding grooves in association with the free NH_2 and COOH groups of the associated peptide (Matsumura et al., 1992; Madden et al., 1992). The interaction of the N-terminal and C-terminal amino acids with the MHC class I molecules are critical and prevent peptides of the incorrect length from forming a stable MHC molecule. This feature of the MHC class I-peptide interactions results in a strong bias for short peptides in the range of 8-10 residues (Falk et al., 1991).

1.8.3 MHC class II antigen processing and presentation.

MHC class II molecules assemble in the endoplasmic reticulum in association with a nonpolymorphic, non MHC encoded type II integral membrane protein called the invariant chain (Cresswell et al., 1990). The invariant chain possesses a short internal segment called the class II associated invariant chain peptide region (Riberdy et al., 1992) which either occludes or closes the MHC class II peptide binding site. The invariant chain is required for the active assembly of MHC class II α and β chains and enables MHC class II molecules to avoid any unproductive interaction with protein peptides in the endoplasmic reticulum. (Roche and Crosswell, 1990; Teyton et al., 1990).

MHC class II molecules generally present peptides which are generated from exogenous antigen. Exogenous antigen is endocytosed in numerous ways into MHC class II expressing antigen presenting cells (Allen et al., 1987; Puri and Factorvich, 1988). By nonspecific adsorption or fluid phase pinocytosis into macrophages; by specific Fc-receptor mediated uptake of antigen-antibody in an immune complex into dendritic cells (Bujdoso et al., 1990; Harkiss et al., 1990)

15

or via high affinity immunoglobulin antigen receptors on B cells (Sallusto and Lanzavecchia, 1994).

Exogenous antigen, once endocytosed, undergoes proteolytic degradation by acid proteases or cathepsins in endocytic vacuoles of the late endosomal/lysosomal pathway (Allen et al., 1987). A novel endocytic compartment distinct from endosomes and lysosomes has recently been discovered (Amigorena et al., 1994; Tulp et al., 1994; West et al., 1994). This compartment for peptide loading of MHC class II molecules is biochemically distinct from conventional endosomes and lysosomes and represents a new stage in the endocytic-lysosomal pathway. The acidic nature of the vacuoles are believed to assist in the efficiency of peptide binding by the MHC class II molecules (Neefjes and Ploegh, 1992). Once peptide is acquired, the MHC class II molecule-peptide complex is long lived on the surface of the cell (Germain and Hendrix, 1991; Davidson et al., 1991). The cytoplasmic tail of the invariant chain has been shown to play an important role at this time in the recruitment of new MHC class II molecules to the endocytic vacuoles (Lotteau et al., 1990) and has also been shown to play a key role in the overall efficiency of MHC class II peptide capture and binding (Viville et al., 1993; Bikoff et al., 1993).

The peptide binding sites of MHC class II molecules are open such that peptides can extend out (Brown et al., 1993) and consequently, MHC class II-associated peptides are typically 12-24 residues in length (Rudensky et al., 1991; Chicz et al., 1992; Hunt et al., 1992b). The binding of the peptides by MHC class II molecules involves polymorphic residues in the MHC molecule and specific side chains of the peptide. The MHC class II groove is lined with structural pockets in which the side chains of some of the specific amino acids of peptides line up (Falk et al., 1991; Hammer et al., 1993). These amino acids are termed anchor residues and two or three are required on a peptide for optimal binding to the MHC class II molecules. The requirement of anchor residues has helped in describing simple motifs which can help to predict the part of a protein which might be effectively presented by a given MHC class II molecule.

1.8.4 Putative presentational elements for γδ T cells.

The specificity, if any, of γδ T cells for antigen in association with antigen presenting molecules is one of the most fundamental questions in γδ T cell immunobiology which remains unanswered. The function of this population of T cells will be more accurately assessed once the ligand these cells recognise has been identified.

Murine and human γδ T cells have been shown to mount strong proliferative responses to killed mycobacteria in the presence of antigen presenting cells. The major stimulatory components of mycobacteria were found in the small molecular weight fraction (2-10kDa) of extracts and were resistant to proteolytic enzymes (Pfeffer et al., 1990, 1992). The chemical nature of this material remains to be elucidated. It is also unknown if the ligand that was recognised was of mycobacterial origin or was an endogenous ligand induced on antigen presenting cells.

γδ T cells do not have a bias for classical MHC class I or class II proteins. Few murine or human γδ T cell clones have been found which recognise antigen in association with MHC class I or MHC class II molecules (Haas et al., 1993). In addition, it has been reported that the specific recognition of MHC molecules by some γδ T cell clones was due to fortuitous cross reactivity of the γδ TCR with a constant region on the side chain of the MHC molecule (Schild et al., 1994). Furthermore, increases in γδ T cell number in mice experimentally infected with malaria was

16

shown not to be due to any classical or non-classical MHC class I presentation. The same level of increase in the γδ T cell population was observed in β2-microglobulin deficient mice (van der Heyde et al., 1993). In addition, γδ T cells do not appear to play any part in graft rejection, their effects potentially being one of suppression of αβ T cell responses (Haas et al., 1993).

Non-classical MHC-like proteins are thought to play a part in the immunopresentation of antigen to γδ T cells (Strominger, 1989). In the mouse, TL region encoded proteins are expressed on epithelial cells in the intestine. The T3b product is expressed on columnar epithelial cells found in close proximity to γδ T cells (Wu et al., 1991). In addition, peptide was found associated with the T3b product (Imani and Soloski, 1991; Vidovic et al., 1989). It was therefore proposed that various murine γδ T cell subsets may recognise antigens presented by different tissue specific TL region encoded proteins (Stominger et al., 1989). The class I gene (T22b) (van Kaer et al., 1991) and the class I gene (27b) (Ito et al., 1990) both encoded within the TL region presented ligands to γδ T cells.

The human MHC class I family includes at least 15 loci other than the classical transplantation antigens (Malissen et al., 1982). Some of these genes encode MHC class I like proteins which may be the human equivalents of the murine TL proteins. Recognition of ligands presented by any of these proteins has not however been reported. Two human γδ T cell clones were found to recognise CD1c which is encoded by one gene of a cluster of five closely related MHC class I-like genes on chromosome 1 (Porcelli et al., 1989; Faure et al., 1990a; Calabi and Milstein 1986).

1.9 Lymphocyte activation.

The adaptive immune response can be split into three distinct stages. Firstly naive lymphocytes become activated following recognition of foreign antigens via their antigen receptor molecules. Secondly, antigen specific cells proliferate and differentiate to become effector cells and thirdly the infectious agent of the immune response is removed.

Activated T lymphocytes undergo replication and produce a single clone of cells which are specific for the antigenic peptide first encountered. All αβ T lymphocytes undergo this process having recognised peptide antigen The nature of the response is thought to be determined by the nature of the signals received during encounter with antigen and antigen presenting cells. The activation of CD4-positive T cells may be the most important as they control the activation of B cells, macrophages and sometimes CD8-positive T cells. It is widely accepted that T cells require two distinct signals to proliferate and subsequently differentiate into armed effector cells (Schwartz, 1992). The first signal comes from the interaction of the TCR with its complementary MHC molecule presenting the required peptide. The second, or co-stimulatory signal, does not induce T cells responses by itself, but is required when the TCR and MHC molecules are engaged. Lack of a co-stimulatory signal induces cell death (Webb et al., 1990) or anergy (Jenkins et al., 1988). Naive CD4-positive T cells respond most efficiently when the signals required for their activation are expressed on the same cell (Liu and Janeway, 1992; Galvin et al., 1992).

Immune activation of B lymphocytes involves the cells leaving the G_0 stage of the cell cycle and replicating to create a number of different clones of progressively more differentiated immunological effector cells. Once activated, B cells become effector cells and differentiate into B cell blasts. These cells initially express the μ heavy chain but in time receive cytokine signals from T cells that induces them to undergo immunoglobulin class switching (Kraal et al., 1982;

17

Butcher et al., 1982). Immunoglobulins produced by the B cell blast progeny have somatic mutations in their variable region. B cell blasts in the germinal centre which produce higher affinity antibodies will continue to proliferate and differentiate, whilst those which produce lower affinities are diminished following the mutations and die by programmed cell death. The continued expression of the gene *bcl-2*, is associated with survival of B cell blasts, whereas failure of continued expression is associated with programmed cell death of germinal centre B cell blasts (Vaux et al., 1988 McDonnell et al 1989).

1.9.1 Co-stimulatory molecules for T cell activation.

For naive T cells to proliferate, the MHC and peptide antigen for which they are specific, must be expressed on a cell which also expresses the protein B7/BB1. B7/BB1 is a ligand for both CD28 which is expressed on naive CD4-positive T cells (Linsley et al., 1991a; Koulova et al., 1991) and for CTLA-4 which is expressed on antigen-experienced T cells (Lindsey et al., 1991b; Brunet et al., 1987). CTLA-4 is related to CD28 but binds B7/BB1 with a higher affinity. CTLA-4 was used *in vitro* and *in vivo* as a soluble, functionally active, fusion protein and inhibited numerous CD4-positive T cell responses to antigen, inducing unresponsiveness or anergy (Ledbetter et al., 1992; Liu et al., 1992b; Linsley et al., 1992; Tan et al., 1993). These results showed that the ligand for B7/BB1 is critical for the co-stimulation of naive CD4-positive T cells The importance of B7 was demonstrated by transfecting nonimmunogenic, transplantable tumour cells with the B7 gene (Townsend and Allison, 1993; Chen et al., 1992; Baskar et al., 1993). These tumour cells were immunogenic when transplanted into mice and moreover, mice which were cleared of the tumour cells had active immunity against identical non-transfected tumour cells. It was also shown that CD28 and CTLA-4 recognises ligands other than B7 (Liu et al., 1993; Hathcock et al., 1993; Freeman et al., 1993b; Lenschow et al., 1993) and a second ligand, B7.2, has been cloned (Freeman et al., 1993a).

HSA can also costimulate growth of naive CD4-positive T cells (Liu et al., 1992a). Soluble CTLA-4 fusion protein and anti-HSA antibodies independently blocked naive CD4-positive T cell responses by 90%. When used together, these reagents completely inhibited the responses. The receptor for HSA is not known. In addition, it has been shown that molecules which costimulate the growth of CD4-positive T cells also appear to costimulate the growth of CD8-positive T cells (Townsend and Allison, 1993; Chen et al., 1992; Baskar et al., 1993).

1.9.2 Co-stimulatory molecules for B cell activation.

B cell responses are regulated by the CD40 ligand (gp39) (Noelle et al., 1992; Hallenbaugh et al., 1992) which is usually expressed by helper T cells (Allen et al., 1993; Aruffo et al., 1993). CD40 is constitutively expressed on B cells and the CD40 ligand is rapidly induced on helper T cells when they encounter antigen (Armitage et al., 1992; Macchia et al., 1993). The interaction of CD40 and ligand activates the B cell which can then respond to soluble cytokines produced by the T cell (Banchereau and Rousset, 1991). The CD40-gp39 interaction is also important for the process of Ig switching (Paul and Seder, 1994).

1.9.3 Co-stimulatory molecule expression on APC.

Dendritic cells and B cells present antigen to CD4-positive T cells. These cell types constitutively express MHC class II molecules in the peripheral lymphoid organs. Dendritic cells found in

tissues are not however constitutively co-stimulatory. Following migration via the afferent lymphatics to lymphoid tissue, these cells differentiate and acquire a co-stimulatory activity (Janeway and Bottomly, 1994). Co-stimulatory dendritic cells are highly efficient activators of naive CD4-positive T cells (Knight and Stagg, 1993) and express the co-stimulatory molecules which bind CD28 and CTLA-4 (Liu et al., 1992b; Larsen et al., 1992). Dendritic cells also express high levels of ICAM-1 and are very effective at forming clusters with T cells (Knight and Stagg, 1993).

B cells are believed to present the peptides formed from soluble protein antigens (Ron et al., 1981) to memory CD4-positive T cells (Fuchs and Matzinger, 1992; Ronchese and Hausmann, 1993). Resting B cells do not express the ligands for CTLA-4 in contrast to activated B cells (Liu et al., 1992b; Hathcock et al., 1993). It was recently shown that expression of CTLA-4 ligands on B cells was induced in a feedback mechanism following binding of the CD40 ligand on CD4-positive T cells by B cells (Ranheim and Kipps, 1993).

1.9.4 Signal transduction by lymphocyte antigen receptors.

T cells and B cells have structurally different receptors for antigen and as a consequence recognise fundamentally distinct forms of antigen, yet the signal transduction events that result from the interaction of their receptors with antigen are similar. In addition to the antigen specific receptors, other molecules contribute to cell activation by either: i) functioning as co-receptor molecules which directly contribute to the formation of the complex between the antigen receptor and the antigen/MHC complex. The known co-receptors on T cells are CD4 and CD8 which bind to MHC class II and class I molecules respectively (Janeway, 1992). The co-receptor on B cells is a complex of three proteins: CD19, complement receptor 2 (CR2) which binds CD23 (Carter and Fearon, 1992) and TAPA-1. ii) increasing the avidity of the interaction between the two cells (e.g. adhesion molecules such as LFA-1). or iii) inducing separate signal transduction events that influence the cellular response (e.g. CD28, CD40).

1.9.4.1 Involvement of protein-tyrosine kinases (PTK) in T cell activation.

The TCR is associated non-covalently with the CD3δ, CD3ϵ, and CD3γ chains and a ζ chain which is either self-associated as a homodimer or associated as a heterodimer with either a η chain or a FcϵRIγ chain (Clevers et al., 1988). The TCR does not have intrinsic PTK activity and is not expressed on the cell membrane independently of the signal transducing subunits (Weiss, 1991). The cytoplasmic domains of the receptor associated chains are considerably larger than the cytoplasmic domains of the TCR and are responsible for coupling the receptor subunits to the intracellular signalling machinery and thereby activating cytoplasmic PTK. The signal transduction functions of the TCR-associated chains were revealed by recent studies with mutant cell lines (Wegner et al., 1992) and chimeric receptors (Irving and Weiss, 1991; Letourneur and Klausner, 1992; Romeo and Seed, 1991). A common cytoplasmic domain motif was found on the TCR associated chains which couples these proteins to intracellular PTK (Weiss, 1993). The consensus sequence is referred to as the antigen recognition activation motif, (ARAM) (Weiss and Littman, 1994) and is sufficient to couple chimeric receptors to early and late signalling events (Irving et al., 1993; Romeo et al., 1992). Multiple copies of ARAM sequences are found within a single receptor and may provide a means of signal amplification.

1.9.4.2 Association of CD4 and CD8 with PTK.

Most encounters of lymphocytes with antigen are thought to involve low affinity interactions of the antigenic receptors. In addition, it has been estimated that as little as a few hundred relevant peptide/MHC complexes are present on an antigen presenting cell (Harding and Unanue, 1990). T lymphocytes use co-receptor molecules that act synergistically with the antigen receptor to induce signal transduction events. It has been shown that binding of the CD4 co-receptor to the same antigen presenting MHC augments signalling via the TCR receptor by up to 100-fold (Dianzini et al., 1992; Miceli et al., 1991). Mechanisms underlying signal amplification have been thoroughly scrutinised in $\alpha\beta$ T lymphocytes, whose integral membrane proteins, CD4 and CD8 serve the essential co-receptor function. Firstly, the extracellular domains of CD4 and CD8 molecules bind to membrane-proximal regions of MHC molecules (Cammarota et al., 1992; Konig et al., 1992). These interactions permit simultaneous binding of both TCR and CD4 or CD8 to the same MHC molecule. Secondly, both CD4 and CD8 bind to the protein tyrosine kinase *lck* through their cytoplasmic domains (Veillette et al., 1991). The mechanism of synergistic activation is believed to be due to interplay between molecules associated with the cytoplasmic tails of the TCR and associated proteins (Diazani et al., 1992).

Two classes of cytoplasmic PTK have been implicated in signalling by the TCR, namely the *src* and *syk*/ZAP-70 families. Members of the *src* family have a unique N-terminal domain with a myristylated glycine at position 2 which is responsible for membrane association and a C-terminal tyrosine phosphorylation site which is the site of negative control of the catalytic activity. Three members of the *src* family are generally expressed in most T cells, *lck, fyn* and *yes*. The clearest documented role for these molecules is that for *lck* which interacts with the cytoplasmic domains of the co-receptors CD4 and CD8 (Rudd, 1990; Veillette et al., 1991). *lck* works in two distinct ways in co-receptor-assisted T cell activation. Enzymatically active *lck* localises to the TCR complex where it acts early in the pathway phosphorylating tyrosines in ARAM sequences of CD3 and ζ chains (Weiss and Littman, 1994). Furthermore, *lck* co-ordinates interaction of the TCR and co-receptors with a single MHC molecule (Saizawa et al., 1987; Collins et al., 1992; Xu and Littman, 1993). CD8 avidity for MHC class I is also increased following TCR stimulation (O'Rourke et al., 1990) although a requirement for *lck* has not been shown. Genetic and biochemical studies have also shown *fyn* to have an important role in signal transduction. The activity of *fyn* increases 2- to 4-fold following TCR stimulation (Tsygankov et al., 1992) and *fyn* PTK activity was detected in TCR immunoprecipitates (Sarosi et al., 1992). In addition, *fyn* activity was found directly associated with the ζ chain (Gauen et al., 1992).

The association between *syk* or ZAP-70 and ARAM containing receptors is well defined. *Syk* and ZAP-70 are not myristylated and hence are not constitutively localised at the plasma membrane. This family of proteins lack a *src*-homology 3 (SH3) domain and the C-terminal negative regulatory tyrosine phosphorylation site which is found in *src* family members. ZAP-70 is a 70kDa PTK expressed exclusively in T cells and NK cells (Chan et al., 1992) whereas *syk* is a 72kDa PTK expressed preferentially in B cells, myeloid cells and thymocytes (Taniguchi et al., 1991). ZAP-70 is not associated with the TCR in the resting state, but following stimulation, is rapidly recruited to the ζ and CD3 chains (Chan et al., 1991) only associating with tyrosine phosphorylated forms of ζ. It is suggested that the TCR interacts with two PTK sequentially. The first, a *src* family member, phosphorylates ARAM sequences which results in the recruitment of ZAP-70 to the membrane complex (Chan et al., 1992).

1.9.4.3 Protein-tyrosine phosphatases (PTPase).

Protein-tyrosine phosphorylation following activation of T cells or cross-linking of cell surface proteins occurs by stimulating the functions of PTK. Protein-tyrosine phosphorylation however can also be enhanced by inhibiting PTPase such as CD45. CD45 is an abundant plasma membrane protein that is differentially expressed on all cells of the hematopoietic lineage except erythroid cells (Trowbridge, 1991). On T cells, the various isoforms of CD45 distinguish different subsets (Bottomly et al., 1989; Byrne et al., 1988). Genetic studies have demonstrated that CD45 is required for TCR signal transduction, including induction of PTK activity (Justement et al., 1991; Pingel and Thomas, 1989). Targets for the CD45 PTPase activity in T cells are the negative regulatory sites of tyrosine phosphorylation in *lck* and *fyn* (Cooper et al., 1986). In CD45-deficient cells, *lck* and *fyn* are hyperphosphorylated at this negative regulatory site (Hurley et al., 1993; Ostergaard et al., 1989; Sieh et al., 1993) whereas in normal cells, CD45 de-phosphorylates this site activating the PTK function (Mustelin et al., 1989). The function(s) of the different extracellular domains of CD45 isoforms have not yet been established though recent studies with chimeric CD45 molecules have indicated that the extracellular and transmembrane domains are not required for CD45 function (Desai et al., 1993; Hovis et al., 1993; Volarevic et al., 1993).

1.9.5 Intracellular signal transduction.

Stimulation of the lymphocyte receptors induces tyrosine phosphorylation of many cytoplasmic and membrane proteins. It is not known which of these proteins are direct substrates of the PTK and the identity of many of these proteins and the functional significance of most of the tyrosine phosphorylations are not known. The most well characterised events following the stimulation of the TCR are the activation of PLC, calcineurin (a calcium/calmodulin-dependant serine phosphatase) and *ras*, all of which are implicated in events leading to the transcriptional activation of the IL-2 gene (Waldman, 1989).

Stimulation of the TCR induces the activation of PLCγ1 via tyrosine phosphorylation (Imboden and Stobo, 1985; Secrist et al., 1991; Weiss, 1991). The activated PLCγ1 hydrolyses phosphatidylinositol (PI) 4,5-bisphosphate into the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Izquierdo and Cantrell, 1992). The second messengers, IP3 and DAG are responsible for the observed TCR induced increase in cytoplasmic free calcium ($[Ca^{2+}]_i$) and activation of protein kinase C respectively (Weiss and Imboden, 1987). The increase in $[Ca^{2+}]_i$ is rapid and sustained and influences calcium/calmodulin dependent events. Work with the immunosuppressive drugs cyclosporin A (CsA) and FK506 has helped elucidate some of the downstream effects of $[Ca^{2+}]_i$. These drugs bind to cytoplasmic proteins known as immunophilins (Schreiber and Crabtree, 1992) and these complexes in turn bind calcineurin with a high affinity, thus inhibiting the phosphatase activity (Liu et al., 1991). It was shown that the phosphatase activity of calcineurin contributes to the activation of transcription factors which are involved in IL-2 transcription (Clipstone and Crabtree, 1992).

Ras is a 21kDa GTP-binding protein with GTPase activity which is rapidly activated following stimulation of the TCR (Downward et al., 1992). The activation of *ras* correlates with the transcriptional activation of the IL-2 gene (Rayter et al., 1992; Woodrow et al., 1993) and activation of the *ras* pathway is not sensitive to CsA treatment.

It has been shown that CD4-positive T cells stimulated by peptide antigen respond differently to

the same cells when stimulated with a superantigen (Liu et al., 1991). It was also suggested that different peptides presented by the same cells may elicit distinct outcomes in the responding T cell (DeMagistris et al., 1992; Jameson et al., 1993; Racioppi et al., 1993; Sloan-Lancaster et al., 1993). In addition, some anti-TCR monoclonal antibodies stimulated cells with only 100 molecules bound per cell while other anti-TCR monoclonal antibodies required ≥10,000 molecules to be bound per cell (Rojo and Janeway, 1988). These results implied that different signals can be delivered to the nucleus of a cell from the same cell surface receptor and it was therefore suggested that the TCR interprets signals through conformational changes induced in the receptor by the binding of the ligand (Janeway and Bottomly, 1994).

1.9.6 Signal transduction by cytokine receptors.

It was originally proposed that each cytokine exerted a specific effect on its own target cell. This, however, is not the case as most cytokines exhibit a wide range of biological effects on various tissues and cells often in a redundant manner. Molecular analysis of cytokine receptors has explained much of this redundancy. Cytokine receptors fall into a family characterised by an extracellular 200 amino acid region of structural homology. This comprises a multichain complex which contains a receptor specific for its ligand and a signal transducing element (Bazan, 1992; Miyajima et al., 1992; Taga and Koshimoto, 1992). IL-6, leukemia inhibitory factor and IL-11 receptors use the same signal transducer (gp130) and so these cytokines have the same function on various different tissues (Taga et al., 1989, 1992; Ip et al., 1992; Yin et al., 1992). IL-3, IL-5 and GM-CSF receptors similarly all interact with the same signal transducing element (Miyajima et al., 1992). Likewise, the IL-2, IL-4 and IL-7 receptor systems share the IL-Rγ chain thereby possibly explaining the similar growth promoting functions of these cytokines.

A general first step in the signalling process of immune and hematopoietic cytokines is ligand induced dimerization of the receptor components. The cytoplasmic regions of the cytokine receptors interact to initiate a signal transduction cascade (Kishimoto et al., 1994). There are no obvious enzymatic motifs (Murakami et al., 1991; Fukunaga et al., 1991) in the cytoplasmic region of the cytokine receptors but a conserved membrane proximal region and dimerization may allow a stable interaction with a signalling molecule. The tyrosine kinase JAK2 has been shown to directly associate with lymphokine receptors and become activated in response to numerous cytokines (Witthuhn et al., 1993; Silvennoinen et al., 1993; Argetsinger et al., 1993). In addition, the tyrosine kinases *fes*, which interacts with the IL-3R and GM-CSFR (Hanazono et al., 1993), and *lck, lyn and fyn* which participate in IL-2 signalling pathways in different cells (Hatakeyama et al., 1992; Torigoe et al., 1992; Kobayashi et al., 1993) have been reported to interact with cytokine receptors.

The binding of IL-2 to the high affinity receptor IL-2R results in signal transduction mediated by the 75kDa protein (IL-2Rβ) and internalisation of the receptor complex. The IL-2Rβ chain is used in two distinct signalling pathways. One region of the IL-2Rβ chain induces cytoplasmic protein tyrosine kinase activation, leading to induction of *c-jun* and *c-fos* (Shibuya et al., 1992). A second region of the IL-2Rβ chain uses a kinase-independant pathway and induces proliferative responses. This signal leads to the induction of the transcription factor c-*myc* which causes the cell to enter into the S-phase of the cell cycle and enter cell division (Shibuya et al., 1992). Studies on the transcriptional activation of acute phase proteins have also provided evidence of two distinct signalling pathways mediating the effects of IL-6 (Akira and Kishimoto, 1992; Kishimoto et al., 1992, 1994). In addition, the receptor common to IL-3, GM-CSF and

IL-5 can deliver different signals from distinct regions of the molecule following stimulation (Sato et al., 1993).

1.10 Lymphocyte homing patterns.

Lymphocytes, which drain to the peripheral lymph nodes from the skin, muscle, gut-associated lymphoid tissues such as Peyer's patches and many other sites, differ in the types of antigens they will have encountered. Separate lineages of lymphocytes, including memory cells have been shown to exhibit specific patterns of recirculation to the skin, gut and lung (Mackay, 1992, 1992a, 1992b; Picker and Butcher, 1992). This occurs due to differences in the expression of adhesion molecules on the lymphocytes extravasating different tissues (Mackay et al., 1992a) and because of these recirculation patterns, lymphocytes are more likely to return to the tissue were they first encountered antigen (Mackay, 1992).

Three families of adhesion molecules have been identified which are involved in the homing of lymphocytes; namely selectins, integrins and chemoattractants. Selectins mediate the attachment and tethering of flowing leucocytes to the vasculature (Lawrence and Springer, 1991; Ley et al., 1991; von Adrian et al., 1991; Lasky, 1992; Mayadas et al., 1993) and are a prerequisite for chemoattractant and integrin mediated cellular adhesion (Arfors et al., 1987; Lawrence and Springer, 1991) Integrins recognise O-linked sialylated carbohydrate residues on serine and threonine rich mucin-like proteins (Rosen, 1993) and integrins are important in leucocyte extravasation (Anderson and Springer, 1987; Larson and Springer, 1990).

1.11 The ruminant immune system.

A general overview of the vertebrate immune system has been discussed with reference to the human and murine species. Ruminants are phylogenetically distinct to mice and man therefore differences in the ruminant immune systems may reflect different ways in which ruminants respond to foreign antigen.

1.11.1 The cellular immune system.

Three distinct subpopulations of ovine T cells have been identified by their expression of either WC1 (T19), CD4 or CD8 (MacKay et al., 1986). The WC1 protein is expressed on $\gamma\delta$ TCR positive lymphocytes and is exclusively expressed in ruminants (MacKay et al., 1989). In the murine and human species, CD4-positive and CD8-positive T cells express the $\alpha\beta$ TCR (Samuelson et al., 1985; Danska, 1989). To date, no monoclonal antibody specific for the ovine $\alpha\beta$ TCR has been produced. Since ruminant $\gamma\delta$ T cells generally do not express CD4 or CD8, it is assumed that ruminant T cells expressing CD4 or CD8 are similar to murine and human T cells expressing CD4 or CD8, and express an $\alpha\beta$ TCR.

The proportion of the various T cell subsets changes markedly during the lifespan of the sheep. Fetal blood has a predominance of CD4-positive and CD8-positive T cells over $\gamma\delta$ T cells whereas during the neonatal period $\gamma\delta$ T cells become the predominant subset. In young sheep $\geq 50\%$ of PBL express the $\gamma\delta$ TCR. This may imply that young sheep have a greater dependence on $\gamma\delta$ T cells rather than $\alpha\beta$ T cells. This is in contrast to humans and rodents where the major T cell subsets express the $\alpha\beta$ TCR. During the post-natal period the $\gamma\delta$ T cell percentage within the lymphocyte population declines and CD4-positive and CD8-positive T cells once again

predominate (MacKay et al., 1986, 1989; Evans, 1993).

The large antigenic load to which neonates are exposed after birth may lead to activation and preferential clonal, or oligoclonal, selection of γδ over αβ T cells (Hein and MacKay, 1991; Hein and Dudler, 1993). The expressed repertoire of γδ T cells in humans and mice is very small, with T cells expressing distinct Vγ and Vδ pairings which populate distinct sites such as skin and gut tissue. In contrast, it has been shown that the repertoire of expressed Vγ and Vδ genes in the peripheral blood of sheep is large compared to the human and mouse species (Hein and Dudler, 1993). In young sheep, the predominance of γδ T cells may be accompanied by a greater diversity in the expressed γδ T cell repertoire.

It has been shown that the protein WC1, originally referred to as T19, is expressed exclusively on γδ T cells in both the ovine species (MacKay et al., 1989) and the bovine species (Clevers et al., 1990). The WC1 protein is not expressed on CD4-positive or CD8-positive T cells, or B cells. During the course of this thesis, a cDNA encoding the bovine WC1 protein was cloned (Wijngaard et al., 1992, 1994; Metzelaar, et al., 1992). It has also been shown in sheep, unlike human and mice, that γδ T cells do not express CD2 (MacKay, 1988; Evans, 1993). This implies that γδ T cells in the ovine species have a decreased dependence on CD2 perhaps for cellular adhesion and activation during an immune response and may rely on other cellular antigens to mediate these effects. In the ruminant species, γδ T cells are prevalent at epithelial sites such as the intestine and skin (Hein and MacKay, 1991) which suggest that they may be involved in immune surveillance of epithelial sites as has been suggested for other species (Janeway et al., 1988).

One reported function of γδ T cells in humans, mice and sheep is cytotoxicity (MacKay et al., 1988). In sheep however, it has been shown by electron microscopy that γδ T cells (Evans, 1993) do not contain electron dense granules. It has been shown in CD8-positive cells of other species that these electron dense granules contain perforin (Kraehenbuehl and Tschopp, 1990). These observations suggest that cytotoxicity may not be the sole function of sheep γδ T cells.

1.11.2 Gut associated lymphoid tissue (GALT).

The GALT consists of the organised lymphoid tissues such as Peyer's patches, mesenteric lymph nodes, and LPL and iIEL (Mowat, 1987). In the sheep, there is a rapid expansion of CD4-positive, CD8-positive and γδ T cells in the lamina propria following exposure to antigens immediately after birth (Gorrell et al., 1988). In the adult sheep, the relative proportions and phenotypes of LPL is similar to that of peripheral blood. In contrast to LPL, IEL are predominantly CD8-positive or γδ TCR positive cells (Gorrell et al., 1988; Press et al., 1991: Gyorffy et al., 1992). In contrast to peripheral blood populations, a large proportion of IEL express MHC class II molecules and lack the CD5 pan T cell marker (Gyorffy et al., 1992). Furthermore, γδ IEL have an immature phenotype (WC1-negative and MHC class I-negative) characteristic of γδ T cells developing in the thymus (MacKay et al., 1986).

1.11.3 Humoral immune system.

The major source of ovine B cells is the ileal Peyer's patch, unlike in murine and human system where these cells are derived mainly from bone marrow (Landsverk et al., 1991). In the ovine species, unlike other species, the placenta is impermeable to maternal immunoglobulins and no transfer of these molecules occurs to the fetus (Brambell, 1970; Carlson and Owen, 1987).

24

1.12 Aims of the thesis.

Research in the sheep has advanced dramatically in recent years with the development of a panel of monoclonal antibodies specific for sheep cell surface proteins. The 1st International Workshop on Ruminant Leucocyte Differentiation Molecules had recently identified monoclonal antibodies which recognise the ovine and bovine homologues of murine and human proteins. These monoclonal antibodies had been clustered according to the CD numbers assigned to murine and human proteins. Numerous monoclonal antibodies were submitted to the workshops which could not be assigned CD numbers because there was no human or murine homologue. The monoclonal antibodies were placed in workshop clusters and analysed. One such cluster was WC1.

The first aim of this thesis was to analyse the WC1 protein in the ovine species. This would be done by use of a number of different anti-WC1 monoclonal antibodies from the 2nd International Workshop on Ruminant Leucocyte Differentiation Molecules. It was intended to analyse the expression of WC1 on ovine leucocytes and, in addition, to establish the WC1 epitope expression on ovine peripheral blood $\gamma\delta$ T cells. It had recently been reported in the bovine species (Davis et al., 1990), that some $\gamma\delta$ T cells expressed epitopes of the WC1 protein on mutually exclusive populations of cells. It was of interest to determine if the same sub-populations of PBL existed in the ovine species. A further aim in the analysis of the WC1 protein was to clone and sequence cDNA encoding this molecule, this is outlined in the appendix.

In the ovine species, $\gamma\delta$ T cells do not express the molecules CD2, CD4 or CD8. These molecules have been shown to be associated with the activation of T cells (Janeway and Bottomly, 1994). In addition, $\gamma\delta$ T cells have long been viewed as the first line of defence against invading pathogens (Haas et al., 1993). If this is the case, $\gamma\delta$ T cells may respond at a faster rate and in a different manner to $\alpha\beta$ T cells. Accordingly, the second major aim of this thesis was to investigate the activation and proliferative response of peripheral blood $\gamma\delta$ T cells following *in vitro* culture with the T cell mitogen ConA, or following culture with antigens to which individual animals have been primed. The responses of $\gamma\delta$ T cells, either in an unfractionated population of PBL or purified, were measured. These responses were compared to the response of $\alpha\beta$ T cells in identical conditions.

The results reported in this thesis attempted to address each of the aims set out above. Novel data on the expression of different WC1 epitopes in the ovine species is reported. In addition, novel data on the kinetics of activation of $\gamma\delta$ T cells and responses to antigen of $\gamma\delta$ T cells are also reported. These data show clearly that $\gamma\delta$ T cells respond in a different manner compared to $\alpha\beta$ T cells. These findings have important implications in understanding the mechanism(s) of activations of $\gamma\delta$ T cells and their role in immune responses.

CHAPTER 2

Materials and Methods

2 MATERIALS AND METHODS

2.1 Animals.

2.1.1 Animals of different age and antigenic burden.

Finnish Landrace sheep of various ages were housed at the Moredun Research Institute, Edinburgh. Blood samples were obtained, by staff at Moredun Research Institute, by venipuncture and collected in Lithium Heparin vacutainers. In addition, lambs kept in gnotobiotic and specific pathogen free (SPF) conditions were also housed at the Moredun Research Institute, Edinburgh. Blood was obtained from age matched gnotobiotic, SPF and normal animals (3-4weeks) and collected in Lithium Heparin vacutainers.

2.1.2 Experimental animals.

All procedures involving experimental animals were carried out in accordance with the regulations laid down in The Animals (Scientific Procedures) Act, 1986. Male and female Finnish Landrace cross Dorset and Grey Faced cross Suffolk sheep were obtained from the Moredun Research Institute, Edinburgh. Animals were immunised with 1 mg ovalbumin (Sigma; Grade V, Cat. No. A5503) and 1 mg heat-killed *M. tuberculosis* (PPD, batch 295, Central Veterinary Laboratory, Weybridge.) emulsified (1:1) in complete Freund's adjuvant (Sigma; Cat. No. F5881) containing H37RA *M. tuberculosis* (ATCC 21577) injected over two sites intramuscularly. A second immunisation with the same antigens in incomplete Freund's adjuvant (Sigma; Cat. No.F5506) was given four weeks later.

2.2 Media.

2.2.1 Medium for lymphocyte culture.

Lymphocyte cultures were maintained in RPMI 1640 (Gibco Biocult.; Cat. no. 074-01800) supplemented with 2 mM L-glutamine (Sigma; Cat. No. G2159), 100 U/ml benzylpenicillin and 100 U/ml streptomycin, 0.05 mM β-mercaptoethanol (Sigma; Cat. No. M6250) and 5mM Hepes (Sigma; Cat. No. H7523). Culture medium consisted of RPMI 1640 with supplements and 10% fetal calf serum (FCS) (Flow Laboratories, Hertfordshire). Wash medium consisted of RPMI with supplements and 1% FCS.

2.2.2 Medium for bacterial culture.

The basic growth medium for *E. coli* strains was Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl (Sigma; Cat. No. S7653) made to pH 7.4 with 5N NaOH (Sigma; Cat. No.S5881)). The selective medium was LB containing 150 µg/ml of ampicillin (LBAmp).

2.2.3 Medium for COS-1 cell culture.

COS-1 cells were maintained in Glasgow's Minimal Essential Medium (G-MEM) (Gibco Biocult; Cat. No. 042-02541) supplemented with 2 mM L-glutamine, 100 U/ml benzylpenicillin and 100 U/ml streptomycin, 0.05 mM β-mercaptoethanol and 5mM Hepes. Culture medium consisted of G-MEM with supplements and 10% FCS, wash medium consisted of G-MEM with supplements and 1% FCS.

2.3 Tissue culture plasticware.

All tissue culture plasticware used for cell culture was supplied by Gibco Biocult, Uxbridge unless otherwise stated.

2.4 Isolation and purification of mononuclear cells.
2.4.1 Peripheral blood mononuclear cells (PBMC).

Ovine peripheral blood was obtained by venipuncture and collected into preservative-free heparin. The final concentration of heparin was 10 units per ml of blood. White blood cells were buffy coated by centrifugation of heparinized whole blood at 1300xg for 15 minutes at 20°C. Buffy coat cells were harvested into sterile PBS with 1% FCS (PBSF) and centrifuged over Lymphoprep (Nyegaard, Oslo, Norway) at 850 xg for 20 minutes at 20°C, with no brake applied at the end of the centrifugation. PBMC were isolated from the Lymphoprep-PBS interface and washed twice with ice cold PBSF. Cells were harvested during each wash procedure by centrifugation at 250 xg for 4 minutes at 4°C. Throughout this thesis, cells were washed and harvested by centrifugation at 250 xg for 4 minutes at 4°C unless otherwise stated.

2.4.2 Mononuclear cells from lymph nodes, spleen or thymus.

Tissues were removed aseptically from animals by standard surgical techniques and were immersed in ice-cold PBSF. Cells were obtained by teasing the tissues apart using forceps and a scalpel blade. The resultant cell suspension was depleted of large debris via sedimentation by gravity for 15 minutes. The supernatant was collected carefully and washed twice with ice-cold PBSF. Mononuclear cells were then purified over Lymphoprep as described in section 2.4.1.

2.4.3 Intraepithelial and lamina propria mononuclear cells from ileal mucosa.

Sections of ileum were isolated by standard surgical techniques and opened by longitudinal section. The tissue was cut into 2-4 cm² portions and washed twice in Hanks' balanced salt solution (HBSS) (Gibco Biocult; Cat. No. 041-04020). Sections were incubated in calcium and magnesium free HBSS (fHBSS) (Gibco Biocult; Cat. No. 041-04170) supplemented with 1 mM EDTA (Sigma; Cat No. E6758) and 5% FCS for 30mins at 37°C with vigorous agitation. The supernatant, containing intraepithelial cells was carefully removed. Intraepithelial mononuclear cells were then isolated over Lymphoprep as described in section 2.4.1. The sections of tissue were then treated to obtain lamina propria cells. The first incubation was repeated for one hour at 37°C and the cell suspension in the supernatant discarded. The sections were then incubated in fresh fHBSS containing 1 mM EDTA, 5% FCS and 50 U/ml collagenase Type XI (Sigma; Cat. No. C7657). Tissues were incubated for 90 minutes to digest collagen. The resulting cell suspension was removed and filtered over nylon wool. Mononuclear cells were subsequently purified by density gradient centrifugation over Lymphoprep as described in section 2.4.1.

2.4.4 Afferent and Efferent mononuclear cells.

Pseudo-afferent and efferent lymphatic cannulations were performed by Dr. John Hopkins (Department of Veterinary Pathology, University of Edinburgh) as previously described (Hall 1967). Pseudo-afferent cannulations required the excision of lymph node at least eight weeks prior to the cannulation, during which time the afferent vessels re-anastamose with the efferent

27

duct. Cannulated sheep were kept in standard pattern metabolism cages and fed hay and water *ad libitum*. Lymph was collected into sterile 250 ml plastic bottles, containing 2.5×10^3 units of heparin and 2.5×10^4 units each of penicillin and streptomycin. Lymph was collected during the same 2 hour period on each day. Lymphocytes were washed twice with ice cold PBSF and subsequently used in assays.

2.4.5 Enrichment of γδ T cells by positive selection using MACS.

Purified lymphocytes were resuspended in PBSF at 4×10^7 cells per ml. These were incubated with the biotinylated anti-WC1 monoclonal antibody, CC15 for 20mins at 4°C and then washed with PBS supplemented with 5mM EDTA. Cells were further incubated with avidin microbeads (Miltenyl Biotec, Eurogenetics UK Ltd.; Cat. No. 481-01) and SA-PE for 15mins on ice and then washed with PBSF. The labelled lymphocyte pellet was resuspended in PBSF and loaded onto a MACS separator (Becton Dickinson, Oxford). This consists of a ferromatrix column located within a strong magnetic field, which was precooled by washing with ice-cold PBSF. The column, with loaded cells, was then washed with 3-4 column volumes of PBSF in the presence of the magnetic field. Cells retained on the column were then washed back to the top of the column by a series of back flushes with PBSF in the absence of the magnetic field, followed by subsequent washing with 3-4 column volumes of PBSF in the presence of the magnetic field. Positively retained cells were subsequently recovered by removal of the magnetic field and elution of the column with PBSF. These cells were analysed by FACS to determine their percentage purity. The purification procedure was repeated until cells were >96% pure.

2.5 Monoclonal antibodies and immunofluorescence staining.

The monoclonal antibodies used in this thesis are shown in Table 1 (Chapter 3). Their specificities, isotype and reference publications are listed.

2.5.1 Titration of working dilution of monoclonal antibodies.

Ascitic fluid and culture supernatant from the monoclonal antibody cell lines were collected. Ascitic fluid was diluted 1in 50 into PFA and then doubling dilutions were made in PFA to a final dilution of 1 in 3200. A doubling dilution of culture supernatant was made into PFA to a final dilution of 1 in 128. 50µl of these dilutions of antibody preparations were then incubated with 1×10^6 PBL, from the species to which the monoclonal antibody was raised, for 20 minutes on ice. Unbound antibody was removed by washing the cell once with ice-cold PFA. The cells were then incubated with 50µl of FITC conjugated $F(ab)_2$ fragment of rabbit anti-mouse immunoglobulin (Dakopatts, Denmark; Cat. No. F313) at the manufacturers recommended working dilution in PFA, for 15 minutes on ice. Cells were then washed twice and analysed by flow cytometry. The optimal working dilution of the monoclonal antibody preparation was determined by the analysis of the histograms of fluorescence intensity versus cell number. The greatest dilution which gave the highest intensity of staining was used routinely throughout this thesis as the working dilution of the monoclonal antibody.

2.5.2 Immunolabelling of cell surface antigens.

The cell surface markers of lymphocytes were stained. Lymphocytes, isolated from any of the tissues or body fluids described above were first washed twice with ice-cold PFA (PBS

28

supplemented with 1%FCS and 0.1% Sodium Azide (Sigma; Cat. No. S8032) and treated as described below.

2.5.2.1 Single colour immunofluorescence.

For single colour immunofluorescence analysis, 1×10^6 cells were incubated with 50 µl of the primary monoclonal antibody at the appropriate dilution in PFA for 20 minutes on ice. Unbound antibody was removed by washing once with ice-cold PFA. The cells were then incubated with 50µl of FITC conjugated F(ab)$_2$ fragment of rabbit anti-mouse immunoglobulin (Dakopatts, Denmark; Cat. No. F313) at the appropriate dilution in PFA for 15 minutes on ice. Subsequently, cells were washed twice with PFA prior to analysis by flow cytometry.

2.5.2.2 Two colour immunofluorescence.

Two colour immunofluorescence analysis requires that the reactivity of each monoclonal antibody is detected by a different fluorochrome. For this type of analysis, 1×10^6 cells were treated in one of the following three ways:

1) Cells were incubated with 50µl of each of the two primary monoclonal antibodies, of different isotypes, at the appropriate dilutions in PFA for 20 minutes on ice. Unbound antibody was removed by washing once with ice-cold PFA. The reactivity of one of the monoclonal antibody used was detected by incubation for 15 minutes on ice with an isotype specific FITC-conjugated secondary antibody. The FITC-conjugated secondary antibodies used were either sheep anti-mouse IgG1-FITC conjugate (The Binding Site, Birmingham; Cat. No. PF273), sheep anti-mouse IgG2a-FITC conjugate (The Binding Site, Birmingham; Cat. No. PF274) or sheep anti-mouse IgM-FITC conjugate (The Binding Site, Birmingham; Cat. No. PF278). The reactivity of the second monoclonal antibody, of different isotype, was developed by incubating the cells with an isotype specific biotin-conjugated secondary antibody for 15 minutes on ice followed by incubation with phycoerythrin conjugated to streptavidin (Pharmacia; Cat. No. STAR4) for 5 minutes on ice. The biotinylated secondary conjugates used were either sheep anti-mouse IgG1-biotin (The Binding Site, Birmingham; Cat. No. PB273), sheep anti-mouse IgG2a-biotin (The Binding Site, Birmingham; Cat. No. PB274) or sheep anti-mouse IgM-biotin (The Binding Site, Birmingham; Cat. No. PB278). The secondary reagents selected were dependent on the isotype of the primary monoclonal antibody and used at appropriate dilutions in PFA. Subsequently, cells were washed with PFA and analysed.

2) The method used routinely throughout this thesis was to biotinylate one of the primary monoclonal antibodies (as described in Section 2.5.3). This allowed for quicker experimentation. It was necessary that the two monoclonal antibodies used were of different isotypes. The reactivity of the non-biotinylated monoclonal antibody was developed as in method 1. The reactivity of the biotinylated monoclonal antibody was detected by incubation with phycoerythrin conjugated to streptavidin for 5 minutes on ice. Cells were then washed with PFA and analysed.

3) A method which allowed for comparison of reactivity of monoclonal antibodies of the same isotype by two colour immunofluorescence involved sequential staining. One of the monoclonal antibodies was biotinylated. Cells were incubated with the non-biotinylated monoclonal antibody for 20 minutes on ice. Unbound antibody was removed by washing once with ice-cold PFA. Reactivity of this monoclonal antibody was detected by incubating the cells

with 50μl of FITC conjugated F(ab)$_2$ fragment of rabbit anti-mouse immunoglobulin for 15 minutes on ice. The cells were washed with PFA and then incubated with 1% normal mouse serum (NMS) (Sigma; Cat.No. M5905), diluted in PFA, for 15mins on ice to block any remaining reactivity of the secondary anti-mouse immunoglobulin. Cells were once again washed in PFA and incubated with an appropriate dilution of the biotinylated monoclonal antibody. The reactivity of the biotinylated monoclonal antibody was detected, following washing in PFA, by incubation with phycoerythrin conjugated to streptavidin for 5 minutes on ice. Finally cells were washed with PFA and analysed.

2.5.3 Immunofluorescence analysis.

All immunofluorescence analysis described in this thesis was carried out using a Becton Dickinson FACScan system, Mountain View, California, USA. After immunolabelling, fluorescence measurements were made on populations of cells defined by setting electronic gates on forward scatter (FSC) and side scatter (SSC) measurements. Dead cells were excluded on the basis of low forward light scatter 10^4 "live gated' cells were analysed per sample. The fluorescence profile of a gated population of cells was displayed as a histogram of fluorescence intensity versus relative cell number. Background fluorescence was determined following reactivity of cells with NMS or NMS-biotin.

2.5.4 Biotinylation of monoclonal antibodies.

Ascitic fluid of the monoclonal antibodies CC15, SBU-T4, SBU-T8 was supplied by Esme Mills (Hybridoma Unit, Department of Veterinary Pathology, University of Edinburgh). Ascitic fluid of the monoclonal antibodies BAQ4A, CACTB1A was a kind gift from Dr. W. C. Davis (Washington State University). IgG was purified from ascitic fluid by precipitation of high molecular weight proteins. 25μl of 1M acetic acid (BDH; Cat. No. 10001) was added per 500μl of ascitic fluid and mixed. To this 100% caprylic acid (Sigma; Cat. No. C2875) was added dropwise (37.5μl of caprylic (octanoic) acid per millilitre of original ascitic fluid with vigorous shaking and incubated at room temperature for 30 minutes. Precipitated proteins were removed by microfuging for 30 seconds. The supernatant was retained and dialysed extensively into PBS over 72 hours. A 1mg ml^{-1} solution of biotin-o-succinimide ester (Sigma; Cat. No. H1759) was prepared in DMSO (Sigma; Cat. No. D2650) and 0.1M NaHCO$_3$ pH 8.3 (BDH; Cat. No. 10247). The protein concentration of the dialysed immunoglobulin was determined and 75μl of the biotin solution added per 1mg of immunoglobulin. This suspension was mixed immediately and incubated with mixing for 4 hours at room temperature. The biotinylated immunoglobulin was then dialysed extensively against PBS and the immunoglobulin concentration determined by spectrophotometry. FACS analysis was used to determine that the monoclonal antibody had retained activity and that the biotinylation procedure was effective.

2.6 *In vitro* proliferation assays.
2.6.1 Irradiation of antigen presenting cells (APC).

PBMC prepared from ovine peripheral blood as described above were resuspended in culture medium at a concentration of 2×10^6 cells ml^{-1} and irradiated using a cobalt-caesium source (Kings Buildings, University of Edinburgh) The level of irradiation which prevented subsequent proliferation of PBMC when cultured with Con A (Sigma Cat. No. C2631) was determined and found to be 3000 rads (data not shown). This amount of radiation was used for treatment of

30

antigen presenting cells in subsequent proliferation assays.

2.6.2 Proliferative response of unfractionated cells.

Ovine mononuclear cells were purified as described in Section 2.4 and resuspended in culture medium. The proliferative response of unfractionated PBMC was measured as follows. 1×10^5 cells in 100μl were added per well to 96-well, round-bottom micro-culture plates (Nunculon, Denmark). Various concentrations of antigens (*Mycobacterium paratuberculosis* (M.tb.) and Ovalbumin (Ova)) were then added to the wells to make a final volume of 200μl. Each concentration of antigen was tested in triplicate. Positive and negative controls were established in the presence of Con A or culture media respectively. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air for 3-5 days and pulsed with 1μCi ^{3}H-thymidine, specific activity 2Ci/mMole, (Amersham Cat. No. TRK310) over the last 5 hours. Cells were collected onto glass filter paper using a Tomtek automated harvester and ^{3}H-thymidine incorporation was assessed by liquid scintillation counting. The data is expressed as the geometric mean for the triplicate cultures with standard deviation included. No irradiated antigen presenting cells were added to the culture.

2.6.3 Proliferative response of purified γδ T cells.

Sheep γδ T cells were purified from peripheral blood of young lambs by positive selection using the anti-WC1 monoclonal antibody CC15 and magnetic activated cell sorting (MACS) as described in detail in Section 2.4.5. The positively selected cells were >96% γδ T cells as judged by immunofluorescence staining. γδ T cells purified from antigen-primed or non-primed sheep were resuspended in medium and 1×10^5 cells in 50μl were added per well to 96-well, flat-bottom micro-culture plates (Nunculon, Denmark). 1×10^5 autologous irradiated PBMC in 50μl were also added to each well to act as antigen presenting cells. Various concentrations of recall antigens (M.tb and Ova) were added to make a final volume of 200μl. Each concentration of antigen was tested in triplicate. Positive and negative controls were established with either Con A and culture media respectively. The proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air for 3-5 days and subsequently pulsed with 1μCi ^{3}H-thymidine and harvested as previously described in Section 2.6.2.1. Unfractionated and γδ T cell depleted populations were cultured in an identical manner to monitor the role of γδ T cells or other PBMC in generating proliferative responses.

2.6.4   Concanavalin A activation of unfractionated PBL and purified γδ T cells.

PBL and γδ T cells prepared from ovine peripheral blood, were resuspended in culture medium at a concentration of 2×10^6 cells per ml. Cell were then cultured in the presence or absence of Con A at a final concentration of 5μgml^{-1}. At various time intervals, viable lymphocytes were harvested by density centrifugation over Lymphoprep as previously described and then washed into PFA. Both single colour and two colour immunofluorescence analysis were performed on harvested viable cells.

2.6.5   Antigen-induced activation of unfractionated PBL and purified γδ T cells.

PBL and γδ T cells prepared from ovine peripheral blood, were resuspended in culture medium at a concentration of 2×10^6 cells per ml. Cells were cultured in the presence or absence of optimum

31

concentrations of antigens. At various time intervals, viable cells were harvested by density centrifugation over Lymphoprep as previously described and then washed in PFA. Both single colour and two colour immunofluorescence analysis was then performed on harvested viable cells to determine the expression of activation markers on the cell surface.

2.6.6 Mixed lymphocyte cultures of unfractionated PBL and purified γδ T cells.

Sheep γδ T cells, as responders, were purified from peripheral blood of young lambs by positive selection using the anti-WC1 monoclonal antibody CC15 and magnetic activated cell sorting (MACS) as described in detail in Section 2.6.2. The positively selected cells were >96% γδ T cells as judged by immunofluorescence staining. 1×10^5 purified γδ T cells cells in 100μl were added to 96-well, round-bottom micro-culture plates (Nunculon, Denmark). For stimulator cells, peripheral blood was obtained from two sheep of a non-matched breed, PBL were purified and then irradiated. Various numbers of irradiated PBL were added to wells containing responding cells. Dilutions of stimulator cells were made from 1×10^6 cells to ~4×10^3 cells per well. Each dilution of stimulator cells was assayed in triplicate. An autologous population of irradiated PBL was added to a set of wells to determine the allo-specificity of any responses. Cultures were made to a final volume of 200μl. Positive and negative controls were established with either Con A or culture media. The proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air for 3-5 days and subsequently pulsed with 1μCi ³H-thymidine and harvested as previously described. Unfractionated and γδ T cell depleted populations of PBL were treated in an identical manner in order to determine the role of γδ T cells or other PBL in generating proliferative responses.

2.7 Affinity purification.
2.7.1 Affinity purification of immunoglobulin.

Saturated supernatant made from the monoclonal antibody producing cell line ST197 was prepared by Esme Mills (Hybridoma Unit, Department of Veterinary Pathology, University of Edinburgh). Immunoglobulin from this supernatant was purified by affinity chromatography on a GammaBind-PLUS affinity column (BDH; Cat. No. GBA 030005). The column was washed with 10 volumes of the fresh culture medium and supernatant then applied to the column at a flow rate of 10ml hr⁻¹. The material was passed through the column once. The column was then washed extensively with washing buffer (0.01M sodium phosphate pH 7.0, 0.15M sodium chloride and 0.01M EDTA). [0.01M sodium phosphate was prepared by mixing 0.01M sodium dihydrogen orthophosphate (BDH; Cat. No. 10249) with 0.01M di-sodium hydrogen orthophosphate (BDH; Cat. No. 30716) until the correct pH was obtained]. Once no protein was detectable in the flow through from the column, as judged by spectrophotometry at 280nm on a Pharmacia LKB Ultraspec, bound material was eluted with 0.05M Acetic acid adjusted to pH 3.0 with ammonium hydroxide (Sigma; Cat. No. A6899). 2ml fractions were collected into 0.9ml 1.0M Tris HCl (Sigma; Cat. No. T6791) pH 9.0 to neutralise the acid (450μl per ml of eluate). Protein containing fractions were identified by spectrophotometry at 280nm. Peak fractions were pooled and dialysed extensively against a sodium bicarbonate buffer pH 8.3 containing 0.5M NaCl. The column was washed with five volumes of cleaning buffer (1.0M acetic acid) and re-equilibrated with washing buffer to pH 7.0 prior to subsequent re-use.

32

2.7.2 Preparation of affinity columns for the purification of ovine WC1.

Affinity purified immunoglobulin (ST197) was coupled to cyanogen bromide (CNBr) activated Sepharose 4B (Sigma; Cat. No. C9142) according to the protocol described in the Pharmacia guide to affinity chromatography (Pharmacia literature, 1983). Briefly, the required amount of CNBr-activated Sepharose 4B was washed and swollen on a scintered glass filter with 1mM HCl (BDH; Cat. No. 10125). The beads were finally washed into coupling buffer (0.1M sodium bicarbonate buffer pH 8.3 containing 0.5M NaCl). Immunoglobulin previously dialysed into the coupling buffer was added to the sepharose and left overnight at 4°C. To block remaining active groups the gel was washed with 1.0M ethanolamine pH 8.0 (Sigma; Cat. No. E9508) and mixed for 16 hrs at 4°C. Excess non-adsorbed protein was removed by washing with coupling buffer followed by a 0.1M acetate buffer pH 4.0 containing 0.5M sodium chloride and finally coupling buffer. Immediately before use the column was pre-eluted with eluting buffer (15mM triethanolamine (Sigma; Cat. No. T1502), 0.5M NaCl and 0.5% sodium deoxycholate (Sigma; Cat. No. D5670) at pH 11.3) and re-equilibrated with neutralising buffer (0.2M phosphate buffer pH 8.5). In order to remove any non-specific material from the lysates applied (Section 2.7.3), pre-columns were made as described above for the ST197 column. The pre-column was coupled to normal mouse sera immunoglobulin and the pre-pre-column contained no immunoglobulin.

2.7.3 Preparation of spleen lysates.

Spleens were obtained from freshly killed animals at the abbatoir and put on ice. Splenocytes were removed from the spleen by dissection of the enclosing connective tissue capsule with a scalpel and forceps. The material remaining was homogenised with ice-cold Hanks Balanced Salt Solution (HBSS) for 1 minute. Large particulate material was left to settle out for 10mins then the supernatant was removed. Peripheral blood lymphocytes or lymphocytes isolated from lymphatic vessels were washed three times with HBSS. The cell suspension of splenocytes or lymphocytes was pelleted by centrifugation at 630g for 15 minutes at 4°C. The supernatant was discarded and the cells resuspended for 30 minutes in ice cold TNT buffer (20mM Tris HCl, 140mM NaCl, 0.5% Triton X100 (Sigma; Cat. No. X-100)) containing a final concentration of 0.2mM PMSF (Sigma; Cat. No. P7626). Cellular debris was removed by centrifugation at 630g for 20 minutes at 4°C, followed by centrifugation at 12000g for 2 hours at 4°C. The spleen lysate was filtered through Millipore prefilters of 0.6µm pore size (Millipore, Ireland; Cat. No. AP 1504700) immediately before addition to the column.

2.7.4 Affinity purification of ovine WC1.

Spleen lysates prepared as described in Section 2.7.3 were applied to the affinity columns at 4°C at a flow rate of 10ml per hour. The material was passed through the column three times. The columns were then washed extensively with washing buffer consisting of 15mM triethanolamine and 0.5% sodium deoxycholate at pH 8.0 until no protein was detectable, by spectrophotometry at 280nm, in the flow through. The bound material was eluted with eluting buffer (15mM triethanolamine, 0.5M NaCl and 0.5% sodium deoxycholate at pH 11.3). 2ml fractions were collected into 0.5M Triethanolamine (TEA) pH 7.9 to neutralise the alkalinity of the eluted material (300µl per ml of eluate). Protein containing fractions were identified by spectrophotometry at 280nm and peak fractions were pooled. The column was re-equilibrated with 5 volumes of neutralising buffer (0.01M sodium phosphate pH 8.5) and then finally washed with 10 volumes of washing buffer.

2.8 Immunohistology of tissues.

Tissues were removed by standard surgical techniques and were snap-frozen in dry ice/isopentane (BDH; Cat. No. 29452). Cryostat sections of tissue cut at 5μm thickness were air-dried onto glass slides and fixed for 10 minutes in acetone at 4°C. Expression of the various ovine lymphocyte surface antigens was detected by staining with monoclonal antibodies listed in Chapter 3 using a commercially available streptavidin-biotin immunoperoxidase staining kit (EURO/DPC, Witney, UK; Strept A-B Universal Kit) according to the manufacturers instructions. Positive staining was visualised by conventional microscopy.

2.9 Protein immunochemistry.
2.9.1 Immunoprecipitation.
2.9.1.1 Iodination of lymphocytes.

Peripheral blood lymphocytes were purified as described and finally washed three times with PBA (PBS supplemented with 0.1% azide). Lymphocytes were radiolabelled with 1mCi of ^{125}I (Amersham, U.K.). Briefly, 100μl of 1mg/ml iodogen in dichloromethane was dried onto a microfuge tube with a stream of gaseous nitrogen. 2×10^7 cells were resuspended in 200μl of PBS then added to the microfuge tube with dried iodogen. 1mCi of ^{125}I was added immediately to the same microfuge tube and the reaction was incubated at room temperature for 2-4 hours, shaking every 15-20mins. Following iodination, the cells were washed twice with PBA.

2.9.1.2 Preparation of lymphocyte lysates.

Labelled or unlabelled lymphocytes were pelleted by centrifugation at 630g for 15 minutes at 4°C. The supernatant was discarded and the cells resuspended for 30 minutes in ice cold TNT buffer (20mM Tris HCl, 140mM NaCl, 0.5% Triton X100 (Sigma; Cat. No. X-100)) containing a final concentration of 0.2mM PMSF (Sigma; Cat. No. P7626). Cellular debris was removed by centrifugation at 630g for 20 minutes at 4°C, followed by centrifugation at 12000g for 2 hours at 4°C.

2.9.1.3   Coupling of monoclonal antibodies to protein A-sepharose beads.

5ml of protein A-sepharose (Sigma; Cat. No. P3391) was washed twice with PBA. 100μl of protein A-sepharose was incubated with 10μl of rabbit anti-mouse Ig (Serotech Cat. No. SERT 100) overnight at 4°C. The protein A-sepharose coupled to rabbit anti-mouse Ig was washed twice with PBA and was then incubated overnight at 4°C with an 100μl aliquot of cold lymphocyte lysate to block any non-specific sites of reactivity. The protein-A sepharose beads were washed free of cold lysate and were then incubated overnight at 4°C with 10μl ascitic fluid of the required monoclonal antibody. The following day the protein-A sepharose beads coupled to rabbit anti-mouse Ig and monoclonal antibody were finally washed twice with PBA.

2.9.1.4 Immunoprecipitation of antigen.

100μl of radiolabelled lysate was incubated with 100μl protein A-sepharose coupled to rabbit anti-mouse Ig overnight at 4°C. This was to remove any non-specific antigen which was radiolabelled. The following day the protein A-sepharose coupled to rabbit anti-mouse Ig was removed by centrifugation. 100μl of the pre-cleared radiolabelled lysate was added to 100μl of

34

protein A-sepharose coupled to the monoclonal antibody of choice. This was incubated overnight at 4°C. The lysate was then removed and the protein A-sepharose/antigen complex was washed. The complex was washed twice in wash buffer I (0.5% sodium deoxycholate, 150mM lithium chloride (Sigma; Cat. No. L0505), 0.1M tris.Cl pH 8.0) then washed once in wash buffer two (0.5% sodium deoxycholate, 0.5M sodium chloride, 0.1M tris.Cl pH 8.0). The series of washes was repeated twice. The protein A-sepharose/antigen complex was resuspended in reducing sample buffer and boiled for 3 minutes. 25µl of the sample was run on a 5-15% SDS-PAGE and the gel then dried. The dried gel was put on X-ray film (Kodak Products, UK) at -70°C for 1-3 weeks and the X-ray film was developed.

2.9.2 Protein quantitation.

Purified protein concentrations were estimated with the Bio-Rad dye-binding assay (Bio-Rad Laboratories; Cat. No. 500-0006) according to the manufacturers instructions. Known concentrations of bovine serum albumin (Sigma; Cat. No. A2153) were used as standards.

2.9.3 ELISA.

100µl of capture monoclonal antibody, ST197, (100µg/ml) was coated onto 96-well flat-bottomed plates (Nunculon, Denmark) overnight at 4°C. The capture monoclonal antibody was removed and retained for further use. The plates were washed x3 with wash buffer (PBS, 0.1% Tween 20 (Sigma; Cat. No. P1379) then incubated with 1% BSA in PBS to block any non-specific reactivity. 100µl aliquots of eluted WC1 antigen were added to the wells in triplicate at various concentrations and incubated overnight at 4°C or at 37°C for 30 minutes. Excess antigen was removed and the wells were washed x3 in wash buffer. Biotin conjugated developing monoclonal antibody (CC15) was added to the wells at a previously determined working dilution and the plates were incubated for 30 minutes at 37°C. The wells were washed x3 in wash buffer. Streptavidin conjugated to alkaline phosphatase (Boehringer Mannheim Chemica, Germany; Cat. No. 1089 161) was added at a dilution of 1:1000 to the wells and the plates incubated for 30 minutes at 37°C. The plates were washed x3 with wash buffer and once with ELISA substrate buffer (0.05M glycine, 0.03M NaOH, 0.25 mM each of $ZnCl_2$ (Sigma; Cat. No. Z3500) and $MgCl_2$ (Sigma; Cat. No. M1028)). The assay was then developed by adding the phosphatase substrate, p-nitrophenyl phosphate (Sigma; Cat. No. 043-H6157) at 0.1M in substrate buffer for 10-30 minutes at 37°C. ELISA plates were read at a wavelength of 405nm, on a Dynatech MR3000 micro ELISA plate reader. Controls were set up using an irrelevant biotinylated monoclonal antibody to develop the captured WC1 antigen and also with no WC1 antigen present.

2.9.4 SDS-PAGE.
2.9.4.1 Preparation of samples for SDS-PAGE.

Lymphocytes were isolated from either peripheral blood or cannulated lymphatic vessels as previously described (Section 2.4) and washed three times in ice cold PBS. Lymphocytes were then harvested by centrifugation at 250g for 4 minutes at 4°C and resuspended at a concentration of 1×10^8 cells per ml in lysis buffer (2% Nonidet P-40 (Sigma Cat. No. N6507) in PBS containing 0.2mM PMSF). The suspension was incubated for 30 minutes on ice. Cellular debris was removed by centrifugation in a microfuge for 10 minutes and the supernatant collected and diluted 1:1 with sample loading buffer (10% (v/v) β-mercaptoethanol; 10% (w/v) SDS (Sigma; Cat. No. L5750); 125 mM Tris.Cl, pH 6.7; 20% (v/v) glycerol (Sigma; Cat. No. G7893); 0.1%

(w/v) bromophenol blue (Sigma; Cat. No. B6896)). The samples were heated in a boiling water bath for 4 minutes prior to use.

2.9.4.2 Running SDS-PAGE.

Protein separation was by discontinuous polyacrylamide gel electrophoresis using a Tris-glycine buffer containing SDS (384 mM glycine; 0.1% SDS; 50 mM Tris.Cl, pH 8). Gels were either non-gradient or 5-15% linear gradient acrylamide gels. The stock acrylamide solution was 30% acrylamide (Bio-Rad Laboratories; Cat. No. 161-0100):0.7% bisacrylamide (Bio-Rad Laboratories; Cat. No. 161-0200)) in 375 mM Tris.Cl (pH 8.6), 0.1% SDS buffer. Stacking gels were made of 3% acrylamide:0.07% bisacrylamide in 125 mM Tris.Cl (pH6.7), 0.1% SDS buffer. Separating gels were either non-gradient 10% acrylamide:0.23% bisacrylamide or gradient 5-15% acrylamide:0.1-0.35% bisacrylamide in 375mM Tris.Cl (pH8.7), 0.1% SDS buffer. Gel solutions were made and then cast by the addition of 50μl of 10% ammonium persulphate (Bio-Rad Laboratories; Cat. No. 161-0700) and 5μl TEMED (Bio-Rad Laboratories; Cat. No. 161-0800). Protein samples were loaded and electrophoresed through vertical slab gels using Bio-Rad Mini-protean II gel equipment. Gels were run at 200V for 30-45 minutes along with Pharmacia Electrophoresis Calibration Kit markers (molecular weight range 14.4 - 94.0kDa). β-galactosidase (118kDa) (Sigma; Cat. No. G8511) and Myosin (205kDa) (Sigma; Cat. No. M3889) were used as comparable high molecular weight markers.

2.9.4.3 Staining of SDS-PAGE.

Coomassie Brilliant Blue G (BBG) (Sigma; Cat. No. B8522) was dissolved in methanol (BDH; Cat. No. 10158) to a final concentration of 0.25%. A solution of 20% methanol-BBG and 5% acetic acid was filtered through Whatman No. 1 filter paper and used to stain gels for 15 minutes at room temperature. Gels were then destained with several changes of 20% methanol, 5% acetic acid. Gels to be silver stained were first fixed in 50% methanol, 10% acetic acid for 15 minutes with constant agitation. This was then replaced by 5% methanol, 7% acetic acid for 30 minutes and finally replaced by 10% glutaraldehyde (BDH; Cat. No. 28682) for 30 minutes. Fixed gels were rinsed well in several changes of Milli-Q water. The gels were then stained with 0.1% silver nitrate (BDH; Cat. No. 10233) for 15 minutes and rinsed in water. The stain was developed in a 3% Na_2CO_3/ 0.02% formaldehyde (Sigma; Cat. No. F1268) solution. When required solid citric acid (Sigma; Cat. No. C0759) was added to stop the reaction. Developed gels were washed in several changes of distilled water, fixed in 10% 'Ilfofix' (Ilford Ltd., Mobberley, Cheshire) for 1 minute and washed well in water.

Coomassie and silver stained gels, when developed, were transferred to 3MM paper and dried at 80°C under vacuum on a Bio-Rad Laboratories Gel Drier (Model 583).

2.9.5 Western Blot analysis.

After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Hybond-C, 0.45μ, Amersham) using a semi-dry electroblotter (Ancos, Denmark) (Khyse-Andersen, 1984). Transfer was performed in 25 mM Tris.Cl, 20% methanol for 45 minutes at a constant current of 120 mA. After blotting, the nitrocellulose membranes were "blocked" using 5% non-fat dried milk (Sainsburys, Savacentre, Edinburgh) in PBS before overnight incubation in primary antibody, diluted in PBS /1% non-fat dried milk. Blots were washed in several changes of blot washing

buffer (1x PBS; 1% non-fat dried milk; 0.1% Tween 20) for 30 minutes at room temperature. Bound primary antibody was detected by incubating for 60 minutes at room temperature with either an anti-mouse (Sigma; Cat. No. B-7264) or anti-sheep (The Binding Site Ltd., Birmingham; Cat. No. PB360) immunoglobulin biotin conjugate (1:1000) followed by alkaline phosphatase conjugated to streptavidin (1:1000). Excess reagent was removed by washing in several changes of buffer over 30 minutes at room temperature. The immunoblots were developed with 0.28 mg/ml nitro blue tetrazolium (Sigma; Cat. No. N5514) and 70µg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma; Cat. No. B6149) in 0.1M Tris.Cl (pH 9.5) as described by Pluzek and Ramlau (1988). Development of blots was halted by washing in water.

2.10 Statistical analysis of data.

Data obtained in this thesis was statistically analysed to determine the significance of any observed differences. Following consultation with Dr. Ian Wilson, University of Reading, data was analysed by one of two methods depending on the experimentation used. Regression analysis was used to determine the significance of observations made over time, were time is a covariate. When regression analysis could not be used, two sample t-tests were used to determine the significance of the difference between two sets of data. The statistical analysis was carried out using the Minitab 10 for Windows™ statistical package (Minitab Inc., 1994). The significance of differences in the data are detailed in the legends to each of the figures and tables which were statistically analysed.

2.11 Microbiological techniques.
2.11.1 Bacterial Strains.

Escherichia coli.(E.Coli.) strain JM101 (supE, thi, Δ(lac-proAB), [F', traD 36, proAB, lacIq Z ΔM15]) (Yanisch-Perron et al., 1985) was used for bacterial transformations.

2.11.2 Preparation of competent *E. coli.*

E. coli, competent for transformation by plasmid DNA were prepared by a modification of the method of Hanahan (1983). 200 µl of a fresh overnight culture of *E. coli* was diluted 1:100 into psi broth (2% tryptone, 0.5% yeast extract, 20mM magnesium sulphate, 10mM sodium chloride, 5mM potassium chloride). Cells were grown with vigorous shaking (230 rpm) at 37°C, until the OD at 550nm reached 0.3. 5 ml of the culture was diluted 1:20 in psi broth and grown until OD 550nm reached 0.48. The culture was chilled briefly on ice, and then centrifuged at 2000xg for 5 minutes at 4°C. The cell pellet was resuspended in 33 ml ice-cold transforming buffer I (35mM sodium acetate, 100mM calcium chloride, 100mM rubidium chloride (Sigma; Cat. No. R2252), 50mM manganese chloride (Sigma; Cat. No. M3634), 15% glycerol, made to pH 5.9 with acetic acid) and held on ice for 10-15 minutes. This was then centrifuged at 2000xg for 5 minutes at 4°C. The pellet was resuspended in 4 ml of Transforming Buffer II (10mM morpholinopropane sulfonic acid (Sigma; Cat. No. M5162), 10mM rubidium chloride, 75mM calcium chloride, 15% glycerol; made to pH 6.7 with potassium hydroxide) and kept on ice for a further 20 minutes. 200µl aliquots were then snap-frozen in liquid nitrogen and stored at -70°C until required.

2.11.3 Transformation of *E. coli* with recombinant plasmid DNA.

For bacterial transformation a 100µl aliquot of competent *E. coli* was thawed on ice. To this, 100-

200ng of DNA was added. The suspension was chilled on ice for 30 minutes prior to heat-shocking at 42°C for 60-90 seconds. Cells were cooled briefly and then each aliquot was made to 1ml with LB broth (containing no antibiotics) and incubated, with shaking, at 37°C for 1 hour. Cells were pelleted at 500xg for 10 seconds and resuspended in 200µl of LB. Initial selection was achieved by plating out cell suspension on plates of LBAmp agar. Antibiotic resistant colonies were picked and investigated for the presence of plasmid DNA containing the required DNA insert by restriction endonuclease analysis.

2.12 Molecular biological techniques.

The manipulation of DNA, including phenol:chloroform extraction of nucleic acids and their subsequent precipitation by ethanol and sodium acetate, were based on the methods of Sambrook et al., 1989.

2.12.1 Miniprep of plasmid DNA.

Transformants were grown to saturation in LBAmp broth and 10 ml of this culture was used for the preparation of plasmid DNA isolated by the alkaline/SDS lysis method of Ish-Horowicz and Burke (1981). The bacteria were pelleted and resuspended in 200µl TNE buffer (25 mM Tris.Cl, pH 8.0; 50 mM NaCl; 1.5 mM EDTA, pH 8.0; 0.8% glucose). The cells were then lysed by adding 400µl sodium dodecyl sulphate (SDS) buffer (0.2N sodium hydroxide, 1% SDS) and incubating for 5 minutes on ice. 300µl of ice-cold 3M sodium acetate (pH 4.7 with acetic acid) was then added to precipitate denatured chromosomal DNA, SDS and protein. After a 5 minute incubation on ice, the material was removed by centrifugation. Plasmid DNA was recovered from the supernatant by precipitation with the addition of 0.6 volumes isopropanol (BDH; Cat. No. 10224). Precipitated plasmid DNA was pelleted by centrifugation, resuspended in SDW and phenol:chloroform extracted. DNA was then re-precipitated by the addition of ethanol and 0.3M sodium acetate followed by centrifugation. The final DNA pellet was resuspended in 20µl of SDW containing a final concentration of 50µg/ml RNAse A (Boehringer Mannheim Chemica, Germany; Cat. No. 109 169). 2-5µl of each sample DNA from a miniprep was analysed by restriction endonuclease digestion followed by agarose gel electrophoresis.

2.12.2 Restriction endonuclease digestion of plasmid DNA.

Samples of plasmid DNA were digested with relevant restriction endonucleases. The digestions were carried out as recommended by the manufacturer. Briefly, a sample of the DNA preparation was incubated for 2-3 hours at 37°C in a final volume of 20-50µl with 1-2.5 Units of the restriction enzyme per µg of purified DNA. Following the incubation, the DNA was visualised by horizontal agarose gel electrophoresis.

2.12.3 DNA agarose gel electrophoresis.

DNA was analysed by horizontal gel electrophoresis using 1.5% agarose (Sigma; Cat. No. A9918) gel in TAE buffer (40mM Tris-acetate; 1mM EDTA, pH 8.0). The agarose was dissolved by heating and allowed to cool to 50°C prior to the addition of ethidium bromide (Sigma; Cat. No. E8751) at a final concentration of 0.1µg/ml. DNA was mixed with loading buffer (5% glycerol, 12.5 mM EDTA (pH 7.9), 0.25% bromophenol blue) and loaded into wells. Gels were run at 5-10 V/cm for 30-60 minutes submersed in TAE and the DNA was visualised on an ultra-violet

(UV) transilluminator. DNA standards were run on every gel for comparison.

2.12.4 Large scale purification of plasmid DNA.

Large scale preparations of plasmid DNA were based on the alkaline lysis methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). Transformed *E. coli* were grown, with shaking at 37°C, overnight in 500mls LBAmp broth. Bacteria were pelleted by centrifugation at 7500xg for 10 minutes and the supernatant removed. Cells were resuspended in 36mls of GTE buffer (50mM glucose; 25mM Tris.Cl, pH 8.0; 10mM EDTA) and 4 mls of freshly made lysozyme (Sigma; Cat. No. L6876) (40mg/ml in GTE) was added. The suspension was mixed thoroughly and incubated at room temperature for 30 minutes. 80mls of freshly prepared alkaline SDS (0.2M NaOH, 1% SDS) was then added and mixed gently. The suspension was incubated on ice for 10 minutes. To this mixture, 60mls of ice-cold 3M potassium acetate (pH 4.7 with acetic acid) was added and incubated on ice for 60 minutes with occasional agitation to break up the precipitate. The suspension was centrifuged (15000xg for 10 minutes) to pellet chromosomal DNA, SDS and protein. The supernatant was transferred to a fresh centrifuge bottle and 0.6 volumes of ice-cold isopropanol added. Nucleic acids were recovered by centrifugation (9500xg for 10 minutes) and the pellet resuspended in 30mls TE (10mM Tris.Cl, pH 8; 1mM EDTA). 30g of solid cesium chloride (Sigma; Cat. No. C3309) were dissolved in this suspension and ethidium bromide was added to a final concentration of 800µg/ml. A cesium chloride gradient was formed by centrifugation at 40000rpm for 18 hours at 20°C in a Beckman Vti 50 rotor (Beckman L8-60M ultracentrifuge). DNA intercalated with ethidium bromide was visualised under UV and collected with a syringe and needle. The ethidium bromide was removed by extraction 6 times with isoamyl alcohol (BDH; Cat. No. 27214). The suspension was finally made up to 10mls with TE buffer. The DNA was then precipitated with 2.5 volumes of 70% ethanol and 0.3M sodium acetate for one hour at 4°C and centrifuged at 9500xg. The DNA pellet was resuspended in TE and precipitated twice in 1 volume 3M ammonium acetate and 2 volumes 95% ethanol. The final pellet of DNA was washed with 70% ethanol, resuspended in SDW and stored at -70°C. The concentration of DNA was measured by spectrophotometry.

2.12.5 Quantitation of DNA.

A suitable dilution of purified DNA in SDW was prepared and the optical density at 260nm and 280nm was measured. Pure DNA gives an approximate OD_{260}/OD_{280} ratio of 1.8-2.0. An OD_{260} of 1.00 corresponds to approximately 50µg/ml of double-stranded DNA. The concentration of purified DNA was also further verified by visualisation on ethidium bromide agarose gel electrophoresis against standards of known DNA concentration.

2.13 Monkey kidney cell line (COS-1 cells) manipulation.
2.13.1 Growth conditions.

COS-1 cells were kindly supplied by Dr. Neil Barclay (University of Oxford). Cells were grown to 90% confluence in tissue culture flasks and then harvested by incubation with 0.01% Trypsin/0.01% Versene (Media Supply) for 10 minutes at 37°C. Flasks were gently tapped and resuspended cells were aspirated and washed free of trypsin/versene by centrifugation. The cells were then innoculated into fresh tissue culture flasks at a dilution of 1 in 4 and incubated at 37°C, 5% CO_2 until required.

2.13.2 Transfection.

COS-1 cells were passaged when only 80-90% confluent. This was done at least twice, to obtain cells in a log phase of growth. Cells passaged for the second time were innoculated into 25cm² flasks at a concentration of $3x10^5$ cells per ml and incubated overnight. The cells were transfected after ~18 hours of culture using DEAE-Dextran (Sigma; Cat. No. D9885). The medium from the cells was aspirated and the cells washed twice with prewarmed G-MEM. DNA (200ng per flask) and DEAE-dextran (250µg/ml final concentration) were made up in G-MEM. A volume of 1ml was added to each of the flasks and the cells incubated for 5 hours at 37°C, 5% CO_2. Following the 5 hour incubation, the DNA/DEAE-dextran mix was removed and the cells washed twice with prewarmed Hepes-buffered G-MEM. Cells were then DMSO shocked for 90secs with 1ml of 10% DMSO in PBS. The DMSO solution was then removed, the cells were washed with prewarmed Hepes-buffered G-MEM and then incubated for the required lengths of time at 37°C, 5% CO_2.

2.13.3 Harvest of transfected COS-1 cells and measure of plasmid DNA expression.

Transfected, mock transfected and non-transfected COS-1 cells were harvested at various time points by incubating with 0.5mM EDTA in PBS for 15 minutes at 37°C. The flasks were tapped gently and the resuspended cells washed into PFA. The cells were aliquoted and immunofluorescently labelled as described in Section 2.5.1.1. Following labelling, the cells were fixed in 2% paraformaldehyde (Sigma; Cat. No. P6148) in PBS for 15 minutes. The cells were then analysed on a FACScan.

2.13.4 Positive selection of transfected cells.

Transfected cells were harvested with 0.5mM EDTA in calcium and magnesium free PBS and then washed twice with PFA. All recovered cells were then incubated for 15mins at 4°C with a working dilution of an appropriate monoclonal antibody. The positive control transfection and recovery system used rat CD2. The anti-CD2 monoclonal antibody OX34 was kindly supplied by Dr. Neil Barclay, University of Oxford. The cells were washed with PBS/0.5mM EDTA and then incubated with Dynabeads M-450 sheep anti-mouse IgG (Dynal Prod. No. 110.01) according to the manufacturers instructions. Cells were harvested by placing the suspension, in a plastic universal, in a strong magnetic field and aspirating the supernatant. The magnetically labelled positive cells were held in place at the side of the tube. This was repeated twice to ensure sufficient enrichment of cells expressing WC1 or CD2 on the cell surface.

2.13.5 Recovery of plasmid DNA from positively expressing transfected cells.

Recovery of the plasmid DNA contained in the recovered cells was by Hirt precipitation (Hirt, 1967). Briefly, cells were recovered as outlined above and pelleted. The pellet was then lysed in 400µl of 0.6% SDS, 10mM EDTA followed by addition of 100µl of 5M NaCl. This was incubated on ice for 15min and the cell debris then pelleted. The supernatant, containing any plasmid DNA was retained and phenol:chloroform extracted twice. Plasmid DNA was then re-precipitated by the addition of ethanol and 0.3M sodium acetate followed by centrifugation. This plasmid DNA was then transformed into recipient competent bacteria *E. Coli* (MC1061/p3). The p3 plasmid contains amber mutated ampicillin and tetracycline drug resistance elements (Seed and Aruffo, 1987) which are only expressed in the presence of a Sup F element such as that

40

present on CDM8 plasmids. Therefore cells containing the plasmid CDM8 are resistant to the drugs ampicillin and tetracycline. The transformed bacteria were grown on an agar plate containing 12.5µg/ml ampicillin and 7.5µg/ml tetracycline. Transformants may contain DNA recovered by Hirt precipitation from the positively selected COS cells. Plasmid DNA from positive colonies was prepared as previously outlined and analysed by restriction endonuclease digestion.

CHAPTER 3

Monoclonal antibodies to WC1 proteins

INTRODUCTION:

Sheep, like other species, have two distinct populations of mature PBL, namely B cells and T cells. These cells are divided into distinct subsets according to function and cell surface phenotype. Three sub-populations of T lymphocytes exist in the sheep and each population expresses one of the following proteins: WC1 (T19); CD4 or CD8 (MacKay et al., 1986, 1988). The WC1 protein was first identified on ovine PBL by the monoclonal antibodies 19-19 (MacKay et al., 1986) and ST197 (McClure et al., 1989) and was referred to as T19. In this thesis all references made to the T19 molecule, or antibody cluster, will use the term WC1. This complies with the international nomenclature defined in the 1st International Workshop on Ruminant Leucocyte Differentiation Molecules (Morrison, 1991) (hereinafter referred to as the 1st Workshop).

It has been established both in the human (Brenner et al., 1986) and murine (Bluestone et al., 1988) species that the majority of lymphocytes expressing the molecules CD4 or CD8 co-express the αβ T cell receptor (TCR) for antigen. It is assumed that a similar situation exists in the sheep. However, at the present time, there is no monoclonal antibody specific for the ovine αβ TCR to confirm this is the case. In the sheep, cells which express WC1 co-express the γ and δ TCR genes (MacKay et al., 1986).

In 1991, fourteen monoclonal antibodies specific for WC1 were submitted to the 1st Workshop. Twelve of the monoclonal antibodies were raised against cattle lymphocytes and two of the monoclonal antibodies were raised against sheep lymphocytes (Morrison and Davis, 1991). The monoclonal antibodies were clustered because they reacted with the same populations of cells and recognised the same proteins. As no human homologue of the WC1 proteins has been identified, a CD number could not be assigned. A temporary Workshop Cluster (WC1) was formed. The results from the workshop showed the reactivity of the fourteen monoclonal antibodies in the WC1 cluster with PBL from both the ovine and bovine species.

In cattle, all the WC1 monoclonal antibodies identified populations of γδ T cells. In particular the monoclonal antibody CC15 reacted with all γδ T cells (Sopp et al., 1991) whereas other WC1 monoclonal antibodies reacted with different numbers of γδ T cells. All the monoclonal antibodies immunoprecipitated two high molecular weight proteins of 215kDa and 300kDa, which were non-covalently linked. In addition, immunohistological analysis revealed that the different monoclonal antibodies recognised cells with the same tissue distribution. In the ovine species only twelve of the fourteen WC1 clustered monoclonal antibodies reacted with PBL. This indicated that some WC1 epitopes are not conserved between the ovine and bovine species. The monoclonal antibody CC15 reacted with the greatest number of lymphocytes, and all γδ TCR positive cells. All the other WC1 monoclonal antibodies reacted with sub-populations of γδ T cells. All of the monoclonal antibodies immunoprecipitated a protein of 215kDa. Other higher and lower molecular weight proteins of 144kDa and 300kDa were also immunoprecipitated by some of the monoclonal antibodies (Hein et al., 1991).

Further monoclonal antibodies to WC1 have since been generated and these were submitted to the 2nd International Workshop on Ruminant Leucocyte Differentiation Molecules (hereinafter referred to as the 2nd Workshop). The Department of Veterinary Pathology at the University of Edinburgh was one of the participants in the 2nd Workshop. In this chapter the results generated in this thesis and submitted to the final proceedings of the 2nd Workshop are discussed (Lund et al., 1993). All of the WC1 monoclonal antibodies investigated here were raised against cattle lymphocytes. The reactivity of the WC1 monoclonal antibodies with ovine PBL was assessed.

The proteins recognised by each of the WC1 monoclonal antibodies were also identified. In addition, the distribution of cells in the major immune compartments reactive with each of the different WC1 monoclonal antibodies was determined.

In this chapter, and elsewhere in this thesis, data is presented from the analysis of immunofluorescently labelled lymphocytes from ovine peripheral blood and other ovine lymphocyte compartments. The results were obtained on a FACScan. The immunofluorescence profile of a population of stained cells is displayed as a histogram of fluorescence intensity versus cell number. The intensity of fluorescence is a consequence of two variables: (i) the level of expression of target molecule on the cell surface and (ii) the affinity of the monoclonal antibody for its epitope.

This chapter will introduce the methodology of the FACScan analysis undertaken in this thesis and will describe the calculations used in determining specific staining by the monoclonal antibodies used both in single colour and two-colour immunofluorescence analysis.

RESULTS:

3.1 Immunofluorescence analysis of ovine PBL.

The lymphocyte population to be analysed by FACScan was identified by a forward scatter (size) versus side scatter (granularity) profile. Figure 3.1 shows the forward scatter versus side scatter profile of ovine PBMC on the FACScan. The different sub-populations of cells can be defined by electronic gates. This allows for the subsequent analysis of individual populations of cells, excluding all other cells in the sample. The various sub-populations of cells within PBMC were gated as follows: P1-lymphocytes and monocytes; P2-granulocytes; P3-lymphoblasts; P4-all leucocytes; P5-gate P1+gate P3. Gates P1, P4 and P5 were routinely used throughout this thesis. Gate P1 was used for the analysis of the lymphocyte population found in peripheral blood, thymus, spleen, mesenteric lymph node, prescapular lymph node, intestinal epithelium, intestinal lamina propria, afferent lymph and efferent lymph. Gate P4 was used for the analysis of whole blood PBMC and gate P5 was used for the analysis of activation of lymphocyte subsets.

3.1.1 Single-colour immunofluorescence.

Single colour FACScan analysis with control monoclonal antibodies of different mouse antibody isotypes was compared to that of NMS and b-NMS (data not shown). No significant differences were observed in the staining profiles with these control reagents. As a consequence NMS and b-NMS were used routinely to determine the non-specific staining of populations of cells. The staining profile for NMS is shown in figure 3.2. In addition, figure 3.2 shows examples of positive cell surface fluorescence using the monoclonal antibodies SBU-1 (anti-ovine MHC class I) and SBU-T4 (anti-ovine CD4). The percentage of cells stained specifically by the test monoclonal antibody is calculated by subtracting the percentage of stained cells with NMS (non-specific staining) from the percentage of cells stained with the test monoclonal antibody (positive staining). The percentage of cells stained with a reagent is determined on those cells with fluorescence intensity greater than that seen with NMS (i.e. to the right of the marker shown on each histogram).

3.1.2 Two-colour immunofluorescence.

Two colour immunofluorescence required the use of different fluorochromes. The reactivity of one monoclonal antibody was detected by incubation with FITC-conjugated anti-isotype specific immunoglobulin reagents and measured in the FL1 channel (green fluorescence). The reactivity of a second monoclonal antibody was measured in the FL2 channel (red fluorescence). FL2 was detected with streptavidin-phycoerythrin (SA-PE) which followed staining with either biotinylated monoclonal antibodies or monoclonal antibodies followed by biotin-conjugated anti-isotype specific immunoglobulin reagents. Two control reagents, NMS or b-NMS, were first used to determine non-specific staining (Fig 3.3A). The specific monoclonal antibodies were then examined against the control sera to enable compensations to be set to account for any residual fluorescence in the overlap of emittance spectrums of FITC and SA-PE fluorescence (Fig 3.3B & 3.3C). Figure 3.3D shows PBL stained with the monoclonal antibody 86D (anti-γδ TCR) detected by FITC-conjugated anti-IgG1 secondary reagent and biotinylated monoclonal antibody CC15 (anti-WC1) detected with SA-PE. The number of WC1 positive cells expressing the γδ TCR was calculated as described in the legend of figure 3.3. This method of calculation was adopted for the analysis of all two colour immunofluorescence staining reported in this thesis.

44

FIGURE 3.1: Scatter profile of ovine PBMC.
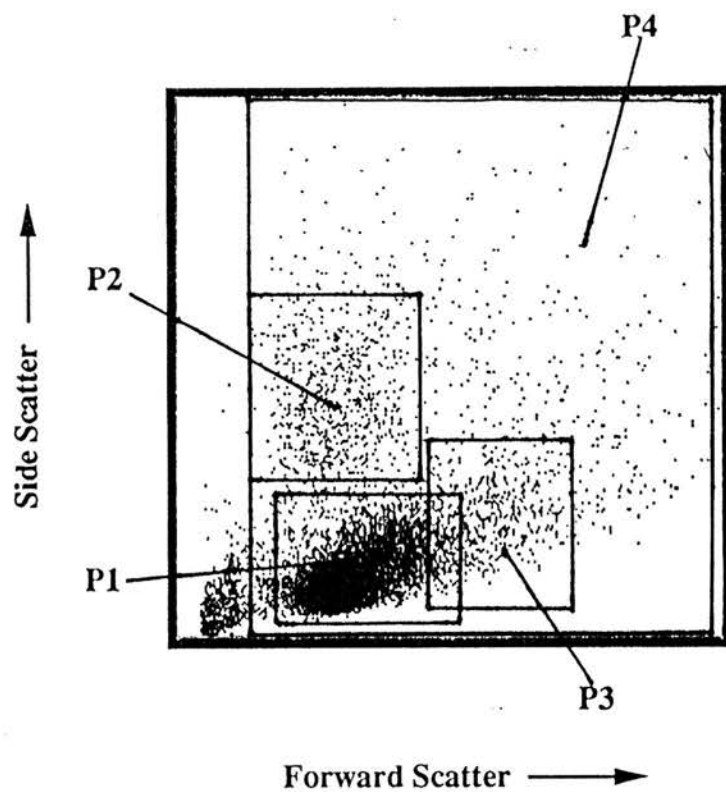
Figure 3.2: **Single colour immunofluorescence.**

PBL were purified from 6 month old sheep (n=6) and stained with NMS, anti-MHC class I or anti-CD4 monoclonal antibodies then anti-mouse FITC. The figures show the single colour immunofluorescence histogram for each of the monoclonal antibodies tested on PBL from one animal. The percentage of positive cells was determined as being that value to the right of the vertical marker shown. The marker was set with reference to the staining of NMS (always <3%). This experiment was repeated eight times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 3.2: Single colour immunofluorescence.
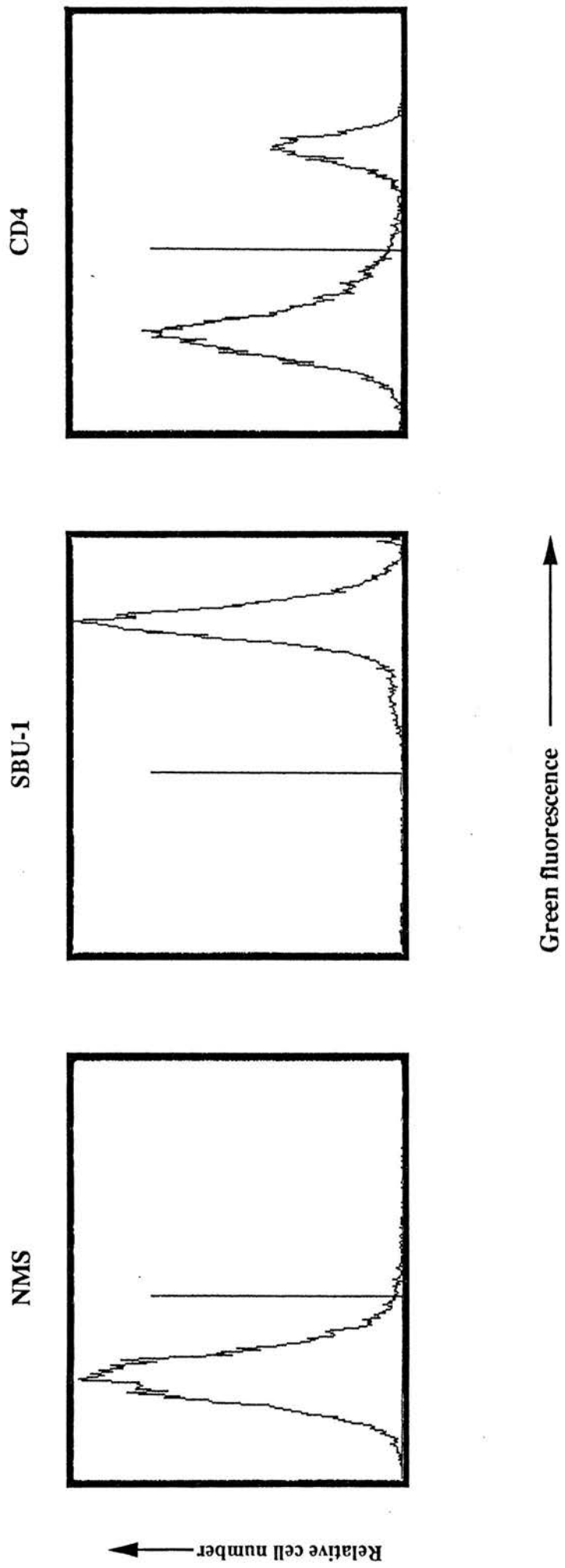
NMS

SBU-1

CD4

Relative cell number

Green fluorescence

Figure 3.3: Two-colour immunofluorescence analysis of monoclonal antibodies CC15 versus 86D.

PBL were purified from 6 month old sheep (n=8) and stained with either NMS or anti-γδ, followed by b-NMS or biotinylated anti-WC1, then streptavidin-phycoerythrin. The figures show the two colour immunofluorescence profiles for each of the combinations of monoclonal antibodies used. Figure 3.3A shows NMS (x-axis) versus b-NMS (y-axis) on which the non-specific staining values were set. Figure 3.3B shows NMS (x-axis) versus biotinylated anti-WC1 (y-axis) and figure 3.3C shows anti-γδ (x-axis) versus b-NMS (y-axis). Figures 3.3B and 3.3C allowed the compensations for FL1 and FL2 fluorescence to be set. Figure 3.3D shows biotinylated anti-WC1 (y-axis) versus anti-γδ (x-axis). Using the other figures of non-specific staining the calculation of the percentage of CC15-positive cells expressing 86D was as follows:

Quadrant 1 (Figure A) + Quadrant 2 (Figure A) = Non-specific staining

Quadrant 1 (Figure D) + Quadrant 2 (Figure D) = Monoclonal antibody staining

Specific staining = Monoclonal antibody staining - Non-specific staining

Percentage of CC15-positive cells expressing 86D:

$$\frac{\text{Quadrant 2 (Figure D) - Quadrant 2 (Figure A)}}{\text{Specific staining}} \quad X \quad 100$$

For determination of the number of 86D-positive cells expressing CC15 the values for quadrant 1 in figure A and figure B are replaced by the values for quadrant 4 in figure A and figure B.

Figure 3.3: Two-colour immunofluorescence analysis of monoclonal antibodies CC15 versus 86D.

(A)

(B)
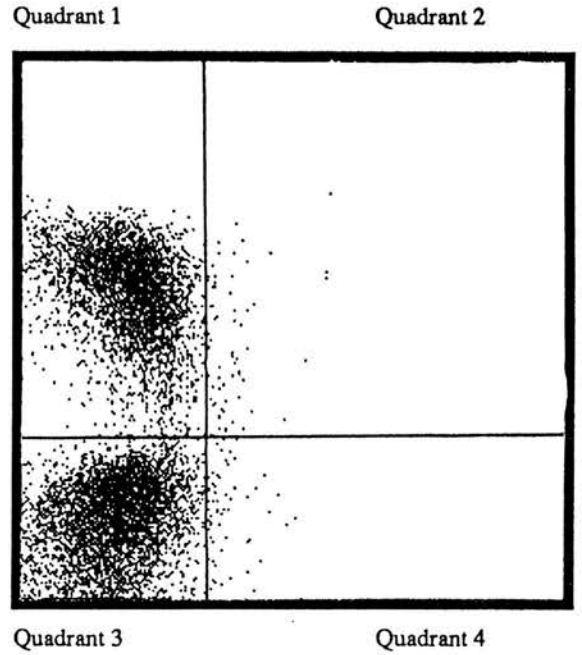
(C)
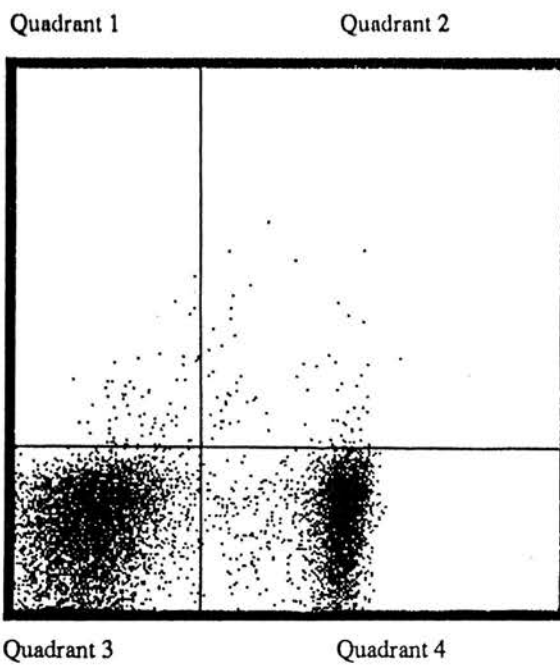
(D)
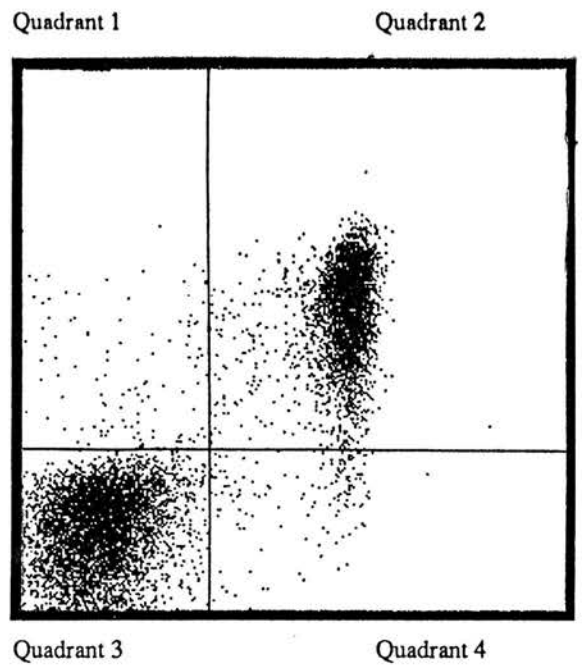
3.2 Monoclonal antibodies to ovine lymphocyte molecules.

The monoclonal antibodies which were used in this thesis are listed in table 3.1 along with their isotype and specificity. The majority of the monoclonal antibodies were sheep specific, derived from fusions performed following immunisation of mice with ovine lymphocytes. Some monoclonal antibodies were generated to lymphocyte antigens of the bovine species and found to cross react with ovine lymphocytes, indicating the conservation of some epitopes across species.

3.2.1 Single colour immunofluorescence analysis of ovine PBL.

Reference immunofluorescence profiles of some of the monoclonal antibodies used in this thesis are shown in figure 3.4. Representative staining patterns were obtained by staining of PBL from a 6 month old lamb. The profiles shown in figure 3.4 match those previously reported in the literature (see references, table 3.1).

3.2.2 CD4-positive T cells; CD8-positive T cell; B cells.

CD4 and CD8 co-receptor molecules are each defined in the ovine species by two monoclonal antibodies. The anti-CD4 monoclonal antibodies ST4 (IgG1) (MacKay et al., 1988) and SBU-T4 (IgG2a) (Maddox et al., 1985), both reacted with populations of ovine PBL of similar size (data not shown). The anti-CD8 monoclonal antibodies ST8 (IgM) (MacKay et al., 1988) and SBU-T8 (IgG2a) (Maddox et al.,1985), both reacted with populations of ovine PBL of similar size (data not shown). Figure 3.5 shows that in two-colour immunofluorescence assays, the pairs of different subset specific reagents stained the same populations of cells. This allowed an interchange of the subset-specific reagents when different isotypes were required. Figure 3.5A shows the two-colour FACScan analysis of cells stained by the monoclonal antibodies ST4 (IgG1) and SBU-T4 (IgG2a). It was seen that both ST4 (IgG1) and SBU-T4 (IgG2a) recognised epitopes on the same populations of lymphocytes. These monoclonal antibodies were used interchangeably in subsequent two-colour immunofluorescence assays. Figure 3.5B shows the two-colour FACScan analysis of cells stained by the monoclonal antibodies ST8 (IgM) and SBU-T8 (IgG2a). It was seen that both ST8 (IgM) and SBU-T8 (IgG2a) recognise epitopes on the same populations of lymphocytes. However, some of the cells which were stained by the monoclonal antibody ST8 at a low intensity did not react with the monoclonal antibody SBU-T8. These monoclonal antibodies were used interchangeably in subsequent two-colour immunofluorescence assays.

The anti-ovine B cell monoclonal antibodies VPM8 (IgG1) **(McConnell, unpublished data)** and DU2.104 (IgM) (Hein, unpublished data) both reacted with similar sized populations of ovine PBL (data not shown). Two-colour FACScan analysis of cells stained by the monoclonal antibodies VPM8 (IgG1) and DU2.104 (IgM) showed that they recognised epitopes on the same populations of lymphocytes (data not shown). These monoclonal antibodies were used interchangeably in subsequent two-colour immunofluorescence assays.

3.2.3 γδ TCR positive T cells.

The monoclonal antibody 86D reacts with the ovine γδ TCR molecule (MacKay et al., 1989). The γδ T cell population in ruminants also express an unique set of cell surface glycoproteins called WC1. The WC1 molecules are detected by a number of monoclonal antibodies of which the monoclonal antibodies CC15 recognises all known WC1 molecules. Figure 3.3 shows the two

45

Table 3.1: Monoclonal antibodies used for the immunofluorescence
analysis of ovine cell surface antigens.

The monoclonal antibodies used routinely in this thesis are listed along with their isotype,
specificity and reference.

TABLE 3.1:   Monoclonal antibodies used for the immunofluorescence analysis of ovine cell surface antigens.

Antibody	Specificity	Isotype	Reference
86D	anti-ovine $\gamma\delta$ TCR	IgG1	MacKay et al., 1989
ST197	anti-ovine WC1	IgG2b	Maddox et al., 1985
CC15	anti-bovine WC1	IgG2a	Clevers et al., 1990
ST4	anti-ovine CD4	IgG1	MacKay et al., 1988
SBU-T4	anti-ovine CD4	IgG2a	Maddox et al., 1985
ST8	anti-ovine CD8	IgM	MacKay et al., 1988
SBU-T8	anti-ovine CD8	IgG2a	Maddox et al., 1985
VPM8	anti-ovine Ig light chain	IgG1	McConnell, unpublished
DU2.104	anti-bovine B cells	IgM	Hein, unpublished
ILA1-11	anti-bovine CD25	IgG1	Naessans et al., 1992
SW73.2	anti-ovine MHC class II (α-chain)	IgG2b	Hopkins et al., 1986
VPM36	anti-ovine MHC class II (β-chain)	IgG1	Hopkins et al., 1993
VPM54	anti-ovine MHC class II (β-chain)	IgG1	Hopkins et al., 1993
73B	anti-ovine CD45R	IgG1	MacKay et al., 1990
SBU1	anti-ovine MHC class I	IgG1	Gogolin-Ewens et al., 1985
NMS	none	ALL	unpublished

FIGURE 3.4: Reactivity of some of the monoclonal antibodies used in this thesis.

Figure 3.5: Two-colour immunofluorescence analysis of monoclonal antibodies to CD4-positive and CD8-positive T cells.

PBL were isolated from 6 month old lambs (n=4) and 10^6 cells were stained with ST4 or ST8 then anti-IgG1 FITC, and biotinylated SBU-T4 or biotinylated SBU-T8 then SA-PE. These cells were analysed by FACScan and the two-colour immunofluorescence profiles shown. The figure shows the positive staining of cells from one animal when compared to the non-specific staining of NMS versus and b-NMS (always <3%). This experiment was repeated four times and the same trends were seen with PBL from each animal and on each occasion tested.
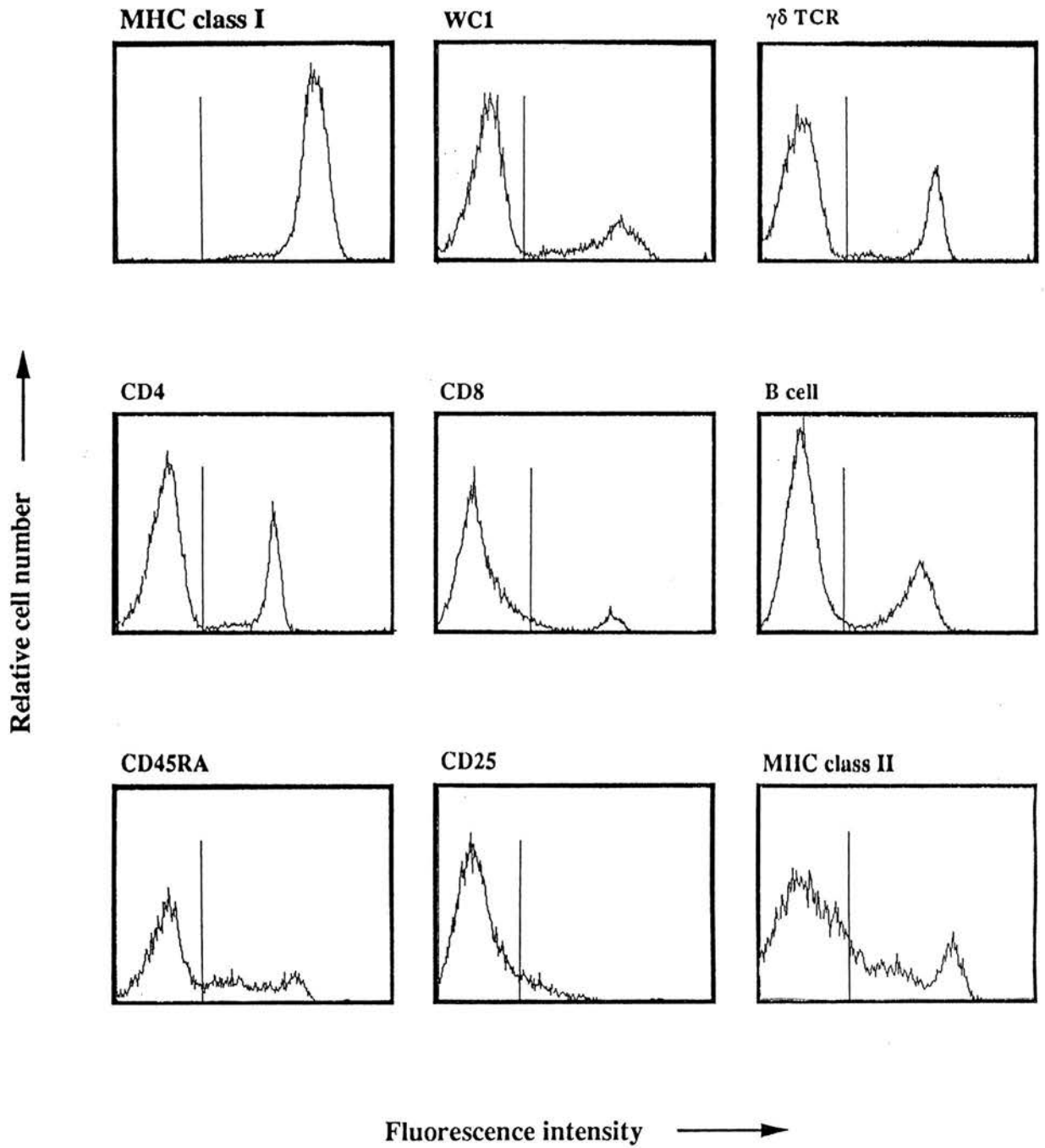
FIGURE 3.5: Two-colour immunofluorescence analysis of monoclonal antibodies to CD4-positive and CD8-positive T cells.
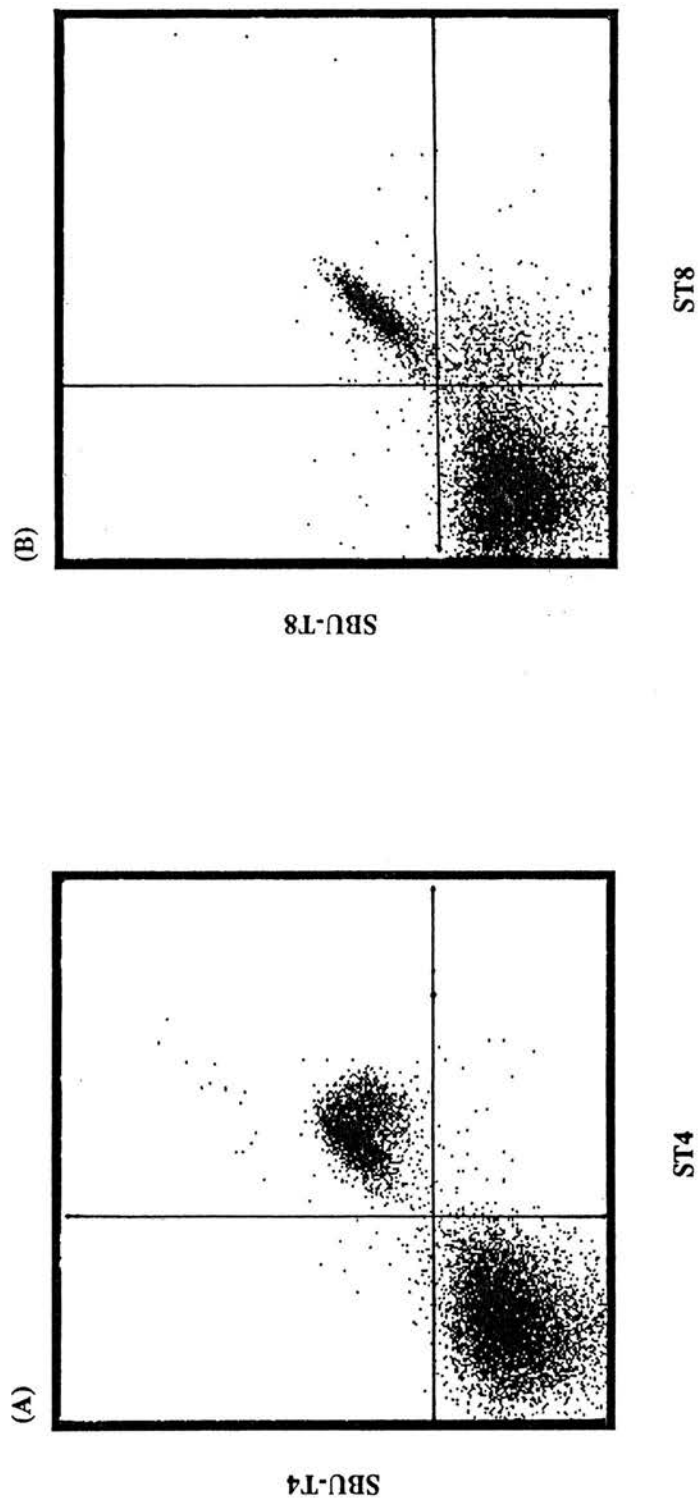
colour immunofluorescence staining profiles of ovine PBL with CC15 and 86D. 98% of CC15 positive cells expressed the γδ TCR and 98% of γδ T cells expressed WC1.

3.3 WC1-clustered monoclonal antibodies.

Table 3.2 lists the monoclonal antibodies in the WC1 cluster which were submitted to the 2nd Workshop. Also listed is the workshop number, the isotype and the donating laboratory of each of the monoclonal antibodies. The monoclonal antibodies were clustered following initial FACScan analysis. A detailed analysis of these WC1 monoclonal antibodies is described in this chapter.

3.3.1 WC1 monoclonal antibodies react with ovine PBL.

Most of the WC1 clustered monoclonal antibodies submitted to the 2nd workshop were raised against bovine lymphocytes (Morrison and Davis, 1991). The reactivity of these monoclonal antibodies with ovine PBL was determined. Table 3.3 shows the percentage of PBL from 6 weeks old lambs and 18 months old adult sheep which reacted with each of the WC1 monoclonal antibodies. The percentage of positively stained calf PBL were included as a positive control, and to allow a comparison between the ovine and bovine species.

All of the WC1 clustered monoclonal antibodies tested, apart from BAQ72A, reacted with PBL isolated from lambs and adult sheep. Smaller percentages of adult PBL reacted with the WC1 monoclonal antibodies compared to PBL from young animals. The 2nd workshop WC1 monoclonal antibody standard, CC15, showed greatest reactivity of all the WC1 monoclonal antibodies and stained 22% of lamb PBL and 8% of adult PBL.

Figure 3.6 shows the single colour FACScan profiles of PBL from 6 month old lambs and 6 month old calves when stained with WC1 monoclonal antibodies. The monoclonal antibody CC15 stained lamb and calf PBL with the same high intensity. This epitope is conserved across species. BAQ89A and CACTB7A (not shown), like BAG25A (shown), reacted with 9-11% of lamb PBL. The patterns of reactivity of BAQ89A, CACTB7A and BAG25A on both ovine and bovine PBL were the same. The monoclonal antibodies CC101 and CC115 (not shown), like CC117 (shown), reacted with 7-9% of lamb PBL and showed similar profiles of staining. The intensity of staining however, was not the same across species. Ovine PBL did not express the epitopes recognised by the monoclonal antibodies CC101, CC115 and CC117 with the same high intensity as was seen in the bovine species. The monoclonal antibodies CACTB15B and CACTB1A reacted with <6% of lamb PBL and showed different immunofluorescence profiles with PBL from the ovine species when compared to those from the bovine species. The size of the populations of PBL stained by the monoclonal antibodies CACTB1A and CACTB15B is smaller in the ovine species than in the bovine species. PBL from lambs were stained with both a high intensity and low intensity by the monoclonal antibodies CACTB1A and CACTB15B. PBL from the bovine species however were stained with an high intensity only.

3.3.2 WC1 monoclonal antibodies react with CC15-positive ovine PBL.

Of all the WC1 monoclonal antibodies tested, CC15 reacted with the greatest number of ovine PBL. This monoclonal antibody was submitted to the 2nd Workshop as a control for reactivity of other WC1 monoclonal antibodies. The reactivity of CC15 with ovine PBL was compared to that

46

Table 3.2: Workshop monoclonal antibodies analysed.

The WC1 specific monoclonal antibodies which were submitted to the 2nd International Workshop on Ruminant Leucocyte Differentiation Molecules and used in this thesis are listed along with the isotype, specificity and donating laboratory.

TABLE 3.2: Workshop monoclonal antibodies analysed.

Antibody	Workshop number	Donating Laboratory	Isotype	Specificty
CC15	45	IAH	IgG2a	Bovine-WC1
CC101	9 & 18	IAH	IgG2a	Bovine-WC1
CC117	30	IAH	IgG2a	Bovine-WC1
CC115	31	IAH	IgG2a	Bovine-WC1
CACTB1A	146	WSU	IgG1	Bovine-WC1
CACTB15B	147	WSU	IgG1	Bovine-WC1
BAQ72A	148	WSU	IgM	Bovine-WC1
BAQ89A	149	WSU	IgG1	Bovine-WC1
CACTB7A	150	WSU	IgG1	Bovine-WC1
BAG25A	151	WSU	IgM	Bovine-WC1

IAH - AFRC Institute of Animal Health, Compton.

WSU - Washington State University, Pullman.

Table 3.3: Reactivity of WC1 monoclonal antibodies with ovine PBL.

PBL were isolated from 6 week old lambs (n=6), 18 month old adult sheep (n=3) and as a control a 6 month old calf (n=1). 10^6 cells were stained with all of the anti-WC1 monoclonal antibodies listed then anti-IgG FITC and analysed by FACScan. The percentages listed are positive cells with respect to NMS used as a negative control. The results shown are the mean values obtained and the standard deviation of the means are shown in the brackets.

TABLE 3.3: Reactivity of WC1 monoclonal antibodies with ovine PBL.

Antibody	Lamb PBL (6 weeks old)	Adult PBL (18 months old)	Calf PBL (6 months old)
CC15	21.6 (6.9)	8.0 (1.9)	25
CC101	7.5 (2.0)	4.5 (1.8)	17
CC117	8.9 (3.2)	2.9 (1.2)	19
CC115	7.3 (2.7)	1.7 (0.9)	17
CACTB1A	5.5 (1.7)	0.9 (0.8)	13
CACTB15B	3.7 (1.7)	4.9 (2.7)	12
BAQ72A	0.0 (0.0)	1.6 (1.3)	9
BAQ89A	8.7 (3.6)	3.1 (1.6)	11
CACTB7A	9.2 (4.4)	2.9 (1.5)	11
BAG25A	10.9 (6.8)	3.4 (1.4)	15

Figure 3.6: Reactivity of WC1 monoclonal antibodies.

PBL were isolated from 6 week old lambs (n=6) and a 6 month old calf (n=1) then stained with all of the anti-WC1 monoclonal antibodies listed then anti-IgG FITC and analysed by FACScan. Single colour immunofluorescence profiles of the monoclonal antibodies CC15, BAG25A, CC117, CACTB1A and CACTB15B on PBL from one animal are shown. This experiment was repeated three times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 3.6: Reactivity of WC1 monoclonal antibodies.

Lamb PBL (6 months old)

Calf PBL (6 months old)

CC15

BAG25A

CC117

CACTB1A

CACTB15B

Relative cell number

Log Fluorescence intensity
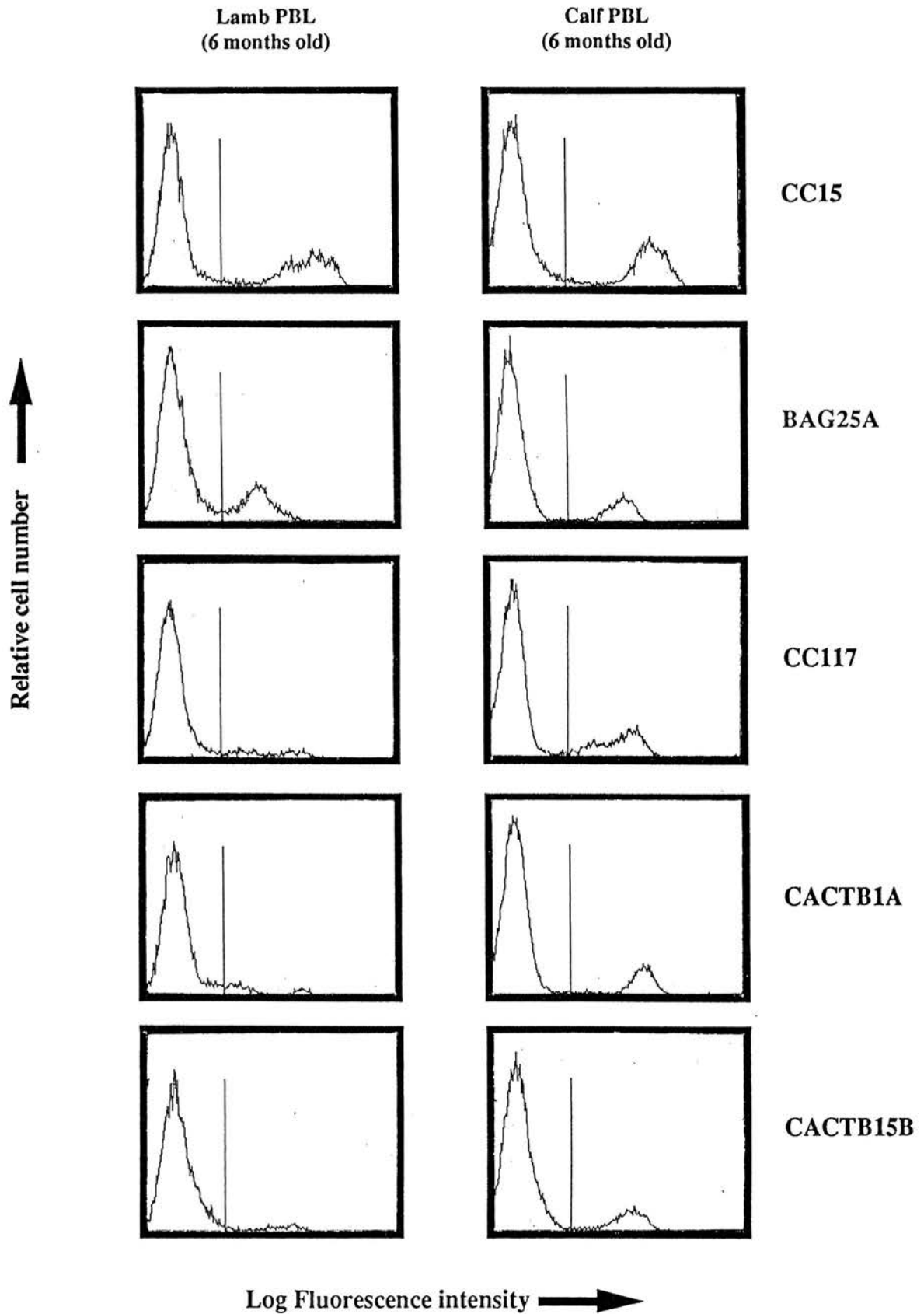
of all cross-reactive WC1 monoclonal antibodies by two colour immunofluorescence. Table 3.4 shows two sets of data: i) the percentage of CC15-positive PBL which reacted with the other WC1 monoclonal antibodies. ii) the percentage of PBL reactive with the WC1 monoclonal antibodies which also reacted with CC15. The results were obtained with PBL from 6 month old lambs. All of the workshop monoclonal antibodies reacted with sub-populations of CC15-positive PBL. The monoclonal antibodies CC101, CC115, CC117, BAQ89A, CACTB7A and BAG25A reacted with 52-67% of the CC15 positive populations. The monoclonal antibody CACTB15B stained only 25% of CC15-positive PBL and the monoclonal antibody CACTB1A stained only 49% of CC15-positive PBL. All of the PBL which reacted with the monoclonal antibodies CC101, CC115 and CC117 also reacted with CC15. In addition, 90-96% of PBL which reacted with the monoclonal antibodies BAQ89A, CACTB7A and BAG25A also reacted with CC15. Representative two-colour immunofluorescence profiles for CC15 versus the monoclonal antibodies CC117, like CC101 and CC117, and BAG25A, like CACTB7A and BAQ89A, are shown in figure 3.7. The profiles demonstrate that all the PBL which reacted with the monoclonal antibodies CC117 and BAG25A are found in the upper right quadrant and therefore express the epitope recognised by the monoclonal antibody CC15. Figure 3.7 also demonstrates that PBL stained at high intensity with CACTB1A or CACTB15B all reacted with CC15, but of the PBL which stained with a low intensity only a proportion reacted with CC15.

3.4 Characterisation of proteins immunoprecipitated from ovine PBL by WC1 monoclonal antibodies.

It was shown in both the bovine species, and the ovine species, that all WC1 monoclonal antibodies submitted to the 1st Workshop reacted with proteins of molecular weight 215kDa. In sheep, some of these monoclonal antibodies also reacted with proteins of 144 and 300kDa. Figure 3.8 shows the proteins immunoprecipitated by the WC1 monoclonal antibodies submitted to the 2nd Workshop. The monoclonal antibodies B7A1 and BAQ4A were submitted to the 1st Workshop and the proteins immunoprecipitated by these monoclonal antibodies were included for a comparison with previous data. All of the monoclonal antibodies, except CC101, reacted with proteins of molecular weights of 140 and 220kDa. The monoclonal antibody CC101 immunoprecipitated the 220kDa form of the protein only. In addition all the monoclonal antibodies, except CACTB1A and CACTB15B, immunoprecipitated a protein of molecular weight 300kDa. The monoclonal antibodies CC15 and BAQ4A routinely immunoprecipitated the highest level of protein whilst the monoclonal antibodies B7A1, BAG25A and BAQ89A immunoprecipitated intermediate levels of the protein and CC101, CACTB7A, CACTB15B and CACTB1A immunoprecipitated low levels of the protein. No protein bands were immunoprecipitated in the presence of NMS.

3.5 Affinity purification of WC1 proteins from ovine PBL.

The reactivity of WC1 monoclonal antibodies with the purified WC1 protein was determined. The WC1 proteins were purified on an affinity column comprising WC1 monoclonal antibody ST197. This section details the preparation of the WC1 affinity column, the purification of WC1 proteins and the detection of purified WC1 proteins.

3.5.1 Preparation of anti-WC1 column.

The monoclonal antibody ST197 was affinity purified from tissue culture supernatent on a

Table 3.4: Two-colour immunofluorescence analysis of reactive WC1 monoclonal antibodies.

PBL were isolated from 6 month old lambs (n=6) and stained with each of the anti-WC1 monoclonal antibodies listed then anti-isotype FITC, and biotinylated anti-WC1 monoclonal antibody CC15 then SA-PE. The percentage of positive cells was calculated with reference to the staining of biotinylated NMS versus NMS. The results shown are the mean values obtained and the standard deviations from the means are shown in the brackets.

TABLE 3.4: Two-colour immunoflourescence analysis of reactive WC1 monoclonal antibodies.

Monoclonal Antibody	Percentage of CC15-positive population reacting with WC1 antibody.	Percentage of WC1-positive cells expressing CC15.
CC101	63.1 (4.3)	100.0 (0.0)
CC117	66.7 (2.6)	100.0 (0.0)
CC115	52.4 (5.0)	100.0 (0.0)
CACTB1A	48.7 (14.5)	65.9 (10.6)
CACTB15B	25.0 (6.4)	80.1 (8.8)
BAQ89A	52.3 (5.4)	94.0 (9.1)
CACTB7A	52.5 (8.6)	90.0 (10.8)
BAG25A	58.4 (6.1)	96.1 (2.4)

Figure 3.7: Two-colour immunofluorescence analysis of WC1 monoclonal antibodies.

PBL were isolated from 6 month old lambs (n=6) and stained with the anti-WC1 monoclonal antibodies CC117, BAG25A, CACTB1A and CACTB15B then anti-isotype FITC, and biotinylated anti-WC1 monoclonal antibody CC15 then SA-PE. The quadrants of the two-colour immunofluorescence profiles shown were determined by the staining of NMS versus b-NMS. This experiment was repeated three times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 3.7: Two-colour immunofluorescence analysis of WC1 monoclonal antibodies.

BAG25A

CACTB15B

Green fluorescence.

CC117

CACTB1A

CC15

CC15

Red fluorescence.
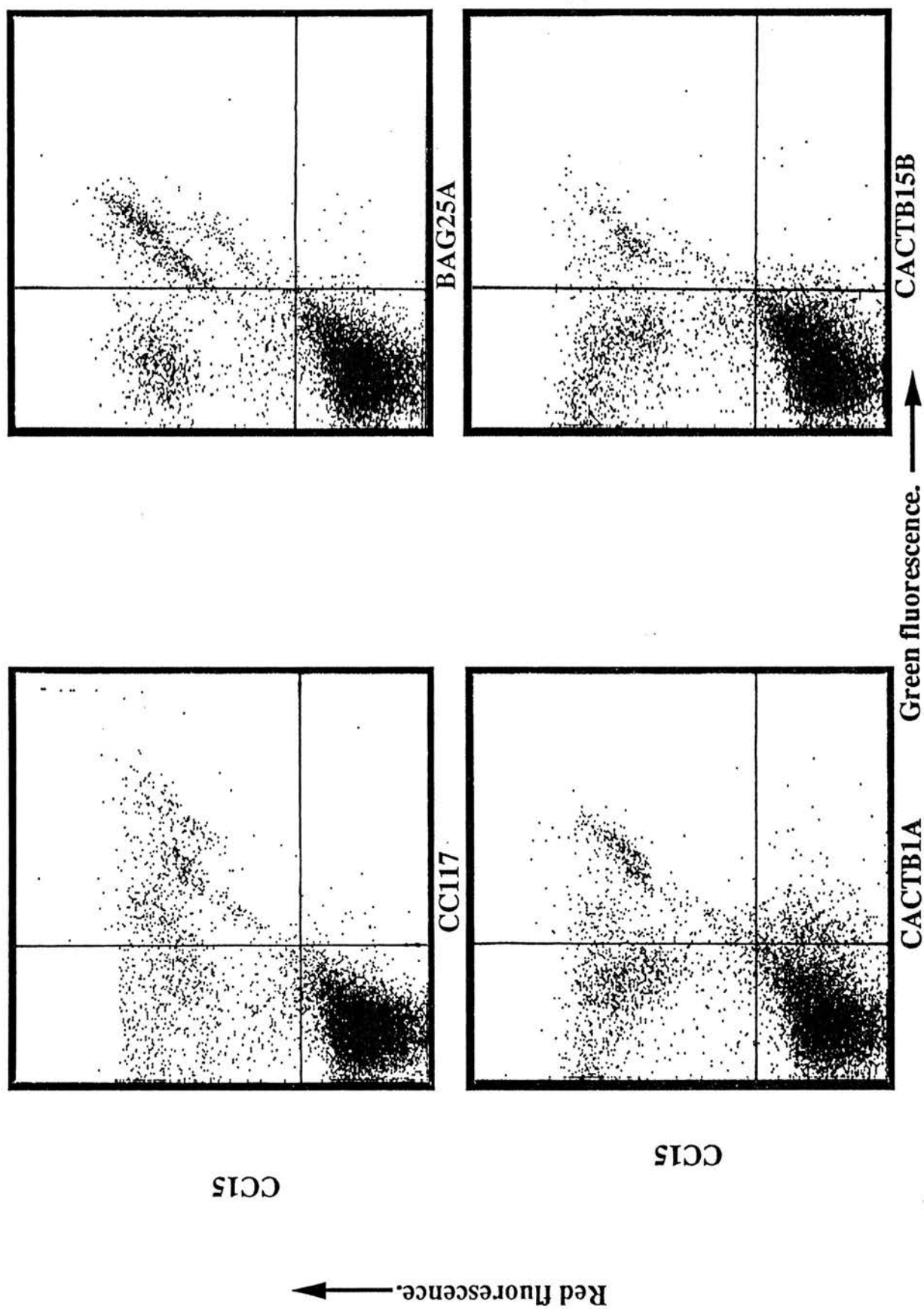
Figure 3.8: Immunoprecipitation of WC1 protein.

An ^{125}I-labelled ovine PBL lysate was prepared and pre-cleared of non-specific material by incubation with agarose beads coupled to anti-Ig. Agarose beads coupled to anti-Ig were pre-adsorbed with cold lysate to prevent non-specific radiolabelled antigen binding. The listed anti-WC1 monoclonal antibodies were then coupled to the pre-adsorbed anti-Ig beads. Anti-WC1 coupled beads were then incubated with the ^{125}I-labelled lysate and the WC1 antigens immunoprecipitated. The proteins immunoprecipitated were then run on a 5-15% SDS-PAGE gel. The relative mobility of the molecular weight markers are shown at the left of the figure. Each of the anti-WC1 monoclonal antibodies used is listed along the top of the figure.

Figure 3.8: Immunoprecipitation of WC1 protein
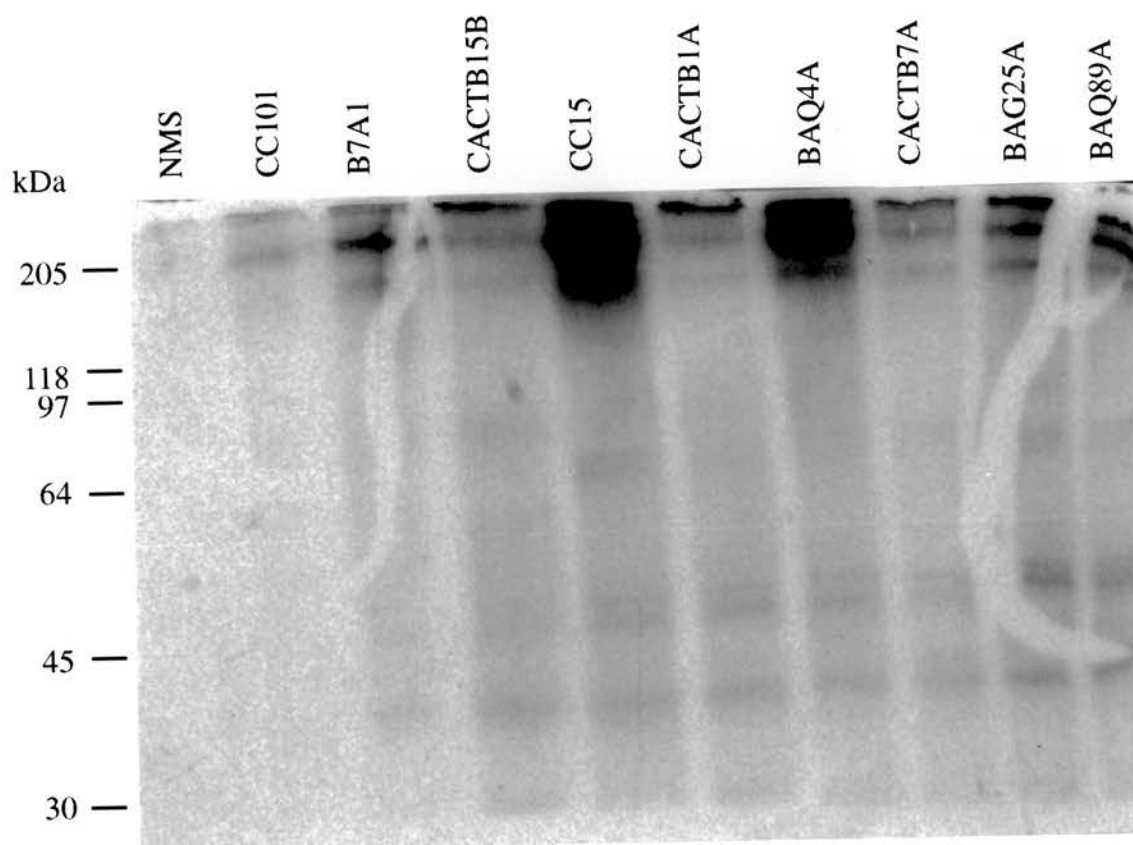
gamma-bind, protein G sepharose column. Approximately 10 litres of supernatent yielded 37.5mg of ST197 monoclonal antibody. Figure 3.9 shows a Coomassie stained SDS-PAGE of ST197 monoclonal antibody culture supernatent prior to, and following, purification. No antibody proteins were visible in the culture supernatent whilst the heavy chain (55kDa) and light chain (28kDa) of the monoclonal antibody were stained in the purified sample. The reactivity of purified ST197 with PBL from the ovine species was determined by FACScan analysis. At a dilution of 1/2000 the monoclonal antibody reacted with PBL (data not shown). Purified ST197 monoclonal antibody was coupled to CNBr activated agarose according to the manufacturers instructions. The amount of protein in solution prior to, and after, coupling to CNBr was measured and the coupling efficiency of the monoclonal antibody ST197 to the agarose calculated to be 95%. Antibodies from NMS were purified in an identical manner on the gamma bind column and then coupled to agarose. The coupling efficiency of NMS antibodies was 87%. The NMS column was used as a pre-column to remove any non-specific proteins. In addition, a pre-pre-column of swollen agarose only was prepared.

3.5.2 Detection of purified WC1 protein.

WC1 protein isolated on the ST197 affinity column was purified from spleen lysates as described in section 2.7. The purified material was analysed by two methods; SDS-PAGE and ELISA.

3.5.2.1 SDS-PAGE of purified WC1 protein.

Purified WC1 protein was eluted from the affinity column and a 15μl aliquot of each fraction analysed on a 5-15% polyacrylamide gel. The proteins present on the gel were detected by silver-stain. Faint bands were detected at 220kDa and 300kDa in fractions 4 through to fraction 12 as shown in figure 3.10. In addition, high molecular weight proteins were also visible in fractions 24-27. A number of other non-specific protein bands of lower molecular weights were observed in all fractions.

3.5.2.2 ELISA of purified WC1 protein.

The specificity of the WC1 ELISA which was developed is shown in figure 3.11. Monoclonal antibody ST197 was used to capture the WC1 proteins in the solution. Captured WC1 protein was subsequently detected with a biotinylated secondary monoclonal antibody, CC15. The optical density readings shown indicated the presence of the WC1 antigen. No signal could be detected in an identical assay developed with a non-specific secondary monoclonal antibody (anti-CD8). These results demonstrated that the ELISA developed was specific for WC1 antigen. Using this ELISA, the presence of WC1 in fractions of material eluted from the affinity columns was determined. Figure 3.12 shows the optical density readings in the capture ELISA of an 50μl aliquot of each of the fractions of material eluted from the affinity columns. The WC1 antigen was detected in fractions 6-13 of the WC1 affinity column. This correlated with the silver stain of an SDS-PAGE of each of the fractions shown in figure 3.10.

3.6 WC1 monoclonal antibodies react with purified WC1 proteins of a similar molecular weight.

The results in section 3.4 and 3.5 suggested that the workshop monoclonal antibodies grouped in the WC1 cluster recognised epitopes on proteins of the same molecular weight. This was

Figure 3.9: Purification of ST197 monoclonal antibody.

The monoclonal antibody cell line ST197 was grown to saturation. From the culture supernatent, monoclonal antibody was purified by affinity chromatography. The protein content of the samples before and after purification was assessed by conventional Coomassie staining of SDS-PAGE. Column 1 shows the protein content of the saturated ST197 supernatent before affinity purification; column 2 shows the molecular weight markers and columns 3, 4, 5 and 6 show the protein content of 1, 3, 5 and 10µl of the purified monoclonal antibody respectively.

Figure 3.9: Purification of ST197 monoclonal antibody
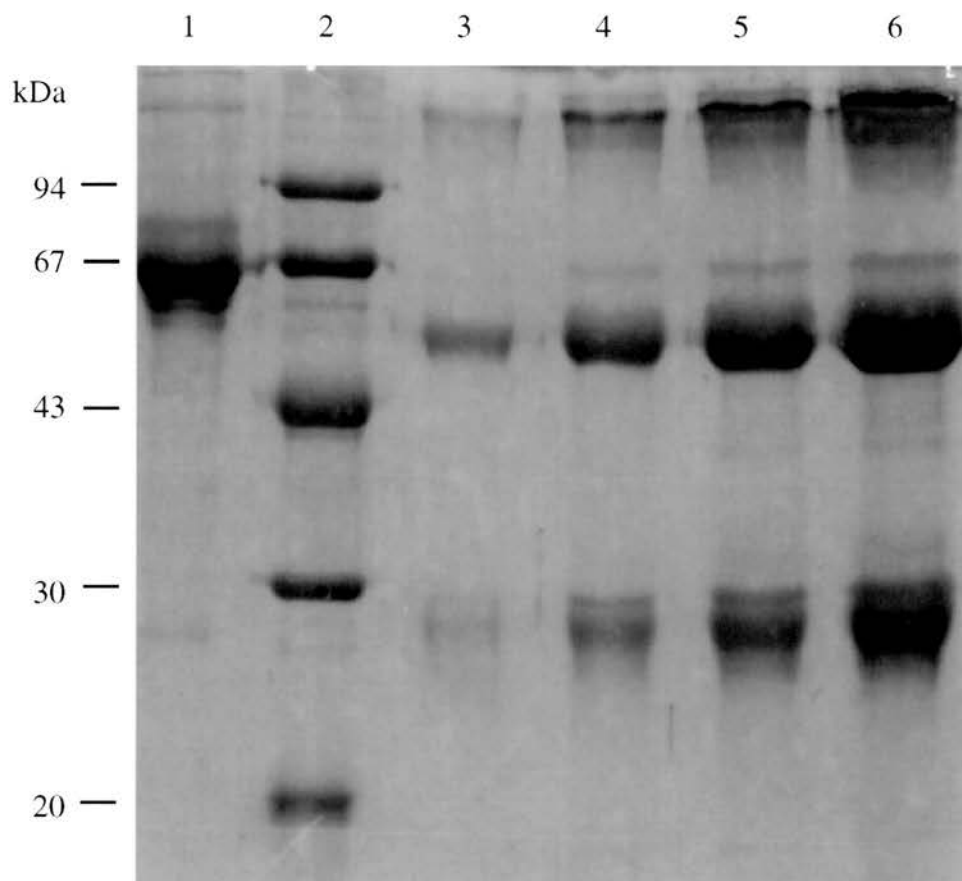
Figure 3.10: Silver stain of purified WC1 protein.

WC1 was purified from spleen lysates by affinity chromatography. 25μl of each eluted fraction from the affinity column was ran on a 5-15% SDS-PAGE. The gel was developed by silver staining. The mobility of the molecular weight markers is shown to the left of the figure. The fraction numbers in ascending order following elution from the column are shown above the respective lanes on the gels. The arrows to the right of the figure indicate the position of faint bands of molecular weights approximating to 220kDa and 300kDa seen in fraction 4 through to fraction 12 inclusive.

Figure 3.10: Silver stain of purified WC1 protein
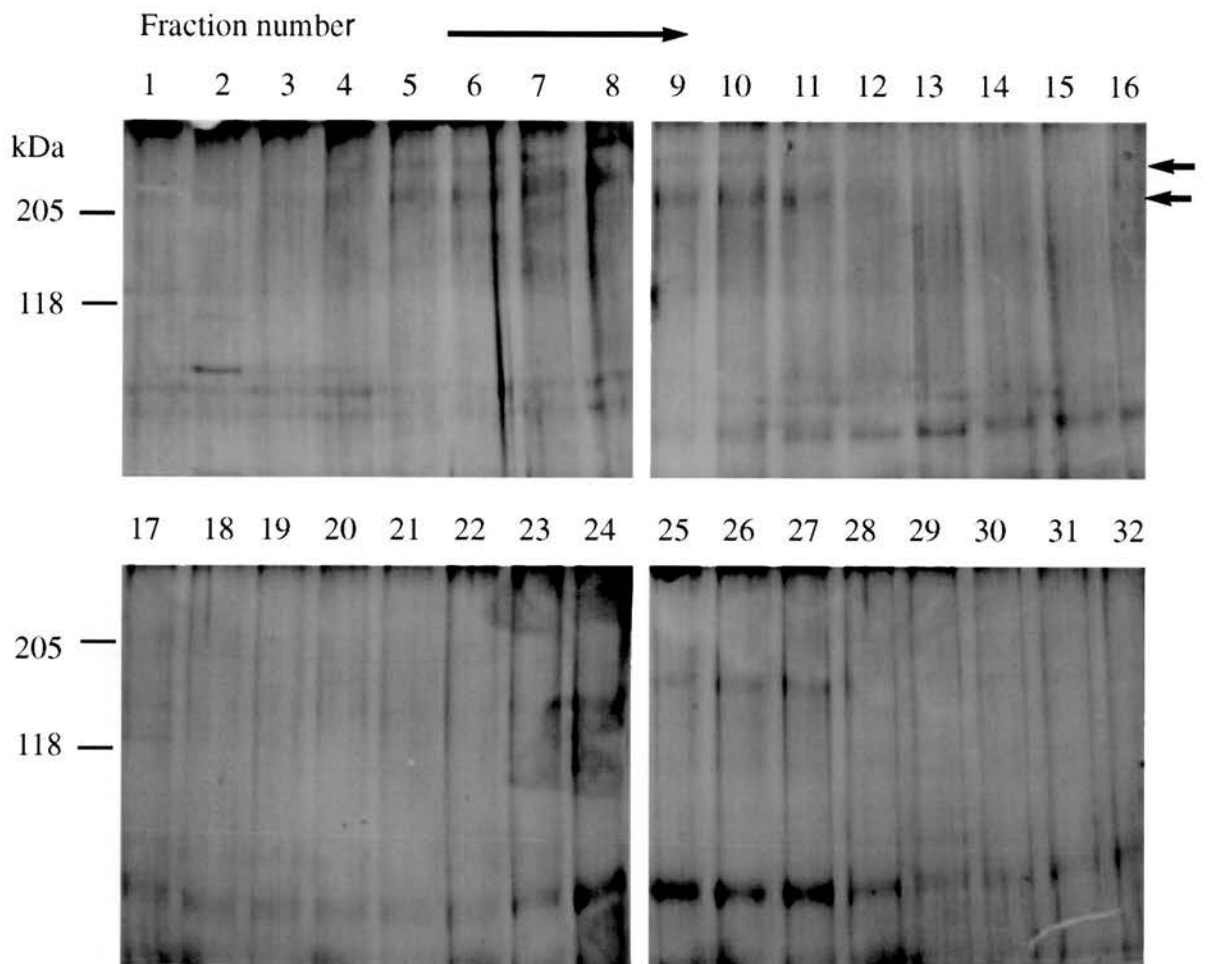
Figure 3.11: WC1 ELISA.

Flat-bottomed 96-well plates were coated with the anti-WC1 monoclonal antibody ST197 and blocked with 1% BSA. 50µl of various dilutions of the purified WC1 protein were added to individual wells. The wells were washed, and any captured WC1 protein detected with the biotinylated WC1 monoclonal antibody CC15. Biotinylated anti-CD8 monoclonal antibody was used as a negative control. Streptavidin-alkaline phoshatase was added to each of the wells and the ELISA developed by addition of the alkaline phosphate colour substrate. Optical density (OD) readings were taken at 405nm. The OD's of the WC1 positive reactions: (———◆———) and (———◇———); and the WC1 negative reactions: (——■——) and (⋯⋯※⋯⋯); are shown.

Figure 3.11: WC1 ELISA.

Legend:
- Developed with anti-WC1
- Developed with anti-WC1
- Developed with anti-CD8
- Developed with anti-CD8

Y-axis: O.D. 405

X-axis: Dilution
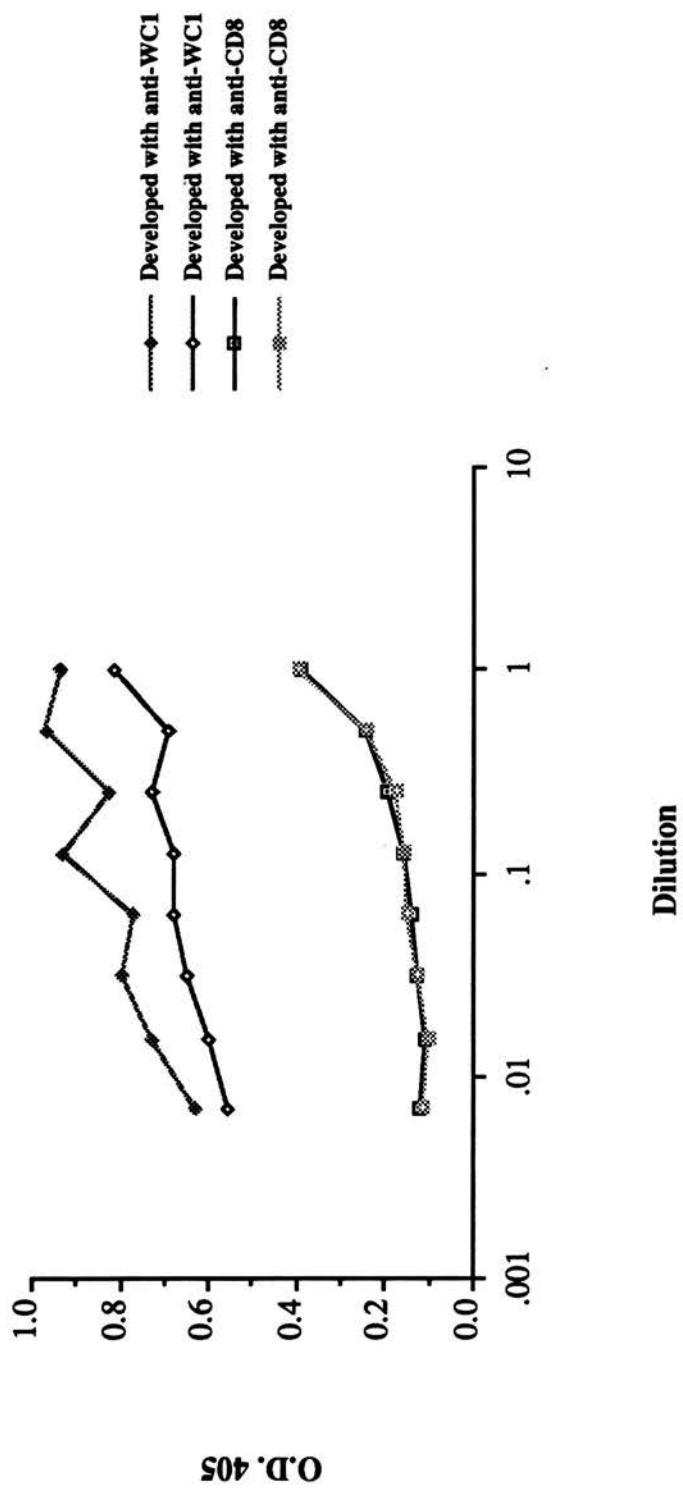
Figure 3.12: **Affinity purification of WC1.**

Flat-bottomed 96-well plates were coated with the anti-WC1 monoclonal antibody ST197 then blocked. 50μl of each fraction eluted from the WC1 affinity column (——◇——), the pre-column (............) and the pre-pre-column (_____) was added to individual wells. The wells were washed, and any captured WC1 protein detected with the biotinylated WC1 monoclonal antibody CC15. Streptavidin alkaline phosphatase was added to each well and the ELISA reaction developed by addition of the alkaline phosphatase colour substrate. Optical density (OD) readings were taken at 405nm. The OD values for the first 25 fractions eluted from each of the columns are aligned on the graph.

Figure 3.12: Affinity purification of WC1 antigen
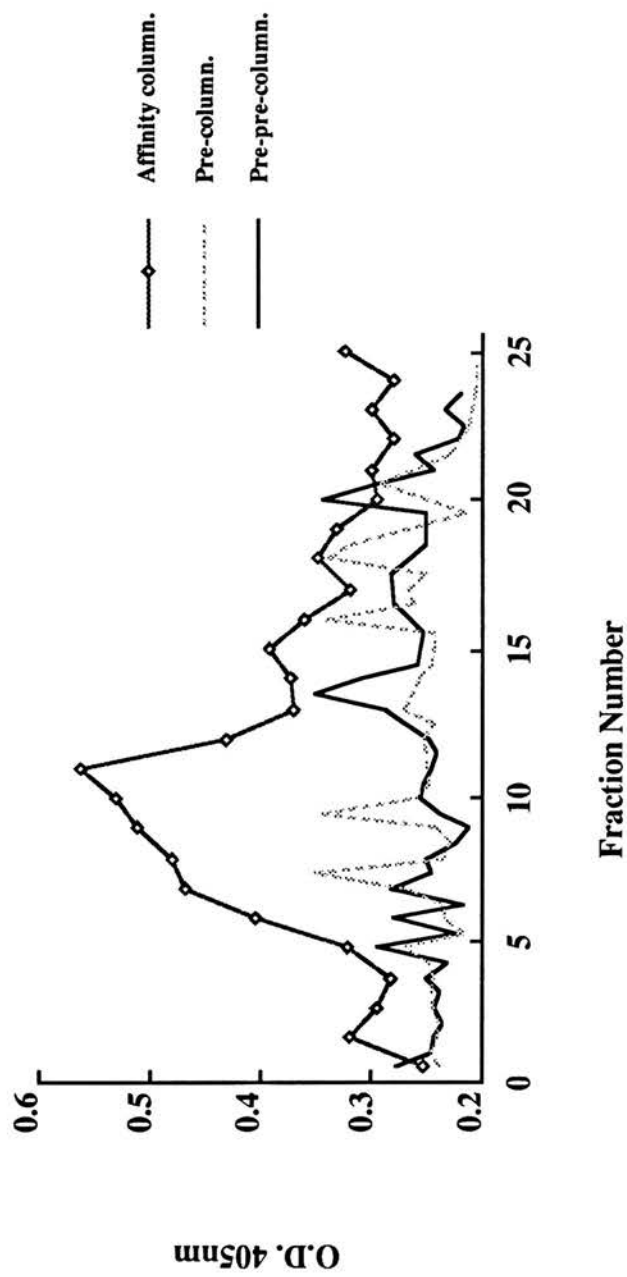
confirmed by Western Blot of purified WC1 proteins. Purified WC1 proteins were separated by SDS-PAGE under both reducing and non-reducing conditions, transferred to a nitrocellulose membrane, and the reactivity of each of the WC1 monoclonal antibodies with the WC1 protein assessed. Figure 3.13A shows that under non-reducing conditions the monoclonal antibody CC15 recognised proteins of molecular weight 140kDa, 220kDa and 300kDa. The monoclonal antibodies ST197, BAG25A, CACTB1A, CACTB7A and BAQ89A recognised protein bands of similar molecular weight but with less intensity than those seen with the monoclonal antibody CC15. The monoclonal antibody BAQ4A reacted with proteins of molecular weight 140kDa, 220kDa and 300kDa and did so with greater intensity than seen with the monoclonal antibody CC15. In contrast, the monoclonal antibodies CC101 and B7A1 reacted a protein with an apparent molecular weight of 144kDa, and along with all the other monoclonal antibodies, except CC15, recognised proteins of lower molecular weights. WC1 proteins with molecular weights lower than 144kDa have not previously been reported and those identified here are thought to be degradation products which occurred as a result of the treatment of the purified material. The epitope recognised by the monoclonal antibody CC15 is possibly conformation dependent, and its loss occurs through degradation of the WC1 molecule. Figure 3.13B shows the results obtained under reducing conditions. None of the monoclonal antibodies reacted with any proteins. This implies reactivity by the monoclonal antibodies with WC1 protein is dependent upon disulphide bonding. This may be a consequence of the high number of cysteine residues found in the protein sequence (see figure 3.20).

3.7 Subsets of WC1 positive T cells have no apparent recirculatory patterns.

Sheep PBL have a number of sub-populations of WC1 positive $\gamma\delta$ T cells as defined by the expression of different WC1 epitopes described in this chapter. The percentage of lymphocytes expressing the different WC1 epitopes was determined in a number of different tissues and body fluids from the sheep. If any specific recirculatory or homing patterns occurred for a subset of WC1 positive cells these would be expected to predominate over the other WC1 cells in a particular tissue. Table 3.5 shows the percentage of lymphocytes in various tissues and lymphoid compartments which expressed each of the WC1 epitopes. Lymphocytes were isolated from the tissues of 6 month old animals and from the lymph fluid of 9 month old animals. The expression of the different WC1 epitopes on lymphocytes found in the peripheral blood of 6 month old animals were included as a reference. There were no distinct recirculatory patterns of any of the WC1 positive subsets in any of the tissues examined. Of all the different compartments examined, the highest numbers of lymphocytes which expressed each of the WC1 epitopes were found in the afferent lymph. Few lymphocytes isolated from both mesenteric and prescapular lymph nodes expressed any of the WC1 epitopes. 18% of the lymphocyte population isolated from the intestinal epithelium expressed the $\gamma\delta$ TCR however <5% of the lymphocyte population were positive for any of the WC1 epitopes.

3.7.1 Histological identification of cells expressing WC1 epitopes.

In the previous section, lymphocytes isolated from different tissues and body fluids were analysed to determine if there was any specific recirculation of lymphocytes which expressed the different WC1 epitopes. However, the procedures employed for purifying lymphocytes from the different tissues did not allow for an examination of the T cell specific areas of the different immune compartments. The tissue distribution of lymphocytes expressing the different WC1 epitopes was therefore determined in the spleen, thymus and lymph node. In all of the tissues examined, the

Figure 3.13: Western blot of purified WC1 protein.

Purified WC1 was ran on a 5-15% SDS-polyacrylamide gel under either reducing (with 5% β-mercaptoethanol) or non-reducing conditions. The material was then transferred from the SDS-PAGE onto a nitrocellulose membrane. Strips of nitrocellulose were then incubated with each of the WC1 monoclonal antibodies and the reactivity of the WC1 monoclonal antibodies was developed with biotinylated anti-IgG and streptavidin-alkaline phosphatase. The relative mobility of the molecular weight markers are shown at the left of the figure and the WC1 monoclonal antibodies used are listed along the top of the figure.

Figure 3.13: Western Blot of purified WC1 protein
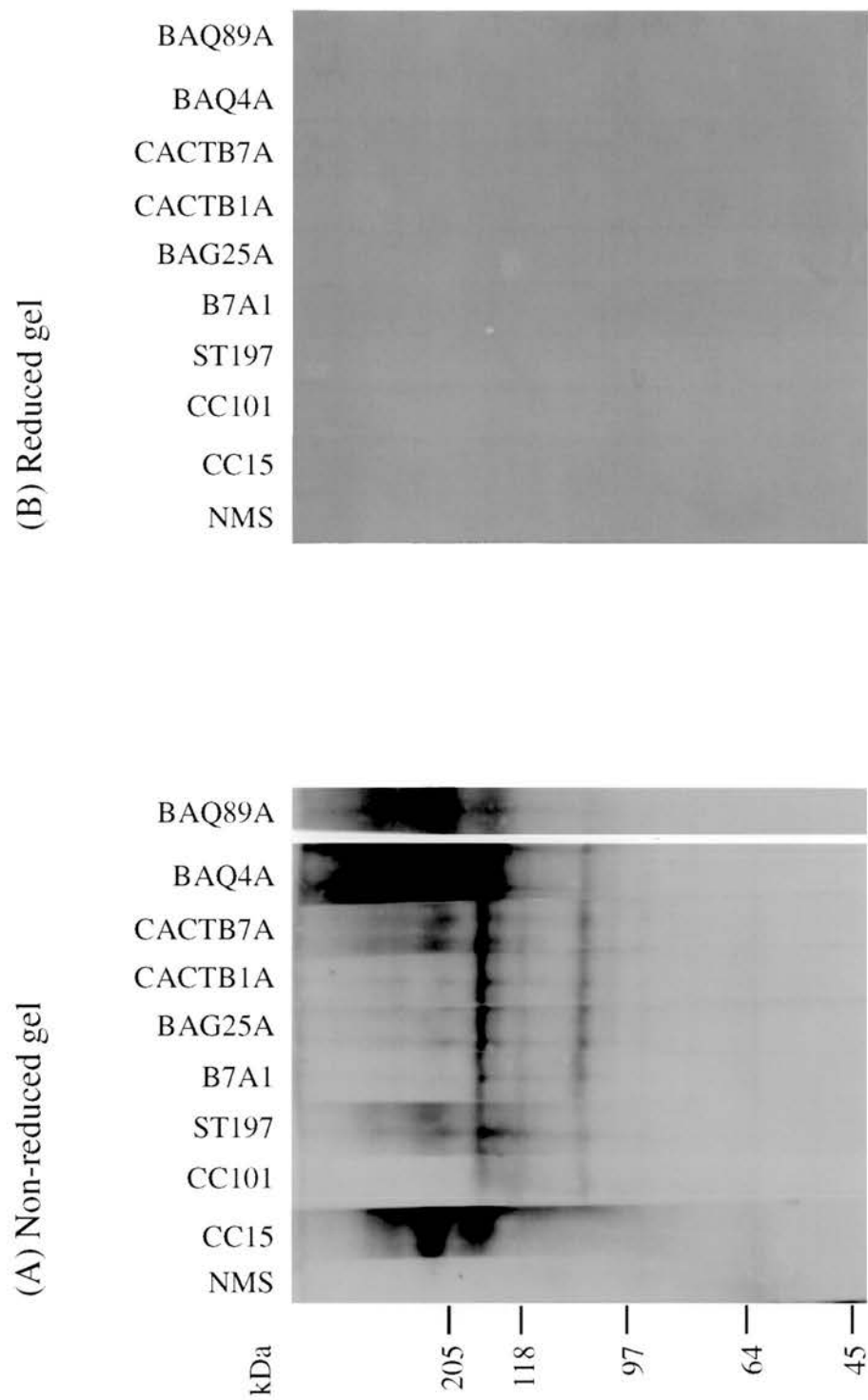
Table 3.5: Tissue distribution of WC1 populations

Lymphocytes were isolated from the listed tissues and peripheral blood of six month old animals (n=5) and the lymphocyte compartments of nine month old animals (n=5). 10^6 lymphocytes from each tissue were stained with each of the monoclonal antibodies listed and then anti-Ig FITC. The percentage of positive cells was calculated with respect to the staining of NMS as a negative control (<3% positive). The results shown are the mean values obtained and the standard deviations of the means are shown in the brackets.

TABLE 3.5: Tissue distribution of WC1 populations.

Percentage of PBL stained by monoclonal antibodies.

Tissue:	CC15	CC101	CACTB1A	CACTB15B	BAQ89A	CACTB7A	BAG25A
Spleen	4.6 (1.9)	3.2 (1.0)	1.1 (0.6)	0.9 (0.3)	2.8 (2.5)	2.7 (1.3)	3.1 (2.2)
Intestinal epithelium	5.0 (2.4)	4.8 (2.3)	0.0 (0.0)	0.0 (0.0)	1.0 (0.1)	0.8 (1.5)	2.8 (2.0)
Lamina propria	4.5 (1.2)	3.4 (0.4)	0.4 (0.4)	0.2 (0.1)	0.4 (0.4)	0.5 (0.5)	1.1 (1.1)
Thymus	5.7 (0.8)	2.5 (1.8)	1.6 (1.6)	0.9 (1.3)	1.1 (0.3)	2.4 (1.5)	3.4 (1.5)
Mesenteric Lymph Node	0.4 (0.5)	0.2 (0.5)	0.5 (0.6)	0.1 (0.2)	0.0 (0.0)	0.2 (0.6)	0.3 (0.3)
Prescapular Lymph Node	1.4 (0.1)	0.9 (0.3)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.9)	0.7 (0.6)
Efferent Lymph	8.0 (4.0)	4.6 (2.2)	2.7 (1.4)	1.8 (1.2)	5.1 (2.9)	4.8 (4.3)	8.7 (3.5)
Efferent Lymph Blasts	8.6 (3.0)	2.3 (1.3)	1.4 (1.2)	1.1 (0.5)	3.4 (2.1)	5.4 (1.3)	8.8 (3.1)
Afferent Lymph	12.2 (2.3)	5.2 (1.0)	5.5 (2.5)	4.8 (2.3)	6.0 (1.5)	6.6 (0.8)	7.7 (1.9)
Peripheral Blood	11.7 (5.3)	3.8 (0.9)	2.8 (1.5)	3.8 (1.6)	5.2 (2.5)	5.0 (3.7)	6.3 (3.1)

reactivity of the monoclonal antibodies CC115 and CC117 was similar to the reactivity of the monoclonal antibody CC101. In addition, the monoclonal antibodies CACTB7A and BAQ89A had similar patterns of reactivity as did the monoclonal antibodies CACTB1A and CACTB15B. The monoclonal antibody BAG25A stained non-specifically all tissue sections.

Figure 3.14 shows the immunoperoxidase staining of lymphocytes with the monoclonal antibodies CC15 (WC1) and 86D ($\gamma\delta$ TCR) in the spleen of a nine month old animal. Stained cells can be seen in the marginal zones, outwith and encircling the periarteriolar sheath and B cell follicular area. This is indicative of cells in transit through the spleen and is consistent with previously reported data (MacKay et al., 1986). Figure 3.15 shows the staining of lymphocytes in the spleen by monoclonal antibodies to the different WC1 epitopes. The number of lymphocytes stained by each of the workshop monoclonal antibodies appeared to be less than that observed with the control monoclonal antibody CC15 (figure 3.14). In addition, the intensity of staining by CC101(A) and CACTB1A(C) appeared to be less than that observed with the control monoclonal antibody CC15 (figure 3.14).

Figure 3.16 shows the distribution of cells in the thymus reactive with the monoclonal antibodies CC15 and 86D. It appeared that there were less WC1-positive cells present than $\gamma\delta$ TCR-positive cells. An increase in WC1 expression has been shown to occur late in thymic development (MacKay et al., 1989). Positive cells were most predominant in the medulla with fewer cells seen in the cortex. Figure 3.17 shows the distribution of cells reactive with the WC1 workshop monoclonal antibodies. The distribution of cells reactive with the WC1 workshop monoclonal antibodies was the same as the distribution of cells reactive with the control monoclonal antibody CC15 (figure 3.16). The intensity of staining of the 2nd workshop monoclonal antibodies and the number of cells which reacted with the 2nd workshop monoclonal antibodies may have been less than that observed with the control monoclonal antibody CC15 (figure 3.16).

Immunohistological staining of the lymph node by the monoclonal antibodies CC15 and 86D is shown in figure 3.18. Positively stained cells are concentrated in the peripheral areas of the cortex adjacent to the subscapular and cortical sinuses and lining the trabeculae. The pattern of reactivity is similar to that observed in the spleen and is indicative of trafficking cells. The staining in the lymph node of the monoclonal antibodies CC101(A), CACTB7A(B) and CACTB1A(C) is shown in figure 3.19. The staining of the WC1 monoclonal antibodies CC101(A) and CACTB1A(C) appeared to be less intense than that of the control monoclonal antibody CC15 (figure 3.18). In addition, all of the workshop monoclonal antibodies appeared to stain fewer cells compared to the control monoclonal antibody CC15 (figure 3.18).

Figure 3.14: **Immunohistological staining of the spleen.**

Cryostat sections of the spleen from a 6 month old sheep were stained with the monoclonal antibodies CC15 (A) and 86D (B) and visualised with alkaline phosphatase. Sections were viewed by conventional microscopy and photographs were taken at x40 magnification. Positive cells are concentrated in the marginal zones. There are no positive cells in the periarteriolar region or in the B cell follicle.

FIGURE 3.14: Immunohistological staining of the Spleen.

(A) CC15
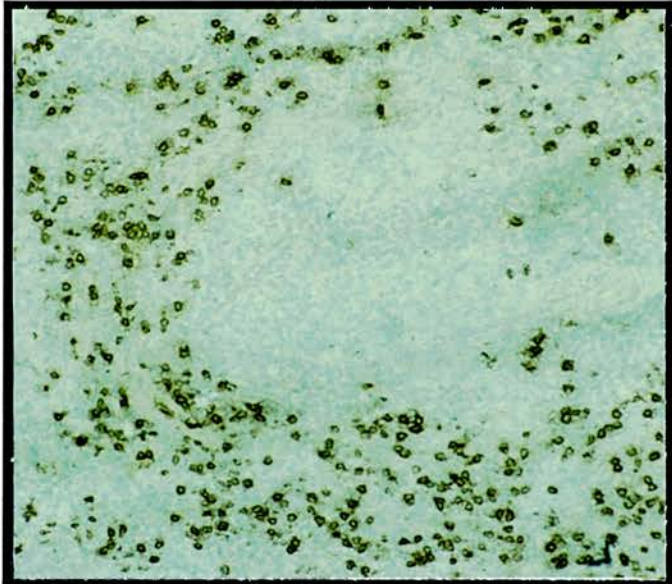
(B) 86D
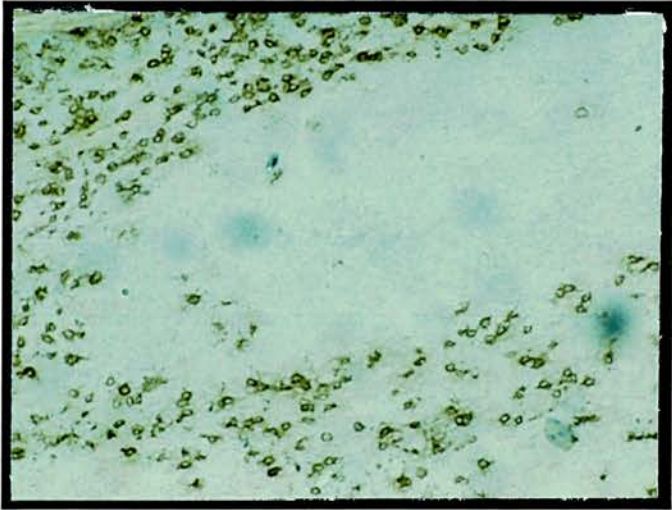
Figure 3.15:   Immunohistological staining of the spleen.

Cryostat sections of the spleen from a 6 month old sheep were stained with the monoclonal antibodies CC101 (A), CACTB7A (B) and CACTB1A (C) and visualised with alkaline phosphatase. Sections were viewed by conventional microscopy and photographs were taken at x40 magnification. Positive cells are concentrated in the marginal zones. There are no positive cells in the periarteriolar region or in the B cell follicle.

FIGURE 3.15: Immunohistological staining of the Spleen.

(A) CC101
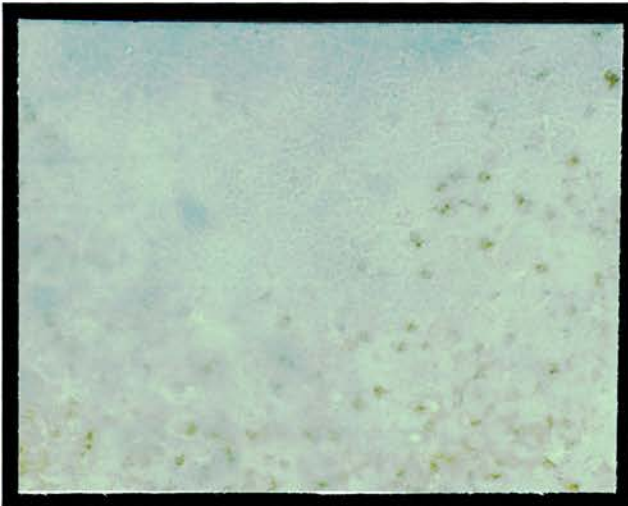
(B) CACTB7A
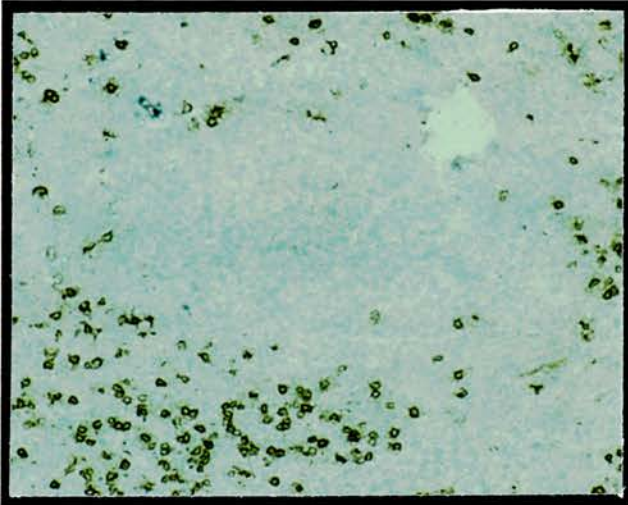
(C) CACTB1A
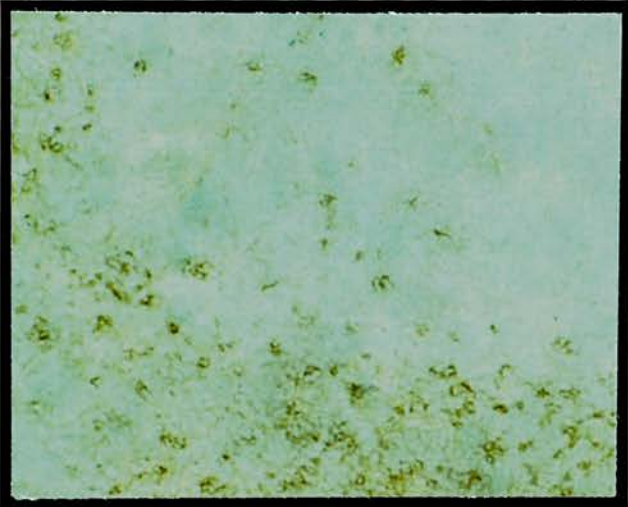
Figure 3.16: Immunohistological staining of the thymus.

Cryostat sections of the thymus from a 6 month old sheep were stained with the monoclonal antibodies CC15 (A) and 86D (B) and visualised with alkaline phosphatase. Sections were viewed by conventional microscopy and photographs were taken at x40 magnification. Positive cells are concentrated in the medulla with fewer positive cells scattered in the cortex.

FIGURE 3.16: Immunohistological staining of the Thymus.

(A) CC15
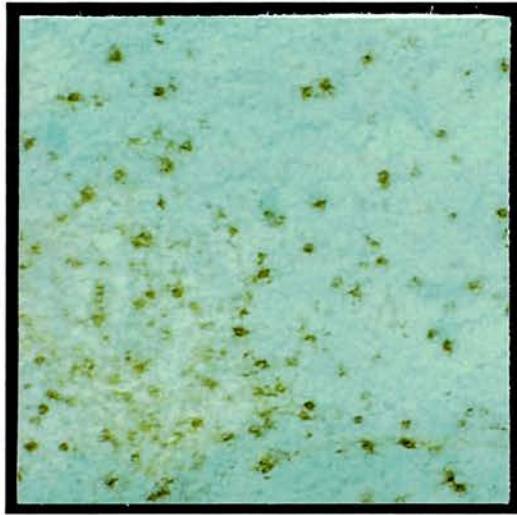
(B) 86D
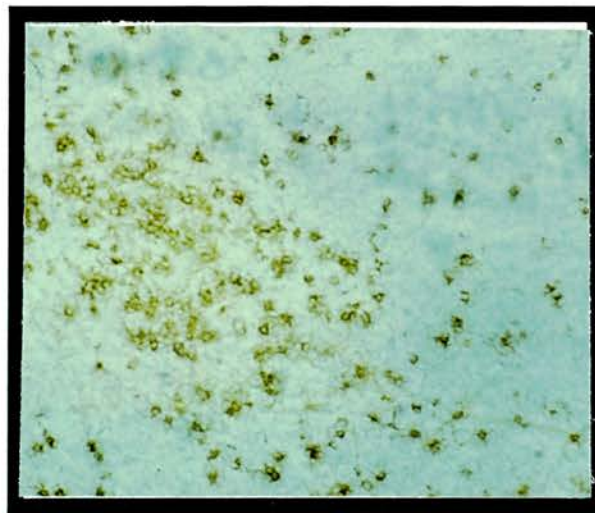
Figure 3.17:   Immunohistological staining of the thymus.

Cryostat sections of the thymus from a 6 month old sheep were stained with the monoclonal antibodies CC101 (A), CACTB7A (B) and CACTB1A (C) and visualised with alkaline phosphatase. Sections were viewed by conventional microscopy and photographs were taken at x40 magnification. Positive cells are concentrated in the medulla with fewer positive cells scattered in the cortex.

FIGURE 3.17: Immunohistological staining of the Thymus.

(A) CC101
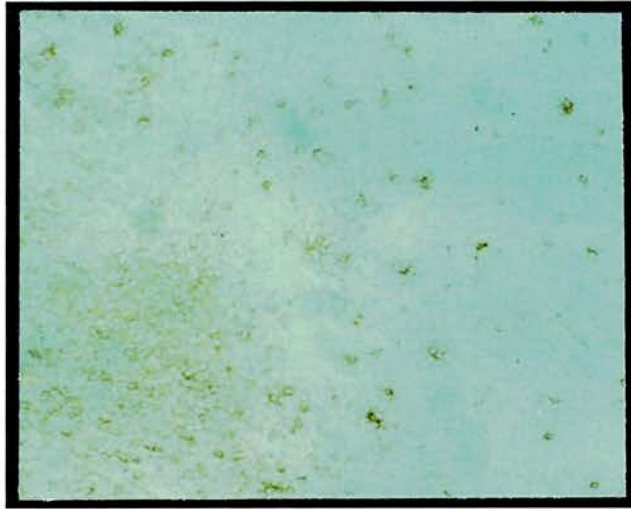
(B) CACTB7A
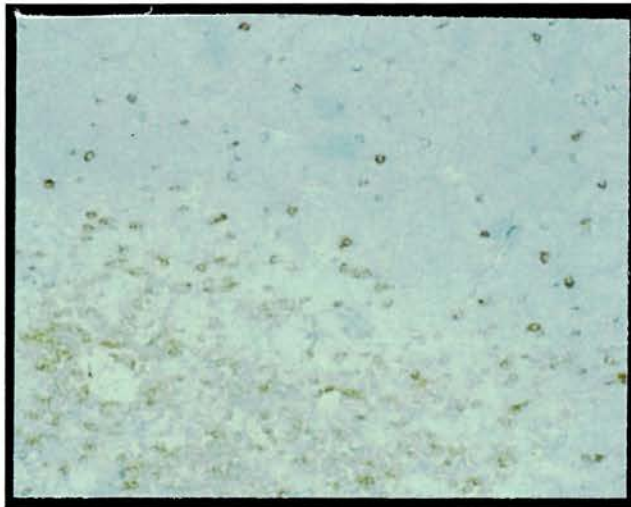
(C) CACTB1A
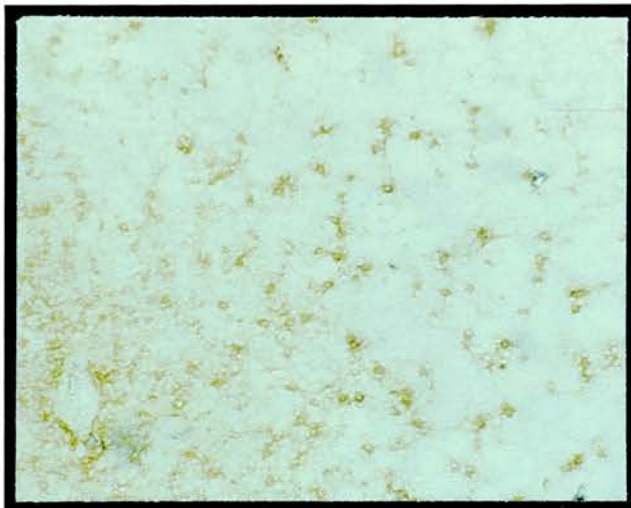
Figure 3.18: Immunohistological staining of the lymph node.

Cryostat sections of the lymph node from a 6 month old sheep were stained with the monoclonal antibodies CC15 (A) and 86D (B) and visualised with alkaline phosphatase. Sections were viewed by conventional microscopy and photographs were taken at x40 magnification. Positive cells are concentrated in the peripheral areas of the cortex adjacent to the subscapular sinuses and the cortical sinuses and lining the trabeculae.

FIGURE 3.18: Immunohistological staining of the Lymph Node.

(A) CC15
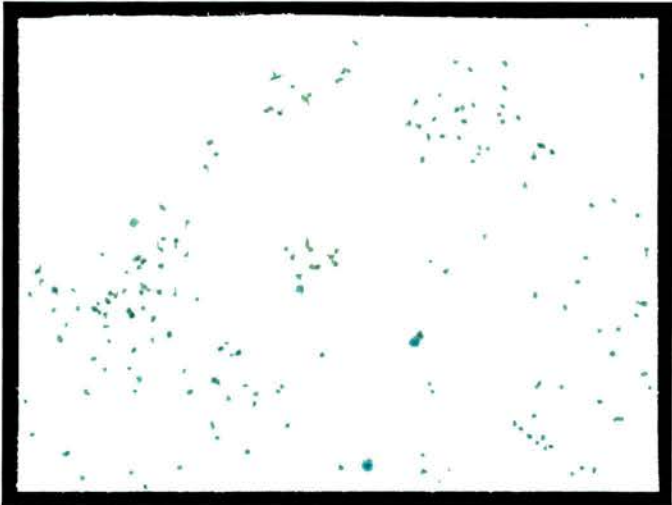
(B) 86D
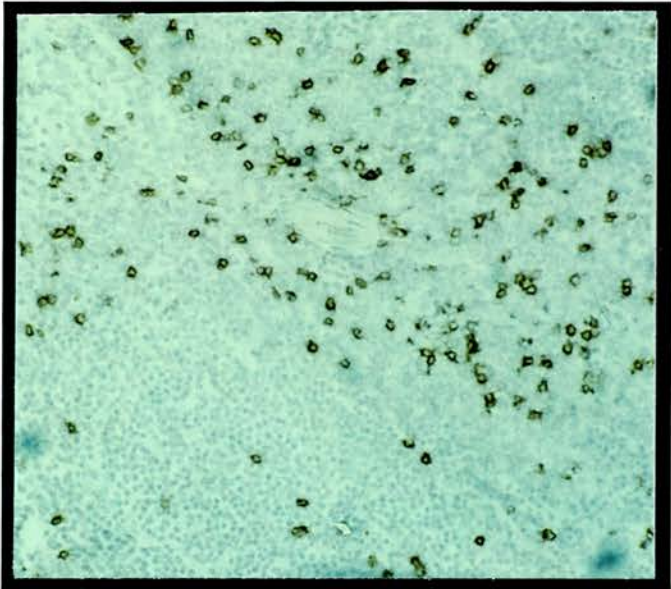
Figure 3.19: Immunohistological staining of the lymph node.

Cryostat sections of the lymph node from a 6 month old sheep were stained with the monoclonal antibodies CC101 (A), CACTB7A (B) and CACTB1A and visualised with alkaline phosphatase. Sections were viewed by conventional microscopy and photographs were taken at x40 magnification. Positive cells are concentrated in the peripheral areas of the cortex adjacent to the subscapular sinuses and the cortical sinuses and lining the trabeculae.

FIGURE 3.19: Immunohistological staining of the Lymph Node.

(A) CC101
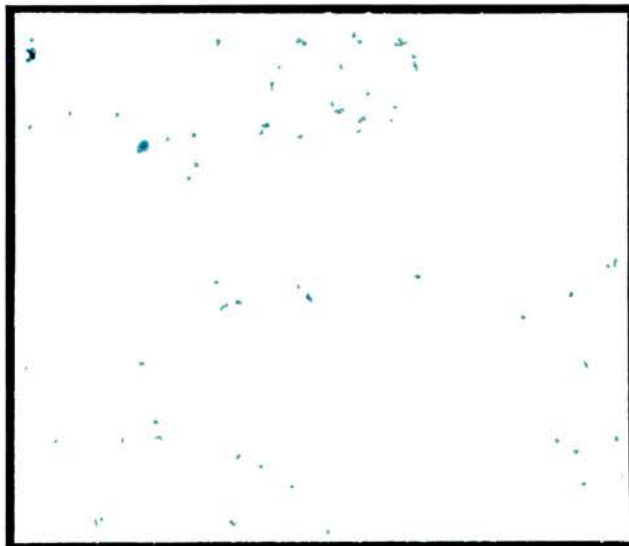
(B) CACTB7A
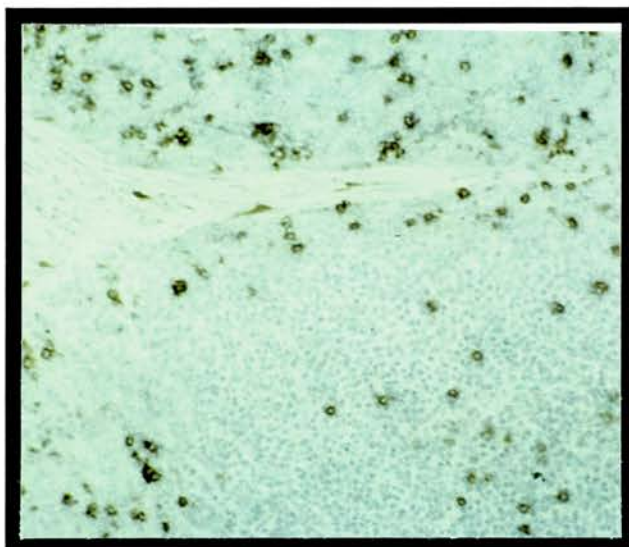
(C) CACTB1A (N3)
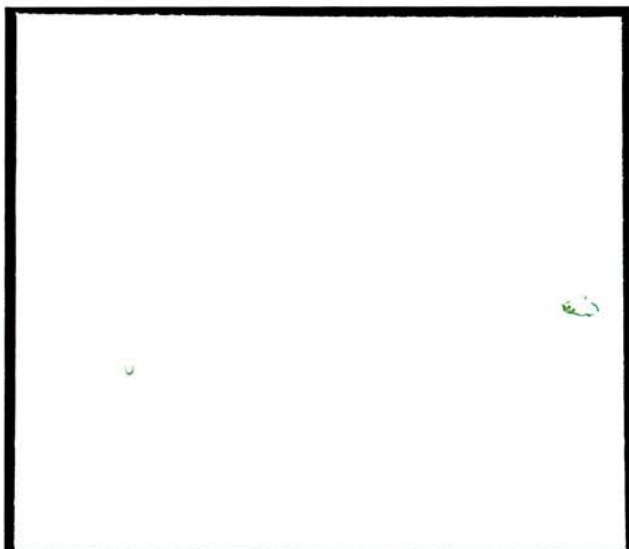
DISCUSSION:

This chapter introduces the use of fluorescence activated cell scanning (FACScan) to distinguish sub-populations of peripheral blood mononuclear cells, in particular sub-populations of lymphocytes. The expression of a number of different cell surface molecules was investigated throughout the course of this thesis. This chapter has explained the methodology used to calculate the level specific expression of these antigens on lymphocytes. Reference staining profiles of some of the monoclonal antibodies have been shown. FACScan analysis of ovine PBL showed that the epitope recognised by the WC1 monoclonal antibody CC15 was expressed on virtually all γδ T cells. The monoclonal antibody CC15 is an IgG2a isotype. This monoclonal antibody, or the monoclonal antibody 86D (anti-γδ TCR), could therefore be used for the identification of the γδ T cell population. It was also shown that the monoclonal antibodies ST4 and SBU-T4 both recognised epitopes on an identical population of lymphocytes. Therefore, these monoclonal antibodies were used interchangeably. The isotype of SBU-T4 is also IgG2a and the isotype of ST4 is IgG1. Similarly, monoclonal antibodies ST8 and SBU-T8 also recognised similar populations of cells though SBU-T8 did not react with cells expressing the ST8 epitope at low levels. SBU-T8 is an IgG2a isotype antibody and ST8 and IgM isotype antibody. The monoclonal antibodies CC15, SBU-T4 and SBU-T8 define the three known T cell populations in the ovine species; γδ, CD4 and CD8 respectively. Because CC15, SBU-T4 and SBU-T8 are of the same isotype, these monoclonal antibodies allowed for direct comparisons of changes in phenotype of the different T cell subsets when stained against monoclonal antibodies of different isotypes. The monoclonal antibodies VPM8 and DU2.104 recognise sheep B cells. DU2.104 is reported however to be a more specific marker (W. R. Hein, unpublished; P.Bird, personal communication). This is thought due to the reactivity of the anti-light chain monoclonal antibody, VPM8, with Fc receptors on some ovine cells. DU2.104 was shown to react only with surface immunoglobulin-positive cells in efferent and afferent lymph, and also peripheral blood (P. Bird, personal communication). DU2.104 is of an IgM isotype and was therefore chosen as a B cell marker in assays requiring monoclonal antibodies other than the IgG1 isotype.

A panel of monoclonal antibodies that recognise epitopes on the WC1 proteins on the cell surface of ruminant γδ T lymphocytes were investigated. These monoclonal antibodies were raised against bovine lymphocytes and their reactivity with ovine lymphocytes was determined here. It was shown that, although the bovine and ovine species are closely related, not all the WC1 epitopes expressed in the bovine species were expressed in the ovine species.

The WC1 epitopes recognised by the cross-reactive monoclonal antibodies were expressed on γδ T cells. All the WC1 monoclonal antibodies reacted with sub-populations of cells stained with the monoclonal antibody CC15. CC15 is believed to recognise an epitope expressed on all WC1 proteins (Clevers et al., 1988; Sopp et al., 1991). The WC1 monoclonal antibodies recognised proteins of molecular weights 144kDa, 215kDa and 300kDa. It was confirmed by Western Blot of purified WC1 protein that all the WC1 monoclonal antibodies recognised proteins of the same molecular weight. The monoclonal antibody CC15 immunoprecipitated proteins of molecular weight 144kDa, 215kDa and 300kDa, and recognised all three in a Western Blot. In contrast, some WC1 monoclonal antibodies only reacted with the two lower molecular weight proteins. In addition, the intensity of protein bands seen with the other WC1 monoclonal antibodies was less than that seen with the monoclonal antibody CC15. This correlated with the FACScan analysis of peripheral blood lymphocytes in that the monoclonal antibody CC15 stained the greatest number of lymphocytes and with the highest intensity. This indicated that either (i) the WC1 monoclonal antibodies had a weaker affinity for the protein than did the monoclonal antibody CC15; or (ii)

the epitopes recognised by the WC1 monoclonal antibodies are expressed at different levels on the surface of γδ T cells. Complex WC1 gene families have been found in cattle, sheep, goats, pigs and horses (Wijngaard et al., 1992, 1994; Metzelaar et al., 1992). The WC1 proteins could be synthesised from the gene pool and different epitopes expressed on different products. The epitope recognised by the monoclonal antibody CC15 may be part of the protein backbone and is expressed on all WC1 molecules.

The function(s) of WC1 is unknown. γδ T cells do not express the co-receptor molecules CD2, CD4 or CD8. It has been proposed that WC1 may fulfil the role of an accessory molecule on the surface of these cells (MacKay et al., 1986; Evans, 1993). Recently, three different bovine WC1 gene constructs have been cloned and sequenced (Wijngaard et al., 1992, 1994: Metzelaar et al., 1992) allowing a putative structure for the WC1 protein to be predicted. Figure 3.20 shows the predicted amino acid sequence of the WC1.1 gene. 91 amino acids (~6.3%) in the extracellular domain of WC1 are cysteine residues. Disulphide bonds can be formed between cysteine residues which cause the protein to fold and so increase the degree of secondary structure. In addition, 61 amino acids (4.2%) in the extracellular domain of WC1 are proline residues. The amino acid proline has one fixed and one flexible peptide bond with adjacent amino acids. This also increases the degree of secondary structure in a protein. It appears that the extracellular domain of WC1 proteins will have extensive secondary structure caused in part by the large number of cysteine and proline residues. This secondary structure could account for the observation that the WC1 monoclonal antibodies did not recognise WC1 protein in a Western Blot when run under reducing conditions.

The WC1 extracellular domain is a single polypeptide chain consisting of 11 non-identical repeats of a 110 amino acid consensus sequence of a cysteine rich region as detailed in figure 3.21 (Wijngaard et al., 1992, 1994). These cysteine rich regions have also been found in a number of other glycoprotein structures including CD5 (Jones et al., 1986; Huang et al., 1987) and CD6 (Aruffo et al., 1991) and the macrophage scavenger receptor cysteine rich (SRCR) domain which may play a role in macrophage-associated immune responses and inflammation (Freeman et al., 1990). The proteins CD5 and CD6 are known to be involved in T cell activation (Geppert et al., 1990). The presence of these domains on the WC1 proteins suggests that the WC1 protein may interact with a corresponding ligand system. CD5 interacts with its ligand CD72 via the SRCR domains (van de Velde et al., 1991) therefore by analogy, WC1 may interact with a membrane bound ligand via its own SRCR domains. It has been proposed therefore, that WC1 may function by controlling tissue-specific homing of γδ T cells (Wijngaard et al., 1994).

It should be noted that no evidence was found in this thesis for a distinct recirculation of any of the WC1 positive populations so far defined in the staining of the major T cell compartments. The greatest number of lymphocytes which expressed the different WC1 epitopes was found in the afferent lymph whilst few lymphocytes in the lymph node expressed any of the WC1 epitopes. However, in both lymphocyte compartments no WC1 sub-population prevailed. In the intestinal epithelium, few of the γδ T cell population expressed any of the WC1 epitopes. This lack of expression of WC1 has been suggested by others (MacKay et al., 1986; McClure et al., 1989) and there are at least three possibilities to explain this observation: (i) a homing of WC1-negative γδ T cells to the intestinal epithelium; (ii) a loss of the WC1 protein upon localisation of the γδ T cell population to the intestinal epithelium; (iii) no monoclonal antibody exists which recognises an epitope on this tissue specific population of γδ T cells.

Figure 3.20:   Predicted amino acid sequence of bovine WC1.

The full amino acid sequence of bovine WC1 is shown in single letter format. The sequence of the pWC1.1 cDNA clone is available in the EMBL database under accession number X63723 (Wijngaard et al., 1994). The structurally important amino acids cysteine (C) and proline (P) are underlined and the transmembrane domain is shown in bold italics. The numbers on the left of each row represent the first amino acid number shown in the row.

Figure 3.20: Predicted amino acid sequence of bovine WC1

```
   8          MAL GRHLSLRGLC VLLLGTMVGG QALELRLKDG VHRCEGRVEV
  51   KHQGEWGTVD GYRWTLKDAS VVCRQLGCGA AIGFPGGAYF GPGLGPIWLL
 101   YTSCEGTEST VSDCEHSNIK DYRNDGYNHG RDAGVVCSGF VRLAGGDGPC
 151   SGRVEVHSGE AWIPVSDGNF TLATAQIICA ELGCGKAVSV LGHELFRESS
 201   AQVWAEEFRC EGEEPELWVC PRVPCPGGTC HHSGSAQVVC SAYSEVRLMT
 251   NGSSQCEGQV EMNISGQWRA LCASHWSLAN ANVICRQLGC GVAISTPGGP
 301   HLVEEGDQIL TARFHCSGAE SFLWSCPVTA LGGPDCSHGN TASVICSGNQ
 351   IQVLPQCNDS VSQPTGSAAS EDSAPYCSDS RQLRLVDGGG PCAGRVEILD
 401   QGSWGTICDD GWDLDDARVV CRQLGCGEAL NATGSAHFGA GSGPIWLDNL
 451   NCTGKESHVW RCPSRGWGQH NCRHKQDAGV ICSEFLALRM VSEDQQCAGW
 501   LEVFYNGTWG SVCRNPMEDI TVSTICRQLG CGDSGTLNSS VALREGFRPQ
 551   WVDRIQCRKT DTSLWQCPSD PWNYNSCSPK EEAYIWCADS RQIRLVDGGG
 601   RCSGRVEILD QGSWGTICDD RWDLDDARVV CKQLGCGEAL DATVSSFFGT
 651   GSGPIWLDEV NCRGEESQVW RCPSWGWRQH NCNHQEDAGV ICSGFVRLAG
 701   GDGPCSGRVE VHSGEAWTPV SDGNFTLPTA QVICAELGCG KAVSVLGHMP
 751   FRESDGQVWA EEFRCDGGEP ELWSCPRVPC PGGTCLHSGA AQVVCSVYTE
 801   VQLMKNGTSQ CEGQVEMKIS GRWRALCASH WSLANANVVC RQLGCGVAIS
 851   TPRGPHLVEG GDQISTAQFH CSGAESFLWS CPVTALGGPD CSHGNTASVI
 901   CSGNHTQVLP QCNDFLSQPA GSAASEESSP YCSDSRQLRL VDGGGPCGGR
 951   VEILDQGSWG TICDDDWDLD DARVVCRQLG CGEALNATGS AHFGAGSGPI
1001   WLDDLNCTGK ESHVWRCPSR GWGRHDCRHK EDAGVICSEF LALRMVSEDQ
1051   QCAGWLEVFY NGTWGSVCRS PMEDITVSVI CRQLGCGDSG SLNTSVGLR
1101   GSRPRWVDLI QCRKMDTSLW QCPSGPWKYS SCSPKEEAYI SCEGRRPKSC
1151   PTAAACTDRE KLRLRGGDSE CSGRVEVWHN GSWGTVCDDS WSLAEAEVVC
1201   QQLGCGQALE AVRSAAFGPG NGSIWLDEVQ CGGRESSLWD CVAEPWGQSD
1251   CKHEEDAGVR CSGVRTTLPT TTAGTRTTSN SLPGIFSLPG VLCLILGSLL
1301   FLVLVILVTQ LLRWRAERRA LSSYEDALAE AVYEELDYLL TQKEGLGSPD
1351   QMTDVPDENY DDAEEVPVPG TPSPSQGNEE EVPPEKEDGV RSSQTGSFLN
1401   FSREAANPGE GEESFWLLQG KKGDAGYDDV ELSALGTSPV TFS
```

Figure 3.21: Predicted amino acid sequences of SRCR region repeats in the extracellular domain of bovine WC1.

The amino acid sequences of the eleven SRCR regions are listed in the single letter code. The sequence of the pWC1.1 cDNA clone is available in the EMBL database under accession number X63723 (Wijngaard et al., 1994). All cysteine (C) residues are underlined. The numbers at the right and left of each row represent the amino acid number of the first and last letters in each row respectively.

Figure 3.21: Predicted amino acid sequences of SRCR region repeats in the extracellular domain of bovine WC1.

```
35    LRLKDGVHRCEGRVEVKHQGEWGTVDGYRWTLKDASVVCRQLGCGAAIGFPGGAYFGPGLGPIWLLYTSCEGTESTVSDCEHSNIKDYRNDGYNHGRDAGVVCSGF      140

141   VRLAGRDGPCSGRVEVHSGEAWIPVSDGNFTLATAQIICAELGCGKAVSVLGHELFRESSAQVWAEEFRCEGEEPELWVCPRVPCPGGTCHHSGSAQVVCSAYSEV      246

247   RLMTNGSSQCEGQVEMNISGQWRALCASHWSLANANVICRQLGCGVAISTPGGPHLVEEGDQILTARFHCSGAESFLWSCPVTALGGPDCSHGNTASVICSGNQQ       352

383   LRLVDGGGPCAGRVEILDQGSWGTICDDGWDLDDARVVCRQLGCGEALNATGSAHFGAGSGPIWLDNLNCTGKESHVWRCPSRGWGQHNCRHKQDAGVICSEFLA      487

488   LRMVSEDQQCAGWLEVFYNGTWGSVCRNPMEDITVSTICRQLGCGDSGTLNSSVALREGFRPQWVDRIQCRKTDTSLWQCPSDPWNYNSCSPKEEAYIWCADSRQ       592

593   IRLVDGGGRCSGRVEILDQGSWGTICDDRWDLDDARVVCKQLGCGEALDATVSSFFGTGSGPIWLDEVNCRGEESQVWRCPSWGWRQHNCNHQEDAGVICSGF          695

696   VRLAGGDGPCSGRVEVHSGEAWTPVSDGNFTLPTAQVICAELGCGKAVSVLGHMPFRESDGQVWAEEFRCDGGEPELWSCPRVPCPGGTCLHSGAAQVVCSVYTEV     801

802   QLMKNGTSQCEGQVEMKISGRWRALCASHWSLANANVVCRQLGCGVAISTPRGPHLVEGGDQISTAQFHCSGAESFLWSCPVTALGGPDCSHGNTASVICSGNH       905

938   LRLVDGGGPCGGRVEILDQGSWGTICDDDWDLDDARVVCRQLGCGEALNATGSAHFGAGSGPIWLDDLNCTGKESHVWRCPSRGWGRHDCRHKEDAGVICSEFLA     1042

1043  LRMVSEDQQCAGWLEVFYNGTWGSVCRSPMEDITVSVICRQLGCGDSGSLNTSVGLREGSRPRWVDLIQCRKMDTSLWQCPSGPWKYSSCSPKEEAYISCEGRRPK    1148

1162  LRLRGGDSECSGRVEVWENGSWGTVCDDSWSLAEAEVVCQQLGCGQALEAVRSAAFGPGNGSIWLDEVQCGGRESSLWDCVAEPWGQSDCKHEEDAGVRCSGVR      1265
```

All WC1 monoclonal antibodies reacted with cells in the same regions of the lymph node, spleen and thymus. The control monoclonal antibody CC15 appeared to react with the greatest number of cells and with the highest intensity of all the WC1 clustered monoclonal antibodies. This correlated with the FACScan analysis of both PBL and of lymphocytes isolated from tissues. In the thymus, all the different WC1 epitopes were expressed predominantly on medullary thymocytes with few reactive cells seen in the cortex. These results confirmed those previously reported by others (MacKay et al., 1986). It is suggested that WC1 is expressed only on mature thymocytes in the medulla, which unlike thymocytes in the cortex, also express MHC Class I (MacKay et al., 1986).

The distribution of WC1 positive cells in the lymph node was indicative of cells in transit and verified the preliminary studies of others (MacKay et al., 1986; Evans et al., 1994). It is known that afferent lymph dendritic cells in the ovine species take up and present antigen (Bujdoso et al., 1989, 1990). In the human and murine species, dendritic cells express all the molecules which are known to present foreign antigen to T cells (Steinman, 1991). In addition, the majority of $\gamma\delta$ T cells in the afferent lymph are activated (Evans et al., 1994). In contrast, $\gamma\delta$ T cells isolated from lymph nodes did not express CD25 (data not shown). It has previously been hypothesised that $\gamma\delta$ T cells may recognise antigen in association with dendritic cells (Bujdoso et al., 1989, 1990; Evans, 1993). The interaction between $\gamma\delta$ T cells and dendritic cells may take place in either the tissue or afferent lymph and subsequently activated $\gamma\delta$ T cells may localise in the lymph node, in sites of transit. $\gamma\delta$ T cells having localised at sites within the lymph node consequently lose their activated phenotype. Alternatively, the majority of $\gamma\delta$ T cells isolated from the lymph node may be derived from the peripheral blood.

This chapter has characterised a panel of monoclonal antibodies specific for WC1. These monoclonal antibodies were submitted to the 2nd International Workshop on Ruminant Leucocyte Differentiation Molecules. The results reported in Chapter 5 detail work done in collaboration with W.C.Davis, at WSU on a selected panel of WC1 monoclonal antibodies. These monoclonal antibodies identified epitopes of the WC1 protein which were expressed on mutually exclusive sub-populations of $\gamma\delta$ T cells in the bovine species. The expression of these WC1 epitopes and the existence of these mutually exclusive populations was investigated in the ovine species.

CHAPTER 4

WC1 epitope expression

INTRODUCTION.

The only known γδ T cell-specific cell surface marker so far described is found in ruminants (MacKay et al., 1989; Clevers et al., 1990). This was previously referred to as T19 but has recently been termed WC1 (Howard et al., 1991). Numerous monoclonal antibodies specific for WC1 protein(s) were analysed at the 1st International Workshop on Leucocyte Differentiation Molecules Workshop (Hein et al., 1991; Sopp et al., 1991). Subsequent to the publication of findings from the 1st Workshop, W.C.Davis of Washington State University (WSU) defined populations of lymphocytes in cattle which expressed different WC1 epitopes (Davis et al., 1990). All WSU WC1 monoclonal antibodies were assigned a number (N1, N2, N3,...) according to the epitopes of the WC1 complex recognised. Some epitopes were recognised by more than one monoclonal antibody. All the available WC1 monoclonal antibodies have not however been assigned N-specificities and a nomenclature has not yet been agreed upon which would refer to all the different epitopes recognised on the WC1 molecules. The following diagram is taken from Davis (1990) and describes the size of the populations of γδ T cells in bovine peripheral blood which expressed the WC1 epitopes N1, N2, N3 and N4. The size of the B cell and αβ T cells populations in the peripheral blood of the bovine species are included for reference.

CD5 — CD2 (CD4 | CD8) — N1/N2 (N3 | N4) — sIgM
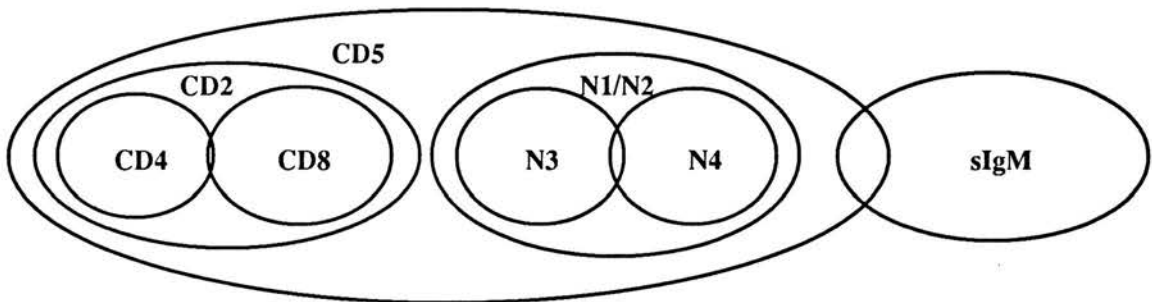
The WC1 positive population was defined as those PBL which reacted with the monoclonal antibodies to the bovine WC1 epitopes N1 and N2. This population could be further split into two mutually exclusive sub-populations with monoclonal antibodies which recognise the WC1 epitopes N3 and N4. The populations of lymphocytes in the bovine species which express the N3 and N4 epitopes are similar in size. A summation of the N3 and N4 sub-populations approximates to the size of the population which expresses the N1 and N2 epitopes. The results submitted to the 1st Workshop however, showed that the WC1 epitopes termed N1 and N2 are expressed on most, but not all, bovine and ovine WC1-positive lymphocytes (Sopp et al., 1991; Hein et al., 1991). The epitope recognised by the monoclonal antibody CC15 is believed to be expressed on all WC1-positive cells though no reference has been made to the reactivity of CC15 on these cells (Davis et al., 1990).

In this chapter, the expression of the WC1 epitopes N1, N2, N3 and N4 was examined on ovine PBL. A comparison was made with the published results for WC1 N-epitope expression by bovine PBL. The expression of the WC1 N-epitopes on γδ T cells from normal, healthy, 6 month old sheep was determined. The WC1 epitope-phenotype measures the number of Nx-positive

PBL which also other N-epitopes. The WC1 N-epitope expression by 6 month old sheep was compared to the expression of the different WC1 N-epitopes on γδ T cells isolated from animals of different age. In addition, the WC1 N-epitope expression on PBL from young healthy lambs was compared to the WC1 N-epitope expression on PBL isolated from young animals which were subject to different antigenic burdens.

The majority of γδ T cells isolated from the peripheral blood of animals are not activated (Evans, 1993; Evans et al., 1994). Accordingly, it is unknown if the expression of WC1 proteins, or the different WC1 N-epitopes, is altered on activated PBL. PBL isolated normal animals were stimulated *in vitro* with the T cell mitogen ConA and the expression of the different WC1 N-epitopes on γδ T cells N-epitope phenotype of γδ T cells determined at various time points following activation.

RESULTS:

4.1 Epitopes of the WC1 proteins.

Table 4.1 lists the monoclonal antibodies made available to the 1st and 2nd International Workshops on Ruminant Leucocyte Differentiation Molecules which have been assigned N-specificities in the bovine species. The cross-reactivity with ovine lymphocytes, isotype and epitope specificity for each monoclonal antibody is also listed. Those monoclonal antibodies cross-reactive with ovine PBL (Hein et al., 1991; Lund et al., 1993; Chapter 3) were analysed in this chapter.

4.1.1 N1 and N2 epitopes are both expressed on similar populations of ovine PBL.

The epitopes N1 and N2 are each only recognised by one monoclonal antibody. These epitopes are expressed on identical populations of bovine PBL (Davis et al., 1990). Two colour immunofluorescence analysis of the N1 and N2 epitope expression on ovine peripheral blood lymphocytes is shown in figure 4.1. 85% of lymphocytes expressing the N1 epitope also expressed the N2 epitope. Similarly, 85% of lymphocytes expressing the N2 epitope also expressed the N1 epitope. There was a small number of cells, 15%, which were positive for only one of the epitopes. This is in contrast to the bovine species where it was reported that all WC1 positive cells expressed both epitopes.

4.1.2 N3 and N4 epitopes are expressed on distinct sub-populations of ovine PBL.

In the bovine species, the WC1 epitopes N3 and N4 are expressed on mutually exclusive populations of lymphocytes. In addition, in the bovine species, N3-positive cells express the N1 and N2 epitopes and similarly N4-positive cells express the N1 and N2 epitopes. Figure 4.2 shows the reactivity of the anti-N3 and anti-N4 monoclonal antibodies with ovine lymphocytes expressing the N1 and N2 epitopes of WC1. The results show that the N3 and N4 epitopes are expressed on sub-populations of N1/N2 positive cells in the ovine species. 30% of the N1/N2 positive population also express the N3 epitope and 60% of the N1/N2 population express the N4 epitope. In the ovine species, the N4 positive population of cells is 2-3 times larger than the N3 population. This is in contrast to the results observed in the bovine species were the N3 and N4 positive populations are equivalent in size. In the ovine species, like the bovine species, a summation of the N3 and N4 populations approximates to the size of that defined by expression of the N1/N2 epitopes. Figure 4.2 also shows that 20% of ovine lymphocytes which expressed the N4 epitope did not express the N2 epitope. It has previously been shown in the bovine species that all lymphocytes which express the N4 epitope co-express the N2 epitope. These results show significant differences between the two ruminant species in their WC1 epitope expression on $\gamma\delta$ T cells.

The N3 and N4 epitopes are expressed on mutually exclusive populations of lymphocytes in the bovine species. Figure 4.3 shows the two colour immunofluorescence staining of N3 versus N4 on sheep lymphocytes. There is little dual expression of the N3 and N4 epitopes. Only 14% of lymphocytes which expressed the N4 epitope also expressed the N3 epitope, and only 26% of those lymphocytes which expressed the N3 epitope also expressed the N4 epitope. This result is similar to that observed in the bovine species.

Table 4.1: N-epitope specificities of WC1 clustered monoclonal antibodies.

The WC1 specific monoclonal antibodies which have been given N epitope specificities are listed along with the isotype, N-epitope specificity. The cross-reactivity of the anti-bovine WC1 monoclonal antibodies with ovine PBL as determined in this thesis and by others (Hein et al., 1991) is listed.

TABLE 4.1: N-epitope specificities of WC1 clustered monoclonal antibodies.

Antibody	Epitope Specificty	Cross-reactive with ovine PBL	Isotype
B7A1	WC1 - N1	Yes	IgM
BAQ4A	WC1 - N2	Yes	IgG$_1$
CACTB1A	WC1 - N3	Yes	IgG$_1$
CACTB15B	WC1 - N3	Yes	IgG$_1$
CACTB32A	WC1 - N3	Yes	IgG$_1$
CACTB35A	WC1 - N3	No	IgM
BAQ53A	WC1 - N3	No	IgM
BAQ72A	WC1 - N3	No	IgM
BAQ89A	WC1 - N4	Yes	IgG$_1$
BAQ159A	WC1 - N4	Yes	IgG$_1$
CACTB7A	WC1 - N4	Ycs	IgG$_1$
BAG25A	WC1 - N4	Yes	IgM

WSU - Washington State University, Pullman.

Figure 4.1: Two-colour immunofluorescence of anti-N1 (B7A1) versus anti-N2 (BAQ4A).

10^6 PBL isolated from 6 month old lambs (n=4) were stained with BAQ4A (anti-N2) and B7A1 (anti-N1) followed by anti-IgG1-FITC, and biotinylated anti-IgM and SA-PE. Cells were analysed by FACScan for two-colour immunofluorescence staining. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS versus b-NMS. This experiment was repeated four times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 4.1: Two-colour immunofluorescence of anti-N1 (B7A1) versus anti-N2 (BAQ4A).
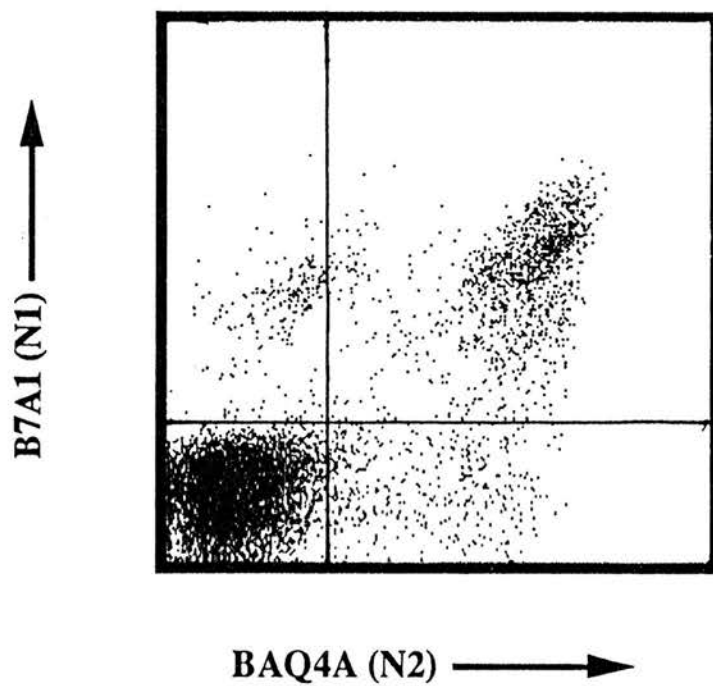
B7A1 (N1) ——

BAQ4A (N2) ——→

Figure 4.2: Two-colour immunofluorescence analysis of monoclonal antibodies to N-epitopes.

10^6 PBL isolated from 6 month old lambs (n=4) were stained with biotinylated BAQ4A (anti-N2) or B7A1 (anti-N1) followed by SA-PE, or biotinylated anti-IgM and SA-PE, and either CACTB1A (anti-N3) or BAQ89A (anti-N4) followed by anti-IgG1-FITC. Cells were analysed by FACScan for two-colour immunofluorescence staining. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS versus and b-NMS. This experiment was repeated four times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 4.2: Two-colour immunofluorescence analysis of monoclonal antibodies to N-epitopes.
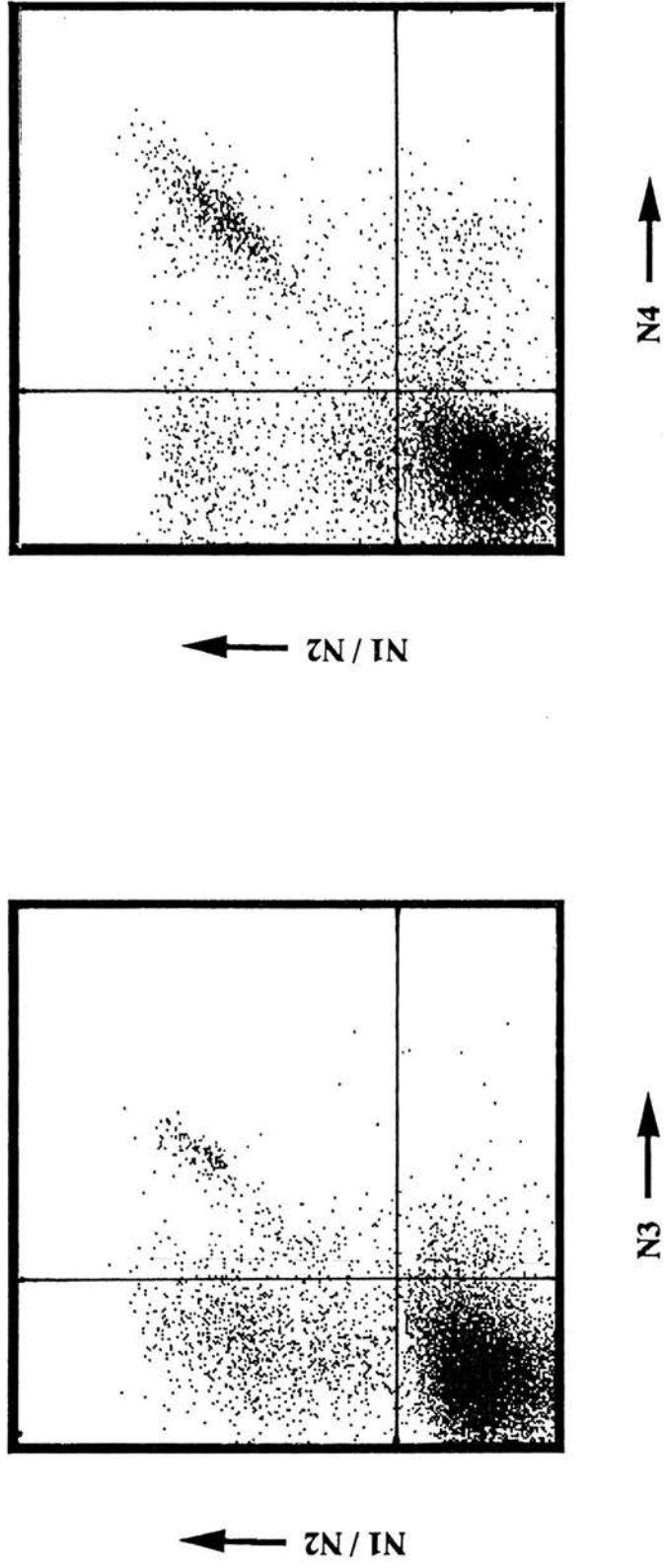
Figure 4.3: Two-colour immunofluorescence analysis of anti-N3 (CACTB1A) versus anti-N4 (BAG25A).

10^6 PBL isolated from 6 month old lambs (n=4) were stained with CACTB1A (anti-N3) and BAG25A (anti-N4) followed by anti-IgG1-FITC, and biotinylated anti-IgM and SA-PE. Cells were analysed by FACScan for two-colour immunofluorescence staining. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS versus and b-NMS. This experiment was repeated four times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 4.3: Two-colour immunofluorescence of anti-N3 (CACTB1A) versus anti-N4 (BAG25A).
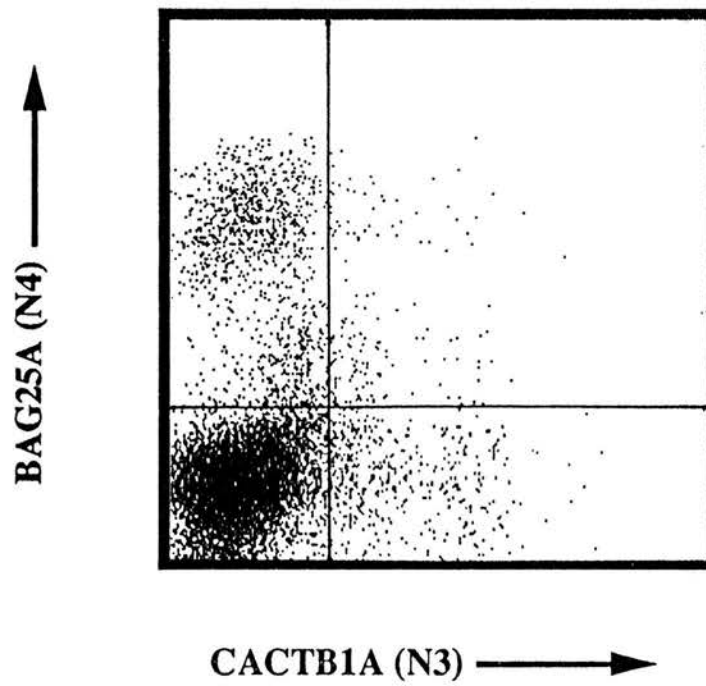
Figure 4.4: Two-colour immunofluorescence analysis of anti-N3 monoclonal antibodies.

10^6 PBL isolated from 6 month old lambs (n=4) were stained with either BAQ4A (anti-N2) or CACTB15B (anti-N3) or CACTB32A (anti-N3) then anti-IgG1-FITC. Cells were then incubated with NMS followed by biotinylated CACTB1A (anti-N3) then SA-PE. Cells were analysed by FACScan for two-colour immunofluorescence staining. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS versus and b-NMS. This experiment was repeated four times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 4.4: Two-colour immunofluorescence analysis of anti-N3 monoclonal antibodies.
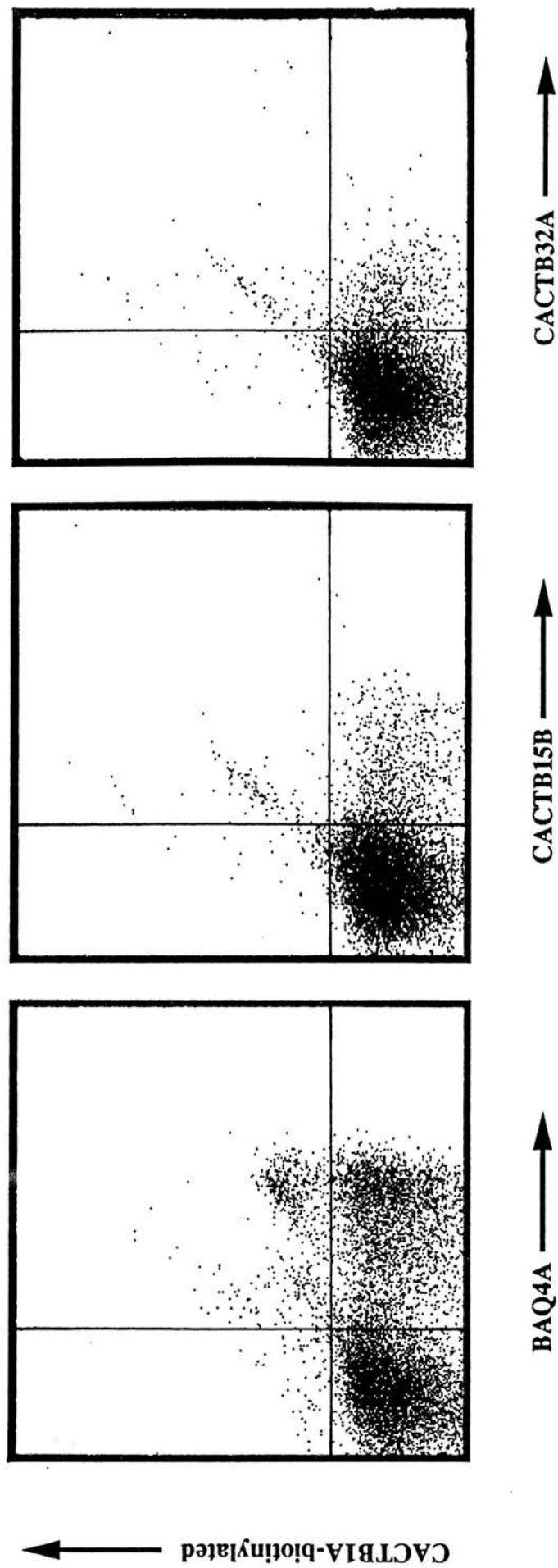
CACTB1A-biotinylated

BAQ4A

CACTB15B

CACTB32A

Figure 4.5: Two-colour immunofluorescence analysis of anti-N4 monoclonal antibodies.

10^6 PBL isolated from 6 month old lambs (n=4) were stained with either BAQ89A (anti-N4), BAQ159A (anti-N4) or CACTB7A (anti-N4) then anti-IgG1-FITC, and BAG25A then biotinylated anti-IgM followed by SA-PE. Cells were analysed by FACScan for two-colour immunofluorescence staining. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS versus and b-NMS. This experiment was repeated four times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 4.5: Two-colour immunofluorescence analysis of anti-N4 monoclonal antibodies
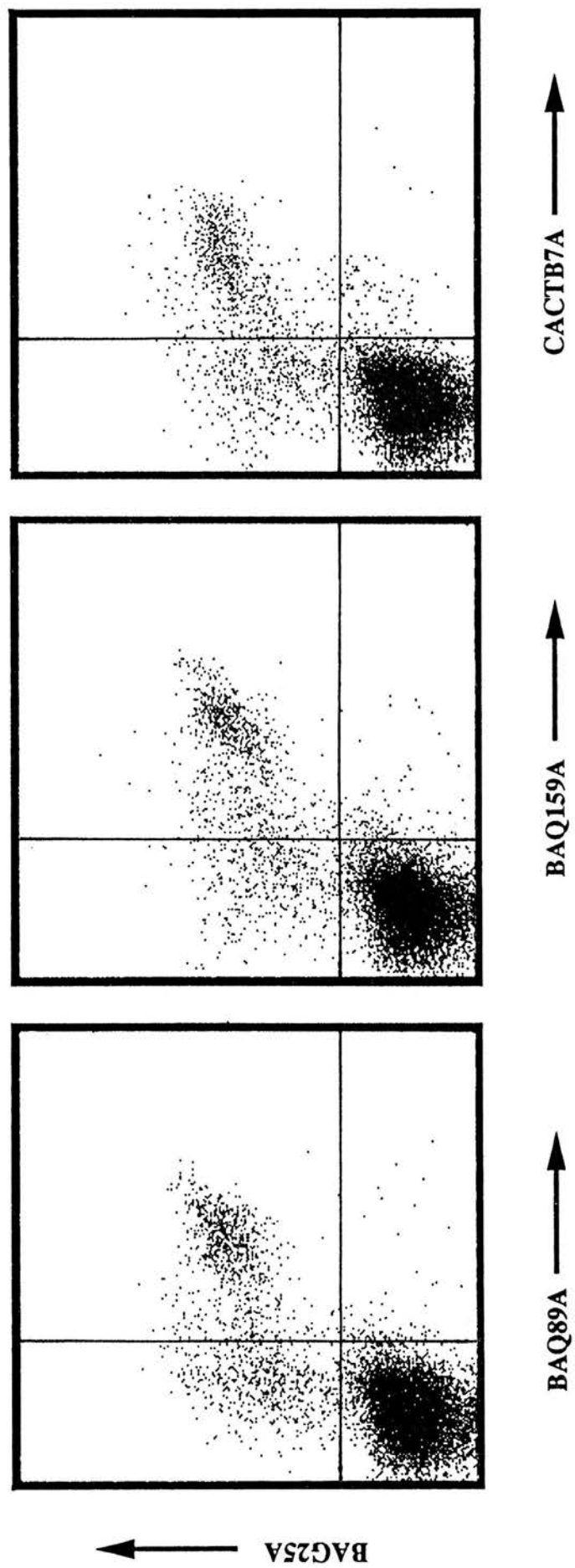
4.2 Changes in the expression of the N-epitopes of WC1.

The results in section 4.1 have shown the expression of different epitopes of the WC1 protein on PBL. The monoclonal antibody CC15 reacted with an epitope found on all WC1 proteins. The epitopes N1 and N2 are expressed on the majority but not all WC1 proteins, and the epitopes N3 and N4 are expressed on WC1 proteins on mutually exclusive populations of cells. These results were obtained using resting PBL from sheep of the same age and status. In this section, the expression of WC1, including the expression of the different N-epitopes, was measured on (i) PBL isolated from animals of different age; (ii) PBL isolated from animals subject to different antigenic burdens; and (iii) ConA stimulated PBL. These different *in vivo* and *in vitro* conditions were used to help determine if there were any distinct functional requirements for different WC1 molecules, as determined by the expression of differetn N-epitopes. Three different variables were measured: a) the size of each N-epitope positive population; b) the level of expression of each of the N-epitopes on the WC1-positive population of PBL as a whole; and c) the WC1 epitope phenotype of PBL.

4.2.1 Changes in the size of the WC1-positive lymphocyte populations expressing the WC1 epitopes N1, N2, N3 or N4.

The size of the PBL populations which expressed the WC1 epitopes N1, N2, N3 or N4 was determined using cells from animals of different ages; using cells from animals with different antigenic burdens; and using cells after culture with ConA. The monoclonal antibody CC15 which recognises all WC1 molecules was included as a control in all assays.

Table 4.2 shows the size of the lymphocyte populations which expressed the WC1 epitopes N1, N2, N3 or N4 at various time points during the first two years of a sheeps life. The percentage of PBL which expressed either N1, N2, N3 or N4 decreased in the peripheral blood of animals of increasing age. This decrease in population percentage with age was significant for all the monoclonal antibodies tested. For example, the N1 positive population decreased from 27% of the lymphocyte population in 0-3 day old lambs to 4% of the lymphocyte population in year old sheep. This low level remained constant in older animals (data not shown).

The size of the different WC1 populations was also shown to vary in animals with different levels of antigenic burden (table 4.3). PBL were isolated from normal animals, animals kept in specific pathogen free (SPF) conditions and animals kept in gnotobiotic conditions and analysed. All samples were taken from lambs which were 4 weeks of age. Gnotobiotic lambs, which have the lowest antigenic burden, had a significantly higher number of lymphocytes expressing the WC1 epitopes CC15, N1, N2 and N4 when compared to normal and SPF lambs. Though there were a greater number of PBL from gnotobiotic lambs expressing the N3 epitope of WC1, this was not significantly different to the number of PBL expressing the N3 epitope of WC1 in normal and SPF lambs. There were no significant differences in the number of lymphocytes expressing any of the WC1 epitopes between lambs which were kept in SPF conditions and lambs which were kept in normal conditions.

The expression of the WC1 epitopes N1, N2, N3 and N4 on PBL was analysed following T cell activation with ConA. The results are shown in table 4.4. All of the populations of lymphocytes which expressed the N1, N2, N3 and N4 epitopes increased in size following culture with ConA. There was an increase in the size of all WC1 positive populations after 24hours of culture of PBL

58

Table 4.2:   Changes in size of N-epitope positive populations of PBL with age.

PBL were isolated from sheep of various ages (n=6) and 10^6 cells from each animal were stained with the monoclonal antibodies CC15, anti-N1, anti-N2, anti-N3 and anti-N4 then anti-IgG FITC and analysed by FACScan. The percentages of PBL which were positive for each of the monoclonal antibodies tested is listed. Staining with the negative control (NMS) was always <3%. The results shown are the mean values of positive cells from 6 different animals in each age group. The errors, in the brackets, are the standard deviation of the means. The significance of the change in number of PBL expressing the various markers with age was determined by regression analysis of the data. A General Linear Model was used to analyse the data with age as a covariate. The probability that the differences in the data are significant is represented at the top of each column.

TABLE 4.2: Changes in size of N-epitope positive populations of PBL with age.

Percentage of lymphocytes expressing different WC1 epitopes.

AGE	*** CC15	*** N1	** N2	** N3	** N4
0-3 Days	30.5 (4.2)	26.7 (4.6)	29.2 (8.2)	13.9 (8.0)	22.2 (7.6)
4 Weeks	23.2 (6.1)	18.1 (5.7)	18.7 (5.7)	7.6 (3.2)	12.1 (1.3)
3 Months	17.9 (6.0)	11.5 (4.4)	14.0 (4.3)	3.8 (1.8)	8.4 (3.4)
6 Months	16.4 (4.3)	9.4 (2.2)	9.5 (2.4)	3.9 (1.4)	8.1 (4.3)
9 Months	12.5 (2.0)	7.9 (2.0)	10.1 (1.1)	2.3 (2.4)	6.3 (1.1)
1 Year	8.4 (3.8)	3.3 (1.2)	4.5 (2.3)	1.0 (1.0)	3.4 (1.9)

* Significant decrease in population size with age.
** $p < 0.01$ *** $p < 0.001$

Table 4.3: Changes in size of N-epitope positive populations of PBL with antigenic burden.

PBL were isolated from lambs housed in normal conditions (n=6), gnotobiotic conditions (n=6) and specific pathogen free conditions (n=6). 10^6 cells from each animal were stained with the monoclonal antibodies CC15, anti-N1, anti-N2, anti-N3 and anti-N4 then anti-IgG FITC and analysed by FACScan. The percentages of PBL which were positive for each of the monoclonal antibodies tested is listed. Staining with the negative control (NMS) was always <3%. The results shown are the mean values of positive cells from 6 different animals in each group. The errors, in the brackets, are the standard deviation of the means. The significant differences between animals housed in different conditions and the number of PBL expressing the WC1 epitopes was determined using two sample t-tests. The probability that the data from normal and SPF lambs are significantly different to the data from gnotobiotic lambs is represented in the table.

TABLE 4.3: Changes in size of N-epitope positive populations of PBL with antigenic burden.

Percentage of lymphocytes expressing different WC1 epitopes.

Antigenic Burden

	CC15	N1	N2	N3	N4
Normal	[23.2 (6.8)] *	[18.1 (5.7)] *	[18.7 (5.7)] *	7.6 (3.2)	[14.0 (6.9)] **
Specific Pathogen Free	[28.1 (8.3)] *	[20.6 (6.7)] *	[20.6 (6.7)] *	3.5 (2.0)	[15.6 (5.9)] **
Gnotobiotic	60.3 (14.1)	42.0 (11.9)	43.3 (8.8)	13.4 (6.4)	30.7 (4.2)

[ ] Significantly different to gnotobiotic lambs.

*p<0.05   **p<0.01

Table 4.4: Changes in size of N-epitope positive populations of PBL following culture with ConA.

PBL were isolated from 6 month old sheep (n=4) and stimulated *in vitro* with 5μg/ml ConA for various lengths of time. Viable cells were harvested over Lymphoprep at the time points indicated. 10^6 viable cells were stained with the monoclonal antibodies CC15, anti-N1, anti-N2, anti-N3 and anti-N4 followed by anti-IgG FITC and analysed by FACScan. The percentages of positive cells is listed. Staining with the negative control (NMS) was always <3%. The results shown are the mean of 4 animals and the errors in the brackets are the standard deviation of the means. This experiment was repeated four times and the same trends were seen with each animal and on each occasion.

TABLE 4.4: Changes in size of N-epitope positive populations of PBL following culture with ConA.

Percentage of lymphocytes expressing the different WC1 epitopes.

Days after stimulation	CC15	N1	N2	N3	N4
0	19.4 (10.7)	12.9 (8.4)	13.4 (8.4)	8.2 (6.0)	10.8 (6.2)
1	38.8 (11.2)	24.7 (11.1)	24.3 (11.2)	11.7 (6.0)	20.2 (8.9)
2	37.7 (13.8)	20.6 (10.1)	22.6 (11.8)	11.1 (5.4)	18.6 (9.7)
3	34.2 (13.4)	20.8 (9.6)	27.5 (8.9)	10.6 (4.2)	18.2 (9.2)
4	37.9 (16.0)	29.9 (14.9)	29.7 (14.6)	17.9 (10.0)	20.4 (10.8)
5	39.7 (16.8)	31.6 (15.8)	32.5 (15.6)	21.4 (13.4)	20.9 (10.6)
6	44.2 (12.3)	N.D.	N.D.	N.D.	N.D.
7	40.6 (17.5)	31.9 (18.4)	34.6 (16.6)	24.9 (15.8)	20.9 (12.6)

with ConA. The data presented in table 4.4 show no significant changes in cell population size. This is due to the large variation in the size of the different WC1-positive T cell populations within resting PBL. Regression analysis of data from the individual animals showed the changes in size of the different WC1 populations were significant.

The percentage of the CC15-positive population which expressed the epitopes N1, N2, N3, and N4 was determined in all of the conditions assayed above. There was no significant change in the level of expression of the WC1 epitopes N1, N2, N3 and N4 on CC15-positive PBL from animals of different ages (data not shown); from animals with different antigenic burdens (data not shown); and following stimulation with ConA (data not shown). The WC1 epitopes N1(80-89%) and N2 (75-86%), like the $\gamma\delta$ TCR (93-98%), were expressed on the majority of CC15 positive cells. The WC1 epitope N3 was expressed on 25-30% of CC15 positive cells and the WC1 epitope N4 was expressed on 45-70% of CC15 positive cells.

4.2.2 Changes in the WC1 epitope phenotype of PBL.

The WC1 epitope phenotype was determined using PBL from animals of different ages. The results showed that there was no significant change either: i) in the number of N1-positive PBL which expressed the epitopes N2, N3 or N4 (data not shown); ii) the number of N2-positive PBL which expressed the epitopes N1, N3 or N4 (data not shown); iii) the number of N3-positive PBL which expressed the epitopes N1, N2 or N4 (data not shown) or; iv) the number of N4-positive PBL which expressed the epitopes N1, N2 or N3 (data not shown). These results were the same for PBL isolated from animals of all ages analysed and were the same as the results reported in section 4.1 for animals of 6 months of age.

Table 4.5 shows the expression of the WC1 epitope phenotype of PBL isolated from sheep with different antigenic burdens. The only differences observed were in the expression of N3 epitope. 20-23% of N4 positive lymphocytes isolated from lambs kept in either normal conditions or SPF conditions, expressed the N3 epitope). However, with PBL isolated from lambs kept under gnotobiotic conditions, significantly fewer, 8.5%, of N4 positive lymphocytes expressed the N3 epitope of WC1. In addition, significantly fewer N1-positive PBL expressed the N3 epitope in gnotobiotic lambs when compared to normal and SPF lambs.

The WC1 epitope phenotype of PBL following stimulation with ConA was determined. The WC1 phenotype was analysed daily on day 0 through to day 7 and again on day 10 following start of culture with ConA. The whole data set was statistically analysed for changes over time using a General Linear Model of regression analysis. Data obtained on days 0, 3, 5, and in some cases day 10 are shown in table 4.6. The only significant change in WC1 epitope expression over time was the expression of N3 and N4 on mutually exclusive populations of $\gamma\delta$ T cells. There was a significant decrease in the dual expression of N3 and N4 on populations of PBL following activation by culture with ConA. 18% of resting, N4-positive PBL expressed the N3 epitope whereas following culture with ConA this decreased to only 8% after 3days. In addition, 29% of resting, N3-positive PBL expressed the N4 epitope whereas only 16% expressed N4 after 3days of culture with ConA.

4.3 An increase in the size of all WC1 populations following *in vitro* activation.

It was reported that the WC1 epitope recognised by the monoclonal antibody 19-19, is lost from

Table 4.5: **Changes in N-epitope phenotype of PBL with antigenic burden.**

PBL were isolated from lambs housed in normal conditions (n=6), gnotobiotic conditions (n=6) and specific pathogen free conditions (n=6). 10^6 cells from each animal were stained. Four different sets of two-colour staining were carried: a) B7A1 (anti-N1) followed by biotinylated anti-IgM then SA-PE and either BAQ4A (anti-N2) or CACTB1A (anti-N3) followed by anti-IgG FITC. b) Biotinylated BAQ4A (anti-N2) then SA-PE and either B7A1 (anti-N1) followed by biotinylated anti-IgM then SA-PE or BAQ4A (anti-N2) or CACTB1A (anti-N3) followed by anti-IgG FITC. c) Biotinylated CACTB1A (anti-N3) followed by SA-PE and, either B7A1 (anti-N1) or BAG25A (anti-N4) followed by biotinylated anti-IgM then SA-PE, or BAQ4A (anti-N2) followed by anti-IgG FITC. d) BAG25A (anti-N4) followed by biotinylated anti-IgM then SA-PE and either BAQ4A (anti-N2) or CACTB1A (anti-N3) followed by anti-IgG FITC. Cells were analysed by FACScan. The positive staining of cells was calculated compared to the non-specific staining of NMS versus and b-NMS. The results shown are the mean values of positive cells from 6 different animals in each group. The errors, in the brackets, are the standard deviation of the means. The significant differences between animals housed in different conditions and the number of PBL expressing the WC1 epitopes was determined using two sample t-tests. The probability that the data from normal and SPF lambs are significantly different to the data from gnotobiotic lambs is represented in the table.

TABLE 4.5: Changes in N-epitopes phenotype of PBL with antigenic burden.

Antigenic burden:

	Percentage of N1+ cells which expressed		
	N2	N3	N4
Normal	83.1 (14.4)	[34.9 (2.1)] *	N.D.
Specific Pathogen Free	81.4 (2.9)	[32.9 (3.3)] *	N.D.
Gnotobiotic	85.9 (9.6)	14.8 (4.7)	N.D.

	Percentage of N2+ cells which expressed		
	N1	N3	N4
Normal	89.4 (9.7)	28.5 (3.2)	66.9 (12.0)
Specific Pathogen Free	90.0 (4.3)	36.7 (14.4)	59.5 (13.1)
Gnotobiotic	89.7 (3.1)	N.D.	68.9 (11.5)

	Percentage of N3+ cells which expressed		
	N1	N2	N4
Normal	93.7 (1.4)	94.5 (7.3)	30.8 (8.4)
Specific Pathogen Free	98.8 (5.4)	91.7 (8.3)	43.7 (19.5)
Gnotobiotic	94.9 (7.3)	N.D.	39.5 (7.4)

	Percentage of N4+ cells which expressed		
	N1	N2	N3
Normal	N.D.	73.6 (20.4)	[20.1 (1.8)] *
Specific Pathogen Free	N.D.	68.1 (13.0)	[23.0 (2.7)] *
Gnotobiotic	N.D.	83.1 (10.7)	8.5 (1.3)

[ ] Significantly different to Gnotobiotic lambs.   * p<0.05

Table 4.6: Changes in N-epitope phenotype of PBL after culture with ConA.

PBL were isolated from 6 month old sheep (n=4) and stimulated *in vitro* with 5µg/ml ConA for various lengths of time. Viable cells were harvested over Lymphoprep at the time points indicated and 10^6 harvested cells were stained. Four different sets of two-colour staining were carried: a) B7A1 (anti-N1) followed by biotinylated anti-IgM then SA-PE and either BAQ4A (anti-N2) or CACTB1A (anti-N3) followed by anti-IgG FITC. b) Biotinylated BAQ4A (anti-N2) then SA-PE and either B7A1 (anti-N1) followed by biotinylated anti-IgM then SA-PE or BAQ4A (anti-N2) or CACTB1A (anti-N3) followed by anti-IgG FITC. c) Biotinylated CACTB1A (anti-N3) followed by SA-PE and, either B7A1 (anti-N1) or BAG25A (anti-N4) followed by biotinylated anti-IgM then SA-PE, or BAQ4A (anti-N2) followed by anti-IgG FITC. d) BAG25A (anti-N4) followed by biotinylated anti-IgM then SA-PE and either BAQ4A (anti-N2) or CACTB1A (anti-N3) followed by anti-IgG FITC. Cells were analysed by FACScan. The positive staining of cells was calculated compared to the non-specific staining of NMS versus and b-NMS. The results shown are the mean of 4 animals and the errors in the brackets are the standard deviation of the means. This experiment was repeated 3 times and the same trends were seen with each animal and on each occasion. The significance of the change in number of PBL expressing the various markers after stimulation was determined by regression analysis of the data. A General Linear Model was used to analyse the data with time as a covariate. The probability that the differences in the data are significant is represented at the top of each column.

TABLE 4.6: Changes in N-epitope phenotype of PBL after culture with ConA.

Days after stimulation	Percentage N1+ cells which expressed			Percentage N2+ cells which expressed		
	$N2^N$	$N3^N$	N4	$N1^N$	$N3^N$	$N4^N$
0	91.0 (7.4)	31.2 (13.9)	N.D.	84.7 3.3	27.6 (5.3)	60.6 (6.6)
3	93.1 (0.2)	22.1 (7.6)	N.D.	88.5 (5.0)	32.4 (4.4)	62.1 (8.1)
5	95.9 (6.7)	25.3 (4.1)	N.D.	82.7 (4.3)	24.5 (5.4)	56.6 (5.7)
10	N.D.	20.2 (5.6)	N.D.	N.D.	N.D.	N.D.

Days after stimulation	Percentage N3+ cells which expressed			Percentage N4+ cells which expressed		
	$N1^N$	$N2^N$	N4*	N1	$N2^N$	N3*
0	93.8 (8.1)	98.7 (5.6)	29.0 (6.9)	N.D.	85.5 (1.7)	18.4 (4.7)
3	90.0 (3.0)	99.0 (0.3)	22.6 (4.1)	N.D.	91.9 (3.1)	7.5 (0.5)
5	93.2 (4.5)	94.5 (9.1)	16.0 (2.3)	N.D.	92.4 (9.1)	9.9 (1.6)
10	99.4 (1.0)	N.D.	16.4 (4.2)	N.D.	N.D.	4.6 (1.7)

Significance of change over time.   * $p<0.005$ N-no significant difference

γδ T cells following their activation (Hein and Mackay, 1991). However, it was shown in results presented here (section 4.2.2) that when stimulated with ConA for various lengths of time, each of the WC1 populations increased in size (table 4.4). In addition, it was shown that there were no significant changes in the expression of the epitopes N1, N2, N3 and N4 on CC15-positive cells during this time (data not shown). In contrast, the intensity of expression of each of the WC1 epitopes on PBL showed marked changes upon activation. Staining profiles of PBL with the monoclonal antibodies CC15 and BAQ4A (N2) at the start of the experiment, and subsequently on day 3 and day 7 are shown in figure 4.6. The changes in these FACS profiles are representative of all the anti-WC1 monoclonal antibodies used in this experiment. The intensity of staining of PBL by each monoclonal antibody decreased until day 3, and then increased throughout the remainder of the assay. However, the intensity of staining of each monoclonal antibody failed to return to starting levels even by day 7 of culture.

Figure 4.6: Reactivity of WC1 monoclonal antibodies with PBL previously cultured with ConA.

PBL were isolated from 6 month old sheep (n=4) then cultured *in vitro* with 5μg/ml ConA for various lengths of time. Viable cells were harvested over Lymphoprep at the start of culture, and on day 3 and day 7 after culture. 10^6 viable cells were stained with the monoclonal antibodies CC15 or anti-N2 followed by anti-IgG FITC and analysed by FACScan. Single colour histograms of positively stained PBL from one animal are shown. Staining with the negative control (NMS) was always <3%. The experiment was repeated four times and the same trends were with PBL from each animal and on each occasion tested.

FIGURE 4.6: Reactivity of WC1 monoclonal antibodies with PBL previously cultured with ConA.

Start of stimulation.

3 days of stimulation.

7 days of stimulation.

CC15 staining

N2 staining

Relative cell number

Fluorescence intensity
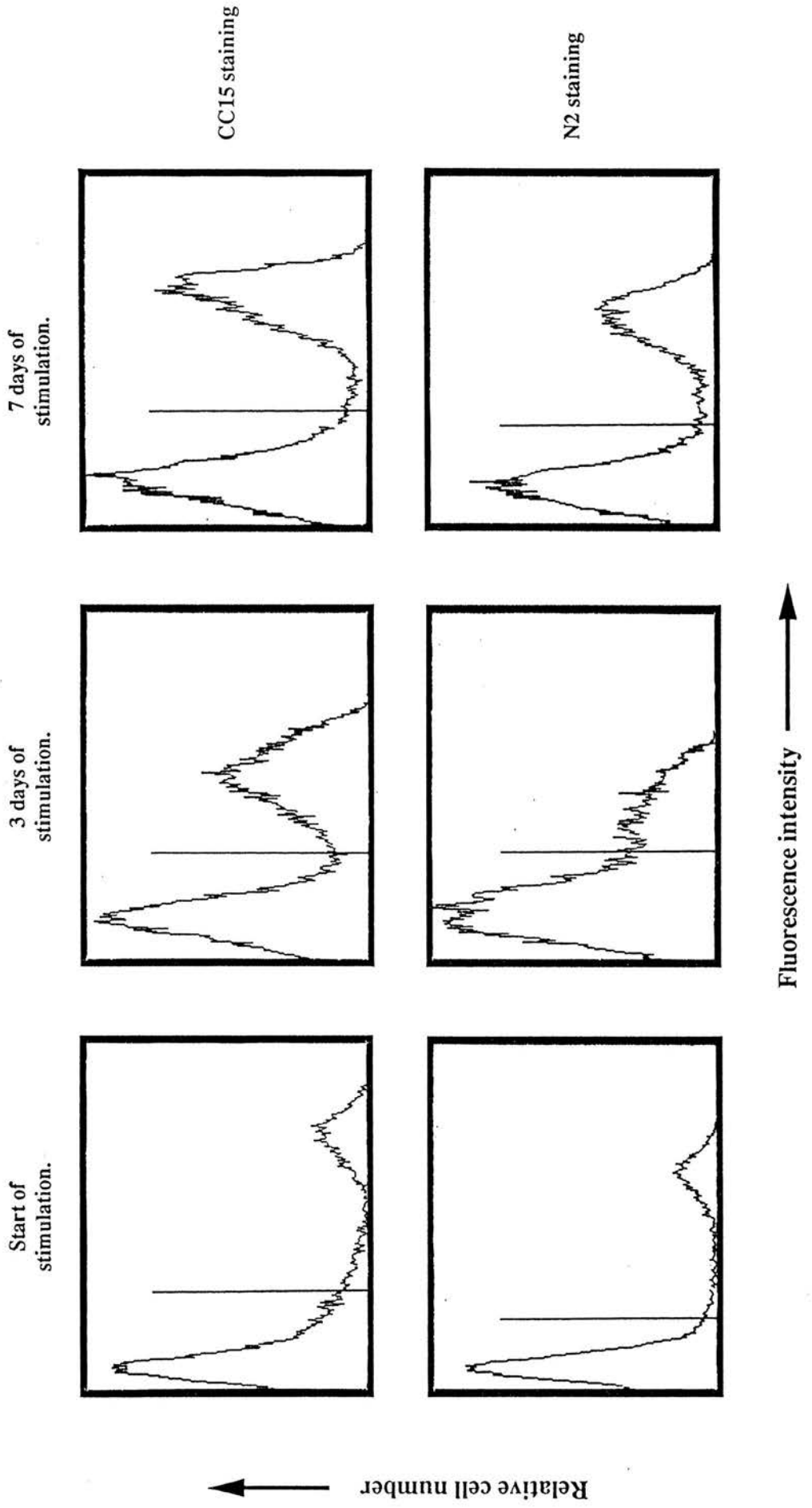
DISCUSSION:

In this chapter, the expression of the WC1 epitopes N1, N2, N3 and N4 was analysed on ovine PBL. It has previously been shown that the epitopes N1 and N2 are expressed on the majority of WC1 positive cells in both the ovine and bovine species (Sopp et al., 1991, Hein et al., 1991). In the bovine species, the epitopes N1 and N2 are expressed on the same population of cells (Davis et al., 1991). The results in this chapter show that a similar situation exists in the ovine species with >90% of reactive cells expressing both the N1 and N2 epitopes. There were a small number of cells which expressed either the N1 or N2 epitope, but not both. In studies with the anti-bovine N3- and anti-bovine N4-grouped monoclonal antibodies, the results showed marked differences in the expression of the N3 and N4 epitopes in the ovine compared to the bovine species. In the bovine species, the epitopes N3 and N4 are expressed on mutually exclusive populations of PBL of similar size although both populations express the two epitopes, N1 and N2. In contrast, in the ovine species the size of the population of cells which expressed the N3 epitope was much smaller than that which expressed the N4 epitope. In addition, in the ovine species, all the anti-N3 monoclonal antibodies recognised identical or closely overlapping epitopes on the WC1 protein. In contrast, all the anti-N4 monoclonal antibodies did not recognise identical or closely overlapping epitopes on the WC1 protein. The anti-N4 monoclonal antibody BAG25A recognised an epitope on ovine PBL which did not overlap with any of the other anti-N4 monoclonal antibodies. In addition, some cells which expressed the epitope recognised by the monoclonal antibody BAG25A did not express the N2 epitope. The epitope recognised by BAG25A was termed N4A and was not analysed any further. The anti-N4 monoclonal antibody BAQ89A was used to stain N4-positive lymphocytes in the ovine species.

The results shown in this chapter suggest there is a restricted expression of the different WC1 epitopes on ovine PBL. The epitopes N1 and N2 were expressed on 90% of WC1 positive ovine PBL. Monoclonal antibodies to the epitopes N1 and N2 immunoprecipitated three proteins of molecular weights 144kDa, 205kDa and 300kDa. In contrast, the epitopes N3 and N4 were expressed on a small number of WC1 positive ovine PBL yet, like the epitopes N1 and N2, the epitopes N3 and N4 were expressed on all three proteins. Figure 4.7 is a summation of the results from chapter 3 and chapter 4 and previous observations (MacKay et al., 1986, 1988, 1989; Evans, 1994; Evans et al., 1994). The diagram shows the representative size of the populations of PBL which expressed the different WC1 epitopes. Each circle represents a population of ovine lymphocytes as defined by the expression of one of the listed epitopes or T cell markers.

Some of the questions regarding the WC1 protein(s) and its epitopes were partially resolved by the recent cloning of three cDNA transcripts of different bovine WC1 mRNAs (Wijngaard et al., 1992, 1994). These WC1 cDNAs were found to be 80% homologous at the nucleotide level. Two of the genes (WC1.1 and WC1.2) were transfected, separately, into mouse L cells and stable transfectants generated. These cells were shown to express the WC1 cell surface protein(s) and were used for FACS analysis of all monoclonal antibodies grouped into the WC1 cluster (MacHugh et al., 1993). The results showed that some monoclonal antibodies recognised both transfectants, whilst other monoclonal antibodies recognised one or the other transfectant. Some monoclonal antibodies reacted with neither of the transfectants. The exact number of WC1 genes expressed in the bovine species is not known, although some reports suggest numerous exist (H. Clevers personal communication; Wijngaard et al., 1992, 1994). In addition, these studies revealed that the monoclonal antibody CC15 recognised epitopes expressed by both of the bovine WC1 cDNA products. The monoclonal antibodies BAG25A and BAQ89A recognised epitopes expressed on

Figure 4.7: **Bubble diagram of WC1 epitope expression on ovine PBL.**

Single colour and two-colour immunofluorescence results were analysed. Each circle represents one population of positively stained PBL. The size of the circle is a representation of the size of each of the populations within ovine PBL.

FIGURE 4.7: Bubble diagram of WC1 epitope expression of ovine PBL.
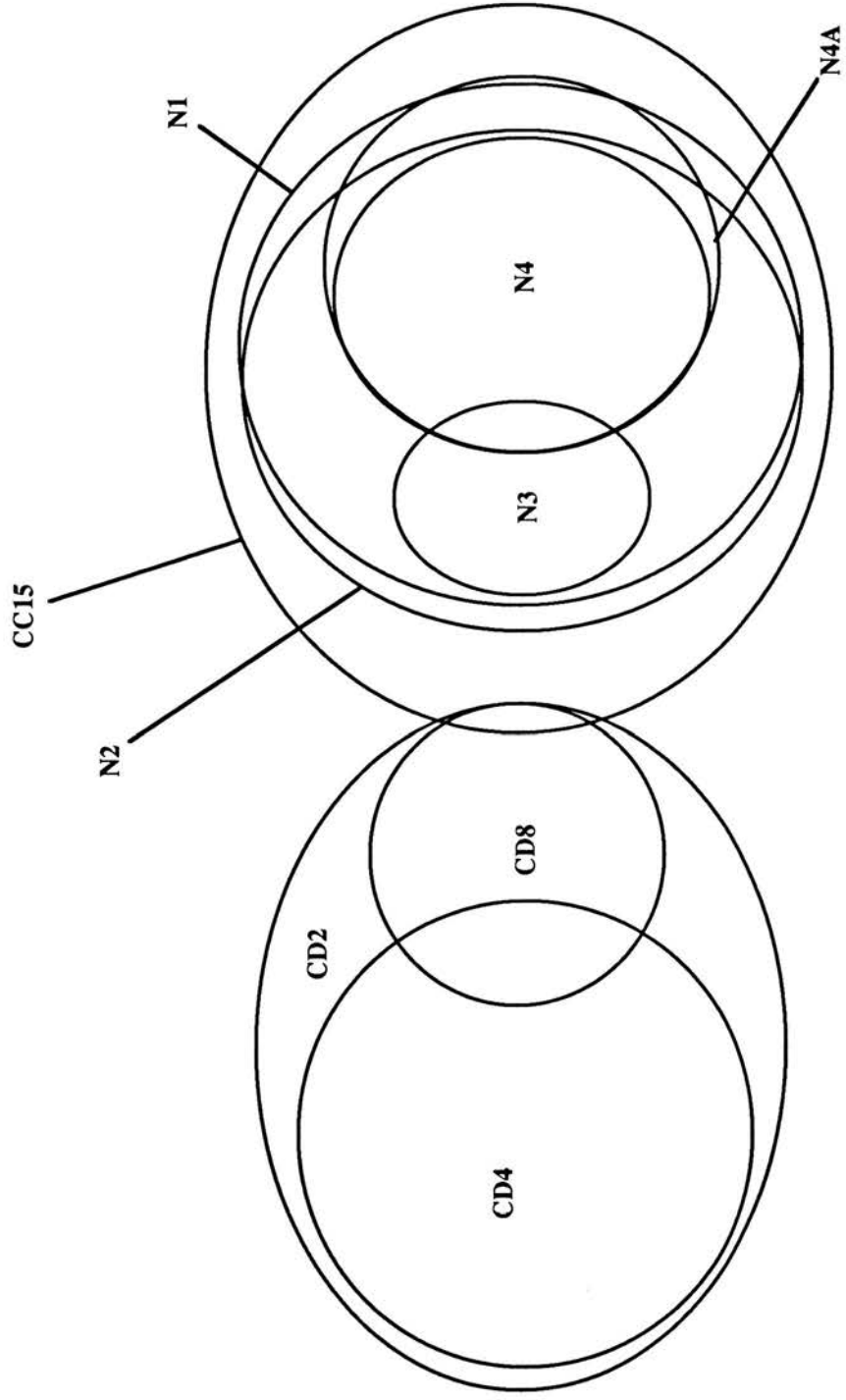
the bovine WC1.1 product only, whilst the monoclonal antibodies B7A1, BAQ4A and CACTB1A recognised epitopes expressed on the bovine WC1.2 product. These results correlated well with the results obtained using FACS analysis of ovine PBL reported here and elsewhere (Hein et al., 1991). It was reported here that in sheep, like cattle, that the epitopes recognised by monoclonal antibodies B7A1 (anti-N1) and BAQ4A (anti-N2) were expressed on the same sub-populations of WC1-positive cells. In addition, the epitopes recognised by the monoclonal antibodies BAG25A (N4A) and CACTB1A (N3) were reported here to be expressed on mutually exclusive populations of lymphocytes in the ovine species.

Recently, DNA sequences homologous to regions of the bovine WC1.1 cDNA were found to be highly represented in a sheep genomic library (Walker et al., 1994). Sequence analysis suggested their DNA segments were each derived from different genes. These results also suggested the existence of a family of between 50 and 100 genes for WC1 in the sheep genome. The differences in the reactivity of the monoclonal antibodies specific for different WC1 epitopes could therefore be due to the expression of antigens encoded by different members of a gene family, or possibly due to variations in the products of a single gene as a result of alternative splicing or post-translational modification. It has been hypothesised by Walker and colleagues (1994) that due to the existence of such a large gene family, individual WC1 proteins may carry out distinct but related functions in the ovine species. In addition, it has been proposed that WC1 may function as a homing molecule for γδ T cells (Wijngaard et al., 1994). The expression of the different epitopes on a homing molecule or different WC1 genes may vary according to the homing requirements of the WC1-positive γδ T cells. It was reported here and elsewhere that γδ T cells in the epithelium of the gut do not express any of the known WC1 epitopes (MacKay et al., 1989; Evans 1994). It will be of interest to determine if this population of cells express any of the WC1 gene products.

It was shown that the percentage of the whole WC1-positive population which expressed each of the different WC1 N-epitopes did not vary on PBL isolated from animals of different ages, on PBL isolated from animals with different antigenic burden or on PBL which were activated *in vitro*.

There were no differences in the WC1 N-epitope phenotype of PBL isolated from animals of different age. However, it was shown on PBL isolated from animals with little or no antigenic burden that the expression of the N3 epitope is less on at least the N1-positive and the N4-positive populations. This may suggest that there is little requirement for the expression of the N3 epitope on γδ T cells in animals undergoing little immunological challenge. Following *in vitro* culture of PBL with ConA, the N4-positive population became N3-negative and the N3-positive population became N4-negative. This may imply that these two sub-populations of γδ T cells carry out different effector functions once activated.

The intensity of staining by all anti-WC1 monoclonal antibodies decreased in the initial stages of culture with ConA, as did all the ratios of N-epitope positive cells to γδ TCR positive cells. The intensity of staining of all anti-WC1 monoclonal antibodies increased after day 3 as did the ratio of N-epitope positive cells to γδ TCR positive cells but neither had returned to starting levels by the end of the assay. The drop in the ratio of N-epitope positive cells/γδ TCR positive cells may be due to one or more of the following reasons: i) there was a loss of some WC1 epitopes from the surface of cells following activation. It has previously been reported that the epitope recognised by monoclonal antibody 19-19 is lost from PBL following activation (MacKay et al., 1989). ii)

there was a selective outgrowth, in the initial stages of activation, of cells which expressed the $\gamma\delta$ TCR but did not express WC1. iii) there is a further set of WC1 proteins whose expression is increased following activation of $\gamma\delta$ T cells. This further set of WC1 proteins do not express any of the epitopes so far identified.

The change in the intensity of expression of the different WC1 epitopes may be due to an initial switch off, or slow down, of *de novo* WC1 synthesis and expression during the initial stages of activation of the $\gamma\delta$ T cell population. Once activated *de novo* synthesis of WC1 is restarted and the intensity of expression of the epitopes subsequently increases.

Collectively, the data presented in chapter 3 and chapter 4, along with that published by others during the course of the thesis has identified WC1 as a protein complex with diversity at least at the genetic level, and possibly at the post-transciptional or post-translational level. This work has also confirmed that, with respect to the WC1 proteins, $\gamma\delta$ T cell immunochemistry in the ovine species is similar in some, but not all, respects to that seen in the bovine species. The function of the WC1 protein complex remains elusive, as does the function of the population of cells which express them. The following chapters in the thesis will describe attempts to identify a functional role for $\gamma\delta$ T cells in the ovine species.

CHAPTER 5

Activation of γδ T cells.

INTRODUCTION:

γδ T cells are a unique subset of lymphocytes which have been found in all species so far examined. They express a T cell receptor (TCR) of γ and δ chains which is thought to be specific for antigen in a manner analogous to the αβ TCR. γδ T cells however, do not express the co-receptor molecules CD4 and CD8 which are involved in αβ T cell activation (Janeway, 1992). In addition, in the ovine species, γδ T cells do not express the adhesion molecule CD2 which has also been implicated in the activation of T cells (MacKay et al., 1989; Evans, 1993). Previous work on ovine peripheral blood γδ T cells has proposed that these observed differences in cell surface phenotype of the T cell subsets may imply differences in activation (Evans, 1993) between γδ T cells and CD4-positive and CD8-positive T cells

It is known that one function for the αβ TCR molecule is to recognise antigenic peptide in association with an MHC molecule expressed on the surface of an APC. The interaction between TCR molecule and the antigen/MHC complex occurs simultaneously with either MHC class I or class II molecules binding the co-receptor molecules CD8 or CD4, respectively. The T cell tyrosine kinases p56[lck] and p59[fyn] are associated with the cytoplasmic portions of the CD4 and CD8 molecules (Abraham et al.,1992; Janeway 1992; Vega et al., 1990; Danielian et al., 1992). Following association of the MHC-peptide complex with TCR and co-receptor, the associated tyrosine kinases are de-phosphorylated and acquire increased kinase activity (Shiroo et al., 1992). This in turn causes activation of intracellular signal pathways which ultimately induce expression of T cell specific genes. Genes involved in the proliferation and clonal expansion of T cells include those encoding IL-2 and the receptor for IL-2 (IL-2R) (Waldmann, 1989).

IL-2 is produced and secreted by T cells when activated and the receptor for IL-2 (IL-2R) is expressed on T cells when activated. The interaction of IL-2 with the IL-2R initiates the growth, differentiation and clonal expansion of T lymphocytes (Minami et al., 1993; Takeshita et al., 1992; Lowenthal and Greene, 1987; Smith, 1988).

The receptor for IL-2 (IL-2R) is a complex transmembrane protein comprising at least three distinct proteins; termed the α, β and γ-chains. IL-2Rα is a highly glycosylated protein of 55kDa and is termed CD25. IL-2Rβ is an IL-2 binding protein of 75kDa. IL-2Rγ is a 64kDa protein whcih was recently identified as the third IL-2 receptor subunit and which plays a pivotal role both in IL-2 binding by IL-2Rβ and in receptor signalling (Minami et al., 1993; Shimizu et al., 1986; Waldmann, 1989). The expression of the gene encoding IL-2Rα can not be detected in resting T cells. However, following mitogenic stimulation, expression of IL-2Rα can be detected within 4-8hrs. The maximum level of expression of the IL-2Rα is reached in 2-4 days (30,000-60,000 IL-2Rα molecules per cell) (Smith, 1988; Depper et al., 1984). The product of the IL-2Rβ gene is expressed at low levels in resting T cells (600 IL-2Rβ-molecules per cell). The level of expression of the IL-2Rβ gene increases in activated T cells though not as dramatically as the level of the IL-2Rα gene expression. The level of IL-2Rα expressed on activated T cells is approximately 20-fold higher than the level of IL-2Rβ expression (Thuy et al., 1987). IL-2Rγ is expressed constitutively in all lymphoid cells (Takeshita et al., 1992).

In the human species, the IL-2R exists in three forms which are distinguished on the basis of affinity for the ligand (Minami et al., 1993). The high affinity IL-2R is comprised of all the subunits (α, β and γ) and has a dissociation constant (Kd) of 10^{-11}M. The intermediate affinity IL-2R contains the IL-2β and IL-2γ subunits (Kd=10^{-9}M) whereas the low affinity receptor

comprises IL-2Rα alone (Kd=10^{-8}M). Binding of IL-2 to the IL-2Rα-chain alone does not induce signaling nor internalisation (Greene et al., 1985; Hatakeyama et al., 1985). Different combinations of the three different IL-2R components have been shown to bind IL-2 however, the only combinations of components which can transduce signals and cause proliferation are the intermediate affinity (β and γ) and high affinity (α, β and γ) receptors.

The IL-2Rβ-chain has the largest cytoplasmic domain and plays a critical role along with the IL-2Rγ-chain in IL-2 induced intracellular signaling. It was recently shown using chimeric receptor constructs that the extracellular domains of the IL-2Rβ and γ chains mediate the heterodimerisation of the IL-2R subunits when IL-2 is bound. It was also shown that this extracellular heterodimerisation is necessary for the heterodimerisation of the internal domains and subsequent T cell signaling and proliferation (Nakamura et al., 1994; Nelson et al., 1994).

The binding of IL-2 to the high affinity receptor results in signal transduction mediated by the 75kDa protein (IL-2Rβ) and internalisation of the receptor complex. The IL-2Rβ subunit has been found physically associated with the tyrosine kinase p56[lck]. Furthermore, IL-2 induces rapid tyrosine phosphorylation of many cellular proteins including the IL-2Rβ-chain (Hatakeyama et al., 1991; Horak et al., 1991). Two alternative intracellular signalling pathways are thought to exist. One leads to the induction of the *c-myc* gene which causes the cell to enter into the S-phase of the cell cycle and onto cell division. The other involves activation of cytoplasmic protein tyrosine kinases (e.g p21[ras]), leading to induction of *c-jun* and *c-fos* (Shibuya et al., 1992).

In this chapter, the rate of activation of the different T cell populations was determined. Because of the features of the different IL-2 receptor components, in particular their differential expression on resting T cells and increased expression following acivation, the analysis of changes in the expression of CD25 will provide a good correlation with the state of activation of a T cell.

As a result of cellular activation, ovine T cells express other cell membrane activation molecules apart from the receptor for IL-2. One such set of proteins are MHC class II molecules. MHC class II molecules are constitutively expressed on the surface of B cells, macrophages, monocytes, dendritic cells and endothelium. Following activation, the expression of MHC class II molecules is increased on ovine T cells (Hopkins et al., 1986). In the human species there are three MHC class II loci each of which classically express products, these are termed DP, DQ and DR (Benoist and Mathis, 1990). In the ovine species however, only the DQ and DR loci have been identified (Dutia et al., 1993; Hopkins et al., 1993). MHC class II glycoproteins in all species so far examined are expressed as noncovalent heterodimers α and β of integral membrane glycoprotein chains. MHC class II molecules are expressed on the surface of APC in association with peptides processed from exogenous proteins. This peptide-MHC complex is recognised by specific T cells so inducing activation of specific T cells.

In this Chapter, the rates of activation of ovine peripheral blood T cells were analysed. The expression of CD25 and MHC class II expression on CD4-positive, CD8-positive or γδ T cells was measured following stimulation with the T cell mitogen ConA. ConA binds to sugar residues on glycoproteins and causes cross-linking of these glycoproteins on the surface of cells (Beckert and Sharkey, 1970). One such glycoprotein is the TCR expressed on T cells. B cells are not activated by treatment with ConA and so the mitogen is T cell specific. Treatment of cells with ConA provides an *in vitro* stimulus regarded as analogous to antigen-specific T cell activation, this results in polyclonal activation, allowing subsequent analysis of complete T cell populations.

RESULTS:

5.1 Increase in size of γδ T cell population following *in vitro* culture of PBL with ConA.

Unfractionated PBL were cultured with the T cell mitogen ConA. At various times after the start of culture, cells were analysed to determine the T cell subset composition of the cultured cells. Table 5.1 shows there was an increase in the percentage of the γδ T cell population after one day in culture. At this time the γδ T cell population increased in size from 16% to 35% of the lymphocyte population. The γδ T cell population remained at this level throughout the period of the assay. The CD4-positive and CD8-positive populations also increased in size, and at a similar magnitude to that of the γδ T cell population, but only after 4-5 days of culture. Statistical analysis showed that there was a significant increase in all T cell populations over the course of the assay but that there were no significant differences in the size of increase of the three T cell populations. The ratio of γδ T cells to CD4-positive plus CD8-positive T cells emphasised the observed differences in the rates of increase in the T cell population sizes on the first day. These results implied that γδ T cells are either better predisposed to *in vitro* culture, or that γδ T cells proliferate and expand at a faster rate than CD4-positive or CD8-positive T cells. The rate of proliferation and expansion of a population of cells will be dependent on the rate of activation of these cells in response to the given stimulus. The rates of expression of CD25 and MHC class II on the T cell subsets was determined.

5.2 CD25 expression by ovine PBL.

During the course of this thesis a monoclonal antibody to bovine CD25 became available (Naessens et al., 1992). Nucleotide sequence analysis revealed a 94% level of homology between ovine and bovine cDNA encoding CD25 (Bujdoso et al., 1992; Weinberg et al., 1988) and because of the predicted amino acid sequence homology between the ruminant CD25 proteins (Bujdoso et al., 1993) it seemed likely that monoclonal antibodies generated to bovine CD25 would cross-react with ovine CD25. This monoclonal antibody was used to measure changes in the expression of the IL-2R on ovine lymphocytes.

Figure 5.1 shows the reactivity of the anti-CD25 monoclonal antibody ILA1-11 with resting and ConA stimulated ovine PBL. Few resting peripheral blood lymphocytes expressed CD25. In contrast, following stimulation with ConA for three days, the majority of PBL expressed CD25. The resting peripheral blood T cells which reacted with the monoclonal antibody ILA1-11 were determined by two colour FACS analysis using T cell subset specific monoclonal antibodies. Figure 5.2 shows the results obtained. CD25 was expressed on <8% of resting peripheral blood γδ T cells, on <8% of resting peripheral blood CD8-positive T cells and on 25% of resting peripheral blood CD4-positive T cells.

5.3 All ovine peripheral blood γδ T cells express CD25 following culture with ConA.

The level of expression of CD25 on the different T cell subsets from unstimulated peripheral blood of sheep was low. However, most peripheral blood lymphocytes expressed CD25 following treatment with ConA. The expression of CD25 on each of the T cell subsets was analysed for 14 days following treatment of PBL with ConA. Two-colour immunofluorescence FACS analysis was performed with subset specific monoclonal antibody versus the anti-CD25 monoclonal antibody.

66

Table 5.1:   Subset composition of ovine PBL after culture with ConA.

PBL were isolated from 6 month old sheep (n=5), cultured with 5µg/ml ConA, and at the time points indicated, viable lymphocytes were harvested. 10^6 viable cells were stained with anti-$\gamma\delta$ TCR, anti-CD4, anti-CD8 or anti-B cell then followed by anti-IgG FITC and analysed by FACScan. The percentages listed are positive cells with respect to NMS used as a negative control (always <5%). The results shown are the mean of 4 animals and the errors in the brackets are the standard deviation of the means. The ratio of CD4-positive and CD8-positive T cells to $\gamma\delta$ T cells is also shown. This experiment was repeated three times and the same trends were seen with each animal and on each occasion. The significance of the change in number of PBL expressing the various markers after stimulation was determined by regression analysis of the data. A General Linear Model was used to analyse the data with time as a covariate. The probability that the differences in the data are significant is represented below each column.

TABLE 5.1: Subset composition of ovine PBL after culture with ConA.

Days after treatment	Percentage of lymphocyte population.				Ratio of CD4-positive + CD8-positive to γδ T cells.
	γδ T cells	CD4+ cells	CD8+ cells	B cells	CD4 + CD8 : γδ
0	15.9 (9.8)	17.5 (4.8)	9.3 (3.4)	39.8 (12.8)	1.69
1	35.0 (11.0)	22.8 (5.9)	13.2 (4.1)	29.5 (19.7)	1.03
2	36.4 (14.3)	26.8 (1.4)	17.8 (1.9)	31.4 (20.3)	1.23
3	34.4 (14.2)	27.7 (5.5)	18.2 (3.5)	23.0 (13.5)	1.33
4	37.4 (15.3)	33.5 (9.4)	22.6 (6.2)	14.0 (8.6)	1.50
5	38.2 (15.7)	36.1 (9.8)	22.8 (6.8)	12.6 (10.0)	1.54
6	38.8 (6.8)	34.8 (9.3)	18.5 (4.9)	12.2 (2.8)	1.37
7	38.7 (16.2)	33.8 (10.4)	20.0 (11.2)	4.6 (4.0)	1.39
	* $p<0.005$	* $p<0.001$	* $p<0.005$	+ $p<0.005$	

* - significant increase in population size

+ - significant decrease in population size

Figure 5.1: Reactivity of monoclonal antibody to CD25 with ovine PBL.

PBL were isolated from 6 month old sheep (n=3) and then cultured with 5µg/ml ConA for 3 days. After culture, viable stimulated lymphocytes were harvested and at the same time fresh unstimulated PBL were isolated from 6 month old sheep (n=3). 10^6 unstimulated cells and 10^6 stimulated cells were stained with anti-CD25 followed by anti-IgG FITC and analysed by FACScan. The figure shows positive staining of PBL from one animal in each group with respect to NMS used as a negative control (always <5%). This experiment was repeated five times and the same trends were seen with each animal and on each occasion.

FIGURE 5.1: Reactivity of monoclonal antibody to CD25 with ovine PBL.
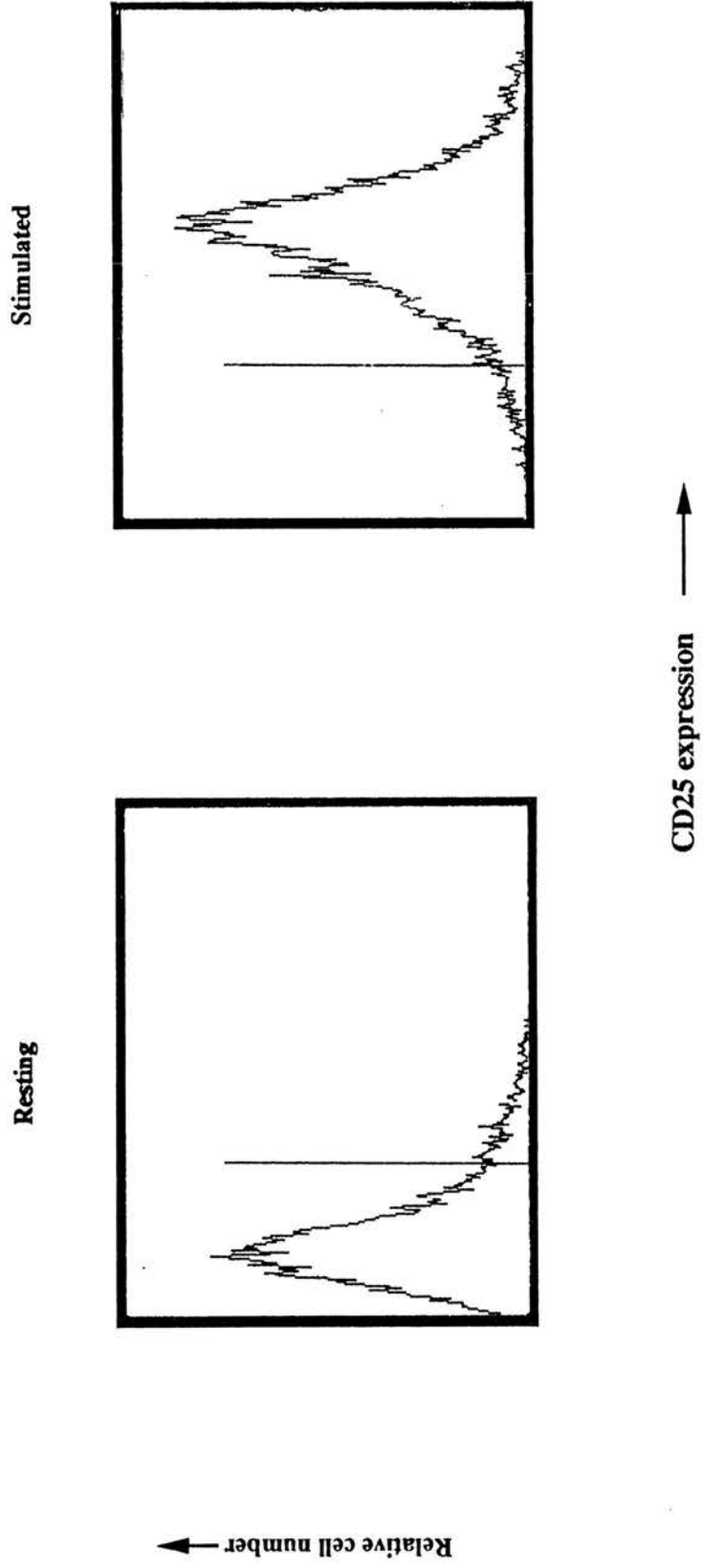
Resting

Stimulated

Relative cell number

CD25 expression ⟶

Figure 5.2: CD25 expression by resting peripheral blood T cell subsets.

PBL were isolated from 6 month old sheep (n=3) and then stained with anti-CD25 followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and two-colour immunofluorescence profile are shown. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS versus and b-NMS (always <5%). This experiment was repeated five times and the same trends were seen with each animal and on each occasion.

FIGURE 5.2: CD25 expression by resting peripheral blood T cell subsets.
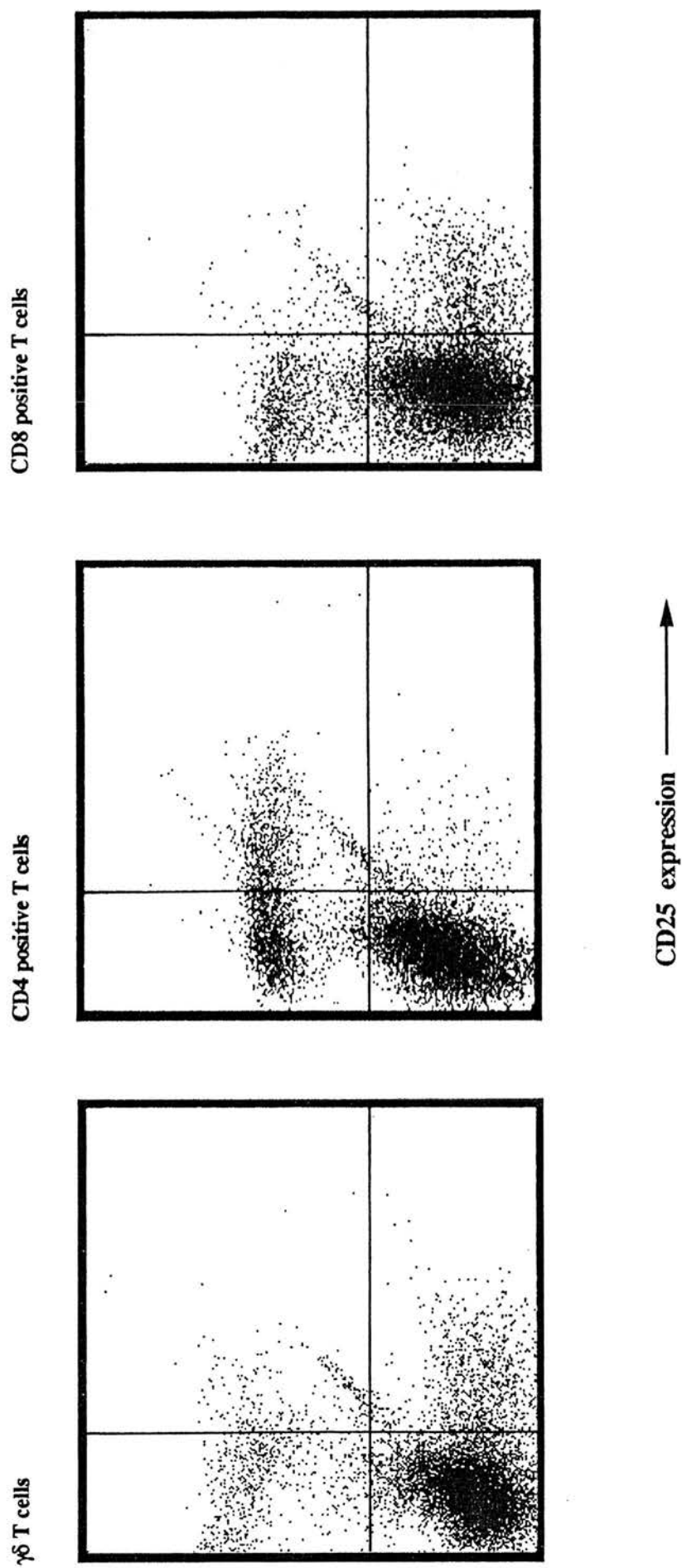
The results in table 5.2 show there was an increase in expression of CD25 on each T cell subset. The rate of expression of CD25 in the first 24hours was significantly greater on γδ T cells compared to CD4-positive and CD8-positive T cells. The maximum level of CD25 expression by the γδ T cell population occurred within 24 hours of treatment with ConA. The CD4-positive and CD8-positive T cell subsets also showed an increase in CD25 expression, but unlike γδ T cells, the maximum level of expression occurred 2-4 days after the start of the culture.

5.3.1 γδ T cells express CD25 and MHC class II with faster kinetics than do CD4 positive and CD8-positive T cells following *in vitro* culture with ConA.

Almost all γδ T cells expressed CD25 within the first 24 hours of activation. The first 24 hours after treatment with ConA showed the biggest changes in levels of CD25 expression by the different T cell subsets with the level of expression of CD25 on γδ T cells significantly higher than that observed on CD4-positive and CD8-positive T cells. Expression of CD25 by the different T cell subsets was therefore analysed at time points within the first 24 hours. The results obtained are shown in figure 5.3. All γδ T cells expressed CD25 within 8 hours of treatment with ConA. In contrast, the greatest number of CD4-positive and CD8-positive T cells which expressed CD25 was not seen until 24 hours after the start of culture with ConA. In addition, not all CD4-positive and CD8-positive T cells expressed CD25 within 24 hours. The rate of increase in expression of CD25 by γδ T cells was significantly different to that of CD4-positive and CD8-positive T cells after 4hours and 8 hours of culture with ConA. There was no significant difference in the rate of increase in CD25 expression at any other time points. Figure 5.4 shows the two-colour immunofluorescence profiles of CD25 versus WC1 or CD4 at the time points 0, 8 and 24 hours. All γδ T cells expressed CD25 at high intensity after only 8 hours in culture with ConA. In contrast, all CD4-positive T cells only expressed CD25 at high intensity after 24 hours of treatment. CD8-positive T cells showed similar two-colour immunofluorescence profiles to CD4-positive T cells (data not shown).

MHC class II expression was compared with the increased expression of CD25 on the different T cell subsets during the first 24 hours of culture with ConA. The monoclonal antibody SW73.2 recognises an epitope on all known ovine MHC class II β-chains (Hopkins et al., 1986) and was used here to detect MHC class II expression. Figure 5.5 shows that the greatest number of γδ T cells which expressed MHC class II occurred within 12 hours of culture with ConA. MHC class II was expressed at significantly higher levels on resting populations of CD4-positive and CD8-positive T cells compared to γδ T cells and concurs with results previously reported (Dutia et al., 1993). The rate of increase in expression of MHC class II molecules by the remaining CD4-positive and CD8-positive T cells appeared to occur at a slower rate than was seen for γδ T cells. The significance of any differences in the rate of increase of MHC class II expression by the different T cell subsets could not be assessed because of the high starting levels of MHC class II expression on CD4-positive and CD8-positive T cells.

5.3.2 Expression of MHC class II β-chain on γδ T cells is different to the expression of MHC class II α-chain on γδ T cells following stimulation with ConA.

Unlike reagents which detect ovine MHC class II β-chains, there is no monoclonal antibody available which recognises an epitope on all ovine MHC class II α-chains. In the ovine species there are monoclonal antibodies which are specific for products of the two different loci (Dutia et

Table 5.2:   CD25 expression on T cell subsets after culture with ConA.

PBL were isolated from 6 month old sheep (n=3) and cultured with 5µg/ml ConA. At the times indicated viable lymphocytes were harvested and 10⁶ cells were stained with anti-CD25 followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the percentage of each T cell subset which expressed CD25 calculated. The table shows the percentage of positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean of three animals with the standard deviation of the mean shown in the brackets. This experiment was repeated 6 times and the same trends were seen with each animal and on each occasion. The significant differences between each T cell population was determined by calculating the rate of increase in expression of CD25 between two points and performing a two sample t-tests. The probability that the rate of increase of CD25 expression by the different T cell subsets is significantly different is represented in the table.

TABLE 5.2: CD25 expression on T cell subsets after culture with ConA.

Days after treatment.	Percentage of lymphocyte population expressing CD25		
	$\gamma\delta$ T cells	CD4+ cells	CD8+ cells
0	7.4 (5.6)	12.6 (2.2)	9.9 (8.9)
1	98.4 (0.7)*	88.8 (2.6)	74.2 (3.7)
2	98.2 (0.4)	95.5 (1.2)	90.2 (2.9)
3	98.0 (0.8)	96.0 (3.5)	90.3 (6.9)
4	98.2 (9.0)	96.2 (1.7)	88.6 (7.4)
5	92.6 (6.7)	97.7 (1.6)	88.4 (6.2)

* significantly different increase compared to CD4 ($p < 0.05$) and CD8 ($p < 0.01$)

Figure 5.3: CD25 expression by T cell subsets of PBL following culture with ConA.

PBL were isolated from a 6 month old sheep and cultured with 5μg/ml ConA. At the times indicated, viable lymphocytes were harvested and 10^6 cells stained with anti-CD25 followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the percentage of each T cell subset which expressed CD25 calculated. The figure shows the percentage of positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean of three animals with the error bars showing the standard deviation of the mean. This experiment was repeated 6 times and the same trends were seen with each animal and on each occasion. The significant differences between each T cell population was determined by calculating the rate of increase in expression of CD25 between each time point and the start of the experiment and performing a two sample t-tests. The probability that the rate of increase of CD25 expression by the different T cell subsets is significantly different is represented in the table.

FIGURE 5.3: CD25 expression by T cell subsets of PBL following culture with Con A.

Lymphocyte subsets.

* significantly different to CD4   ++ p<0.001   ++ p<0.005
+ significantly different to CD8   ** p<0.005   * p<0.05

Figure 5.4: CD25 expression by T cell subsets of PBL following culture with ConA.

PBL were isolated from 6 month old sheep (n=3) and cultured with 5µg/ml ConA. At the times indicated, viable lymphocytes were harvested and 10^6 cells stained with anti-CD25 followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the two-colour immunofluorescence profiles are shown. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS versus and b-NMS (always <5%). This experiment was repeated five times and the same trends were seen with each animal and on each occasion.

FIGURE 5.4: CD25 expression by T cell subsets of PBL following culture with Con A.

Figure 5.5: MHC class II expression by T cell subsets of PBL following culture with ConA.

PBL were isolated from 6 month old sheep (n=3) and cultured with 5µg/ml ConA. At the times indicated, viable lymphocytes were harvested and 10^6 cells stained with anti-MHC class II followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the percentage of each T cell subset which expressed CD25 calculated. The figure shows the percentage of positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean of three animals with the error bars showing the standard deviation of the mean. This experiment was repeated five times and the same trends were seen with each animal and on each occasion.

FIGURE 5.5: MHC class II expression by T cell subsets of PBL following culture with Con A.

Lymphocyte subsets.
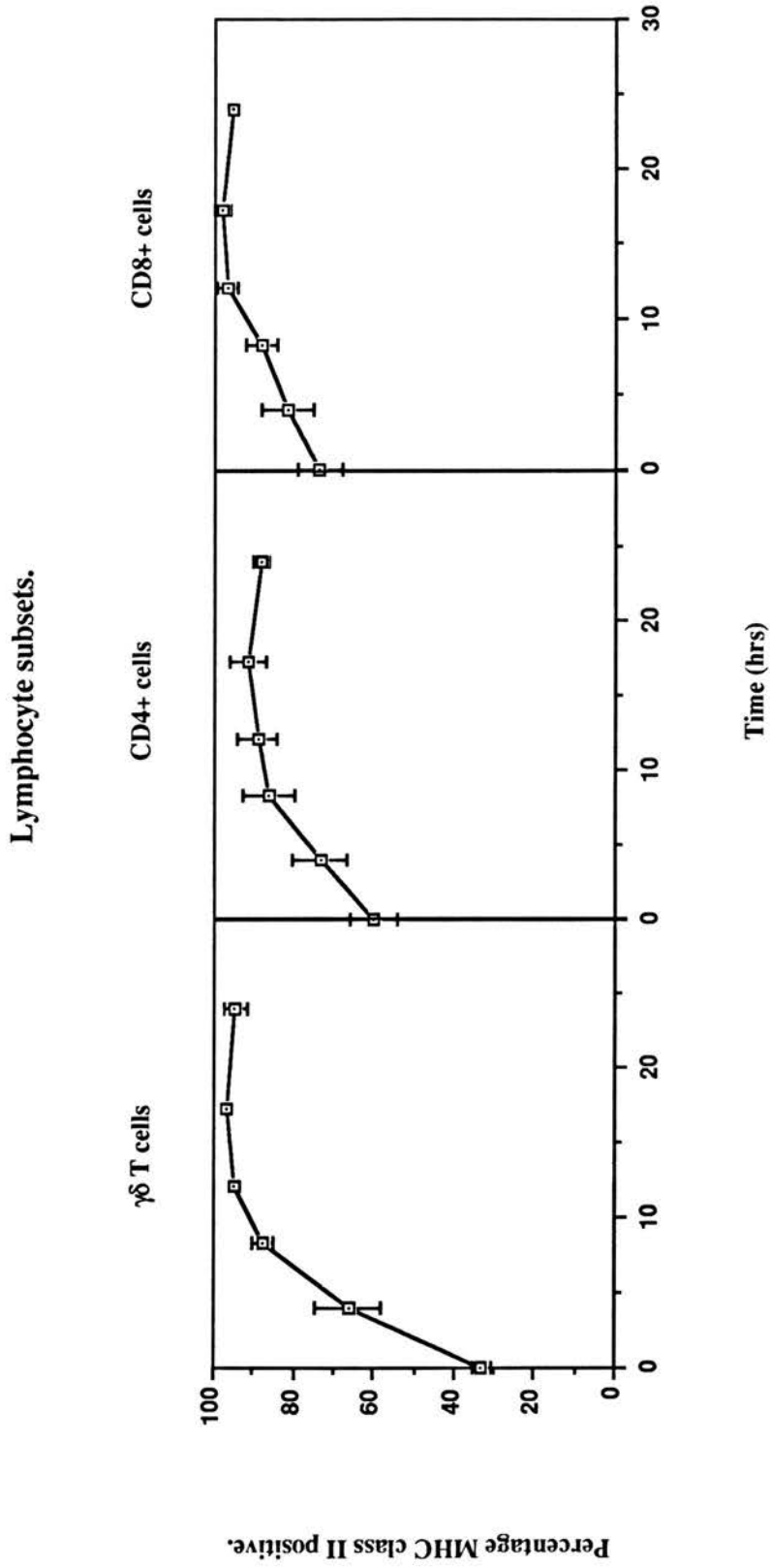
al., 1993; Hopkins et al., 1993). The monoclonal antibody VPM36 recognises products of the DR locus and monoclonal antibody VPM54 recognises products of the DQ locus. The changes in the levels of expression of these different α-chains on the T cell subsets was determined during the first 24 hours of culture with ConA. The results in table 5.3 showed differences in the expression of the α and β chains on the different T cell subsets. There were no significant changes in the level of expression of both DQ and DR α-chains on any T cell subset following stimulation with ConA. In contrast, there was a significant increase in the level of MHC class II β-chain expression. This was similar to results previously reported (see figure 5.5).

5.3.3 All subsets of γδ T cells express CD25 with similar kinetics.

The monoclonal antibody CC15 has been used to define the γδ T cell population expressing CD25 in all experiments so far. CC15 reacts with a WC1 epitope on cells which also express the γδ TCR (chapter 3). The WC1/γδ TCR positive population can be divided into two mutually exclusive populations expressing either of the determinants N3 or N4, these cells defined by the co-expression of determinants N1 and N2 (chapter 4). The kinetics of activation were investigated for each of the WC1/γδ TCR positive T cell sub-populations. PBL were treated with ConA and the expression of CD25 on the different populations of cells analysed. Table 5.4 shows that all sub-populations of WC1/γδ TCR positive cells expressed CD25 with similar kinetics and with kinetics similar to those seen with the monoclonal antibody CC15 which recognises all WC1 positive cells.

5.4 Activation of γδ T cells is a specific response to ConA.

In the above experiments (section 5.3), all viable cells were harvested on Lymphoprep prior to immunostaining and FACS analysis. Because of this procedural step, and the *in vitro* culture, a number of possible explanations can be given for the results obtained other than a difference in the kinetics of activation of γδ T cells and, CD4-positive and CD8-positive T cells. These are: i) the different peripheral blood T cell subsets are not equally viable during *in vitro* culture. ii) γδ T cells may be concentrated in the harvested population. i.e. populations of CD4-positive and CD8-positive T cells may pass through the Lymphoprep either when resting or once activated. iii) γδ T cells may be non-specifically activated by the isolation procedures or culture. This section of the chapter reports the results obtained in testing these three possibilities.

5.4.1 All T cell populations remain viable during the first 24 hours of culture with ConA.

The viability of the different T cell subsets during *in vitro* culture was measured with fluorescein di-acetate (FDA). Upon internalisation by viable cells FDA, which is lipid soluble, is cleaved by non-specific esterases into a non-lipid soluble moiety and emits green fluorescence (Balding et al., 1983). This enables two-colour immunofluorescence to be performed using T cell subset specific monoclonal antibodies detected with red fluorescence. The data is shown in Table 5.5. A significantly greater proportion of the γδ T cell population remained viable for the period of the assay. 87% of γδ T cells were viable after 24hrs of culture whereas only 79% of CD4-positive and 74% of CD8-positive T cells were viable after 24hrs of culture. Whilst the level of viabiliy of γδ T cells remains significantly higher in culture than the level of viability of either CD4-positive or CD8-positive T cells this represents only a very small proportion of the T cell populations. It should also be noted that the measure of viability used in this experiment does not take account of cells which may have died and not being detectable on the FACS using the gates already set. The

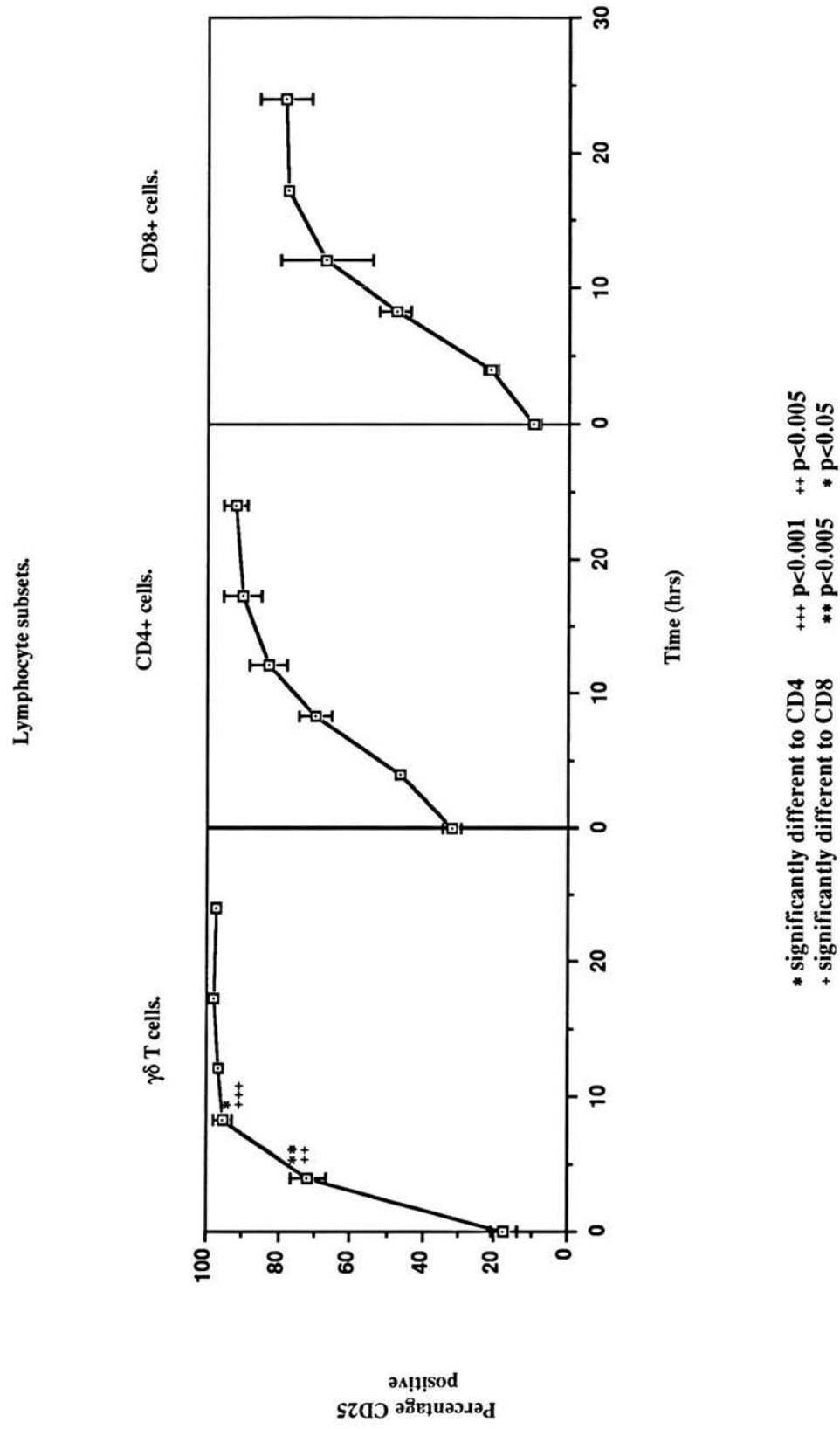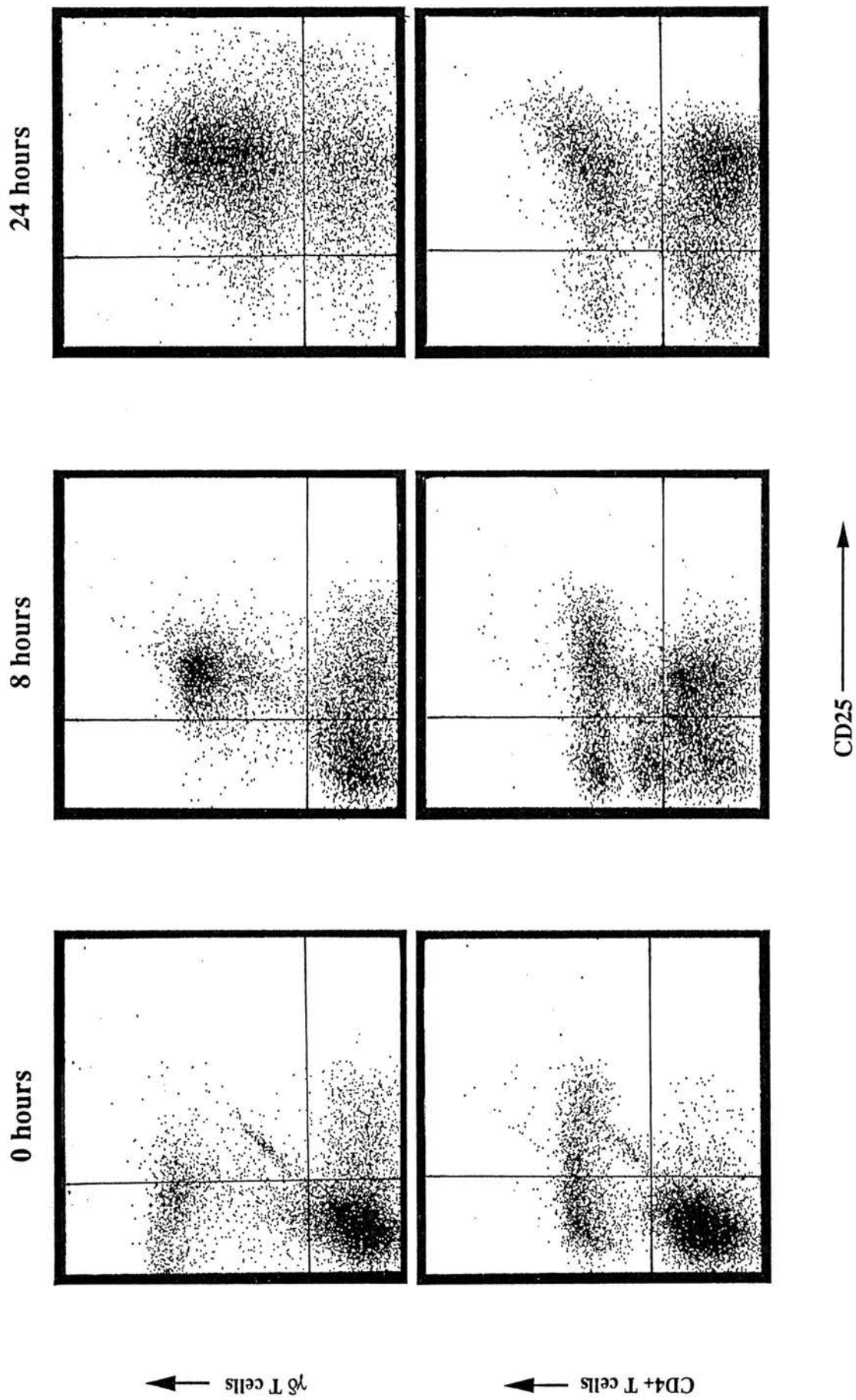**Table 5.3:** MHC class II α- and β-chain expression by T cell subsets of PBL following culture with ConA.

PBL were isolated from 6 month old sheep (n=3) and cultured with 5µg/ml ConA. At the times indicated, viable lymphocytes were harvested and 10^6 cells stained with anti-MHC class IIα-chain or anti-MHC class IIβ-chain followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the percentage of each T cell subset which expressed MHC class II α- or β-chains calculated. The figure shows the percentage of positive staining of cells compared to the non-specific staining of NMS versus and biotinylated NMS (always <5%). The results shown are the mean values obtained and the standard deviation of the means are shown in the brackets. This experiment was repeated three times and the same trends were seen with each animal and on each occasion. The significance of the change in number of PBL expressing the various markers after stimulation was determined by regression analysis of the data. A General Linear Model was used to analyse the data with time as a covariate. The probability that the differences in the data are significant is represented below each column.

TABLE 5.3: MHC Class II α-and β-chain expression by T cell subsets of PBL following culture with ConA.

Hours after treatment.	MHC Class II β-chain expression.			MHC Class II DQ α-chain expression (VPM36).			MHC Class II DR α-chain expression (VPM54).		
	γδ T cells	CD4+ cells	CD8+ cells	γδ T cells	CD4+ cells	CD8+ cells	γδ T cells	CD4+ cells	CD8+ cells
0	21.7 (3.8)	47.7 (7.5)	45.6 (11.3)	20.0 (6.6)	21.8 (6.7)	26.3 (3.5)	17.6 (5.6)	49.1 (8.0)	41.6 (11.3)
4	27.4 (4.2)	49.1 (5.8)	48.9 (6.7)	10.7 (4.4)	N.D.	12.5 (1.7)	7.9 (2.7)	29.4 (10.9)	23.1 (6.6)
8	54.7 (7.2)	64.0 (1.4)	70.2 (7.9)	7.3 (2.6)	12.8 (3.6)	11.1 (1.4)	12.7 (2.9)	34.8 (13.7)	29.4 (6.7)
12	83.7 (3.6)	75.6 (4.1)	77.4 (2.9)	11.5 (4.2)	18.2 (4.5)	10.7 (3.9)	13.5 (2.6)	28.9 (5.7)	25.1 (2.6)
18	88.6 (2.6)	81.2 (3.5)	86.4 (2.1)	3.6 (1.7)	15.5 (1.6)	7.9 (1.9)	18.1 (7.3)	27.7 (6.3)	28.0 (11.6)
	*	*	*						
	p<0.001	p<0.001	p<0.001	N.S.D.	N.S.D.	N.S.D.	N.S.D.	N.S.D.	N.S.D.

* - significant increase in expression over time
N.S.D. - no significant change in expression over time

Table 5.4: CD25 expression by WC1 subsets following culture with ConA.

PBL were isolated from 6 month old sheep (n=3) and then cultured with 5μg/ml ConA. At the times indicated, viable lymphocytes were harvested and 10^6 cells stained with anti-WC1 or anti-N1 or anti-N2 or anti-N3 or anti-N4 followed by anti-IgG FITC and biotinylated anti-CD25 then SA-PE. The cells were analysed by FACScan and the percentage of each N-epitope positive subset of cells which expressed CD25 was calculated. The figure shows the percentage of positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean values of three animals and the standard deviation of the means are shown in the brackets. This experiment was repeated three times and the same trends were seen with each animal and on each occasion.

TABLE 5.4: CD25 expression by WC1 subsets after culture with ConA.

Percentage of WC1 positive T cell populations expressing CD25.

Hours after treatment.	CC15+ cells	N1+ cells	N2+ cells	N3+ cells	N4+ cells
0	6.8 (1.4)	2.7 (1.1)	7.5 (1.8)	10.2 (6.9)	5.7 (1.3)
4	8.9 (0.9)	6.8 (2.7)	7.4 (3.4)	12.0 (6.7)	6.5 (2.1)
8	65.7 (6.9)	70.0 (9.0)	68.5 (8.5)	85.0 (4.5)	66.6 (3.2)
12	85.5 (2.0)	86.2 (4.4)	86.0 (2.5)	100.0 (0.0)	79.0 (3.7)
18	89.9 (0.8)	97.1 (2.9)	93.9 (1.3)	100.0 (0.0)	94.5 (2.6)

Table 5.5:   Viability of lymphocyte subsets after culture with ConA

PBL were isolated from 6 month old sheep (n=5) and cultured with 5μg/ml ConA. At the times indicated, viable lymphocytes were harvested on Lymphoprep and analysed elsewhere. Cells which passed through the Lymphoprep were collected and 10^6 cells were stained with either biotinylated anti-$\gamma\delta$, biotinylated anti-CD4, biotinylated anti-CD8 or biotinylated anti-B cell followed by SA-PE and FDA then analysed by FACScan. The percentages listed are positive cells with respect to NMS versus b-NMS used as a negative control (always <5%). The results shown are the mean values of five animals and the standard deviation of the means is shown in the brackets. This experiment was repeated twice and the same trends were seen with each animal and on each occasion. The significance of any differences in viability of each T cell population was determined by performing a two sample t-test. The probability that the differences in viability of the T cell population is significantly different is represented in the table.

TABLE 5.5: Viability of lymphocyte subsets after culture with ConA.

Viability of lymphocyte populations.

Hours after treatment.	γδ T cells	CD4+ cells	CD8+ cells
0	100 (0.0)	100 (0.0)	100 (0.0)
4	[99.2 (0.3)] **	94.4 (1.3)	91.1 (3.5)
6	[98.5 (1.1)] *	89.7 (4.1)	72.9 (15.0)
8	[97.4 (0.4)] **	93.7 (0.8)	86.3 (6.8)
10	[97.2 (1.4)] *	87.1 (3.3)	78.4 (11.5)
24	[87.0 (2.2)] **	79.3 (5.1)	74.4 (6.4)

[ ] significantly different to CD4+ and CD8+ cells

* p<0.05   ** p<0.01

differences in viability may have better been addressed by continually monitoring the cell number also.

5.4.2 A proportion of each lymphocyte population was not harvested on Lymphoprep.

It was proposed that not all CD4-positive and CD8-positive T cells were harvested on Lymphoprep, unlike γδ T cells. Table 5.6 shows the T cell composition of the lymphocytes which passed through the Lymphoprep following treatment with ConA. The size of the γδ T cell and CD4-positive T cell populations which passed through the Lymphoprep did not change significantly during the course of the assay. In contrast, the size of the CD8-positive T cell population which passed through the Lymphoprep decreased significantly from 18% after 4hours in culture with ConA to 8% after 18hours in culture with ConA.

The level of expression of CD25 and MHC class II on the T cells which passed through the Lymphoprep was also determined and the results are shown in table 5.7. There was an increase in CD25 and MHC class II expression by all T cell populations. The increase in expression of CD25 was significantly different on the different T cell subsets. Fewer CD4-positive and fewer CD8-positive T cells expressed CD25 following culture with ConA compared to γδ T cells. A similar pattern was seen with the expression of MHC class II on the different T cell subsets though no significant differences were observed.

These results in table 5.6 and table 5.7 indicated that after treatment with ConA, there was no selective bias for harvesting CD25-positive or/and MHC class II-positive γδ T cells on Lymphoprep following culture with ConA.

5.4.3 CD25 is not expressed on T cell subsets when cultured without ConA.

The level of expression of CD25 increased on all T cell subsets following culture with ConA. To determine the specificity of this increase, the level of CD25 expression on the T cell subsets following *in vitro* culture with medium alone was determined. Figure 5.6 shows that 12% of the γδ T cell population expressed CD25 at the start of the culture whereas 33% expressed CD25 after 24 hours of culture. The CD4-positive and CD8-positive T cell subsets showed no significant changes in their level of CD25 expression. There was no significant increase in the expression of CD25 by γδ T cells after 8 hours of *in vitro* culture in medium alone though the increase in CD25 expression was significant after 16 hours and 24 hours.

The results in this section have indicated that the activation of the T cell subsets appears to be a specific response to the culture of the cells with ConA. It would appear that the γδ T cell population becomes activated quicker than either the CD4-positive or CD8-positive T cell populations in response to ConA stimulation.

5.5 No change in the expression of CD4 and CD8 by γδ T cells following activation.

The proteins CD4 or CD8 are expressed on resting and activated αβ T cells in the human and murine species, and presumably the ovine species. CD4 and CD8 are required as co-receptor molecules for αβ T cell responses to antigen. No co-receptor molecules have generally been identified on γδ T cells therefore the expression of CD4 and CD8 molecules on γδ T cells was investigated following the activation of these cells. The changes in expression of the co-receptor

69

Table 5.6:   Composition of cells passing through Lymphoprep.

PBL were isolated from 6 month old sheep (n=5) and cultured with 5μg/ml ConA. At the times indicated, viable lymphocytes were harvested on Lymphoprep and analysed elsewhere. Cells which passed through the Lymphoprep were collected and 10^6 cells were stained with anti-$\gamma\delta$ TCR, anti-CD4, anti-CD8 or anti-B cell followed by anti-IgG FITC and analysed by FACScan. The figure shows the percentage of positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean values obtained and the standard deviation of the means are shown in the brackets. This experiment was repeated twice and the same trends were seen with each animal and on each occasion. The significance of the change in number of each T cell subset which passed through Lymphoprep was determined by regression analysis of the data. A General Linear Model was used to analyse the data with time as a covariate. The probability that the differences in the data are significant is represented below each column.

TABLE 5.6: **Composition of cells passing through Lymphoprep.**

Hours after treatment.	Percentage of each cell population found in lymphocyte gate.		
	$\gamma\delta$ T cells	CD4+ cells	CD8+ cells
0	0	0	0
4	8.6 (3.1)	32.2 (6.5)	18.4 (1.5)
6	16.0 (2.3)	43.2 (3.9)	10.5 (4.4)
8	24.2 (7.4)	44.0 (7.0)	10.9 (3.3)
10	24.5 (12.3)	40.4 (6.2)	13.0 (1.2)
18	12.0 (3.5)	33.2 (4.7)	8.3 (1.7)
	N.S.D.	N.S.D.	* $p<0.05$

* - significant decrease over time

N.S.D. - no significant change over time

Table 5.7: CD25 and MHC class II expression of lymphocytes passing through Lymphoprep.

PBL were isolated from 6 month old sheep (n=3) and cultured with 5µg/ml ConA. At the times indicated, viable lymphocytes were harvested on Lymphoprep and analysed elsewhere. Cells which passed through the Lymphoprep were collected and 10^6 cells stained with either anti-MHC class II or anti-CD25 followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the percentage of each T cell subset which expressed either CD25 or MHC class II was calculated. The table shows the percentage of positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean values of three animals and the standard deviation of the means is shown in the brackets. This experiment was repeated twice and the same trends were seen with each animal and on each occasion. The significance of any differences in the number of each T cell population which expressed either CD25 or MHC class II was determined by performing a two sample t-test comparing the different T cell populations. The probability that the differences in viability of the T cell population is significantly different is represented in the table.

TABLE 5.7: CD25 and MHC class II expression of lymphocytes passing through Lymphoprep.

Hours after treatment.	CD25 expression.			MHC Class II expression.		
	γδ T cells	CD4+ cells	CD8+ cells	γδ T cells	CD4+ cells	CD8+ cells
0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
4	11.7 (2.8)	24.1 (12.8)	6.7 (4.6)	39.2 (2.2)	44.5 (5.4)	50.1 (8.7)
8	[64.7 (0.3)] *	49.0 (4.3)	15.6 (11.4)	65.8 (10.2)	65.3 (7.5)	76.2 (3.8)
12	[79.3 (3.1)] *	54.0 (10.1)	22.6 (12.2)	70.0 (3.6)	62.2 (6.1)	69.7 (11.8)
18	[69.2 (5.2)] **	39.0 (3.0)	12.5 (3.7)	100.0 (0.0)	92.7 (10.3)	41.7 (16.4)

[ ] significantly greater than CD4+ and CD8+

* p<0.05 ** p<0.01

Figure 5.6: CD25 expression by T cell subsets of PBL following culture in the absence of ConA.

PBL were isolated from 6 month old sheep (n=3) and cultured in RPMI medium with no ConA. At the times indicated, viable lymphocytes were harvested and 10^6 cells stained with anti-CD25 followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the two-colour immunofluorescence profiles are shown. The figure shows the positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean of three animals with the error bars showing the standard deviation of the mean. This experiment was repeated twice and the same trends were seen with each animal and on each occasion. The significance of any differences in the number of each T cell population which expressed either CD25 was determined by performing a two sample t-test comparing the various time points with the value at the start of the experiment. The probability that the differences in viability of the T cell population is significantly different is represented in the table.

FIGURE 5.6: CD25 expression by T-cell subsets following culture in the absence of ConA.

Percentage of cells expressing CD25

Hours after start of culture

γδ T cells.
CD4 positive T cells.
CD8 positive T cells.
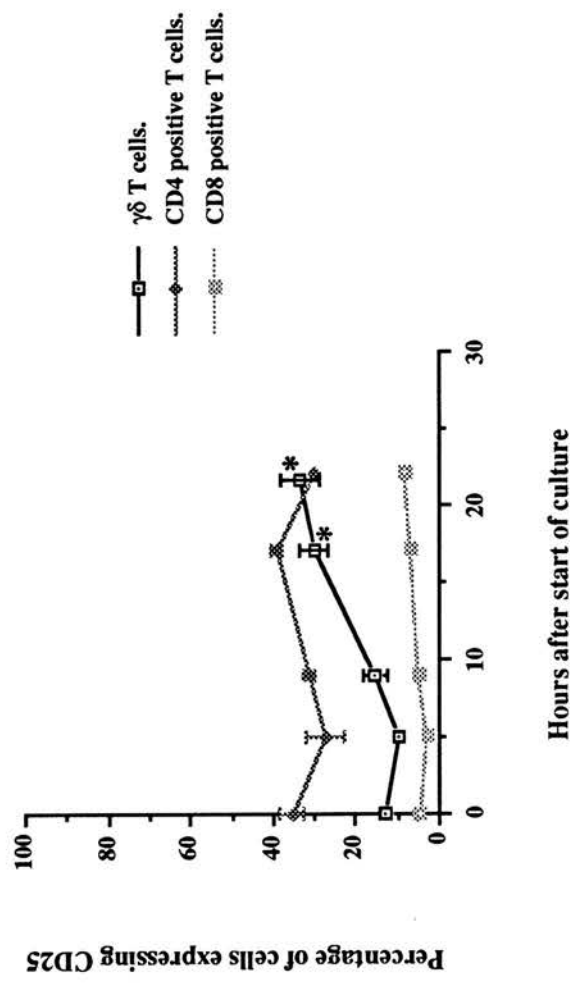
* - significant increase in size from start of experiment p<0.05

molecules CD4 and CD8 was measured and compared with the changes in expression of CD25 during the first 24 hours after stimulation with ConA. Figure 5.7 shows the results obtained. There was no significant change in the levels of co-receptor molecule expression by γδ T cells. The γδ T cell population remained 8-10% CD4-positive and 5-7% CD8-positive during the assay period. In the same assay, the size of the γδ T cell population which expressed CD25 increased from 17% to 90%.

5.6 CD45RA and CD25 expression on resting peripheral blood T cells does not influence the kinetics of γδ T cell, CD4-positive and CD8-positive T cell activation.

The different populations of lymphocytes in resting peripheral blood differ in the expression of a number of cell surface proteins. Two of these proteins are markers of activation (CD25) (Tsudo et al., 1986, 1987) and antigenic experience (CD45) (Clement, 1992). T cells having responded to antigen have a different cell surface phenotype (Horgan et al., 1992) and will respond more rapidly to the same antigen than naive T cells from the same animal. The best studied marker delineating naive and memory T cells is CD45 (Leucocyte Common Antigen) and its various isoforms (Merkenschlager et al., 1989). Figure 5.2 showed that <8% of resting peripheral blood γδ T cells and <8% CD8-positive T cells expressed CD25 whereas 25% of CD4-positive T cells expressed CD25. Figure 5.8 shows that only 1% of peripheral blood γδ T cells expressed the naive phenotype marker CD45RA. In contrast, 66% of CD4-positive and 83% of CD8-positive T cells expressed CD45RA.

It was proposed that the differences in CD25 and CD45RA phenotype of resting peripheral blood T cell subsets may account for the observed differences in rates of activation. To determine if this was the case, peripheral blood lymphocytes were depleted of all CD45RA positive and all CD25 positive cells and cultured as before with ConA. Figure 5.9 shows the histogram profiles of lymphocytes before and after the depletion of CD45RA positive cells and CD25 positive cells. All positively stained cell were removed. The remaining lymphocytes, which now had the same phenotype with respect to CD45RA and CD25, were treated with ConA as previously described. The expression of the activation markers CD25 and MHC class II was analysed on the different subsets as well as the re-expression, if any, of CD45RA. Table 5.8 shows the percentage of each subset of cells which were positive for the three markers; CD25, MHC class II and CD45RA. The level of expression of CD25 and MHC class II increased on all T cell subsets. This increase in expression occurred at a significantly faster rate on γδ T cells compared with CD4-positive and CD8-positive T cells between 8hours and 4 hours after the start of culture. The expression of CD45RA did not change significantly on any of the T cell subsets.

Figure 5.7: Expression of T cell markers by γδ T cell after culture with ConA.

PBL were isolated from 6 month old sheep (n=3) and cultured with 5μg/ml ConA. At the times indicated, viable lymphocytes were harvested and 10^6 cells stained with either anti-CD25, anti-CD4 or anti-CD8 followed by anti-IgG FITC and biotinylated anti-WC1 then SA-PE. The cells were analysed by FACScan and the two-colour immunofluorescence profile are shown. The figure shows the positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean values of 3 animals with the error bars showing the standard deviation of the mean. This experiment was repeated three times and the same trends were seen with each animal and on each occasion. The significance of the change in number of γδ T cells which expressed CD4 or CD8 was determined by regression analysis of the data. A General Linear Model was used to analyse the data with time as a covariate. The probability that the differences in the data are significant is represented below each column.

FIGURE 5.7: Expression of T cell markers by γδ T cells after culture with ConA.
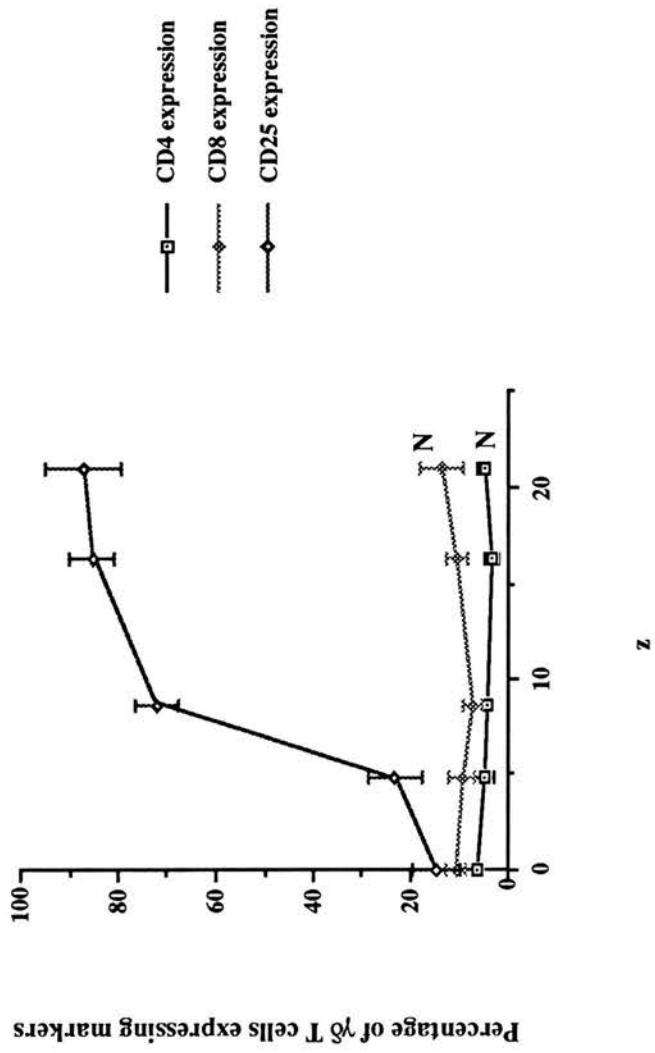
N - no significant difference from the start of the experiment

Figure 5.8: **CD45RA expression by resting peripheral blood T cell subsets.**

PBL were isolated from 6 month old sheep (n=3) and stained with anti-CD45RA followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the two-colour immunofluorescence profile are shown. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS versus and b-NMS (always <5%). This experiment was repeated five times and the same trends were seen with PBL from each animal tested and on each occasion.

FIGURE 5.8: CD45RA expression by resting peripheral blood T cell subsets

γδ TCR positive T cells

CD4 positive T cells

CD8 positive T cells

CD45RA expression ⟶
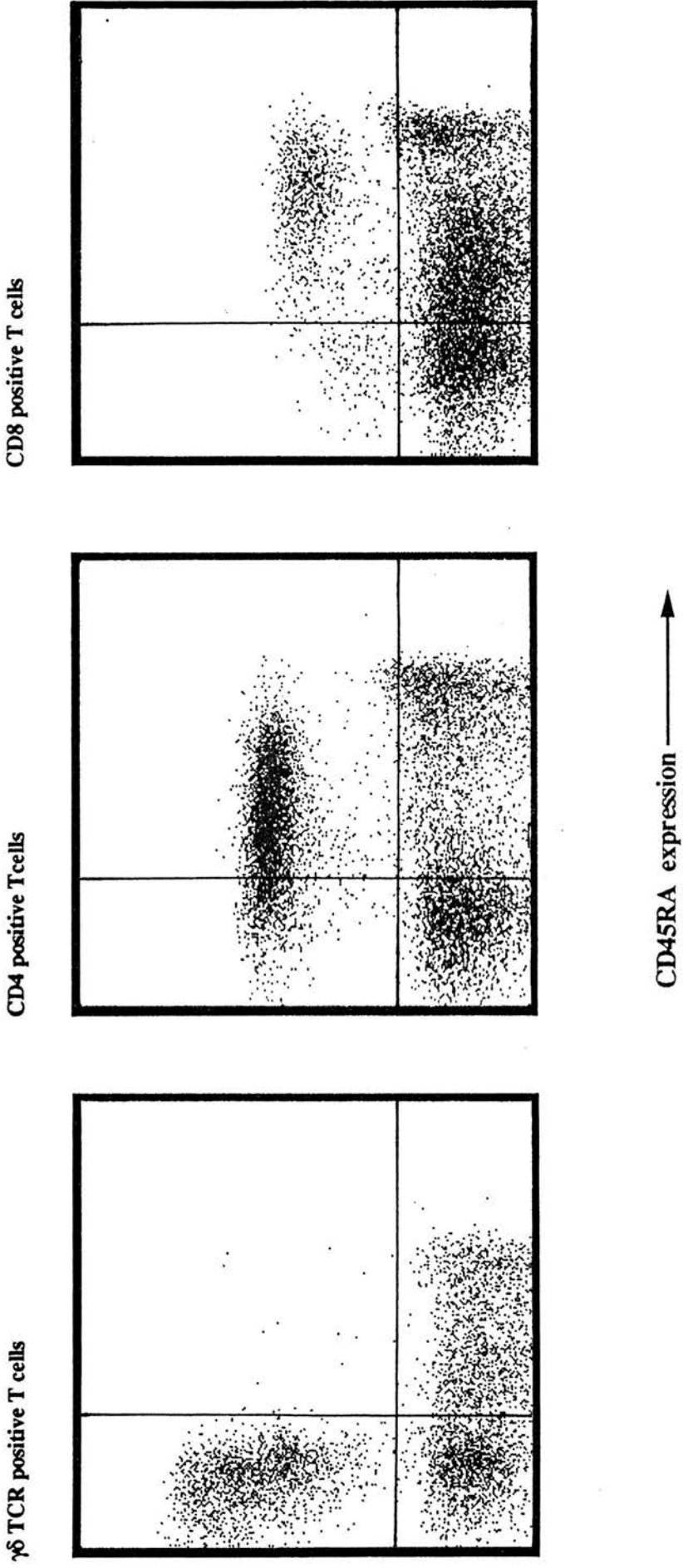
Figure 5.9: Single colour immunofluorescence analysis of MACS depleted PBL.

PBL were isolated from 6 month old sheep (n=3) and stained with anti-CD25 and anti-CD45 followed by biotinylated anti-IgG and streptavidin-microbeads and SA-PE. Single colour immunofluorescence profile of cells before fractionation and after depletion of CD25-positive and CD45RA-positive cells by passage through a MACS column are shown. The figure shows the positive staining of PBL from one animal compared to the non-specific staining of NMS (always <5%). This experiment was repeated three times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 5.9: Single colour immunofluorescence analysis of MACS depleted PBL.
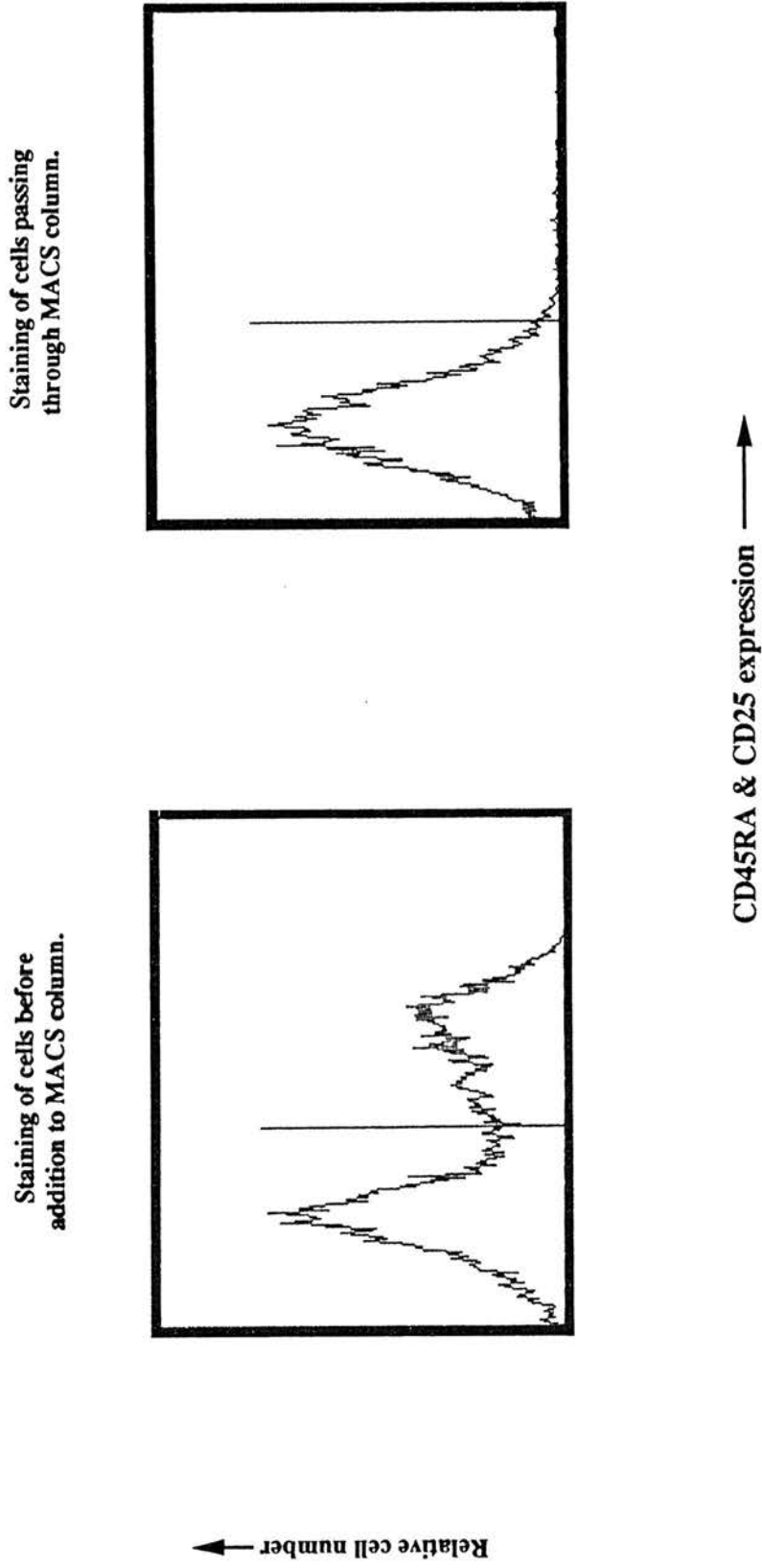
Staining of cells before
addition to MACS column.

Staining of cells passing
through MACS column.

Relative cell number

CD45RA & CD25 expression

Table 5.8:   Phenotype of MACS-depleted PBL after culture with ConA.

PBL were isolated from 6 month old sheep (n=3) and depleted of CD25-positive cells and CD45RA-positive cells and cultured with 5μg/ml ConA. At the times indicated, viable lymphocytes were harvested and 10^6 cells stained with either anti-CD25, anti-MHC class II or anti-CD45RA followed by anti-IgG FITC and either biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8 then SA-PE. The cells were analysed by FACScan and the percentage of each T cell subset which expressed either CD25, MHC class II or CD45RA was calculated. The figure shows the percentage of positive staining of cells compared to the non-specific staining of NMS versus and b-NMS (always <5%). The results shown are the mean values of three animals. The standard deviation of the mean is shown in the brackets. This experiment was repeated twice and the same trends were seen with each animal and on each occasion. The significance in the rate of expression of CD25 by each T cell subset was determined by performing a two sample t-test comparing the level of expression at different time points. The probability that the differences in rates are significant is represented in the table. The significance of any re-expression of CD45R was determined by regression analysis of the data. A General Linear Model was used to analyse the data with time as a covariate. The probability that the differences in the data are significant is represented below each column.

TABLE 5.8: Phenotype of MACS depleted PBL after culture with ConA.

Hours after treatment.	CD25 expression.			MHC Class II expression.			CD45RA expression.		
	γδ T cells	CD4+ cells	CD8+ cells	γδ T cells	CD4+ cells	CD8+ cells	γδ T cells	CD4+ cells	CD8+ cells
0	2.6 (2.1)	17.6 (6.8)	1.4 (1.0)	19.0 (5.5)	59.5 (7.8)	73.8 (7.0)	0.9 (0.7)	0.4 (0.3)	1.7 (2.0)
4	23.1 (3.8)	38.6 (10.2)	17.6 (8.2)	31.8 (9.9)	71.4 (3.8)	81.8 (3.7)	0.9 (0.4)	0.5 (0.1)	1.5 (0.5)
8	[77.5 (1.9)] *	63.1 (10.1)	37.5 (10.8)	[85.6 (6.2)] *	92.6 (1.0)	96.2 (1.7)	1.3 (0.7)	1.4 (1.0)	1.8 (0.3)
16	86.5 (3.1)	69.1 (11.8)	54.7 (7.7)	94.5 (2.4)	93.8 (0.9)	99.7 (0.4)	2.1 (0.9)	3.4 (0.5)	4.2 (0.8)
24	90.6 (2.5)	76.9 (12.5)	58.0 (9.8)	96.7 (3.2)	97.0 (2.1)	99.7 (0.5)	4.8 (4.0)	4.1 (1.9)	9.3 (5.0)
							N.S.D.	N.S.D.	N.S.D.

[ ] rate of increase from 4hrs to 8hrs significantly different to CD4+ and CD8+   * p<0.05

N.S.D no significant difference

DISCUSSION:

Following *in vitro* stimulation with the T cell mitogen ConA, the γδ T cell population within unfractionated ovine PBL increased in size during the first day of culture. Comparatively similar increases in size of the CD4-positive and CD8-positive T cell sub-populations were also seen, though this took up to 4 days. The ratio of CD4-positive plus CD8-positive T cells subsets to γδ T cells emphasises the marked differences in rates of population expansion observed. These differences in population sizes following stimulation suggested that there may be differences in the rates of activation of the different T cell subsets. An analysis of CD25 (IL-2Rα) and MHC class II expression by the different T cell subsets was performed. The cell surface expression of these proteins is increased upon activation. Initial experiments showed differences in CD25 expression by the γδ T cell subset compared to CD4-positive and CD8-positive T cells after 24 hours culture of PBL with ConA. An analysis of the kinetics of activation during the first 24 hours of culture with ConA revealed a faster rate of activation by γδ T cells compared to CD4-positive and CD8-positive T cells. Similar results were seen for both of the markers analysed. γδ T cells can be divided into subsets based on the type of WC1 epitopes expressed on their cell surface. The various sub-populations of γδ T cells all showed similar kinetics of activation following treatment with ConA.

The expression of the MHC class II β-chain on activated γδ T cells showed similar kinetics to that of CD25 expression. The monoclonal antibody SW73.2 recognises all ovine MHC class II β-chains products from both of the identified loci (Hopkins et al., 1986). MHC class II α-chain expression could not be analysed with one monoclonal antibody. The products of the two different loci were identified with two different monoclonal antibodies (Dutia et al., 1993; Hopkins et al., 1993). MHC class II β-chains were expressed on all T cell subsets following stimulation with ConA. In contrast, the products of the two MHC class II α-chain loci were expressed on less than 30% of each T cell subset after culture with ConA. Each MHC class II protein comprises an α-chain and a β-chain. These results therefore, indicate that more MHC class II loci may exist in the ovine species than have so far been identified. Possibly a DP loci exists as in the human species.

Control experiments revealed the following: i) a small increase in CD25 expression on all lymphocyte subsets following culture without ConA. ii) each lymphocytes subset remained equally viable during culture. iii) each subset of cells which passed through the Lymphoprep expressed a similar level of CD25 and MHC class II. These results clearly indicated that the apparent faster rate of activation by γδ T cells was not the result of the purification procedure, or culture conditions used. Therefore, it can be stated that in response to the mitogen ConA, γδ T cells become activated at a much faster rate than do CD4-positive or CD8-positive T cells. Recent observations (Verhagen et al., 1993) concurred with the results presented in this chapter. In these results however, a period of preculture was required before differences in the activation of γδ T cells compared to CD4-positive or CD8-positive cells was observed when cultured with ConA. In our observations, no preculture of PBL was required and furthermore, culture of PBL in medium alone resulted in a modest increase in CD25 expression by γδ T cells in contrast to CD4-positive or CD8-positive T cells did.

It may be assumed from the observations presented in this chapter that activation of γδ T cells *in vivo* will occur more quickly than activation of CD4-positive or CD8-positive T cells. Recent observations (A. Anderson, personal communication) suggest this to be the case. The appearance

of CD25-positive γδ T cells in an inflamed joint occurred earlier, and more markedly, than did the appearance of CD4-positive or CD8-positive T cells expressing CD25.

In the human species, naive cells can be distinguished from recently activated cells by the expression of two of the different CD45 isoforms, CD45RA and CD45RO respectively (Clement, 1992). The external domain of CD45 is divided into four sub-domains (Pingel and Thomas 1989). The differential exon usage of CD45 results in changes in the O-linked carbohydrate region in the amino terminus of the external domain. These changes will result in differences in the structure of the protein. If the different isoforms of CD45 bind different ligands, then expression of the different epitopes may have a significant impact on lymphocyte function. Resting peripheral blood γδ T cells express both CD45RA and CD25 at low levels whereas resting peripheral blood CD4-positive and CD8-positive T cells express much higher levels of CD45RA and CD25. It was shown here that these phenotypic differences in the two T cell lineages did not account for the differences observed in rates of activation between these cells. PBL depleted of all CD45RA-positive and CD25-positive cells were cultured in the presence of ConA. γδ T cells expressed higher levels of CD25 at a faster rate than did CD4-positive and CD8-positive T cells.

The concentration of ConA used routinely throughout this thesis was 5μg/ml. It should however be noted that the responses of T cell subsets to ConA may not be optimal as no titration of a dose response was carried out. The results detailed in this chapter therefore only detail the responses of T cell subsets under one set of conditions. The responses of the different T cell subsets may be different in alternative conditions.

There are a number of explanations for the observed differences in CD25 and MHC class II β-chain expression by γδ T cells, CD4-positive T cells and CD8-positive T cells. Firstly γδ T cells may be in a pre-activated state compared to CD4-positive and CD8-positive T cells. This was suggested by the CD45 isoform expression of γδ T cells compared to CD4-positive and CD8-positive T cells and by the expression of CD25 on γδ T cells when cultured without ConA. The majority of ovine γδ T cells do not express CD45RA and therefore display a memory phenotype. In contrast, the majority of the CD4-positive and CD8-positive T cell populations express CD45RA. Secondly, different signal transduction pathways may exist for the two T cell lineages. In the ovine species, γδ T cells do not generally express the proteins CD2, CD4 or CD8 (MacKay et al., 1989; Evans, 1993). This suggests that there is either no associated p56[lck] or p59[fyn] activity and no requirement for dephosphorylation by the CD45 associated phosphatase or that dephosphorylation of these molecules occurs by another signal transduction pathway. Furthermore, it has been reported that human γδ and αβ T cells exhibit differences in their TCR associated signal transducing element, the CD3 complex (Brenner et al., 1988). Since ConA induces some of its mitogenic effects on T cells via proteins such as those of the CD3 complex, variability in this structure between cell types may result in different responses to stimuli. Thirdly, the transcriptional and translational control of, at least CD25 and MHC class II molecules may be different between the two T cell subsets. It was shown that the faster rate of activation observed by Verhagen (1993) was not as a result of an accumulation of CD25 mRNA or translated products. It was proposed that the faster rate of activation by γδ T cells may be as a result of accumulation of specific transcription factors for the CD25 gene such as NFκB and KBF1. The transcription factors NFκB and KBF1 bind the CD25 κB sequence (Hemar et al., 1991). Alternatively, γδ T cells may constitutively express higher levels of the CD25 specific transcription factors. This constitutive expression by γδ T cells would enable an increase in the level of expression of this molecule to occur very rapidly.

The results presented in this chapter have focussed on the response of γδ T cells to mitogenic stimulation. These intriguing results led to the proposition that in an antigen-specific response, γδ T cells would similarly respond at a quicker rate than do CD4-positive or CD8-positive T cells. The following chapter investigated the response of γδ T cells to antigen.

CHAPTER 6

γδ T cell responses to antigen

INTRODUCTION:

The T cell receptor for antigen (TCR) consists of a heterodimeric structure of either $\alpha\beta$ or $\gamma\delta$ polypeptide chains (Brenner et al., 1986). The receptor for antigen on $\alpha\beta$ T cells recognises fragments of antigen presented on the surface of APC in association with the classical presentational element MHC class I or MHC class II molecules (Kronenberg et al., 1986). Associated non-covalently with the TCR are five polypeptide chains ($\gamma,\delta,\varepsilon,\zeta,\eta$) which comprise the CD3 complex. The CD3 complex has been implicated in signal transduction (Clevers et al., 1988). CD3 proteins are invariant in sequence and assemble in the same combination on T cells, irrespective the TCR expressed by the cell.

Most cell-mediated immune responses appear to be carried out by the $\alpha\beta$ T cell population. Because of the similarity between $\alpha\beta$ and $\gamma\delta$ T cell receptor genes, and their predicted proteins, it has been assumed that $\gamma\delta$ T cells would recognise antigen in an analogous manner to $\alpha\beta$ T cells. However, this has not been established (Haas et al., 1993). In addition, it has been suggested that the contribution of $\gamma\delta$ T cell to the immune system is distinct to that of $\alpha\beta$ T cells (Schild et al., 1994). The only examples of $\gamma\delta$ T cell antigen recognition are those observed with cloned $\gamma\delta$ T cell lines (Haas et al., 1993). These $\gamma\delta$ T cell lines recognise a diverse array of antigens including tetanus toxoid (Kozbor et al., 1989), a synthetic polypeptide polyglutamate tyrosine (GAT) (Vidovic et al., 1989), bacterial superantigens such as staphylococcal enterotoxin A (Rust et al., 1990) and mycobacterial antigens (Kabelitz et al., 1990, Janis et al., 1989) including mycobacterial heat shock proteins (O'Brien et al., 1989). Some reports have proposed that $\gamma\delta$ T cells respond to self proteins including immunoglobulin (Wright et al., 1989) and autologous stress proteins (O'Brien et al., 1991).

Antigen recognition by $\alpha\beta$ T cells involves non-covalent interaction between CD4 and MHC class II molecules, or between CD8 and MHC class I molecules. Since the majority of $\gamma\delta$ T cells do not express CD4 or CD8, it is suggested that $\gamma\delta$ T cells do not respond to antigen in a manner analogous to $\alpha\beta$ T cells. Work with many of the proposed antigen-specific $\gamma\delta$ T cell clones detailed above concurred with this and showed no MHC restriction (Holoshitz et al., 1993; Schild et al., 1994)

It has further been hypothesised that $\gamma\delta$ T cells recognise antigen in association with presentational elements other than MHC molecules. There is evidence that $\gamma\delta$ T cell clones recognise antigen via presentational elements which are non-polymorphic (Tonegawa et al., 1989; Janeway et al., 1988). These molecules are similar to the classical MHC class I molecules and are termed MHC class Ib molecules. Genes for these molecules are found within the MHC locus. In humans these loci are HLA-E, HLA-F and HLA-G (Rothbard et al., 1989). In mice these are the Qa and Tla loci (Flaherty et al., 1990). MHC class Ib recognition by $\gamma\delta$ T cells was seen with cloned mouse $\gamma\delta$ T cell lines. These $\gamma\delta$ T cell lines were found to recognise non-polymorphic molecules mapping to the Tla region of the mouse genome (Ito et al., 1989, Vidovic et al., 1989). CD1 has also been proposed as a presentational element for human $\gamma\delta$ T cells (Porcelli et al., 1991). This molecule is evolutionarily related to classical MHC class I molecules, but is encoded by genes outwith the MHC locus (Calabi and Milstein, 1986). The CD1 molecules, like MHC class I, associate non-covalently with β_2-microglobulin.

Distinct differences between $\alpha\beta$ and $\gamma\delta$ T cells becomes apparent when the alloreactivity of these populations is studied. Allorecognition, is the *in vitro* cross-reactivity of lymphocytes with

foreign MHC and peptide. It is believed that a structure, or shape, on the MHC/peptide complex of allogeneic cells is recognised by the TCR. This structure, or shape, is similar to that of the conventional MHC/antigen which the T cell would recognise. Most of an allogeneic response is due to the recognition of structures, or shapes, on MHC class II molecules although a small amount of proliferation to MHC class I can occur (Sherman and Chattopadhyay, 1993). A large proportion of αβ T cells show alloreactivity in a mixed lymphocyte culture, in contrast, γδ T cells do not show alloreactivity (Cabone et al., 1991).

Sheep have a higher proportion of peripheral blood γδ T cells and these have a greater diversity within the variable region of their TCR than do either the human or murine species (Hein and Dudler, 1993). This suggests that γδ T cells in the ovine species have developed, or retained, different levels of genetic and functional complexity, and that the ovine species relies more on the function of γδ T cells within its immune system. It was recently reported in sheep (Bujdoso et al., 1993) that 60-70% of γδ T cells in the afferent lymph expressed the receptor for IL-2. This observation might be explained by the recirculatory patterns of γδ T lymphocytes which may concentrate activated γδ T cells into the afferent lymphatics (Bujdoso et al., 1993). Alternatively, γδ T cells may recognise ligands at the epithelial sites from which these lymphatics drain and become activated. Subsequently, these cells may drain through the afferent lymphatics to disseminate the immunological response to the antigen.

This chapter has concentrated on determining the antigen specificity of the γδ T cell population in the sheep. If the role of γδ T cells within the immune response is similar to that of αβ T cells it will be necessary to define a requirement for foreign antigen by γδ T cells and that which results in an active and proliferative response by γδ T cells. Secondly any potential requirement for presentational elements by γδ T cells which may be required for specific recognition of antigen need to be identified. The activation and proliferation of ovine γδ T cells both as a discrete population of cells and also as part of an unfractionated population of PBL was assessed in response to both antigen and allogeneic PBL.

RESULTS:

6.1 Proliferative response of unfractionated PBL to antigen.

Sheep were immunised with both ovalbumin (Ova) and *Mycobacterium paratuberculosis* purified protein derivative (M.tb-PPD). The secondary *in vitro* proliferative response by PBL from these primed animals was investigated. Figure 6.1 shows the typical dose response seen by PBL from each of the animals tested. The optimum concentration of Ova to which the PBL responded was 500µg/ml whilst that of M.tb-PPD was in the range 6.4µg/ml - 66.7µg/ml.

6.2 Counts per minutes (CPM) versus stimulation index (SI).

In considering responses to antigen, a common way to present proliferation data is to express the results as a stimulation index (SI). The SI is calculated as the number of times greater the response to the stimuli is than the response to medium alone. This method of representation is appropriate when comparing the responses of the same population of cells to different stimuli. However, this method is not useful when comparing the response of different populations of cells to the same stimuli. Figure 6.2 shows the proliferative responses of the various T cell populations to ovalbumin. The same data are presented as the SI (Fig 6.2A) and as specific CPM values (i.e. CPM experimental minus CPM control) (Fig 6.2B). The data expressed as SI showed little difference between the responses to antigen by the γδ T cell and αβ-T cell populations. However when the data was expressed as specific CPM values, the αβ-T cell population showed significant responses to antigen whereas γδ T cell responses to antigen were insignificant. The use of specific CPM was regarded as a more accurate representation of the data and is used to represent data in all of the proliferation assays reported in this chapter.

6.3 Lymphocyte populations change in size after culture with antigen.

Ovine PBL were cultured with 500µg/ml ovalbumin and the phenotype of viable lymphocytes was analysed before and at various times after the start of culture. Table 6.1 shows the subset composition of the viable lymphocyte population on each day of culture with or without antigen. When PBL were cultured in the presence of ovalbumin there was a significant increase in the size of the γδ T cell population on day 2 with no corresponding increase in the CD4-positive and CD8-positive T cell populations. This was reflected in the CD4-positive plus CD8-positive T cell/γδ T cell ratio on day 2 of culture which was significantly less than that seen at the start of the experiment. A similar trend was seen when cells were cultured in the presence of *M.tb.*-PPD (data not shown). When cells were cultured in the absence of antigen no significant increase in the γδ T cell population was seen on day 2 and no significant changes were seen in the CD4-positive plus CD8-positive T cell/γδ T cell ratio during the culture period. At all time points, and when cells were cultured in the presence or absence of antigen, the γδ T cell population were positive for cell surface expression of WC1 (data not shown).

6.3.1 All γδ T cells become activated when unfractionated PBL are cultured with antigen.

γδ T cells become the predominant T cell population following culture of ovine PBL from animals primed with antigen. Accordingly, the activation of γδ T cells in response to antigen was determined. The assay for T cell activation was cell surface expression of CD25 and MHC class II

Figure 6.1: Proliferative response of unfractionated PBL to antigen.

10^5 purified ovine PBL were cultured in round-bottomed microculture plates with various concentrations of either (A) ovalbumin (—◆—) or (B) *M.tb*.-PPD (—◆—). Cultures were incubated for 5 days and the proliferation measured by the uptake of ^{3}H-thymidine over the last 5 hours of culture. The proliferative response to medium alone is also shown (—□—). The data is expressed as the arithmetic mean of cpm from triplicate cultures. This experiment was repeated eight times and the same trends were seen on each occasion.

FIGURE 6.1: Proliferative response of unfractionated PBL to antigen.

(A) Response to Ovalbumin.
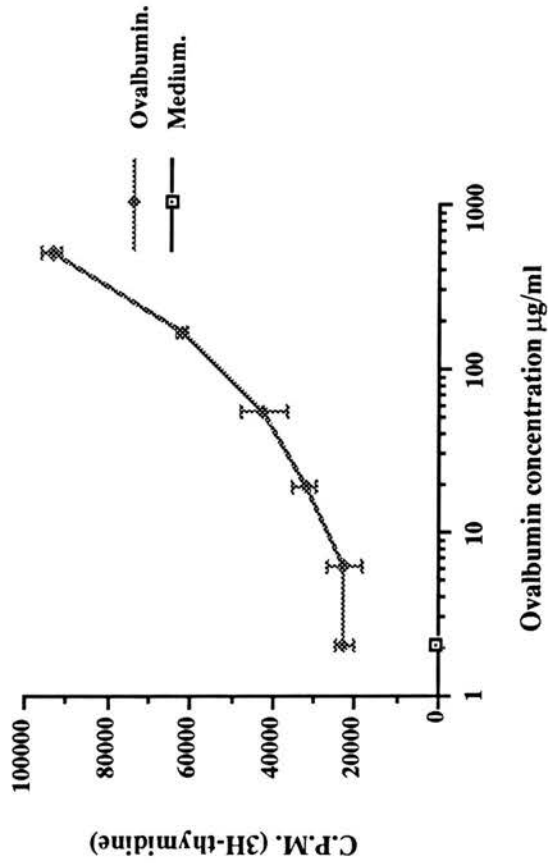
(B) Response to *M.tb.*-PPD
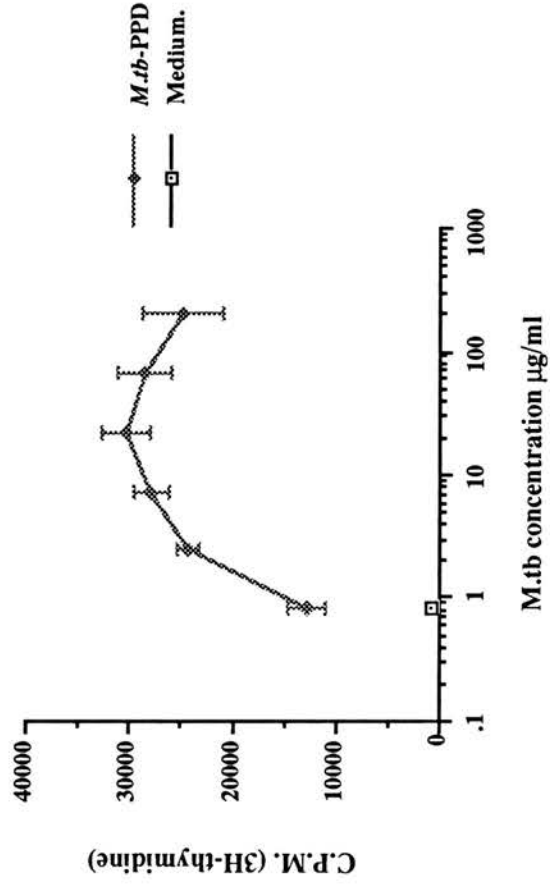
Figure 6.2:   **Counts per minute versus stimulation index.**

Unfractionated PBL (■), γδ positive T cells (◪) and γδ depleted T cells (◪) were prepared from antigen primed sheep (n=3). 10^5 cells of each population were cultured with optimum concentrations of antigen. In addition, γδ positive T cells were cultured in the presence (▓) or absence (◪) of 10^4 irradiated autologous PBL as antigen presenting cells. Cultures were incubated for 3, 4 or 5 days. The proliferative responses were measured by the incorporation of ^{3}H-thymidine over the last 5 hours of culture. Figures 6.2A and 6.2B show the same sets of data from one animal. The stimulation index (fig 6.2A) is calculated as the number of times the response to antigen is greater than the response in medium alone. Figure 6.2B show the specific CPM values which are the CPM of the response to antigen minus the CPM of the response in medium alone. The values are given as the arithmetic mean of triplicate cultures; error bars in figure 6.2B show the standard deviation. This experiment was repeated five times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 6.2: Counts per minute versus stimulation index.
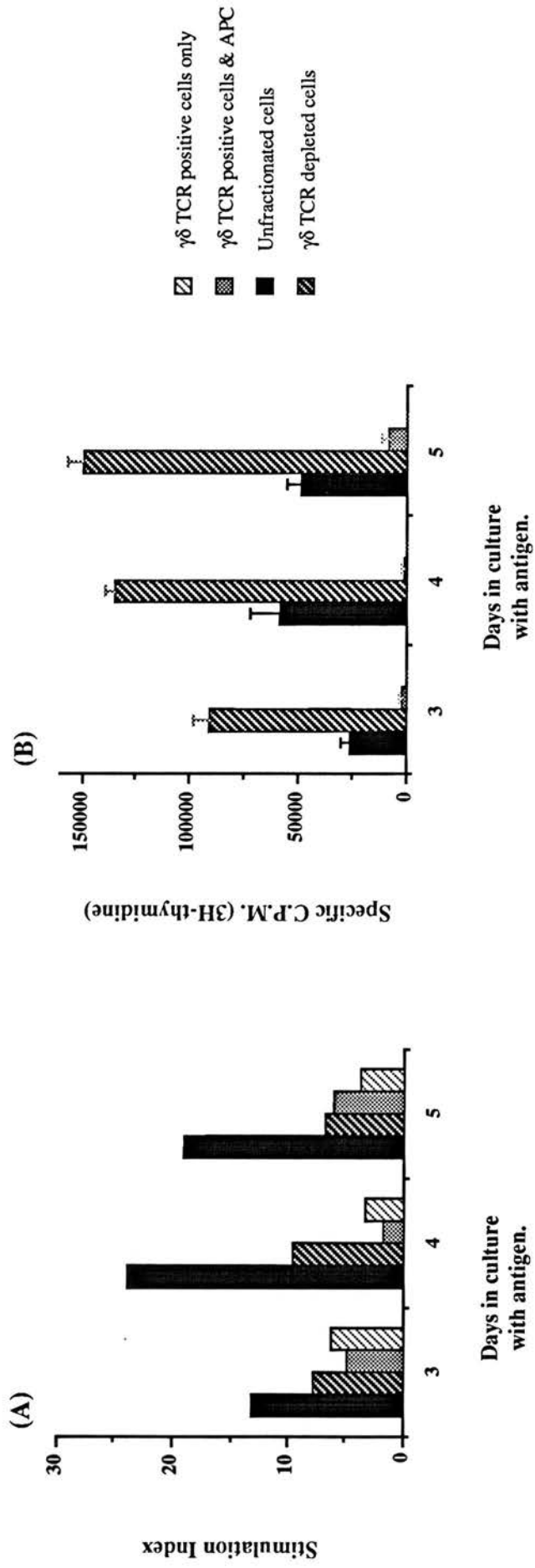
TABLE 6.1: T cell composition of PBL after culture with antigen.

Ovalbumin

Days after start of culture.	Percentage of T cells in lymphocyte population.			Ratio of CD4-postive plus CD8-positive to γδ T cells
	γδ	CD4	CD8	$\dfrac{CD4 + CD8}{\gamma\delta}$
0	20.9 (3.9)	31.3 (5.4)	18.0 (5.1)	2.36
1	23.5 (4.4)	34.0 (5.3)	19.7 (2.5)	2.29
2	[44.0 (8.1)] *	20.5 (4.3)	13.6 (1.3)	[0.78] **
3	27.1 (6.4)	32.9 (6.0)	22.1 (5.7)	2.03
4	33.0 (7.2)	24.4 (5.2)	19.2 (4.1)	1.32
5	33.0 (7.8)	30.7 (7.0)	19.1 (8.4)	1.51

No Antigen

Percentage of T cells in lymphocyte population.			Ratio of CD4-postive plus CD8-positive to γδ T cells
γδ	CD4	CD8	$\dfrac{CD4 + CD8}{\gamma\delta}$
20.9 (3.9)	31.3 (5.4)	18.0 (5.1)	2.36
22.7 (3.2)	36.4 (6.3)	19.6 (1.0)	2.47
26.0 (8.4)	35.3 (4.1)	18.2 (8.3)	2.06
24.2 (5.4)	38.6 (7.8)	24.9 (4.1)	2.62
22.8 (8.7)	36.9 (8.6)	24.5 (3.5)	2.69
26.0 (9.9)	39.9 (10.8)	25.7 (3.1)	2.52

[ ] significantly different from start of experiment
* $p<0.05$ ** $p<0.005$

molecules. Figure 6.3 shows the proportion of each T-cell subset which expressed the CD25 after culture with antigen. The results show that >80% of γδ T cells expressed CD25 after culture with either ovalbumin or *M.tb.*-PPD. Only 40-45% of CD4-positive T cells, and 20-25% of CD8-positive T cells, expressed CD25 in the same cultures. The results also showed that the greatest number of γδ T cells expressed CD25 on day 1. An analysis of the kinetics of activation during the first 24 hours of culture showed that the greatest number of γδ T cells expressed CD25 only after 24 hours of culture with antigen (data not shown). The greatest number of CD4-positive, and CD8-positive T cells, which expressed CD25 was seen on day 2 of culture. Similar trends were seen for MHC class II expression on the T cell subsets after culture with antigen (data not shown). It was also observed that γδ T cells did not remain more viable in *in vitro* culture with antigen than did any of the other T cell subsets (data not shown). In addition, no significant increase in activation of any T cell subset was seen when PBL were cultured without antigen (data not shown). This implied that the activation of the γδ T cell subset was due to the presence of the antigen and not due to an artifact of the tissue culture system used.

6.4 Isolation of purified populations of γδ T cells.

The majority of ovine γδ T cells become activated when unfractionated PBL are cultured with antigen. This is indicative of antigen-specific activation of γδ T cells. The distinction between antigen-specific polyclonal activation of γδ T cells or by-stander activation of these cells as a consequence of antigen specific αβ T cell activation was investigated.

Ovine γδ T cells were purified from the peripheral blood of animals previously immunised with ovalbumin and *M.tb.*-PPD. Selection of γδ T cells was made via the WC1 surface proteins using the biotinylated anti-WC1 monoclonal antibody CC15 linked to magnetic microbeads. It was shown in chapter 3 that the anti-WC1 monoclonal antibody CC15 recognises an epitope expressed on all γδ T cells. In addition, it was shown during the course of this thesis (data not shown) and previously (Evans, 1993) that purification of γδ T cells via the WC1 molecule does not activate these cells. SA-PE was used to identify those cells labelled with magnetic beads. Figure 6.4 shows the immunofluorescence profiles of MACS fractionated cells. During purification, WC1-positive cells were retained on the MACS column, whilst those cells not expressing WC1 passed through the column. The purification procedure always yielded WC1-positive T cell populations of >95% purity which were used in subsequent assays.

Single colour FACS analysis of the purified WC1-positive cells is shown in Figure 6.5. The majority (96%) of the cells expressed the γδ TCR epitope recognised by the monoclonal antibody 86D. A small percentage cells reacted with either the anti-CD4, anti-CD8, or anti-B cell monoclonal antibodies. The CD4-positive, CD8-positive, and B cell marker-positive purified cells are described as the 'contaminating population' and on each occasion represented 10-12% of the positively selected cells. Figure 6.6 shows that all of the purified cells which stained with either anti-CD4, anti-CD8, or anti-B cell monoclonal antibodies also expressed the WC1 protein. The intensity of WC1 expression on these contaminating cells was lower than that seen on the other positively selected WC1 cells. The MACS purified γδ T cells were used as purified populations in the following assays. The percentage of purified WC1-positive cells which expressed CD4, CD8, or the B cell marker was similar to observations with peripheral blood (data not shown). Two-colour immunofluorescence showed that there was always a small percentage of peripheral blood WC1-positive cells which expressed CD4, CD8, or the B cell marker.

Figure 6.3:   CD25 expression by T cell subsets after culture with antigen.

Purified ovine PBL were cultured with either 500µg/ml ovalbumin (———◆———), 66.7µg/ml *M.tb.-*
PPD (········※········) or no antigen (———□———) for the times indicated. Viable cells were harvested and 10⁶
cells and stained with biotinylated anti-WC1, biotinylated anti-CD4 or biotinylated anti-CD8
followed by SA-PE and anti-CD25 followed by anti-mouse IgG1 FITC and analysed by FACScan.
The percentage of each T cell subset which expressed CD25 was calculated at the various time
points. The values shown were determined with reference to staining of NMS versus b-NMS
(always <5%). The figure shows the mean values obtained from three animals. The error bars
show the standard deviation of the results. This experiment was repeated five times and the same
trends were seen with each animal and on each occasion tested.

FIGURE 6.3: CD25 expression by T cell subsets after culture with antigen.
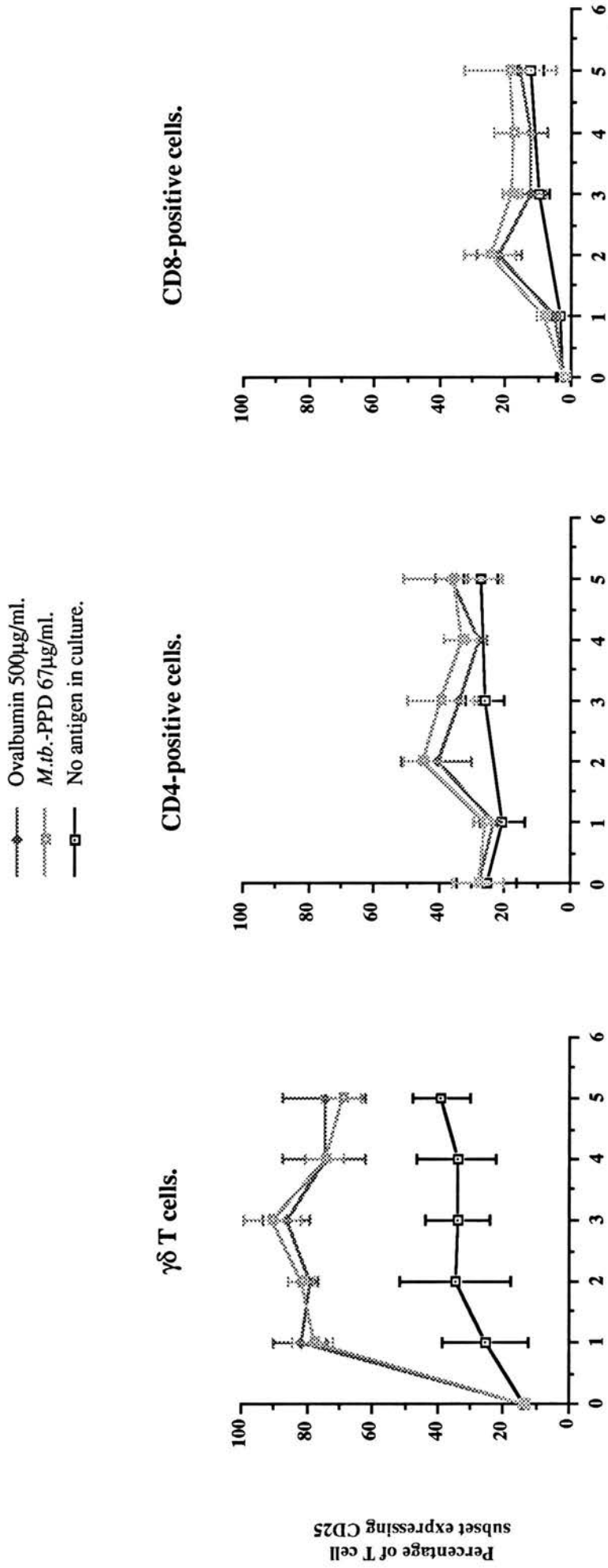
Figure 6.4: **Purification of γδ T cells.**

Flow cytometric analysis of ovine PBL before and after positive selection of γδ T cells. Each histogram shows PBL from one animal stained with biotinylated anti-WC1 monoclonal antibody then SA-PE. Cells analysed by FACScan were: i) before addition to the MACS column (Pre-MACS); ii) cells positively retained on the MACS column; and iii) cells which had passed through the MACS column. The percentage of positive cells was determined with respect to NMS as a negative control (always <5%). This experiment was repeated ten times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 6.4: Purification of γδ-T cells
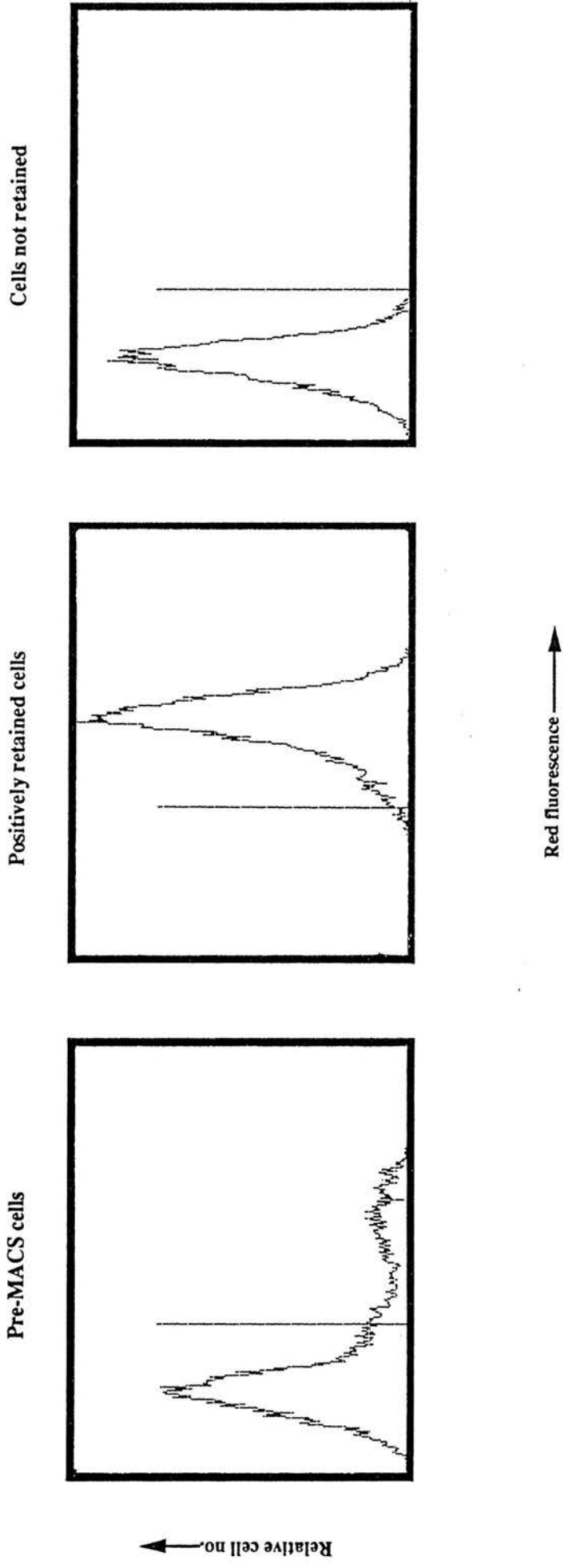
Pre-MACS cells

Positively retained cells

Cells not retained

Relative cell no.

Red fluorescence

Figure 6.5: **Single colour immunofluorescence of positively selected γδ T cells.**

Flow cytometric analysis of positively selected γδ T cells. Positively selected γδ T cells were stained with either NMS, anti-γδ TCR, anti-CD4 or anti-CD8 followed by anti-isotype specific FITC. The histograms show the percentage of positive PBL from one animal with respect to NMS as a negative control (always <5%). This experiment was repeated ten times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 6.5: Single colour immunofluorescence of positively selected γδ T cells

B cell

86D

CD4

CD8

Green Fluorescence ⟶

⟵ Relative cell no.

Figure 6.6: Two-colour immunofluorescence of positively selected γδ T cells.

Two-colour flow cytometric analysis of positively selected γδ T cells from one animal. Cells were stained with either NMS, anti MHC class I, anti-CD4, anti-CD8 or anti-WC1 followed by anti-isotype specific FITC. Cells were selected on the MACS column by reactivity with biotinylated anti-WC1 monoclonal antibody and were therefore already stained with SA-PE. The quadrants were set with respect to the NMS staining as shown. This experiment was repeated five times and the same trends were seen with PBL from each animal and on each occasion tested.

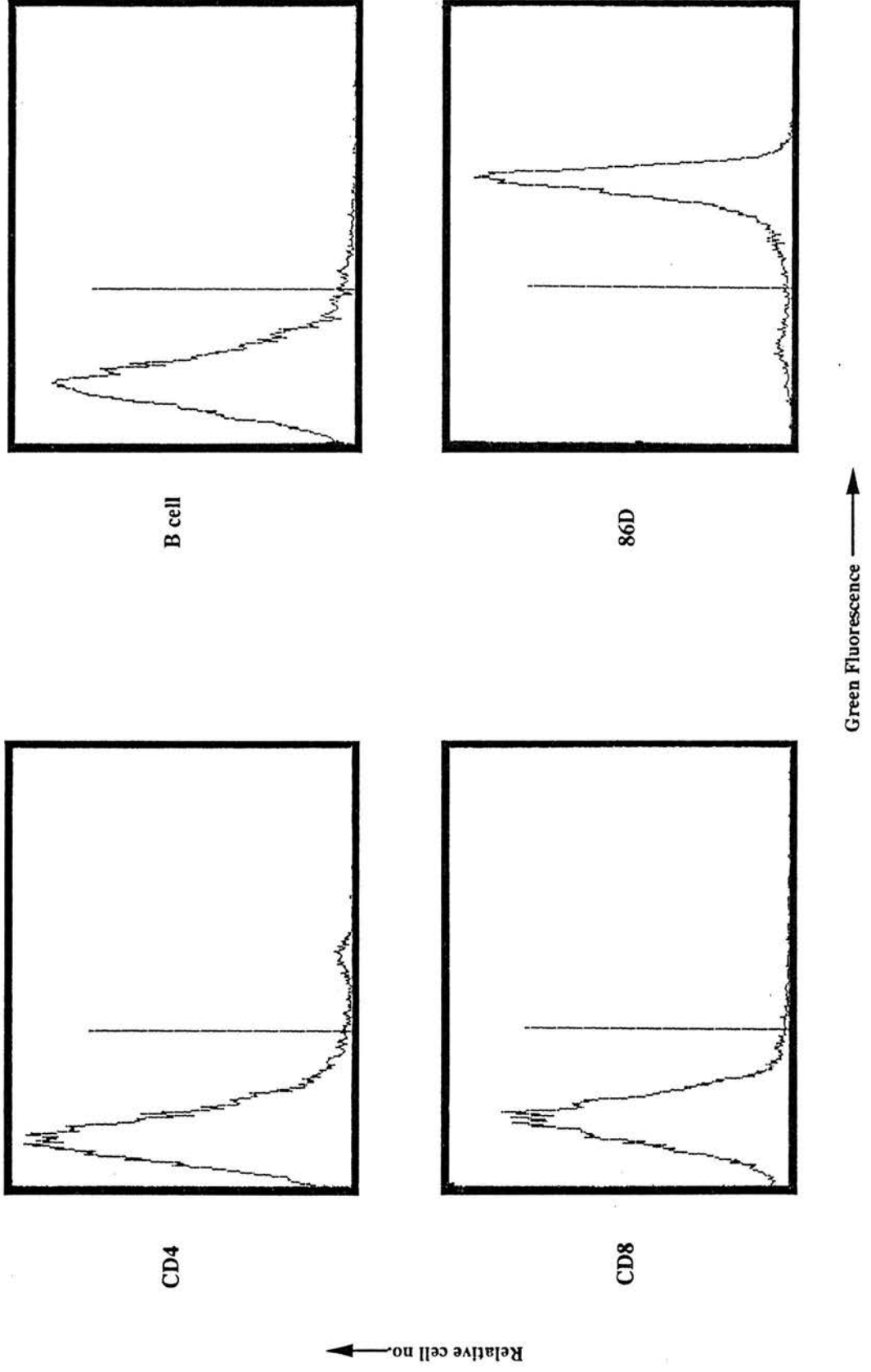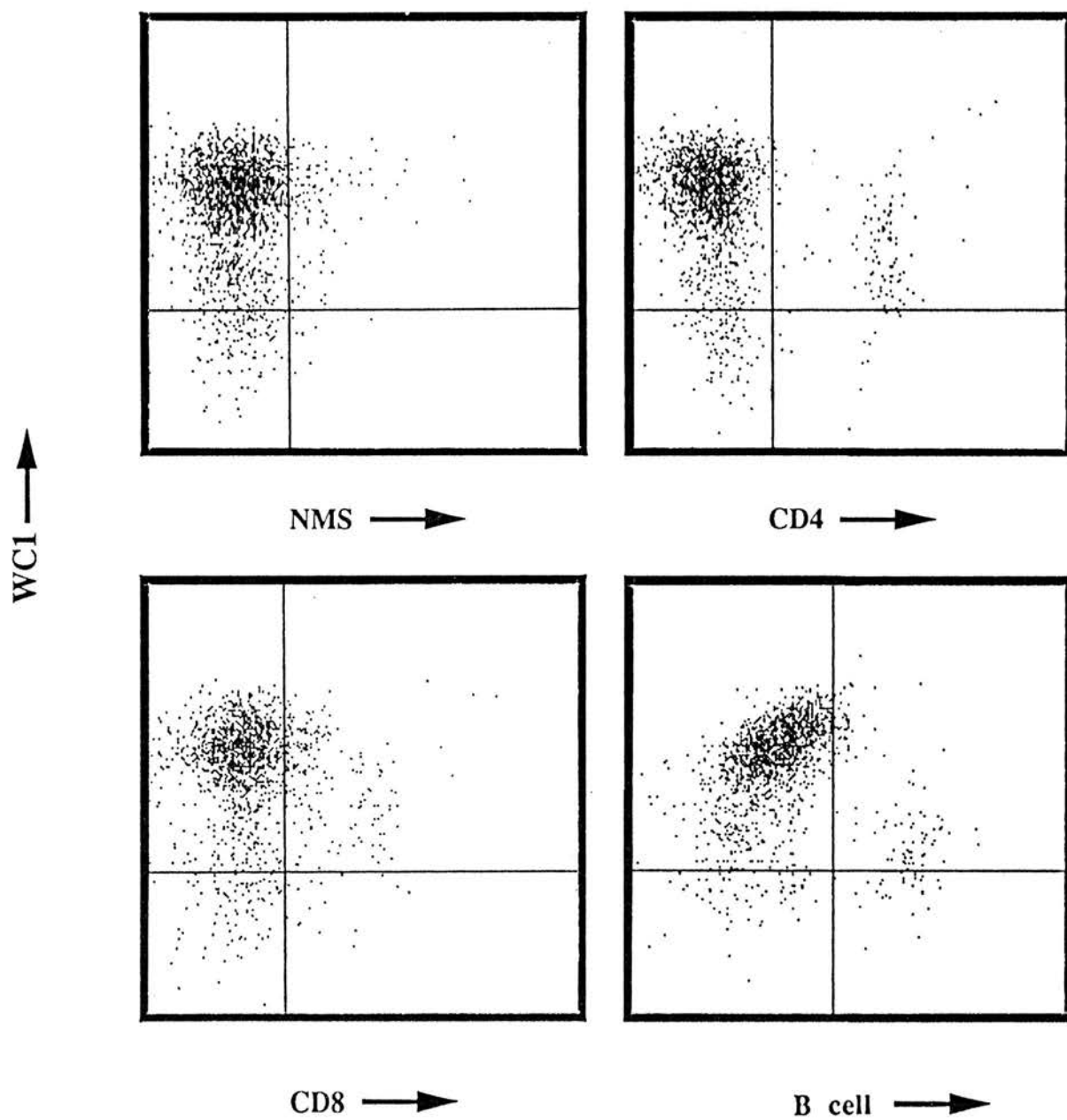FIGURE 6.6: Two-colour immunoflourescence of positively selected γδ T cells.

The only other T cells which will be found in the γδ-depleted lymphocyte population are CD4-positive and CD8-positive αβ T cells. The γδ-depleted lymphocyte population is termed the αβ T cell population. The presence of other peripheral blood mononuclear cells subsets is not neglected.

6.4.1 Purified γδ T cells proliferate in response to ConA.

γδ T cells purified by MACS separation are cell-surface labelled with monoclonal antibody, magnetic beads and streptavidin-phycoerythrin. It was determined that this had no affect on the ability of γδ T cells to proliferate under normal circumstances. Unfractionated cells, and purified γδ T cells, were assayed *in vitro* in the presence, and absence, of the T cell mitogen ConA to determine proliferative responses. The results are shown in figure 6.7. Purified γδ T cells responded significantly in the presence of ConA (Fig 6.7A). The response of purified γδ T cells in the presence of ConA was however, less than the proliferative response seen with unfractionated cells (Fig 6.7B). These results indicated that the procedure for purification of γδ T cells did not abrogate proliferative responses by these cells to ConA. It had previously been shown (Evans, 1993) that ConA stimulation of the purified γδ T cells had no effect on the phenotype of this population. In experiments in this thesis, similar results were observed. The γδ T cell population constituted 96% or more of the cells both before and after stimulation with ConA (data not shown).

6.4.2 Purified γδ T cells do not proliferate in response to antigen.

γδ T cells as part of an unfractionated population of PBL were shown to become activated following *in vitro* culture in the presence of antigen. In addition, purified γδ T cells were shown to proliferate in response to ConA. In this section, the proliferative response of purified γδ T cells to antigen was determined. The response of purified γδ T cells to antigen was measured both in the presence and absence of irradiated autologous PBL as antigen presenting cells (APC). The proliferative responses of purified γδ T cells were compared to those of unfractionated PBL and the αβ-T cell population. Figure 6.8 shows the proliferative responses of the different cell populations when each was cultured with an optimum concentration of either ovalbumin or *M.tb.*-PPD. The response by γδ T cells to the antigen, whether APC are present or not, was very low and often negligible. In addition, when γδ T cells are removed from PBL, the proliferative response of the remaining αβ-T cell population was significantly greater than that seen by the unfractionated population. The background level of ^{3}H-thymidine incorporation by the αβ-T cell population was also higher than seen by γδ T cells or unfractionated PBL (data not shown).

The response by ovine PBL to antigen was enhanced following the removal of γδ T cells. This may be due to either an increase in the number of antigen specific cells in the resultant culture or removal of a regulatory function mediated by γδ T cells. The proliferative response of αβ T cells reconstituted with various numbers of γδ T cells was examined to determine if any regulatory function could be observed. The αβ T cell population constitutes PBL which have been depleted of γδ T cells. All known APC found in peripheral blood will be present in the αβ T cell population therefore there is no requirement for autologous APC to be added to this assay. Figure 6.9 shows the results obtained when cells from three different animals were cultured with ovalbumin. Though reconstitution of αβ T cells with γδ T cells affected the actual observed counts of proliferative responses to antigen these observations were not significant. The observed increase in proliferation appeared to be dependent on the number of γδ T cells added back to the

Figure 6.7: Proliferative response of purified $\gamma\delta$ T cell populations in the presence of ConA.

Unfractionated ovine PBL and purified $\gamma\delta$ T cells were prepared and 10^5 cells from each purification were incubated with 5µg/ml ConA or in medium alone. Cultures were incubated for 5 days and proliferation was measured by the uptake of ^{3}H-thymidine over the last 5 hours of culture. Figure 6.7A shows the proliferative responses of purified $\gamma\delta$ T cells to ConA and medium. Figure 6.7B compares the proliferative responses of purified $\gamma\delta$ T cells in the presence of ConA to that of unfractionated PBL in the presence of ConA. Data are given as the arithmetic mean of triplicate cultures. Each symbol represents data from one animal. Errors were always <15% of the proliferative values.

FIGURE 6.7: Proliferative response of purified γδ T cells in the presence of ConA.
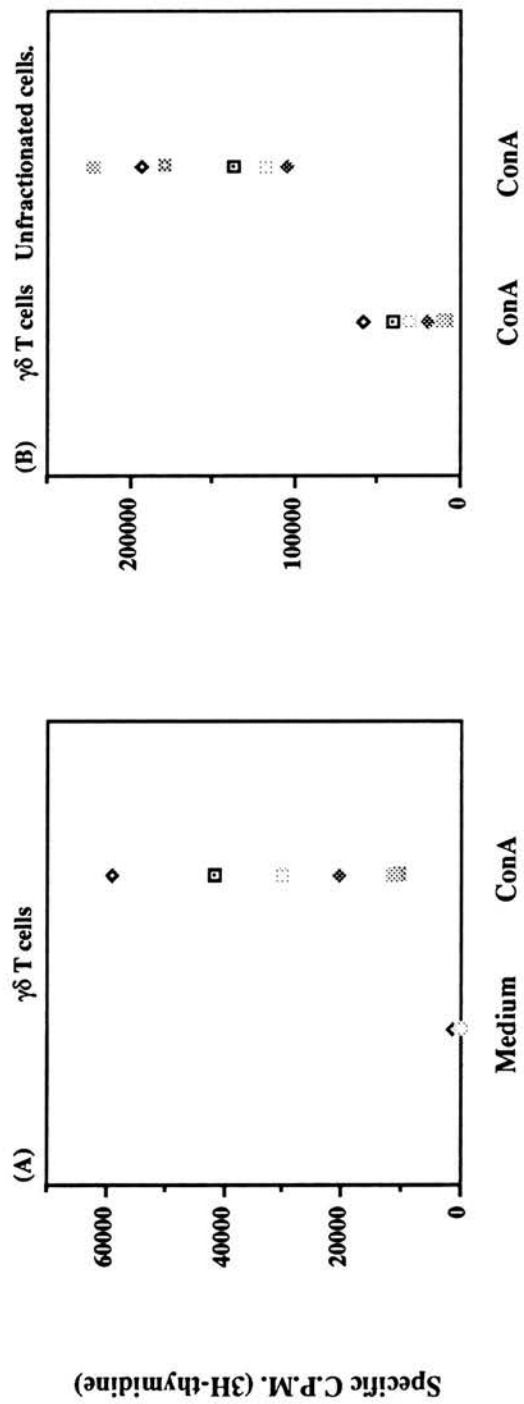
Figure 6.8: Proliferative response of T cell subsets to antigen.

PBL were isolated from immunised animals (n=3) and $\gamma\delta$ positive T cells (▨), $\gamma\delta$-depleted T cells (▨) and unfractionated lymphocytes (■) were prepared. 10^5 cells/well of each population were cultured with 500µg/ml of ovalbumin (A) or 66.7µg/ml *M.tb.* (B). In addition, $\gamma\delta$ positive T cells were cultured in the presence (▦) or absence (▨) of 10^4 irradiated autologous PBL as antigen presenting cells. Cultures were incubated for 1 to 5 days and proliferation was measured by the uptake of ^{3}H-thymidine over the last 5 hours of culture. Data are given as the arithmetic mean of the specific CPM values. Error bars show the standard deviations of the mean. This experiment was repeated five times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 6.8: Proliferative response of T cell subsets to antigen.
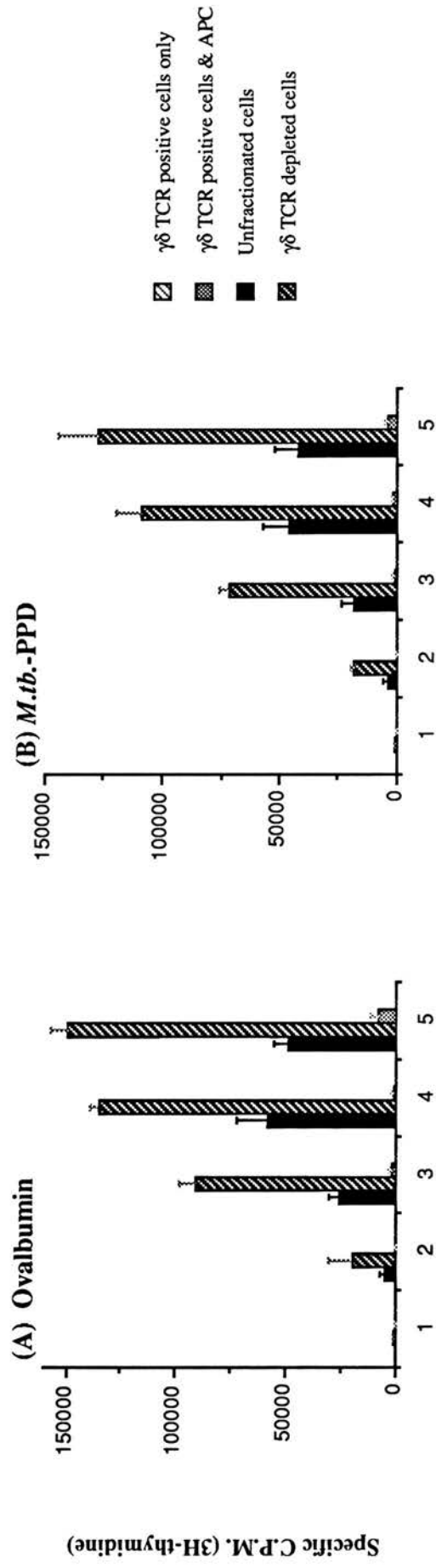
Figure 6.9:   Proliferative response of T cell subsets to ovalbumin.

Ovine PBL were isolated from immunised animals (n=3) and populations of $\gamma\delta$ T cells and $\gamma\delta$-depleted T cells prepared. 10^5 $\gamma\delta$-depleted T cells per well were cultured with 500µg/ml of ovalbumin in the presence (—◇—) or absence (·······※·······) of different numbers of $\gamma\delta$ T cells. The proliferative response of the same populations of cells to medium was included as a control (—□—). Cultures were incubated for 5 days and proliferation was measured by the uptake of ^{3}H-thymidine over the last 5 hours of culture. Data are given as the arithmetic mean of the specific CPM values. Error bars show the standard deviations of the mean. This experiment was repeated twice and similar trends were seen on each occasion. The significance of any differences in the proliferative responses in the presence of different numbers of $\gamma\delta$ T cells was determined by performing a two sample t-test comparing the proliferative response of test assays to the proliferative response of $\gamma\delta$ T cells only. The probability that the proliferative responses in the presence of different numbers of $\gamma\delta$ T cells are significantly different is represented in the figure.

FIGURE 6.9: Proliferative response of T cell subsets to ovalbumin.

—□— Response to medium.

——— $\alpha\beta$ T cells only.

—◊— $\alpha\beta$ T cells plus $\gamma\delta$ T cells.
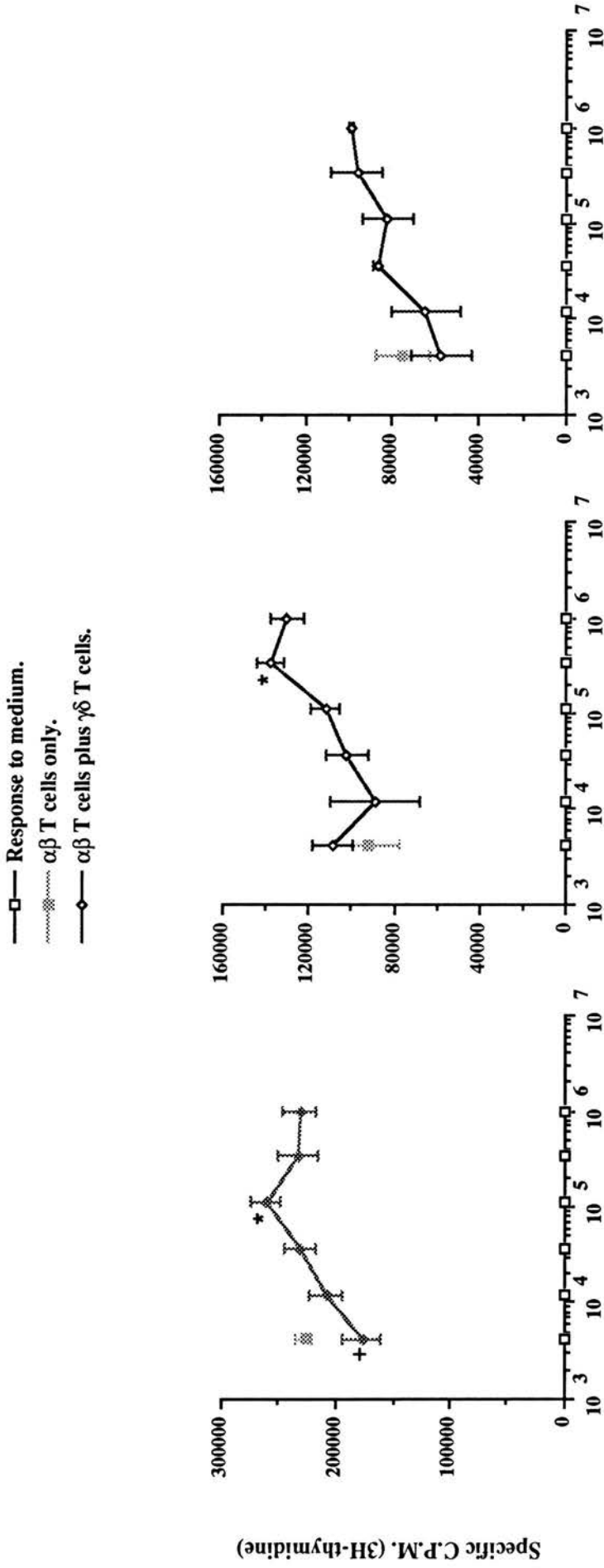
Number of $\gamma\delta$ T cells added to assays.

\+ significantly less than $\alpha\beta$ T cells only $p<0.05$

* significantly greater than $\alpha\beta$ T cells only $p<0.05$

Specific C.P.M. (3H-thymidine)

culture. When reconstituted *in vitro* at concentrations similar to those observed in the peripheral blood (<25%) γδ T cells inhibited the proliferative responses of the αβ T cell population in two out of three sheep though only one of these observations was statistically significant. In contrast, when reconstituted *in vitro* at concentrations in excess of those observed *in vivo* (30-90%) sheep γδ T cells enhanced the responses of the αβ T cell population in all animals examined. It should be noted though that only two of these observations were significantly greater than the control.

6.5 CD25 expression by purified γδ T cells.

It has been shown that the level of CD25 and MHC class II expression on γδ T cells, within a population of unfractionated PBL, increased following culture in the presence of antigen. Purified γδ T cells however did not proliferate when exposed to antigen, both in the presence and absence of APC (Fig.6.8). The expression of CD25 by purified γδ T cells and by γδ T cells within unfractionated PBL was compared following culture with antigen or ConA. Table 6.2 shows the percentage of γδ T cells in each population which expressed CD25 during the first 24 hours of each culture.

The data shows that 76% of γδ T cells within unfractionated PBL expressed CD25 in response to antigen. In contrast, only 48% of purified γδ T cells expressed CD25 following culture with antigen. 84% of purified γδ T cells and 98% of γδ T cells within unfractionated PBL expressed CD25 after 24 hours in culture with ConA. The rate of increase in CD25 expression on purified γδ T cells was significantly slower than the rate of increase in CD25 expression on γδ T cells within unfractionated PBL in all of the conditions assayed. In additon, the final percentage of purified γδ T cells which expressed CD25 at the end of the assay was significantly lower than the final percentage of γδ T cells within unfractionated PBL which expressed CD25.

6.5.1 γδ–depleted T cells, precultured with antigen, activate γδ T cells in the absence of antigen.

Most γδ T cells within unfractionated PBL become activated following culture with antigen. To test if this activation was through direct recognition of antigen, the response of γδ T cells when in culture with antigen pulsed γδ depleted T cells was determined. All known APC found in peripheral blood will be present in the γδ depleted T cell population therefore there is no requirement for autologous APC to be added to this assay. γδ depleted T cell were cultured with antigen, extensively washed and subsequently cultured with γδ T cells at the γδ T cell:αβ T cell ratio of unfractionated PBL. Table 6.3 shows that there was a marked increase in the expression of CD25 on γδ T cells after culture with antigen pulsed γδ depleted T cell. The increase in CD25 expression on purified γδ T cells occurred at a significantly greater rate during the first 12hours than on γδ T cells within unfractionated PBL when cultured with antigen. 35% of purified γδ T cells expressed CD25 after 6 hours in culture with antigen pulsed γδ depleted T cells whereas only 20% of γδ T cells within unfractionated PBL expressed CD25 after 6 hours in culture with antigen. The number of γδ T cells (72-76%) which expressed CD25 after 24 hours was similar following both treatments.

6.5.2 γδ T cells express CD25 at a higher intensity following culture with ConA compared to culture with antigen.

Purified γδ T cells and γδ T cells within unfractionated PBL expressed markers of activation

Table 6.2: CD25 expression by γδ T cells.

Populations of unfractionated PBL and purified γδ T cells were prepared (n=3) and cultured in the presence of either antigen, 5μg/ml ConA, or neither. Cells were harvested at the time points indicated and stained with biotinylated anti-WC1 followed by SA-PE and anti-CD25 followed by anti-mouse IgG1 FITC. The cells were analysed by FACScan and the percentage of γδ T cells expressing CD25 calculated with respect to NMS and b-NMS staining (always <5%). Data is the mean of three animals with errors shown in brackets. This experiment was repeated three times and the same trends were seen with PBL from each animal and on each occasion tested. The significance of any differences in the expression of CD25 by γδ T cells was determined by performing a two sample t-test comparing the expression of CD25 by γδ T cells within an unfractionated population to the expression of CD25 by purified γδ T cells in the absence of APC. The probability that the expression of CD25 by different γδ T cell preparations is significantly different is represented in the figure.

TABLE 6.2: CD25 expression by γδ T cells

Hours after start of culture.	Unfractionated γδ T cells plus ConA.	Purified γδ T cells plus ConA.	Unfractionated γδ T cells plus Antigen.	Purified γδ T cells plus Antigen.	Unfractionated γδ T cells plus Medium	Purified γδ T cells plus Medium.
0	17.5 (3.6)	8.3 (0.9)	13.8 (1.0)	8.3 (0.9)	14.7 (1.3)	8.3 (0.9)
6	71.6 (4.8)	[9.1 (4.0)]***	20.4 (1.6)	[13.1 (5.1)]*	18.4 (2.6)	8.3 (2.0)
12	95.4 (2.5)	[35.1 (16.4)]***	44.4 (12.5)	[16.1 (8.8)]**	17.3 (5.9)	10.7 (2.3)
18	96.4 (1.2)	[59.5 (12.2)]***	64.1 (3.4)	[31.7 (17.4)]*	14.1 (4.3)	10.7 (0.0)
24	97.8 (0.5)	[83.9 (2.4)]**	75.7 (5.1)	[47.8 (24.6)]*	15.7 (1.5)	18.3 (3.2)

[ ] significantly different to unfractionated   * p<0.05   ** p<0.005   *** p<0.001

Table 6.3:   **CD25 expression by γδ T cells.**

Populations of γδ-depleted T cells were prepared (n=3) and cultured in the presence of antigen for 2 days. Viable cells were harvested and cultured with purified γδ T cells. The final percentage of γδ T cells in the culture matched the percentage of γδ T cells within unfractionated PBL at the start of the experiment. At the time points indicated, viable cells were harvested and stained with biotinylated anti-WC1 followed by SA-PE and anti-CD25 followed by anti-IgG1 FITC. The cells were analysed by FACScan and the percentage of γδ T cells which expressed CD25 was calculated with respect to the staining of NMS and b-NMS. Data is the mean values from three animals with the standard deviation from the mean shown in brackets. This experiment was repeated three times and the same trends were seen with PBL from each animal and on each occasion tested. The significance of any differences in the expression of CD25 by γδ T cells was determined by performing a two sample t-test comparing the expression of CD25 by γδ T cells within an unfractionated population to the expression of CD25 by purified γδ T cells in the presence of autologous antigen pulsed PBL. The probability that the expression of CD25 by different γδ T cell populations is significantly different is represented in the figure.

TABLE 6.3: CD25 expression by γδ T cells

Percentage of γδ T cells expressing CD25.

Hours after start of culture.	Purified γδ T cells added to autologous αβ T cells previously cultured with antigen.	γδ T cells in unfractionated PBL plus antigen.
0	13.2 (2.0)	13.8 (1.0)
6	[35.1 (4.8)] **	20.4 (1.6)
12	[66.5 (5.0)] *	44.4 (12.5)
18	58.1 (14.1)	64.1 (3.4)
24	72.2 (9.2)	75.7 (5.1)

[ ] significantly different to unfractionated PBL * p<0.05 ** p<0.01

following culture with antigen. However, purified γδ T cells did not exhibit any proliferative responses to the same antigen. In contrast, purified γδ T cells when cultured with ConA expressed markers of activation and a proliferative response was measured. Figure 6.10 shows a comparison in the level of CD25 expressed by γδ T cells within unfractionated PBL in response to either antigen or ConA. γδ T cells expressed CD25 at high intensity after 24 hours of culture in the presence of ConA. This was associated with a proliferative response. In contrast, γδ T cells expressed CD25 at intermediate intensities when cultured with antigen and no proliferative responses were observed. Similar results were seen with purified γδ T cells when cultured with either antigen or ConA (data not shown). These results indicated that proliferation by γδ T cells only occurred when high levels of CD25 were observed.

6.6 Mixed lymphocyte reactions.

γδ T cells showed no proliferate response when cultured with soluble protein antigen in the presence or absence of APC's. This suggested that either i) antigen is not presented to γδ T cells in a manner analogous to αβ T cells (i.e. via MHC molecules) or ii) PBL do not express the correct antigen presenting molecules for γδ T cells and were therefore the wrong choice of APC.

Allorecognition of MHC molecules is believed to occur in an MLR, where a population of lymphocytes is cultured with and reacts to a population of irradiated allogeneic lymphocytes. It has previously been reported in the human and murine species that γδ T cells have very little alloreactivity. The alloreactivity of sheep γδ T cells was therefore assessed to determine if γδ T cells have the capacity to recognise classical MHC molecules.

Allorecognition by cells within the unfractionated PBL population was shown. Ovine PBL proliferated in response to two different populations of allogeneic PBL in a mixed lymphocyte reaction (data not shown). The proliferative response of purified γδ T cells to each of the allogeneic PBL populations was determined. Figure 6.11 shows the response of γδ T cells to different numbers of allogeneic PBL after five days in culture. The responses of αβ T cells and unfractionated PBL to allogeneic PBL were included for a comparison of the response by different populations of cells. γδ T cells showed little, if any, response to allogeneic PBL. The proliferative responses of γδ T cells to allogeneic PBL were not significantly greater than the response to autologous PBL. In contrast, the responses of αβ T cells and unfractionated PBL to allogeneic PBL were significantly greater than the responses to autologous PBL. The response by αβ T cells to autologous PBL was higher than the responses seen by unfractionated PBL to the same stimulatory cells.

Figure 6.10: Comparison of CD25 expression on γδ T cells.

PBL from immunised sheep (n=3) were incubated with either 500μg/ml ovalbumin or 5μg/ml ConA for 24 hours. Viable cells were harvested and stained with biotinylated anti-WC1 followed by SA-PE and anti-CD25 followed by anti-mouse IgG1 FITC. Cells were analysed by FACS and the quadrants were determined by staining of NMS versus b-NMS. Two clour immunofluorescence profiles of PBL from one animal are shown. This experiment was repeated five times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 6.10: Comparison of CD25 receptor expression on γδ T cells.
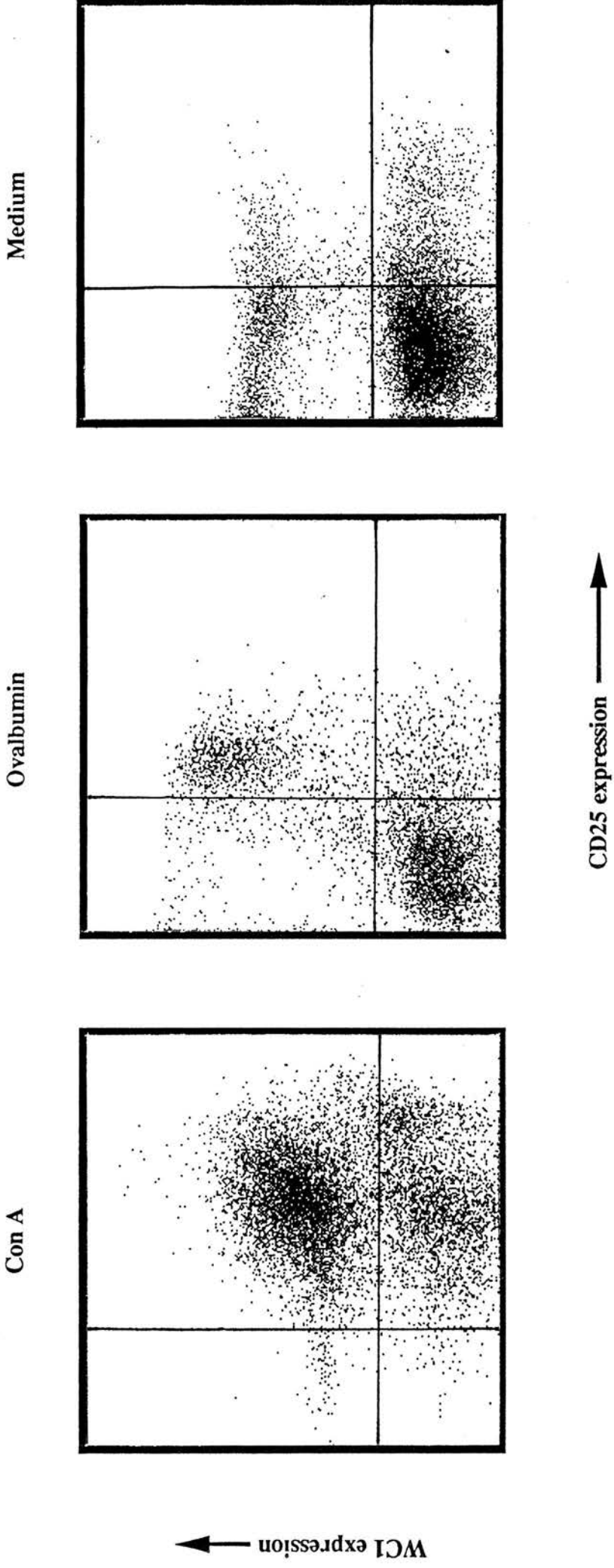
Figure 6.11: **Allogeneic and autologous proliferative responses of T cell subsets.**

Populations of $\gamma\delta$ positive T cells, $\gamma\delta$-depleted T cells and unfractionated lymphocytes were isolated from ovine PBL (n=3). 10^5 cells/well of each population were cultured with different numbers of irradiated allogeneic PBL from two different animals (—□—) and (⸺◆⸺), and with different number of autologous PBL (⸺✳⸺). Cultures were incubated for 5 days and the proliferative response was measured by the uptake of ^{3}H-thymidine over the last 5 hours of culture. The data are given as the geometric mean of the specific CPM values; errors shown are the standard deviations of these values. This experiment was repeated five times and the same trends were seen with PBL from each animal and on each occasion tested.

FIGURE 6.11: Allogeneic and autologous proliferative responses of T cell subsets.

Response to allogeneic PBL.
Response to allogeneic PBL.
Response to autologous PBL.

γδ-T cells.

Unfractionated PBL.

αβ-T cells.

Specific C.P.M. (3H-thymidine)
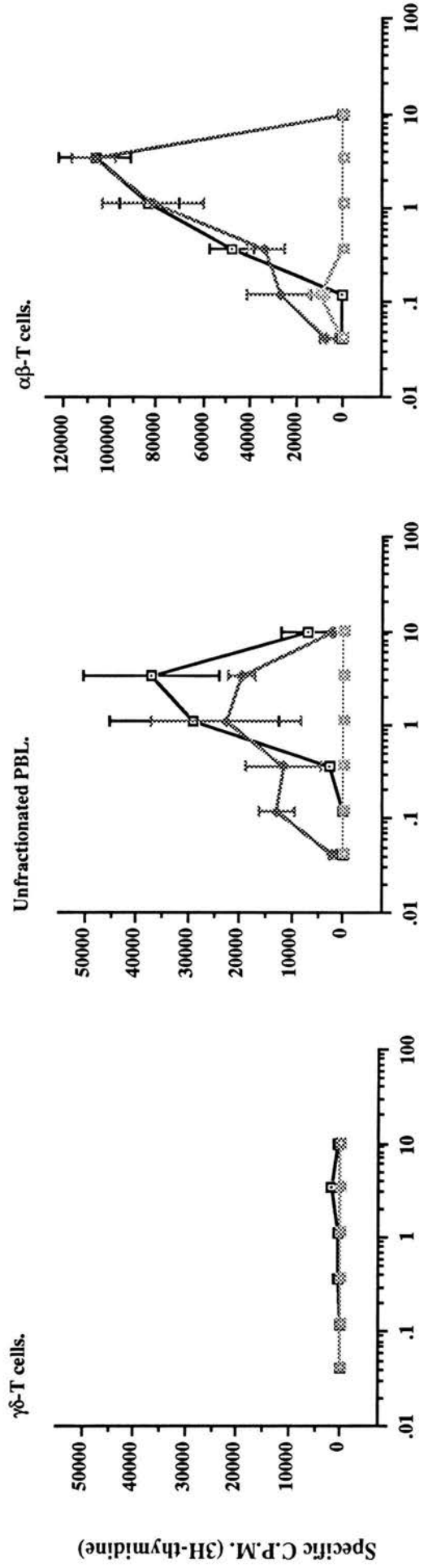
Ratio of stimulator cells to effector cells

DISCUSSION:

γδ T cells were discovered by identification of their rearranged TCR genes, and not from any observed biological function. The gene sequences of the γδ TCR are similar to those of the αβ TCR. Accordingly, the functions and specificities of γδ T cells were predicted to be similar to those of αβ T cells. Research into αβ T cells function has therefore defined a paradigm which dictated the experimentation, theory and approach to identifying the function of γδ T cells.

The results presented in this chapter indicated that responses by ovine peripheral blood γδ T cells to antigen are different to the responses by ovine peripheral blood αβ T cells to antigen. γδ T cells did not proliferate when cultured *in vitro* with antigen, whilst unfractionated PBL and PBL depleted of γδ T cells showed a significant proliferative response to antigen. However, all γδ T cells within an unfractionated population of PBL showed an increase in expression of CD25 in the presence of antigen whereas only 50% of purified γδ T cells expressed CD25 following culture with antigen. In the presence of allogeneic lymphocytes, the proliferative responses by γδ T cell were very small when compared to those of unfractionated PBL and αβ T cell populations. When γδ T cells became activated in response to antigen they all expressed an intermediate level of CD25 and did not proliferate. In contrast the proliferation of γδ T cells in response to ConA was coupled with an high intensity of CD25 expression. It would appear that there is critical threshold level of expression of CD25 above which cells will proliferate.

Following exposure to antigen, all γδ T cells within an unfractionated population of PBL became activated. It was therefore expected that purified γδ T cells would become activated and proliferate in a similar manner. In contrast, purified γδ T cells did not proliferate in response to antigen but did become activated though at a slower rate than γδ T cells within an unfractionated population of PBL. It was recently shown that γδ T cells could differentiate *in vivo* into a functional nonproliferative state during a normal immune response (Spaner et al., 1993). In contrast to observations reported in this chapter however, there was an initial proliferative response by γδ T cells. It should be noted that these results were obtained with a *scid* mouse carrying a single γδ TCR transgene and that this single gene rearrangement would account for an infinitely small part of the γδ TCR repertoire of the mouse.

There are several possible explanations for purified γδ T cells becoming activated but not showing any measurable proliferative response. Three follow; Firstly, some of the contaminating CD4-positive or CD8-positive T cells may have specifically recognised the antigen and subsequently activated γδ T cells in a bystander manner. It was shown that purified γδ T cells were ≥96% WC1-positive and that some of these cells expressed the molecules CD4 or CD8. It was also shown that 96% of the WC1-positive cells also expressed the γδ TCR. The TCR usage of the WC1-positive cells which coexpressed either CD4 or CD8 is unknown, these cells may express an αβ TCR molecule which is specific for antigen. Secondly, some γδ T cells may have recognised antigen directly in a manner analogous to immunoglobulin, but may still require costimulation by other cells within PBL. It is known that the complementarity determining regions 3 (CDR3) of αβ TCR molecules are critical for contact between the TCR and the antigenic peptide bound in the MHC groove (Jorgensen et al., 1992). These regions are also critical in immunoglobulin binding of antigen (Davies et al., 1990). It has been found that the lengths of CDR3 in α and β chains are nearly identical, both the length and distribution within the chain however are very limited. In contrast, the CDR3 lengths on the δ and γ TCR resembled those of the immunoglobulin heavy chain (long and variable) and light chain (short and less variable) respectively. It was therefore

proposed that γδ TCR may recognise antigen in a manner similar to that of immunoglobulins. Any MHC restricted antigen recognition so far observed by γδ T cell clones may simply be due to fortuitous cross-reactivity with a structural feature of the MHC molecule. Finally, purified γδ T cells may recognise antigen directly or via presentational molecules when in culture but may not respond because they are unable to synthesise the cytokines required to enter the cell cycle and undergo DNA synthesis. It has been shown that numerous bovine and ovine γδ T cell clones can not be induced to synthesise the cytokines IL-2, IL-4 and γ-IFN (W.I.Morrisson and H. Takamatsu, personal communication).

When αβ T cells were reconstituted with purified γδ T cells at cell concentrations higher than would normally occur in the peripheral blood of an animal, the proliferative responses to antigen appeared to be enhanced though most of these data points were not significantly greater than the control response of αβ T cells. It should be noted though that there was a measurable increase in proliferative response which correlated with an increase in the number of γδ T cells added to the culture. Activated or stimulated T cells are known to secrete numerous cytokines (Ottenhoff, 1991; Liew, 1989; Beckman, 1992) and, in this thesis, γδ T cells were shown to become activated in the presence of antigen. Therefore, when more γδ T cells are added to cultures of αβ T cells and antigen, there will be an increasing amount of cytokine secreted by cells in the cultures. Consequently, the proliferative response of αβ T cells would be enhanced.

67% of purified γδ T cells became activated within 12 hours when cultured with αβ T cells which had previously been exposed to antigen. This was a faster response than that by γδ T cells in unfractionated PBL and cultured with antigen. The activation of γδ T cells in the absence of antigen has not previously been reported to our knowledge. The activation of γδ T cells may be a response to either a factor secreted by cells which have specifically recognised antigen or to a cell-surface factor expressed by cells which have specifically recognised antigen. It has been reported that γδ T cells specifically recognise autologous and mycobacterial heat shock proteins (O'Brien et al., 1989). In the results reported in this chapter, γδ T cells may have recognised and responded to an autologous 'stress protein' expressed on antigen specific cells αβ T cells.

The intensity of expression of CD25 on γδ T cells correlated with the proliferative responses of these cells. There was a threshold level of expression of CD25 above which cells proliferated. CD25 is the α-chain of the IL-2R complex and exhibits low affinity for IL-2 (Waldmann, 1993). It is known that expression of CD25 is increased on T cells when activated but for the IL-2R to bind IL-2 with high affinity, expression of the β-chain and the γ-chain of the IL-2R complex must be increased too (Tsudo et al., 1986, 1987). γδ T cells when cultured with ConA showed an increase in intensity of CD25 expression; this may correlate with an increase in the IL-2R β-chain and IL-2R γ-chain expression and subsequent proliferation caused by binding of IL-2. In contrast, γδ T cells when cultured with antigen showed an increase in intensity of CD25 expression; this may not have correlated with an increase in IL-2R β-chain and IL-2R γ-chain expression and therefore subsequent proliferation did not occur.

The results observed in this chapter may also be explained by differences in the signal transduction mechanisms of αβ and γδ T cells. There is a milieu of proteins on the surface of lymphocytes of which at least three have been shown to be associated with cell association, signal transduction and activation of αβ T cells, namely CD2, CD4 and CD8. These proteins are not expressed on the surface of most ovine peripheral blood γδ T cells (MacKay et al., 1989) however, human and murine peripheral blood γδ T cells do express CD2. It is assumed there are numerous

other proteins involved in the process of T cell activation and proliferation and that the requirements for these proteins vary from cell to cell. It was seen that purified $\gamma\delta$ T cells did not proliferate in response to antigen. This may be due to: i) the lack of a cell surface protein on $\gamma\delta$ T cells which is required for antigen specific proliferation in a manner analogous to $\alpha\beta$ T cell; ii) the lack of a ligand on $\gamma\delta$ T cells specific for co-stimulatory molecules present on antigen stimulated PBL. B7/BB1 is expressed on antigen presenting cells and has been shown to be essential for $\alpha\beta$ T cell antigen specific proliferation. The ligands for this B7/BB1 are CD28 which is expressed on naive CD4+ T cells (Linsley et al., 1991a; Koulova et al., 1991) and CTLA-4 which is expressed on antigen-experienced T cells (Linsley et al., 1991b; Brunet et al., 1987). There is no citation detailing the expression of either of these two molecules on the $\gamma\delta$ T cell population. or iii) the lack of internal signal transduction molecules in the cytoplasm of $\gamma\delta$ T cells which are interconnected with the TCR molecule. Molecules such as p56^{lck} or p59^{fyn} or even transcription factors such as NFκB or KBF1 (Hemar et al., 1991). $\gamma\delta$ T cells may become activated upon recognition of antigen in a manner analogous to $\alpha\beta$ T cells but lack a second or additional signal which will trigger a switch from the resting G0 phase enabling the proliferation of the cells.

PBL depleted of $\gamma\delta$ T cells ($\alpha\beta$ T cells) proliferated in response to both antigen and allogeneic cells with far greater magnitude than either unfractionated PBL or purified $\gamma\delta$ T cells. This intriguing observation may have been due to one or a combination of the following factors. Firstly the $\gamma\delta$ T cell population may have a suppressive role on the response of other PBL. The proliferative responses of $\alpha\beta$ T cells in medium alone were much higher than unfractionated cells. Secondly, each assay performed used the same number of effector cells. By removing $\gamma\delta$ T cells, the PBL population is depleted of 10-15% of the starting cells and the number of antigen specific CD4-positive, and CD8-positive, $\alpha\beta$ T cells is therefore proportionately increased in the assay.

CHAPTER 7

Final discussion

7 FINAL DISCUSSION:

In this thesis a number of novel observations were made about the peripheral blood γδ T cell population of sheep. These observations can be divided into two main areas. The first set of observations details aspects of the γδ T cell phenotype, in particular the expression of the WC1 molecule. The second set of observations details the specific and non-specific responses of γδ T cells following culture with either mitogen or antigen.

7.1 Expression of WC1 epitopes by peripheral blood γδ T cells.

The cell surface glycoprotein WC1 was identified as a unique molecule expressed on ruminant γδ T cells (MacKay et al., 1986, 1989; Clevers et al., 1990). In this thesis it was shown that, although the bovine and ovine species are closely related, not all the WC1 epitopes expressed in the bovine species were expressed in the ovine species. All WC1 monoclonal antibodies recognised epitopes of WC1 which were expressed on subpopulations of γδ T cells. In addition, some WC1 monoclonal antibodies recognised WC1 epitopes which were expressed on mutually exclusive populations of γδ T cells. All of the WC1 monoclonal antibodies immunoprecipitated one or more proteins of molecular weights, 144kDa, 215kDa or 300kDa and the intensity of protein bands seen using the different WC1 monoclonal antibodies correlated with the intensity of staining seen in FACS analysis of PBL. It was also shown, by FACS analysis and immunohistology, that no one sub-population of WC1 positive cells predominated in any tissue and that all WC1 monoclonal antibodies reacted with cells in the same regions of the lymph node, spleen and thymus. In the thymus, WC1 epitopes was expressed predominantly on medullary thymocytes with few reactive cells seen in the cortex. This distribution is indicative of mature thymocytes and it has been proposed that WC1 is a marker of maturation of γδ T cells (MacKay et al., 1989). The distribution of WC1 positive cells in the lymph node and the spleen was indicative of cells in transit and verified preliminary studies of others (MacKay et al., 1986, 1989; Evans et al., 1994). These observations may help in delineating a function for γδ T cells. Two potential roles for γδ T cells have been suggested here. In the first role γδ T cells may function as antigen presenting cells. In the afferent lymph, γδ T cells were shown to be activated (Bujdoso et al., 1993) and furthermore it was shown in this thesis that activated γδ T cells have increased levels of MHC class II of expression. γδ T cells may actively take up antigen in the afferent lymph (or in the tissues which drain into the afferent lymph) and subsequently pass to the subscapular and trabecular regions of the lymph node were they may present antigen to incoming lymphocytes. In an alternative role, γδ T cells, due to their observed distribution in the lymph node, may be the first cells to respond to antigen-bearing, antigen presenting cells such as afferent lymph dendritic cells. γδ T cells may then either respond in an analogous manner to αβ T cells or may help in sequestering antigen specific lymphocytes to the lymph node.

The function of the WC1 protein is unknown. It was first proposed that WC1 may fulfil the role of an accessory molecule on the surface of γδ T cells (MacKay et al., 1986; Evans, 1993) as ovine γδ T cells do not express any of the known co-receptor molecules which are involved in αβ T cell activation (MacKay et al., 1988, 1988a). More recently complex WC1 gene families have been found in cattle, sheep, goats, pigs and horses (Wijngaard et al., 1992, 1994; Metzelaar et al., 1992) and it has been proposed that WC1 may control tissue-specific homing of γδ T cells (Wijngaard et al., 1994). Genes encoding WC1 were also found in the human and mouse genomes though no expressed product was been observed (Wijngaard et al., 1994). Wijngaard and colleagues (1992, 1994) cloned and sequenced three different bovine WC1 gene constructs which were 80%

homologous at the nucleotide level. A putative structure for the WC1 protein was subsequently predicted in which the extracellular domain appears to be a single polypeptide chain consisting of 11 non-identical repeats of a 110 amino acid consensus sequence of a cysteine rich region. The exact number of WC1 genes expressed in the bovine species is not known, although some researchers suggest they could be very numerous (H. Clevers personal communication; Wijngaard et al., 1992, 1994). Recently, DNA segments homologous to part of the bovine WC1 cDNA were found to be highly represented in a sheep genomic library (Walker et al., 1994). Sequence analysis of these gene segments revealed that the proteins products were WC1-like, and it was suggested that each DNA segment corresponded to a different gene. These results, and studies by others (W. Kimpton, personal communication), suggested the existence of a large family of between 50 and 100 genes for WC1 in the sheep genome. It was proposed by Walker and colleagues (1994) that individual WC1 proteins may carry out distinct but related functions in the ovine species. The differences in the reactivity of the different monoclonal antibodies to WC1 observed through the course of this thesis may be due to the expression of different members of the WC1 gene family.

Cysteine rich regions analogous to WC1 were found in the extracellular domains of CD5 (Jones et al., 1986; Huang et al., 1987), CD6 (Aruffo et al., 1991) and the macrophage scavenger receptor cysteine rich (SRCR) domain (Freeman et al., 1990). CD5 is known to be involved in T cell activation (Geppert et al., 1990) and interacts with its ligand, CD72, via the SRCR domains (van de Velde et al., 1991). Therefore, by analogy, WC1 may be involved in the activation of γδ T cells and may interact with a membrane bound ligand via the SRCR domains. The specificities of each of the different SRCR domains is unknown although it has been proposed that they may differ (H. Clevers, personal communication).

It is interesting to speculate on a function for WC1 from the results obtained in this thesis. No specific homing of any of the WC1 subpopulations identified by monoclonal antibodies used in this thesis was observed in any of the tissues analysed. It was seen in this thesis, and by others (MacKay et al., 1989; Evans 1993), that γδ T cells in the epithelium of the gut do not express any of the identified WC1 epitopes. The monoclonal antibodies used in this thesis and by others (MacKay et al., 1989; Evans 1993) will only recognise a small number of WC1 epitopes. It will be of interest to determine if γδ T cells in the epithelium of the gut do express any WC1 gene products. In addition, it was shown in this thesis, that the proportion of γδ T cells in sheep peripheral blood which expressed each of the WC1 epitopes remained constant on PBL isolated from animals of different ages. Changes were however detected in the WC1 phenotype of PBL isolated from animals with varying levels of antigenic burden and of activated PBL. The changes were observed with the mutually exclusive WC1 subpopulations N3 and N4. In normal animals 30% of the N3-positive population express N4 and 20% of the N4-positive population express N3. With PBL isolated from antigenic naive lambs and with PBL stimulated with ConA, these WC1 subpopulations became more segragated (i.e fewer cells expressed both the N3 and N4 epitope). Though it is difficult to intrpret the functional significance of these observations, the results may suggest that these two sub-populations of γδ T cells may carry out different functions in the sheep.

The tissue distribution of all the different WC1 epitope-positive cells and the WC1-epitope phenotype of γδ T cells may remain constant in healthy animals and only be changed in diseased animals when cells are stimulated or carrying out effector functions. It is known that the size of the peripheral blood γδ T cell population is increased in a number of diseased states in the murine

and human species (Haas et al., 1993).

If WC1 functions a homing molecule then it will be necessary to identify a ligand. It will then be of interest to determine if the different WC1 genes encode molecules with different homing properties, and secondly, if the various SRCR domains of the WC1 proteins bind different ligands. An ideal way to identify the ligands recognised by WC1 will be to use the protein product of the different WC1 genes. The different SRCR domains of the bovine WC1.1 gene have been cloned into an expression vector which will couple the SRCR domain to an immunoglobulin heavy chain (H. Clevers, personal communication). The product will be a chimeric SRCR-Ig molecule with the immunoglobulin heavy chain acting as a reporter protein. Using purified SRCR-Ig, the distribution of the ligand(s) for the different SRCR domains can be determined. These ligands may subsequently be identified and the function of the WC1 protein postulated. This would potentially delineate a function for γδ T cells in the ruminant species.

7.2 Specific and non-specific responses of γδ T cells.

A series of experiments carried out in this thesis examined the response of γδ T cells to the T cell mitogen ConA and to the antigens Ova and *M.tb.*-PPD. The four most significant observations made were: 1) γδ T cells became activated at a much faster rate than did CD4-positive or CD8-positive T cells following culture with either the antigens ovalbumin or *M.tb.*-PPD, or the T cell mitogen ConA. 2) Purified γδ T cells, though activated, did not proliferate when cultured with antigen, both in the presence and absence of APC. 3) γδ T cells became activated in the absence of antigen when cultured with αβ T cells which had previously been exposed to antigen. 4) PBL depleted of γδ T cells proliferated in response to both antigen and allogeneic cells with far greater magnitude than unfractionated PBL.

The concentration of ConA used routinely throughout this thesis was 5µg/ml. It should however be noted that the responses of T cell subsets to ConA may not be optimal as no titration of a dose response was carried out. The results detailed in this chapter therefore only detail the responses of T cell subsets under one set of conditions. The responses of the different T cell subsets may be different in alternative conditions.

The activation of γδ T cells following culture with either ConA, ova or *M.tb.*-PPD was measured by the increase in cell surface expression of CD25 and MHC class II molecules. The activation was shown by control experiments to be mitogen or antigen specific. Verhagen and colleagues (1993) also reported a faster rate of activation of γδ T cells compared to CD4-positive or CD8-positive T cells. Results presented in this thesis and elsewhere (Bujdoso et al., 1993), along with those of Verhagen (1993), have lead to the proposal that *in vivo,* γδ T cells may become activated in response to a stimuli at a faster rate than either CD4-positive or CD8-positive T cells. Recent observations (A. Anderson, personal communication) suggested this to be the case. The level of CD25 expression on γδ T cells infiltrating an inflamed joint of a sheep which had artificially induced arthritis increased at a faster rate and more markedly than did the level of CD25 expression on CD4-positive or CD8-positive T cells.

It was interesting to note that following stimulation of PBL, MHC class II β-chains were expressed on all T cell subsets whereas, the products of the two MHC class II α-chain loci so far identified in the ovine species were expressed on less than 30% of each T cell subset. Each MHC class II protein comprises an α-chain and a β-chain therefore this observation lead us to propose that

more MHC class II loci must exist in the ovine species than have so far been identified

A number of explanations for the differences in rates of activation between γδ T cells and, CD4-positive T cells and CD8-positive T cells may be proposed. Firstly, γδ T cells may be in a pre-activated state compared to CD4-positive and CD8-positive T cells. This was suggested by the observation that there was a modest increase in CD25 expression by γδ T cells within PBL when cultured in the absence of ConA or antigen as a stimuli. In contrast, CD4-positive and CD8-positive T cells did not increase their expression of CD25. It has also been seen that resting peripheral blood γδ T cells in the bovine species express low levels of CD25 (H. Takamatsu, W.I. Morrison, personal communication). Secondly, different signal transduction pathways may exist for the three T cell lineages. The cell surface phenotype of γδ T cells is different to the cell surface phenotype of CD4-positive or CD8-positive cells (MacKay et al., 1989). Thirdly, the transcriptional and translational control of genes including those used when cells become activated and undergo proliferative responses may be different in the three T cell subsets.

These observations of a faster rate of activation by γδ T cells compared to αβ T cells fit well with theories regarding the role of γδ T cells put forward by others (Kaufamn, 1990, Pfeffer et al., 1990, 1992). In these hypothesis, γδ T cells are proposed to act as the first line of defence in the immune response to infections. It is reasonable to assume if this were the case, then γδ T cells would respond to at a faster rate than αβ T cells to the pathogenic challenge.

Purified γδ T cells did not proliferate when cultured with antigen, both in the presence and absence of autologous PBL as APC. γδ T cells within an unfractionated population of PBL, and purified γδ T cells alone, did however become activated following culture with antigen. The intensity of expression of CD25 on γδ T cells following culture with antigen was lower than the intensity of expression of CD25 on γδ T cells following culture with ConA. This suggested that there may be a threshold level of expression of CD25 on γδ T cells which signifies a proliferative response. Cells expressing CD25 at a lower intensity may remain non-proliferative.

An explanation for the lack of proliferation by γδ T cells in the assays reported here may lie in the choice of APC. In all the assays presented in this thesis, irradiated autologous PBL were used as APC for γδ T cells. Unfortunately there are no positive controls in the ovine species for antigen recognition by γδ T cells, as is the case for γδ T cells other species. It was unknown if autologous PBL were the correct APC to be used in such assays. It is also not possible to categorically decide that the activation of γδ T cells in the cultures with antigen was in fact a direct response to the antigen or some other factor present in the culture system. It was observed that during *in vitro* culture in the absence of antigen, γδ T cells expressed increased levels of CD25. This has also been observed in analysis of the responses of γδ T cells from the bovine species (H. Takamatsu, W.I. Morrison, personal communication). It appears that γδ T cells show a modest increase in their level of CD25 expression simply as a consequence of the isolation of PBL. An hypothesis presented here for these observations is that on the luminal surfaces of the peripheral blood vasculature, ligands specific for surface molecules of γδ T cells may be expressed. When in contact with these ligands, activation of γδ T cell may be suppressed. The vasculature of other lymphoid vessels such as the afferent lymph may not contain the necessary ligands for suppression of γδ T cell activation and this may explain the observation that γδ T cells express higher levels of CD25 in the afferent lymph compared to peripheral blood or efferent lymph.

An alternative reason for the lack of proliferation by γδ T cells may be that γδ T cells are unable

to synthesise the cytokines required to enter the cell cycle and undergo DNA synthesis. It has been shown that numerous bovine and ovine γδ T cell clones can not be induced to synthesise the cytokines IL-2, IL-4 and γ-IFN (W.I.Morrisson and H. Takamatsu, personal communication)

Result presented in this thesis showed that γδ T cells became activated when cultured with αβ T cells which had previously been exposed to antigen. This occurred at a faster rate than was seen for the activation of γδ T cells within unfractionated PBL when in culture with antigen. Activation also occurred to a greater extent than was seen on unfractionated PBL, and purified γδ T cells alone, following their culture in the absence of antigen. The activation of ovine peripheral blood γδ T cells in the absence of exogenous antigen has not previously been reported and may be a response to either a factor secreted by cells which have specifically recognised antigen or to a factor expressed by cells which have specifically recognised antigen. One such factor may be self-ligands which are expressed by APC. It has been reported that γδ T cells specifically recognise autologous and mycobacterial heat shock proteins (O'Brien et al., 1989).

7.3 Future prospects.

A significant absence in γδ T cell immunology is the identification of the presentational element or ligand recognised by the γδ TCR molecule. It was assumed that γδ T cells recognised antigen in a similar manner to αβ T cells: i.e recognising peptide in association with the presentational molecules MHC class I and MHC class II. However, it has been shown that most γδ T cells recognise ligands independent of MHC molecules (Haas et al., 1993; Allison and Havran, 1991; Raulet, 1989; Kronenberg, 1994) and that γδ T cells can recognise single molecule ligands rather than peptides bound to larger molecules (Schild et al., 1994; Weintraub et al., 1994; Sciammas et al., 1994). Furthermore it was recently shown that γδ TCR heterodimers appear more immunoglobulin like in their CDR3 region (Rock et al., 1994). A significant point to stress is that although numerous ligands for individual γδ T cell clones have been identified (Haas et al., 1993; Kronenberg, 1994) and recognition of whole molecules by individual γδ T cell clones has been reported (Schild et al., 1994; Weintraub et al., 1994; Sciammas et al., 1994) these observations account for only a limited number of the possible range of γδ TCR specificities. All experiments performed in this thesis used PBL as APC for γδ T cells. PBL express both MHC class I and MHC class II molecules which are the classical antigen presenting molecules for αβ T cells (Kronenberg et al., 1986). As has been reported by others (Schild et al., 1994; Weintraub et al., 1994; Sciammas et al., 1994) during the course of this thesis, γδ T cells may not recognise antigen in a manner analogous to αβ T cells therefore this choice of APC may not only be incorrect, but APC may not be required for stimulation of γδ T cells.

In the sheep, γδ T cells are known to be activated to a greater extent in the afferent lymph compared to peripheral blood or efferent lymph (Evans et al., 1993). CD25 is expressed at an intermediate intensity although, as previously proposed in this thesis, this does not necessarily mean that the γδ T cells are proliferating. It is unknown what ligand is responsible for activating γδ T cells, which migrate via afferent lymph, and on which cells this ligand is found. The ligand recognised by γδ T cells, may not only be different from that recognised by αβ T cells, but also may vary from tissue to tissue. It is known that afferent lymph contains a population of cells not seen in other lymphatic and peripheral vessels. These are dendritic cells and are potent antigen presenting cells (Steinman, 1991). In addition to MHC class I and MHC class II molecules, ovine afferent lymph dendritic cells express other presentational elements such as CD1 (Bujdoso et al., 1989). It may be hypothesised that either in the afferent lymph, or in the tissues, dendritic cells

stimulate γδ T cells either via antigen presenting molecules and peptide or via the expression of a ligand specific for γδ T cells. γδ T cells are subsequently found with an activated phenotype in the afferent lymph (Bujdoso et al., 1989; Evans et al., 1993). It will be of great interest to determine the proliferative response of γδ T cells to antigen in the presence of purified afferent lymph dendritic cells. In the sheep, cannulation of the afferent lymphatics allows for the collection of large numbers of afferent lymph dendritic cells, which may readily be purified by conventional means for use in *in vitro* assays (Bujdoso et al., 1989).

Because of the large number of γδ T cells in ruminants such as the sheep, these experimental animals will play an important role in defining the function of γδ T cells within the immune system. These studies will supplement those ongoing in the murine system.

Functional studies of γδ T cells in the murine system are beginning to define a role for this population of cells. A potential way to identify the function of γδ T cells is to analyse their function in animals devoid of other T cell subsets (e.g. αβ T cells). αβ-depleted mice have been generated by treatment of animals from birth and onwards with an anti- αβ TCR monoclonal antibody (Carbone et al., 1991). Transgenic mice have also been generated by genetic recombination or disruption of genes in embryonic stem cells (Ishida et al., 1990; Mombaerts et al., 1992; Philpott et al., 1992). Using αβ T cell depleted animals, and transgenic animals, it may be possible to determine both a specific ligand for γδ T cells and also a fundamental function for this population of lymphocytes. It has already been shown with transgenic mice that αβ T cells can develop normally in γδ T cell deficient mice (Ishida et al., 1990); that the homing of γδ T cell subsets to epithelia is independent of γδ TCR expression (Bonneville et al., 1990); and that negative selection, (Bonneville et al., 1990a; Dent et al., 1990) and positive selection, (Pereira et al., 1992; Wells et al., 1991,1993) of γδ T cells occurs. It has also been demonstrated that there are different requirements for MHC class I expression for the development of γδ T cells in transgenic animals (Correa et al., 1992; Pereira et al., 1992) and that MHC class II molecules are not involved in the positive selection of intraepithelial Vδ 4+ γδ T cells (Schleussner and Ceredig, 1993). In addition, it was shown that the tyrosine kinase p56[lck] is required for the thymic development of γδ T cells in transgenic mice (Penninger et al., 1993). The function of γδ T cells can be more directly ascertained in animals transgenic animals devoid of the αβ T cell subset and in αβ-depleted animals. It has already been shown that γδ T cells are not helper cells for B cells (Philpott et al., 1992); that γδ T cells show no proliferative response to allogeneic cells (Carbone et al., 1991); and that γδ T cells are required for recovery from Listeria and Malaria infection (Mombaerts et al., 1993; Tsuji et al., 1994). More recently, it was shown in γδ TCR transgenic animals that γδ T cells expressing the TCR Vγ1.1Jγ4Cγ4 conferred resistance to acute T cell leukaemias (Penninger et al., 1995). This resistance occurred independent of MHC class I or MHC class II molecules, the TAP-2 peptide transporter protein or heat-shock proteins.

The results obtained with transgenic mice however are questioned by some (Routtenberg, 1995). It should be noted, that when a particular gene or cell population is deleted from an individual, the individual will compensate for this loss by the increased expression of molecules or cells which carry out similar functions. The transgenic animal will therefore be unnatural and the significance of any results may therefore be difficult to interpret. Similarly, it may be difficult in some instances to speculate as to the requirement of a molecule or cell population by merely deleting it from the individual.

The development of the transgenic mouse has given the immunologist an immensely powerful

tool which will undoubtedly help in deciphering the function of γδ T cells within the immune system and the potential of the transgenic animal should not be overlooked.

APPENDIX

COS cell cloning system

Results and Discussion.

The initial aim of this thesis was to clone and sequence the ovine WC1 gene. Two approaches were taken. The first involved the direct molecular cloning of the WC1 cDNA. An already prepared cDNA library was to be used in the COS cell transfection system (Seed and Aruffo, 1987; Aruffo and Seed, 1987) with the aim of identifying the cDNA encoding WC1 by selective panning with the anti-WC1 monoclonal antibodies. A positive control for transfection and cDNA recovery for this system used cDNA encoding rat CD2, and the monoclonal antibody OX34, which is specific for rat CD2 (kindly provided by Dr. Neil Barclay, University of Oxford). The second approach was to affinity purify the WC1 protein and obtain the N-terminal amino acid sequence. This would be used to generate a WC1 cDNA probe. The affinity purification of the WC1 protein is described in Chapter 3.

A.1 Establishing the cDNA transfection and recovery system.
A.1.1 *Kpn* I and *Xba* I restriction endonucleases linearise the plasmid vector CDM8 containing the cDNA encoding rat CD2.

cDNA encoding rat CD2, ligated into the shuttle vector CDM8 (CDM8-CD2) (a gift from Dr. Neil Barclay, University of Oxford), was used to establish the molecular cloning system as previously described (Seed and Aruffo, 1987; Aruffo and Seed, 1987). The shuttle vector, CDM8, with stuffer DNA, is ~4.8Kb in size and the rat CD2 cDNA ~1.2Kb. When rat CD2 cDNA was inserted into the vector instead of the stuffer fragment, the vector is ~5.0Kb in size. The restriction endonucleases *Xba* I, *Kpn* I and *BamH*I were used to digest preparations of the supplied plasmid construct. Figure A.1 shows the ethidium bromide staining bands of CD2-CDM8 following restriction digestion with the named enzymes. In lanes 1 and 2 (*Xba* I), and 3 and 4 (*Kpn* I), one major band and numerous smaller bands were seen. The major band was of ~5.0kbp and corresponds to the expected size of the linearised CDM8-CD2 plasmid. The other bands were comparable to the bands observed with uncut plasmid control (lanes 7 and 8). Figure 1 also shows that the restriction enzyme *BamH*I cleaves the CDM8-CD2 plasmid at three sites (lanes 5 and 6). Any of the restriction enzymes used here can therefore be used to determine that CDM8-CD2 is present in the prepared sample.

A.1.2 Polymerase chain reaction analysis of the CD2 cDNA contained within the CDM8-CD2 construct.

To further confirm that the plasmid DNA prepared and analysed in A.1.1 was CDM8-CD2, a polymerase chain reaction (PCR) of the CD2 construct was developed. Figure A.2 shows the results of a PCR when $MgCl_2$ was titrated. The expected PCR product of 750bp was seen in lanes 5 and 6 which correspond to 4mM and 5mM $MgCl_2$ respectively. This result was taken as confirmation that the designated CDM8 vector did in fact contain rat CD2. The plasmid preparations used in this and the previous experiment were used as control plasmids in future restriction digests.

A.1.3 COS cells maximally express CD2 48 hours after transfection.

The conditions for transfection where as previously described by Seed and Aruffo (1987) with minor modifications as detailed in Chapter 2. Cells were transfected with the CD2-CDM8 plasmid and incubated for various lengths of time. At various time points after transfection, the expression

Figure A.1   Restriction endonuclease digestion of CDM8-CD2.

The two different plasmid preparations were used. Lanes 1, 3, 5 and 7 shows results with a miniprep of transformed *E.Coli*. and lanes 2, 4, 6 and 8 show results with a large scale preparation of transformed *E.Coli*. A sample of each DNA preparation was incubated with the restriction enzymes *Xba*I (lanes 1 and 2), *Kpn*I (lane 3 and 4) and *BamH*I (lanes 5 and 6) according to the suppliers instructions and with no restriction enzyme (lanes 7 and 8). The DNA was then analysed on a 1% agarose gel by ethidium bromide staining following electrophoresis. DNA size markers are shown to the left of the figure.

Figure A.1: Restriction endonuclease digestion of CDM8-CD2.
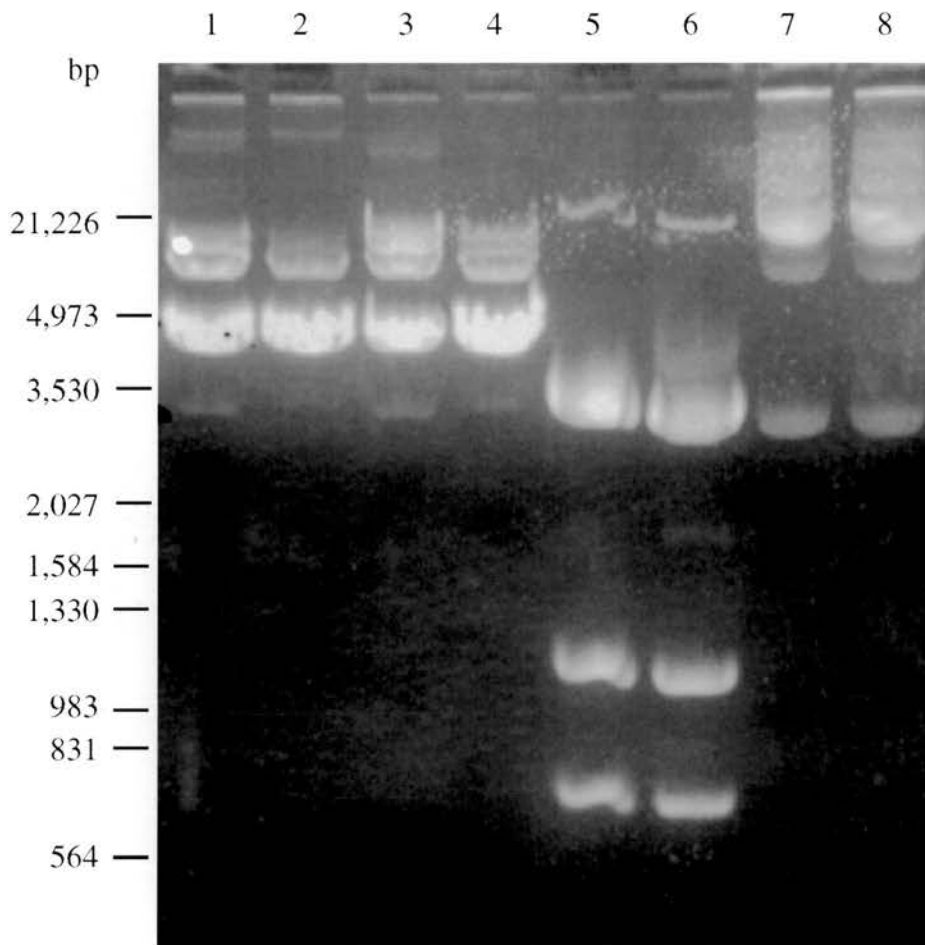
Figure A.2 Polymerase chain reaction of rat CD2.

CDM8-CD2 plasmid DNA was analysed by PCR. Lanes 1 through to 6 are PCR reactions performed with increasing final concentrations of $MgCl_2$ in the PCR reaction of 0, 1, 2, 3, 4 and 5mM respectively. The PCR products of each reaction were analysed on a 1% agarose gel by ethidium bromide staining following electrophoresis. DNA size markers are shown to the left of the figure.

Figure A.2: Polymerase chain reaction of rat CD2.
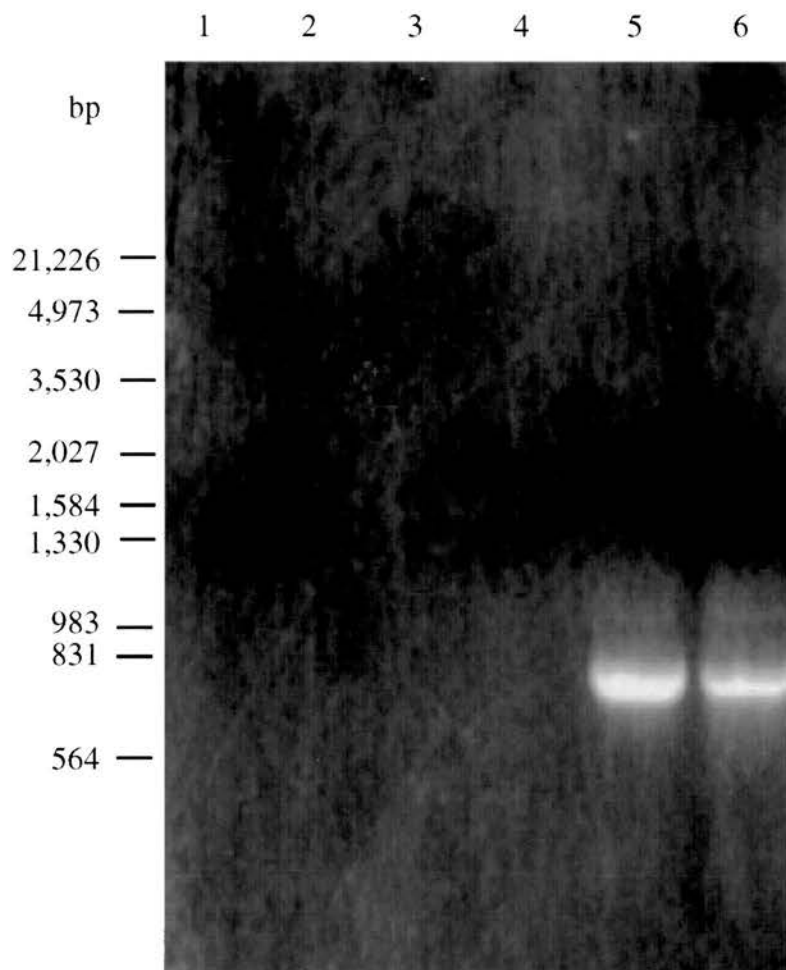
of CD2 was measured by FACS to determine the optimum time to be used for harvesting of transfected cells and subsequent recovery of plasmid DNA. Cells were stained with the monoclonal antibody OX34 (a gift from Dr. Neil Barclay, University of Oxford) which is specific for rat CD2. Figure A.3 shows the single colour immunofluorescence profiles of COS cells at various time points after transfection with the CDM8-CD2 plasmid. After 48 hours 40% of the harvested cells expressed the rat CD2 molecule. This decreased throughout the remainder of the assay with only 20% expressing rat CD2 72 hours after transfection. None of the mock transfected cells were reactive with the anti-CD2 monoclonal antibody (data not shown). The number of reactive cells decreased at time points after 72 hours and the number of viable cells also decreased. This is indicative of the transient expression system used by the CDM8 vector (Seed and Aruffo, 1987; Aruffo and Seed, 1987)

A.1.4 CD2 encoding cDNA can be recovered from transfected cells.

Following transfection and culture for 48 hours, COS cells were harvested and plasmid DNA recovered from transfected cells. It was possible to select the cells expressing the protein encoded by the transfected DNA using a monoclonal antibody specific for rat CD2 which was coupled to magnetically labelled beads. These positively selected cells were subsequently lysed and the plasmid DNA recovered by Hirt precipitation (Hirt, 1967). This plasmid DNA was then transformed into competent MC1061/p3 bacteria. The plasmid CDM8 contains a SupF element. Sup F plasmids can be selected in nonsuppressing host bacteria containing a second plasmid, p3. This plasmid contains amber mutated ampicillin and tetracycline drug resistance elements (Seed and Aruffo, 1987; Aruffo and Seed, 1987). These mutations are suppressed in the presence of a Sup F element and therefore bacteria transformed with a Sup F containing plasmid are resistant to the drugs ampicillin and tetracycline.

The transformed bacteria were grown on agar plates containing 12.5μg/ml ampicillin and 7.5μg/ml tetracycline. The following day, plasmid DNA was prepared from selected colonies and treated with the restriction endonuclease *Kpn*I prior to analysis by agarose gel electrophoresis. The results are shown in figure A.4. In each case (lanes 1-5), two bands were seen: a band of ~5.0kb corresponding to linearised CDM8-CD2 plasmid; and a larger size band which corresponds to uncut plasmid DNA (lane 7) non digested plasmid. Lane 6 shows the comparative agarose gel electrophoresis patterns of the control plasmid DNA prior to transfection and recovery. The similarity of these results shows that the transfection, expression and subsequent recovery of plasmid DNA from COS cells had been successfully established using the control plasmid. The next section deals with the transfection of the ovine cDNA library.

A.2 COS cells do not appear to express WC1 following transfection with an ovine cDNA library.

The protocol used to transfect and recover plasmid DNA encoding CD2 was used in an attempt to isolated cDNA encoding the ovine WC1 protein. A cDNA library, prepared from ConA stimulated ovine lymphocytes, by Dr. Raymond Bujdoso (University of Edinburgh) was used. COS cells where grown to optimal conditions and transfected with the CDM8 plasmid containing ovine cDNA. As a positive control, COS cells were transfected with CDM8-CD2 and as a negative control, no DNA was added to the transfection medium. Cells where left for 48 hours post transfection and then harvested. To determine whether the transfection protocol had been successful, some COS cells from each transfection experiment were stained with the specific

Figure A.3 Time course of COS cell transfection with CD2-CDM8.

COS cells were transfected with the control plasmid CD2-CDM8 and cultured for various lengths of time. The cells were then harvested and stained with anti-rat CD2 monoclonal antibody followed by anti-Ig FITC then analysed by FACScan. Single colour immunofluorescence profiles are shown. The figure shows positive cells to the right of the marker which was set on the non-specific staining of NMS (always <3%). This experiment was repeated three times and the same trends were seen on each occasion.

FIGURE A.3: Time course of COS cell transfection with CD2-CDM8.
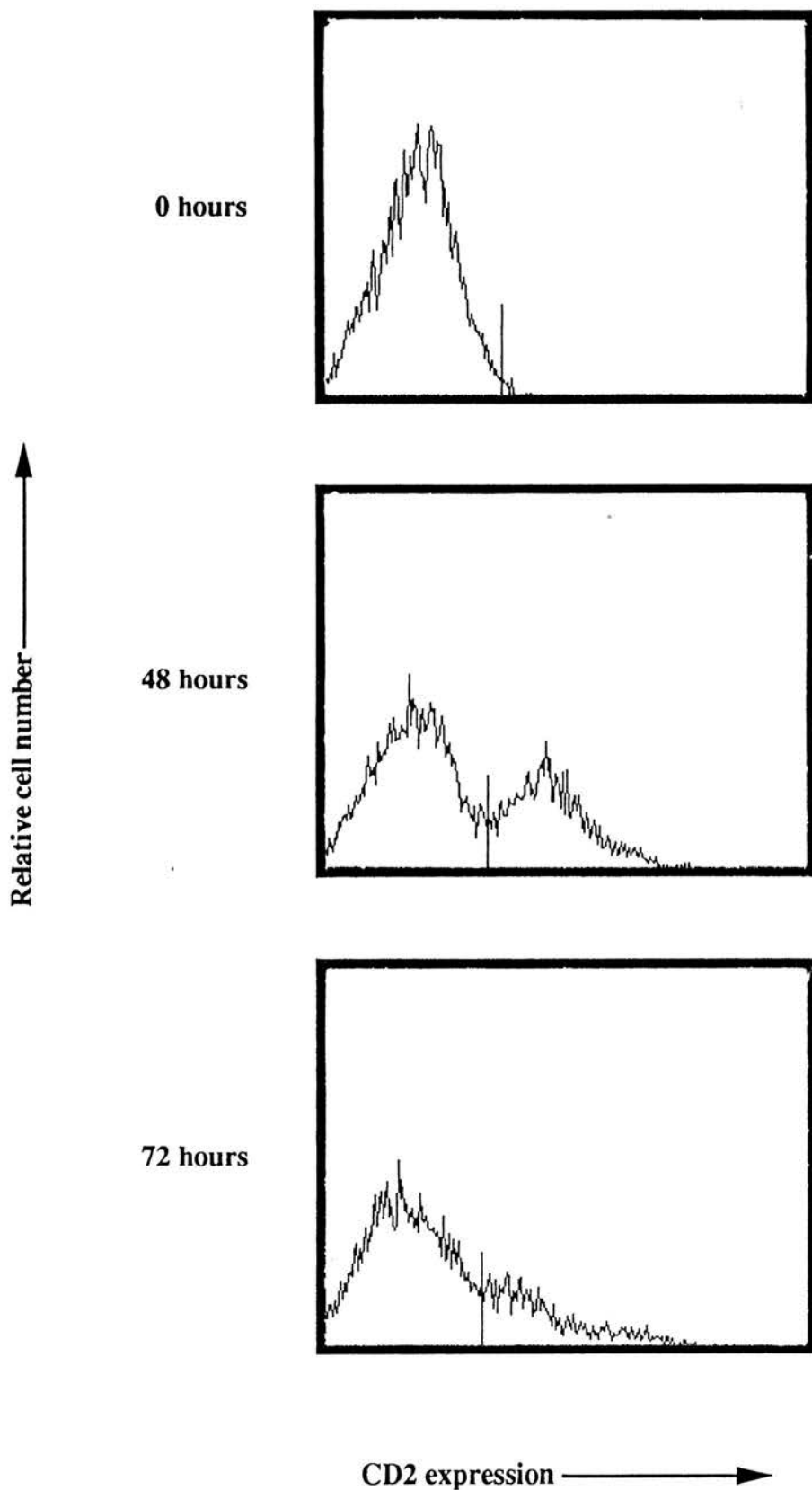
Figure A.4 *Kpn* I restriction digest of recovered CDM8-CD2.

Plasmid minipreps were made from five different colonies of *E.Coli* which were transformed with plasmid DNA recovered from transfected COS cells. The plasmid minipreps were incubated with the restirction enzyme *Kpn*I (lanes 1-5) according to the suppliers instructions. Control CDM8-CD2 plasmid was also incubated with the restirction enzyme *Kpn*I (lane 6) according to the suppliers instructions and with no restirction enzyme (lane 7). The DNA was then analysed on a 1% agarose gel by ethidium bromide staining following electrophoresis. DNA size markers were run in lane 8 and are also shown to the left of the figure. The plasmid DNA in each reaction was analysed on a 1% agarose gel by ethidium bromide staining following electrophoresis.

Figure A.4: Kpn 1 restriction digest of recovered CDM8-CD2
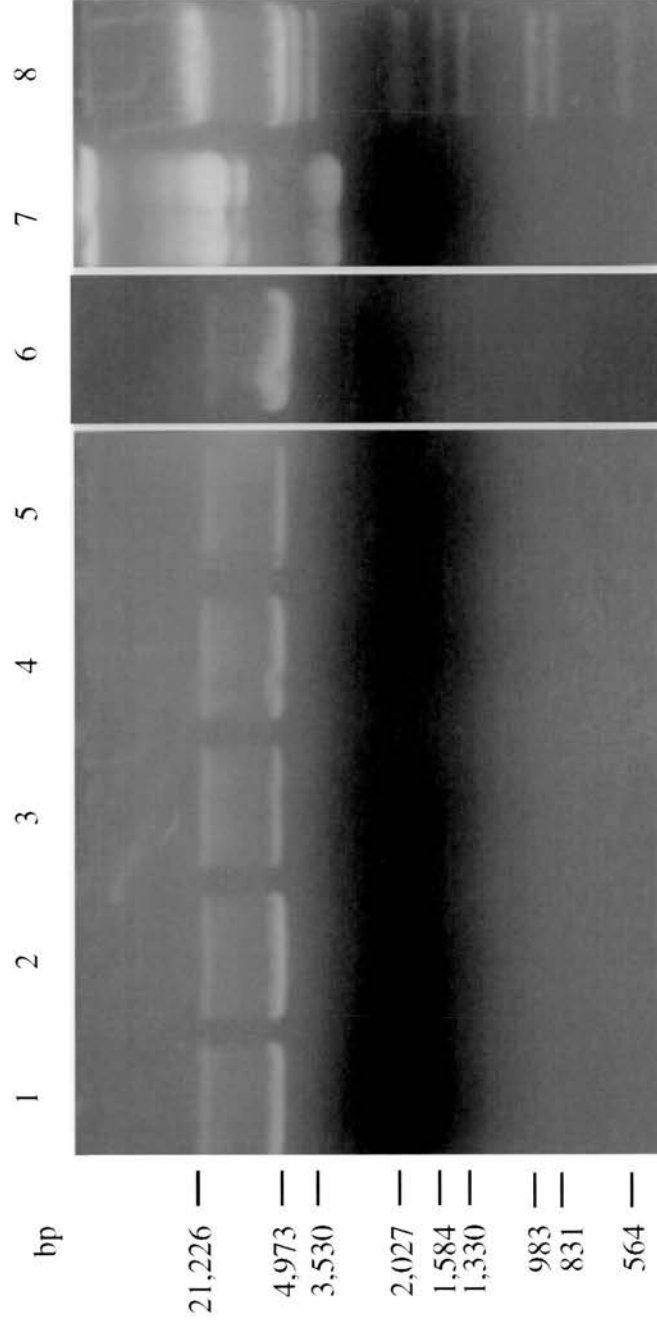
monoclonal antibodies and analysed by FACS. Figure A.5A shows the FACS profiles of COS cells transfected with CDM8-CD2 with the monoclonal antibody OX34. As was previously observed, 40% of cells transfected with the CD2 cDNA expressed this protein. COS cells transfected with the ovine cDNA library did not react with the monoclonal antibody specific for WC1 (figure A.5B).

It was unknown at the time that a collaborative project between the International Laboratory for Research into Animal Diseases (ILRAD), Kenya and the Department of Immunology, Utrecht Hospital, Netherlands was already underway to clone the gene encoding bovine WC1. Their project was successful and the results were recently published (Wijngaard et al., 1992, 1994; Metzelaar et al., 1992). The knowledge of a successful conclusion to this work became apparent during my initial attempts to clone ovine WC1. It was also revealed that a second collaborative project was underway with the Basel Institute for Immunology which was in the process of cloning the ovine gene. Due to these circumstances, the work in this thesis aimed at cloning the ovine WC1 gene was curtailed.

Figure A.5   Reactivity of transfected COS cells.

COS cells were transfected with the ovine cDNA library (figure A.5A) and the control plasmid CD2-CDM8 (figure A.5B) and cultured for 48 hours. The cells were then harvested and stained with either anti-WC1 (figure A.5A) or anti-rat CD2 (figure A.5B) monoclonal antibody followed by anti-Ig FITC then analysed by FACScan. Single colour immunofluorescence profiles are shown. The figure shows positive cells to the right of the marker which was set on the non-specific staining of NMS (always <3%). This experiment was repeated three times and the same trends were seen on each occasion.

Figure A.5: Reactivity of transfected COS cells.

(5A)

WC1 expression ⟶

(5B)

CD2 expression ⟶

Relative cell number
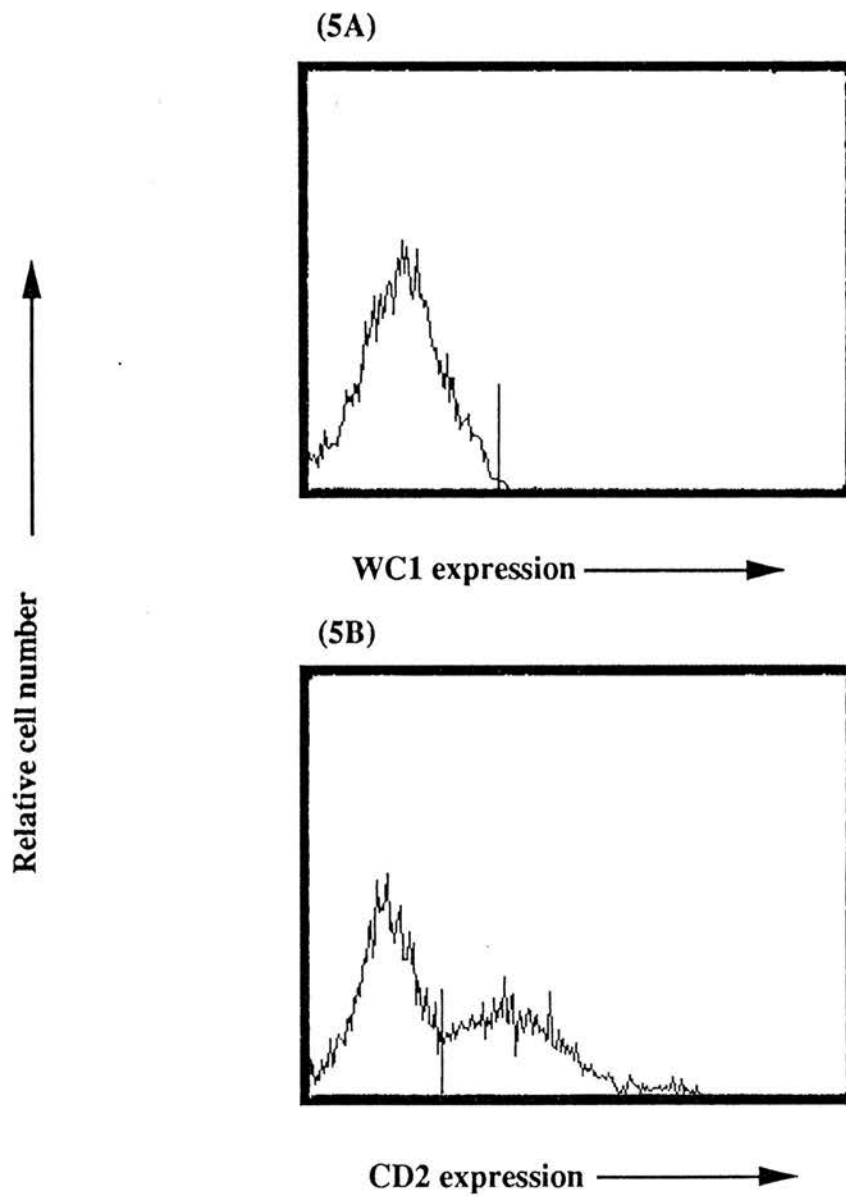
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6.7 Different rates of interleukin 2 receptor expression by ovine γ/δ and α/β T cells

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Abstract

We have used the monoclonal antibodies IL-A111, CACT116A, to investigate the expression of interleukin 2 receptors (IL-2R) by ovine peripheral blood T cells. Monoclonal antibodies (mAbs) IL-A111 and CACT116A reacted with concanavalin A (Con A) activated ovine lymphocytes and both mAbs did so with similar FACS (fluorescence activated cell sorting) profiles and fluorescence intensity. Following activation by Con A all three major ovine T cell subsets, namely CD4$^+$, CD8$^+$ and γ/δ T cells, express the IL-2R. All γ/δ T cells express IL-2R within 12 h following activation by Con A in vitro whereas α/β T cells do not express the IL-2R until 24 h after the start of culture with the mitogen. In freshly isolated peripheral blood a small percentage of lymphocytes, which are almost all CD4$^+$, express the IL-2R.

Introduction

Cross-linking of the antigen receptor on T cells results in the expression of several activation molecules including the interleukin 2 receptor (IL-2R). The function of this molecule is to bind IL-2 and allow the activated T cell to progress through the cell cycle during clonal expansion. In the human system high affinity IL-2R comprise at least two proteins: a 55 kDa protein designated CD25 (or α chain) and a 75 kDa protein which is believed to be responsible for signal transduction. Monoclonal antibodies (mAbs) have been generated which are known to react with bovine IL-2Rα chain (Naessens et al., 1992). cDNA encoding CD25 protein has been cloned and sequenced from several species including ovine (Bujdoso et al., 1992) and bovine (Weinberg et al., 1988). Based on the predicted homology of CD25 proteins between the ruminant species we have used workshop antibovine temporary cluster TC24 (CD25) mAbs to investigate the expression of IL-2R by peripheral blood T cells in the ovine species.

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Materials and methods

Monoclonal antibodies

The mAbs IL-A111 and CACT116A (both IgG_1 isotype) which were included in the temporary workshop cluster TC24 (CD25 cluster) were used in this study. Ovine T cell subset-specific monoclonal antibodies used were SBU-T4 and SBU-T8, which recognize OvCD4 and OvCD8, respectively, and are both of the IgG_{2a} isotype (Maddox et al., 1985). Ovine γ/δ T cells were identified by the WC1 mAb CC15 (IgG_{2a} isotype) which reacts with all of these cells in sheep. MHC Class II expression was identified by the mAb SW73.2 (IgG_{2a} isotype) which reacts with β chains of all known ovine MHC Class II molecules (Hopkins et al., 1986).

Preparation of peripheral blood mononuclear cells (PBMC) and immunofluorescence staining

PBMC were prepared from heparinized venous blood by centrifugation over Lymphoprep. Cells were cultured with 5 μg ml^{-1} Con A for the specified time in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} benzylpenicillin, 100 U ml^{-1} streptomycin, 5×10^{-5} M 2-mercaptoethanol and 2 g l^{-1} sodium bicarbonate.

Immunofluorescence staining and FACScan analysis of unstimulated or Con A-activated PBMC were as described elsewhere (Bujdoso et al., 1989).

Results and discussion

We have generated by polymerase chain reaction a 1.3 kb cDNA that contains the entire 825 bp coding region of the IL-2R protein CD25. The nucleotide sequence of this cDNA is detailed elsewhere (Bujdoso et al., 1992). Figure 1 shows the bestfit alignment of the predicted amino acid sequence of ovine CD25 in comparison with the predicted amino acid sequences of bovine, human and murine CD25. The precursor sequence of ovine CD25 (Bujdoso et al., 1992) has 94%, 72% and 63% homology with bovine (Weinberg et al., 1988), human (Nikaido et al., 1984) and murine (Miller et al., 1985) CD25 protein, respectively. Because of the predicted amino acid sequence homology between the ruminant CD25 proteins it seemed likely that mAbs generated to bovine CD25 would cross-react with ovine CD25. The mAbs IL-A111 and CACT116A were used to test this prediction. Our preliminary experiments showed that both mAbs reacted with native CD25 protein expressed on Con A-activated ovine PBMC. Approximately 60% of cells were stained by both mAbs. The control staining for these experiments was the reactivity of the mAbs IL-A111 and CACT116A with unstimulated PBMC

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       -21                    +1                    20                    40
OVINE  MEPSLLMWRFFVFIVVPGCVTEACHDDPPSLRNAMFKVLRYEVGTMINCDCKAGFRRVSAV---
BOVINE MEPSLLMWRFFVFIVVPGCVTEACHDDPPSLRNAMFKVFRYEVGTMINCDCKTGFRRVSAV---
HUMAN  MDSYLLMWGLLTFIMVPGCQAELCDDDPPEIPHATFKAMAYKEGTMLNCECKRGFRRIKSGSLY
MURINE MEPRLLMLGFLSLTIVPSCRAELCLYDPPEVPNATFKALSYKNGTILNCECKRGFRRLKEL-VY


                              60                    80                    100
       MRCVGDSSHSAWNNRCFCNSTSPAKNPVKPVTPGSEEQRERKPT-DAQSQTQPPEQADLPGHCE
       MRCVGDSSHSAWENRCFCNSTSPAKNQVKQVTPAPEEHREKKHT-DAQNQTQPPEEADLPGHCE
       MLCTGNSSHSSWDNQCQC-TSSATRNTTKQVTPQPEEQKERKTT-EMQSPMQPVDQASLPGHCR
       MRCLGNS----WSSNCQC-TSNSHDKSRKQVTAQLEHQKEQQTTTDMQKPTQSMHQENLTGHCR


                              120                   140                   160
       EPPPWEHEREPLKRVYHFTLGQTVHYQCAQGFRALHTGPAETTCTMIHGEMRWTRPRLKCISEG
       EPPPWEHEREPLKRVYHFTLGQTVHYQCAQGFRALQTSPAETTCMMINGELRWTRPRLKCIREG
       EPPPWENE--ATERIYHFVVGQMVYYQCVQGYRALHRGPAESVCKMTHGKTRWTQPQLICTGEM
       EPPPWKHE--DSKRIYHFVEGQSVHYECIPGYKALQRGPAISICKMKCGKTGWTQPQLTCVDER


                              180                   200                   220
       ANSQAPDEAEPPESTEAPPGSGTFLTTRMAGTTDFQKPTDVVATLDTFIFTTEYQIAVAGCILL
       EHGQASDDAEPQESTEAPPGSGTFLPTRMAGTTNFQKPTDEIATLDTFIFTTEYQIAVAGCTLL
       ETSQFPGEEKPQASPEGRPESET---SCLVTTTDFQIQTEMAATMETSIFTTEYQIAVAGCVFL
       EHHRFLASEESQGSRNSSPESET---SCPITTTDFPQPTETTAMTETFVLTMEYKVAVASCLFL


                              240
       LSSILLLSCLTWQRRWKKNRRTI
       LASILLLSCLTWQRKWKKNRRTI
       LISVLLLSGLTWQRRQRKSRRTI
       LISILLLSGLTWQHRWKKSRRTI
```

Fig. 1. The predicted amino acid sequences (in single letter symbols) of ovine, bovine, human and murine CD25 protein in a best-fit alignment.

which showed that approximately 10% of unstimulated cells bound both mAbs, suggesting that a significant number of activated cells are already, and probably continually, present in the peripheral blood of sheep (data not shown). mAbs IL-A111 and CACT11 6A are known to react with mouse L cells transfected with cDNA encoding bovine IL-2R α chain (Naessens et al., 1992) which strongly suggests these reagents react with the equivalent molecule expressed by ovine cells. The availability of known antibovine CD25 mAbs will complement those mAbs raised specifically to the ovine CD25 protein (Verhagen et al., 1992).

To determine which subset of T cells in peripheral blood expressed IL-2R in the absence of any in vitro stimulation, two colour immunofluorescence analysis using T cell subset-specific mAbs and IL-A111 mAb was carried out using PBMC isolated from lambs 6 weeks old. Figure 2 shows that the majority of CD25 expression in peripheral blood of these young lambs occurs on CD4$^+$ T cells as is the case in other species (Taga et al., 1991). Very few CD8$^+$ cells or γ/δ TCR cells express IL-2Rα chains in animals of this age. We have noticed in subsequent experiments using peripheral blood obtained from older animals that the percentage of CD4$^+$ cells expressing CD25 was greater than that shown here for relatively young animals. The failure to see significant IL-2R expression by CD8$^+$ or γ/δ TCR$^+$ cells may indicate that these
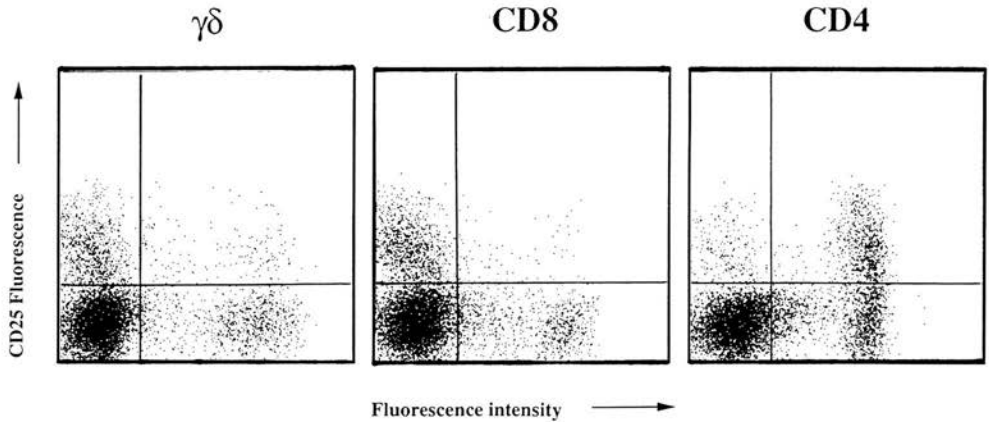
γδ   CD8   CD4

Fig. 2. Two colour immunofluorescence profiles of sheep PBMC stained with either CC15 (to detect γ/δ T cells), SBU-T8 or SBU-T4, and in each case with IL-A111. mAbs were detected by isotype specific second layer reagents and analysed by FACScan. Background gates were set with irrelevant IgG_1 and IgG_{2a} monoclonal antibodies.

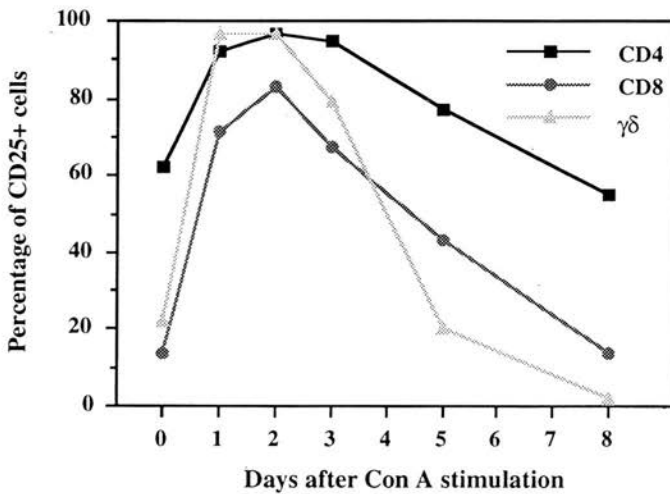Fig. 3. Sheep PBMC were cultured with 5 μg ml^{-1} Con A for 8 days. At time zero, and every 24 h thereafter, viable cells were harvested from culture and stained with either CC15 (to detect γ/δ T cells), SBU-T8 or SBU-T4, and in each case with IL-A111. mAbs were detected by isotype specific second layer reagents and analysed by FACScan. The percentage positive cells represents those cells coexpressing CD25 and a subset specific molecule. Background gates were set with use of irrelevant IgG_1 and IgG_{2a} mAbs.

cells do not express this particular receptor molecule, or that the mAb IL-A111 does not react with the IL-2Rα chains expressed by these cells. To test these alternatives PBMC were cultured with Con A for 7 days during which
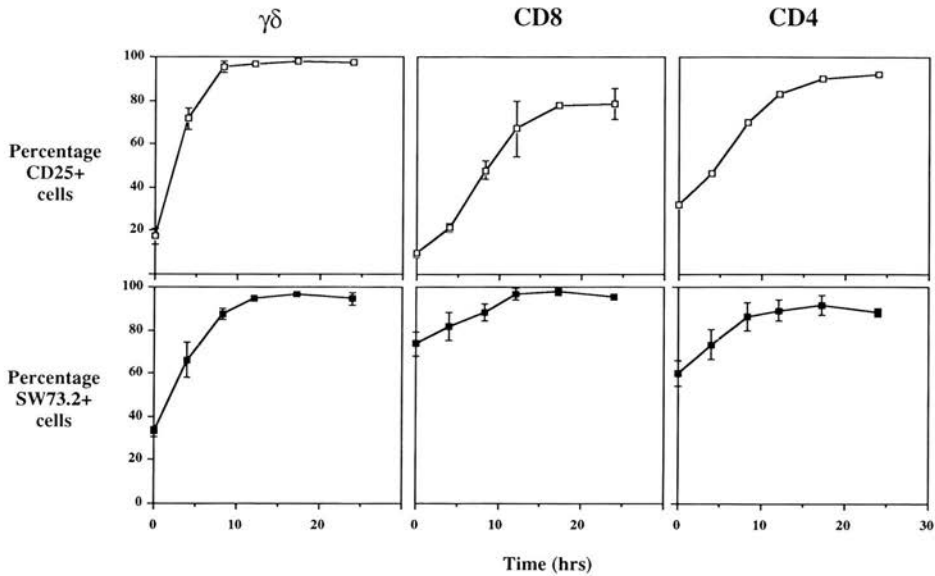
Fig. 4. Sheep PBMC were cultured with 5 μg ml^{-1} Con A for 24 h. At time zero and at the times shown viable cells were harvested from culture and stain with either CC15 (to detect γ/δ T cells), SBU-T8 or SBU-T4, and in each case with IL-A111 or SW73.2. mAbs were detected by isotype specific second layer reagents and analysed by FACScan. The percentage positive cells represents those cells coexpressing CD25 and a subset specific molecule. Background gates were set with use of irrelevant IgG$_1$ and IgG$_{2a}$ mAbs.

time the IL-2R expression by CD4, CD8 and γ/δ T cells was assessed at 24 h intervals by two colour immunofluorescence analysis. Figure 3 shows the percentage of each T cell subset expressing CD25 following activation by Con A. All three T cell subsets showed IL-2R expression following activation by Con A, and maximal numbers of cells within each subset expressed CD25 after 24–48 h of culture with the mitogen. The percentage of cells which expressed IL-2R within each subset decreased after 48 h of culture and returned to levels seen before the addition of Con A by Days 5–8. It is clear from these data that, firstly, all of the major T cell subsets of the sheep can express IL-2Rα chains, and secondly, that the acquisition, and the loss, of cell surface expression of this receptor protein appears to occur more rapidly with γ/δ T cells compared with both CD4$^+$ and CD8$^+$ T cells.

To investigate the apparent different rates of IL-2R expression by γ/δ and α/β T cells the time course for CD25 expression by Con A-activated cells was investigated over 24 h. Figure 4 shows that all γ/δ T cells expressed the IL-2R by 12 h following the start of culture with Con A. In contrast, the maximal number of CD4$^+$ and CD8$^+$ cells which expressed the IL-2R was not seen until 24 h after the start of culture with mitogen. It can also be seen that not

all the α/β T cells express CD25 after activation. This was most evident with CD8$^+$ cells where only approximately 80% of the cells manage to express CD25. Figure 4 also shows that the rapid expression of IL-2R by γ/δ T cells was not restricted to this activation molecule. MHC Class II molecules detected by the mAb SW73.2 showed as equally a rapid rise in expression as CD25. The maximal number of γ/δ T cells which expressed MHC Class II molecules was reached at the same time as the maximal number of cells which expressed the IL-2R. Significantly higher percentages of CD4$^+$ and CD8$^+$ T cells, compared with γ/δ T cells, already expressed MHC Class II molecules but the rate of expression of these molecules by the remaining α/β T cells occurred at a slower rate than was seen for γ/δ T cells.

These observations suggest the rate of activation is more rapid in γ/δ T cells than it is in α/β T cells. γ/δ T cells generally lack the coreceptor molecules CD4 and CD8 which are intimately associated with tyrosine kinases and distinct activation pathways. The likelihood exists that different activation pathways involving other regulatory molecules exist in γ/δ T cells which account for their more rapid rate of activation. Our future experiments will focus on these mechanisms.

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6.12 Expression of T19 (WC1) molecules by ovine lymphocytes

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Abstract

Workshop clusters WC1 and WC2 monoclonal antibodies (mAbs) were tested for their reactivity with ovine peripheral blood mononuclear cells (PBMC). The populations of ovine lymphocytes defined by these mAbs were found to be part of the population of T19$^+$ (WC1$^+$) cells, and the γ/δ T cell receptor (TCR) (WC2) expressing T cells. The expression of cell surface WC1 antigens following mitogen stimulation of ovine PBMC was studied. Whilst the size of the WC1 populations increased during culture with concanavalin A, the changes in the ratio of WC1:γ/δ TCR percentages observed suggested either a loss of WC1 molecules or a selective expansion of WC1$^-$ cells.

Introduction

In the ovine species the T19 molecule defines a distinct population of T cells which expresses a γ/δ T cell receptor (TCR) (Mackay et al., 1986) in association with CD3 and is negative for CD2, CD4, and CD8. A similar population of cells has since been identified in the bovine species by use of antiovine and antibovine T19 monoclonal antibodies (mAbs) (Mackay and Hein, 1989; Clevers et al., 1990). The mAbs recognize non-covalently linked protein species with molecular weights of 215 kDa and 300 kDa (Clevers et al., 1990; Morrison and Davis, 1991; Hein et al., 1991a). Results obtained from the first workshop on ruminant leukocyte differentiation molecules placed all the anti-T19 mAbs into workshop cluster WC1 and mAbs to the γ/δ TCR WC2 (Hein et al., 1991; Howard et al., 1991b). These clusters are referred to in the Second Workshop 2 as temporary clusters TC22 and TC36, respectively. The antigen is often referred to as T19 and the antibody cluster as WC1. In this paper all references made to either the antigen or antibody cluster will use the term WC1. Results from the First Workshop showed that,

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of all the WC1 clustered mAbs, CC15 reacted with most peripheral blood mononuclear cells (PBMC) in cattle and sheep (Mackay et al., 1991; Sopp et al., 1991). This mAb was submitted to the Second Workshop as a WC1 standard. It has also been shown that the WC1$^+$ population in cattle, defined by the coordinately expressed determinants termed N1 and N2, can be further split into two subpopulations with mAbs describing the determinants N3 and N4 (Davis et al., 1990). The determinants N1, N2, N3 and N4 are defined by mAbs to the WC1 complex. mAbs recognize determinants expressed on subpopulations of the CC15 defined WC1$^+$ cells and all immunoprecipitate proteins of the same molecular weight. mAbs that recognize the same determinants, or determinants on the same molecular complex, either block labelling of PBMC by other mAbs or yield diagonal staining patterns upon dual staining of PBMC. Not all WC1 mAbs have been assigned N specificities and a nomenclature has not yet been agreed upon to refer to the different determinants recognized on WC1 molecules. The ovine and bovine species are closely related in evolutionary terms and there is a strong likelihood that the N3 and N4 subpopulations may exist in the sheep. Here we report the reactivity of some of the TC22 and TC36 mAbs with resting and activated ovine peripheral blood lymphocytes. Our results suggest that the N3 population is not as prominent in the sheep as it is in cattle.

Materials and methods

Monoclonal antibodies (mAbs)

The WC1 (TC22) and WC2 (TC36) mAbs used are listed in Section 1. The ovine γ/δ T cell receptor mAb used was 86D (Mackay et al., 1989).

Immunofluorescence staining and FACScan analysis

Immunofluorescence staining and FACScan analysis of PBMC were carried out as described elsewhere (Bujdoso et al., 1989). PBMC were dual stained with unconjugated WC1 or WC2 clustered mAb followed by isotype-specific immunoglobulin–FITC and then normal mouse sera. Cells were then stained with either biotinylated CC15 or 86D followed by streptavidin–phycoerythrin and analyzed by FACScan. The proportion of the CC15 or 86D defined population reacting with the cross-reactive WC1 and WC2 mAbs, respectively, is given as a percentage of those populations. Background gates were set with use of irrelevant IgG$_1$ and IgG$_{2a}$ mAbs.

Preparation and culture of peripheral blood mononuclear cells (PBMC)

PBMC were prepared from heparinized venous blood by centrifugation over Lymphoprep. Cells were cultured for the times indicated with 5 μg ml^{-1} concanavalin A (Con A) in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} benzylpenicillin, 100 U ml^{-1} streptomycin, 5×10^{-5} M 2-mercaptoethanol and 5 mM HEPES.

Results

Reactivity of WC1 mAbs with ovine lymphocytes

WC1 (TC22) mAbs submitted to the Second Workshop on ruminant leukocyte differentiation molecules were analyzed for their reactivity with ovine lymphocytes. Table 1 and Fig. 1 show the results obtained with some of these mAbs by single colour immunofluorescence analysis of PBMC from a lamb approx. 6 months old and an adult sheep approx. 18 months old. The reaction patterns of the same mAbs on calf PBMC were included as a positive control and for a comparison of the results between the ovine and bovine species. All of the WC1 mAbs tested, apart from BAQ72A, reacted with PBMC from young and adult sheep. Smaller percentages of positive cells were seen using PBMC from older sheep. CC15, the workshop standard, reacted with 32% of lamb cells and gave a profile similar to that observed with bovine PBMC. BAQ89A and CACTB7A, like BAG25A (shown), reacted with 14–20% of lamb PBMC and were seen to have similar patterns of reactivity on both ovine and bovine PBMC. In contrast, the mAbs CC101 and CC117, like CC115 (shown), all stained 10–12% of lamb lymphocytes with similar immunofluorescence profiles though the reactivity was not the same across species. The mAbs CACTB15B and CACTB1A only reacted with small populations of lamb PBMC; the immunofluorescence profiles seen were different from lamb to lamb. In addition, the size of the population defined by these mAbs within ovine PBMC is much smaller than that defined within bovine PBMC.

Of all the WC1 clustered mAbs tested, CC15 reacted with the greatest number of ovine PBMC. Two colour immunofluorescence analysis was performed using CC15 versus other cross-reactive WC1 clustered mAbs to determine the subgroup profiles of these reagents. Table 2 and Fig. 2 show the results obtained using PBMC from an approx. 6-month-old lamb. All the workshop mAbs reacted with subpopulations of CC15$^+$ cells though none reacted with the same percentage as CC15. BAQ89A, CACTB7A, BAG25A (shown), CC101, CC117 (shown) and CC115 reacted with 58–67% of the population defined by CC15, whilst CACTB15B stained only 20% of CC15$^+$ cells. Cells stained at high to intermediate intensity with CACTB1A all reacted with

Table 1
Reactivity of WC1 and WC2 (TC22 and TC36) monoclonal antibodies with ovine lymphocytes

Monoclonal antibody	Lamb PBMC (approx. 6 months)	Adult PBMC (approx. 18 months)	Calf PBMC (approx. 6 months)
WC1 (TC22)			
CC15 (45)	32	8	25
CC101 (9&18)	10	5	17
CC117 (30)	12	4	19
CC115 (31)	10	3	17
CACTB1A (146)	6	2	13
CACTB15B (147)	4	6	12
BAQ72A (148)	0	0	9
BAQ89A (149)	14	4	11
CACTB7A (150)	14	4	11
BAG25A (151)	20	4	15
WC2 (TC36)			
86D	34	8	19
CACTB6A (136)	10	3	14
CACT16A (142)	0	0	16
CACT17A (143)	0	0	16
CACTB12A (144)	0	0	16
CACTB44A (145)	0	0	17
CACT71A (141)	0	4	7
CACT18A (139)	0	0	28
CACT61A (140)	0	0	26
CACT77A (167)	0	6	4
CACT26A (166)	0	2	8
CACT63A (189)	0	0	5

Cells were stained for single colour fluorescence analysis with the mAbs listed and analysed by FAC-Scan. The percentages listed are positive cells with respect to normal mouse sera used as a negative control. The results shown are a representative set of data from one of five different experiments which all showed the same trends as those seen here.

CC15, but of those cells stained with a low intensity of CACTB1A only a proportion reacted with CC15 (data not shown).

Reactivity of WC2 mAbs with ovine lymphocytes

Of all the WC2 (TC36) mAbs tested only CACTB6A showed clear reactivity with ovine cells where 10% of lamb and 3% of adult sheep PBMC were immunofluorescently labelled by the mAb (Table 1 and Fig. 1). The immunofluorescence profiles observed with this mAb on sheep PBMC were different to those seen on bovine PBMC. In particular, CACTB6A did not stain ovine PBMC with the high intensity fluorescence observed on bovine cells. CACT71A, CACT77A and CACT26A reacted with 2–6% of adult sheep cells
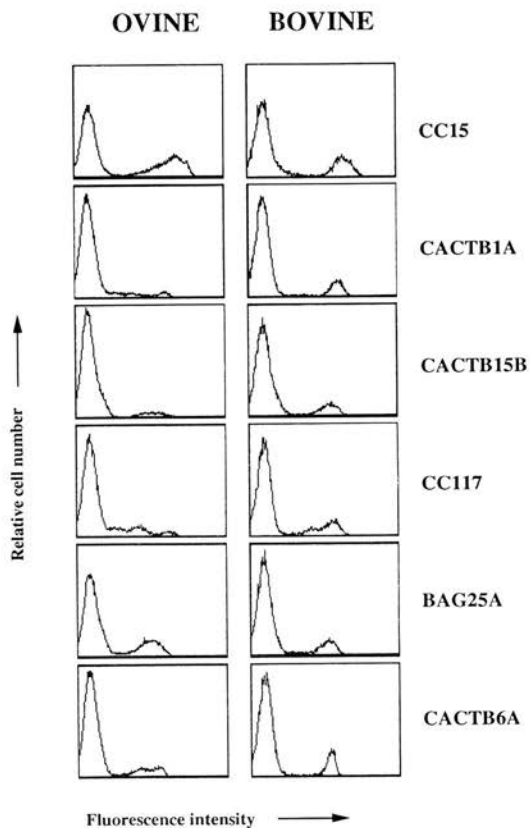
Fig. 1. Representative single colour immunofluorescence profiles of the reactivity of WC1 and WC2 mAbs with ovine PBMC isolated from a 6-month-old lamb and a 6-month-old calf. These immunofluorescence profiles show the staining patterns for some of the mAbs listed in Table 1. The results shown are a representative set of data from one of five different experiments which all showed the same trends as those seen here.

but failed to stain both ovine and bovine cells with high intensity immunofluorescence. PBMC from lambs were not reactive with these mAbs and they were not analyzed further. The mAb 86D identifies the γ/δ TCR molecule in ruminants. Table 2 and Fig. 2 show the results of two colour fluorescence analysis of 86D versus CACTB6A. The staining pattern shows clearly that CACTB6A defines a subpopulation of cells within the 86D population.

Changes in WC1 expression by Con A activated lymphocytes

WC1 is reported to be lost from ovine WC1$^+$ cells following activation (Hein and Mackay, 1991). Figure 3(a) shows the reactivity of some of the WC1 defined workshop mAbs and 86D (anti-γ/δ TCR) with ovine PBMC

Table 2
Two colour immunofluorescence analysis of WC1 and WC2 monoclonal antibodies with lamb PBMC

Monoclonal antibody	Percentage of CC15 population reacting with WC1 monoclonal antibody
WC1 (TC22)	
CC101 (9 and 18)	67
CC117 (30)	64
CC115 (31)	58
CACTB1A (146)	48
CACTB15B (147)	20
BAQ89A (149)	58
CACTB7A (150)	62
BAG25A (151)	65
	Percentage of 86D population reacting with WC2 monoclonal antibody
WC2 (TC36)	
CACTB6A (136)	21

The results shown are a representative set of data from one of three different experiments which all showed the same trends as those seen here.

CC117

BAG25A

CACTB15B

CACTB6A
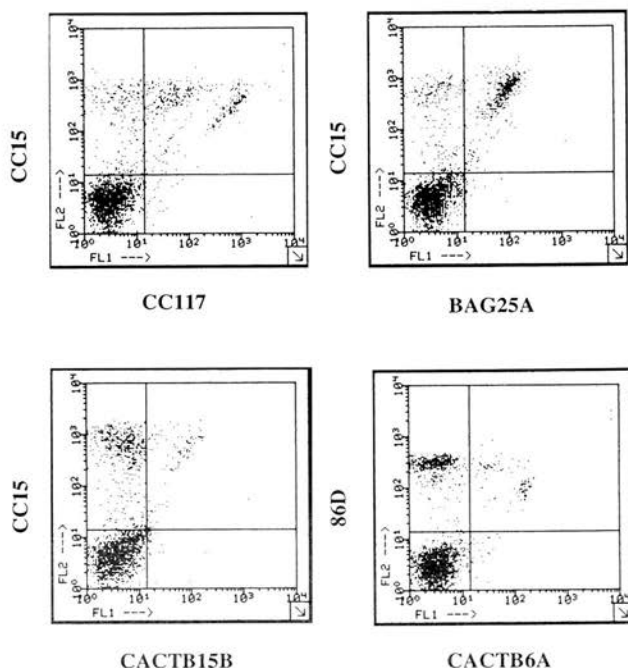
Fig. 2. Immunofluorescence profiles of two colour immunofluorescence analysis of the cross-reactive WC1 and WC2 mAbs. The FACS profiles shown here are for some of those reactivities shown in Table 2. The results are a representative set from three experiments all of which showed the same trends as shown here.
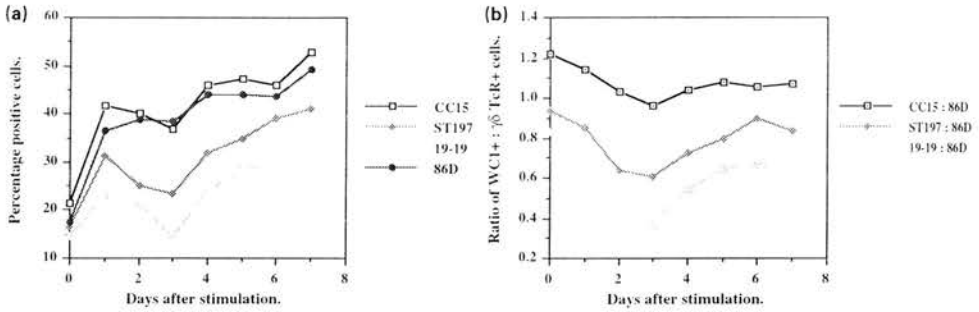
Fig. 3. Single colour fluorescence analysis of Con A stimulated ovine PBMC. Sheep PBMC were cultured with 5 μg ml^{-1} Con A for 7 days. At time zero, and the shown times, viable cells were harvested from culture and stained with either 86D (anti-γ/δ TCR), CC15 (workshop standard), 197 and 19-19 (these are the two mAbs in the WC1 cluster which have been raised against sheep lymphocytes). This was followed by antimouse immunoglobulin–FITC and analysis by FACScan. Normal mouse serum was used as a negative control. (a) Percentage staining observed with CC15, ST197, 19-19 and 86D. (b) Ratio between the percentage cells stained with the WC1 clustered mAbs and the percentage of cells stained with 86D (WC1:γ/δ TCR).

after their stimulation with the T cell mitogen Con A. It can be seen that the cell populations defined by the different WC1 mAbs and 86D all increased in size during the first 24 h of culture. The same was seen with all WC1 clustered mAbs (data not shown). The increase is more marked than that seen with the CD4$^+$ and CD8$^+$ T cell populations (data not shown). These observations support the notion of a faster rate of activation or proliferation by γ/δ compared with α/β T cells (Bujdoso et al., 1993).

After a slight fall in the percentage of WC1$^+$ cells between Days 1 and 3 the percentage of cells in the culture reacting with the WC1 mAbs increases thereafter during the culture period. Figure 3(b) shows the ratio of WC1:γ/δ TCR staining of PBMC following activation by Con A. Over the first 3 days the ratio falls, returning to starting levels over the next 4 days. These data suggest that the WC1 molecule, or WC1 epitopes, may be lost during expansion of γ/δ TCR$^+$ cells, or that there is a more rapid proliferation of WC1$^-$ cells during the initial period of culture.

Discussion

The mAb CC15 is believed to define a population of cells which represent all of the T19$^+$ (WC1) cells in cattle. This would also appear to be the case in the sheep for at least two reasons. Firstly, of all the WC1 mAbs tested, CC15 defines the largest numbers of PBMC in the ovine species. Secondly, the populations of cells defined by WC1 clustered mAbs tested here are subpopulations of CC15$^+$ cells. In cattle the N3 and N4 defined populations are of approximately equal size and their percentages summate to equal that of

N1$^+$/N2$^+$ cells, the N1$^+$/N2$^+$ population being slightly smaller in size than the CC15 population. The mAbs defining the N4 population in bovine (BAQ89A and BAQ159A from the First Workshop and BAG25A and CACTB7A from the Second Workshop) also define a population of cells in ovine though the mAbs recognize different epitopes (our own unpublished observations). BAG25A consistently reacts with the largest number of cells and using two colour immunofluorescence analysis it can be shown that no other N4 grouped mAb blocks the reactivity of BAG25A or has its own reactivity blocked by BAG25A. Instead, all other anti-N4 mAbs reacted with subpopulations of the BAG25A defined cells. Of the mAbs defining the N3 population in bovine (BAQ53A, BAS1A, CACTB32A, CACTB35A from the First Workshop and CACTB1A, CACTB15B, BAQ72A from the Second Workshop) only CACTB32A (very weakly), CACTB1A and CACTB15B react with ovine cells. From two colour immunofluorescence analysis it was seen that biotinylated CACTB1A reactivity was blocked by preincubation with either CACTB15B or CACTB32A. These N3 cross-reactive mAbs showed different staining profiles on ovine PBMC compared to those seen with bovine PBMC. Furthermore, the size of the N3 population in ovine PBMC is much less than the N4 population although the ovine N3 and N4 percentages still summate to the percentage of N1$^+$/N2$^+$ cells as is the case in bovine (our unpublished observations).

In summary, our results reported here indicate that the CC15 defined, sheep WC1$^+$ PBMC comprise mainly N4$^+$ cells with a small N3$^+$ population and possibly a third N3$^-$/N4$^-$ minor population. The reason(s) for the differences observed in the staining patterns across species with the N3 mAbs, and the mAbs CC101, CC117 and CC115 such as lower staining intensities and smaller number of positive cells are unclear. This may be a result of (1) different post-translational modifications of some protein determinants in sheep compared with cattle, (2) different levels of WC1 antigen expression in ovine compared with bovine, (3) a weak cross-reactivity of the mAbs, (4) simply a low representation of the homologous cell subpopulations in different species.

We have investigated the expression of WC1 on ovine T cells in an attempt to determine if this molecule is lost from the surface of cells following activation. The WC 1 population increased in size following stimulation with Con A. However, it could not be determined if the reductions in the ratios of WC1:γ/δ TCR was a result of loss of cell surface WC1, conformational changes of the molecule leading to determinant loss, or selective and rapid outgrowth of WC1$^-$ cells during the initial phase of the culture. This may be resolved using homogeneous populations of WC1$^+$ cells and WC1 mRNA studies together with an analysis of the cell surface expression of WC1.

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Antigen recognition and activation of ovine γδ T cells

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SUMMARY

We have investigated several aspects of γδ T cells in sheep. γδ T cells of sheep express a unique transmembrane protein termed T19 but lack the expression of particular cell-surface molecules such as CD2, CD4 and CD8 which are typically associated with αβ T cells. The majority of γδ T cells isolated from animals of all ages examined lacked the expression of CD45RA. A faster rate of activation by γδ T cells compared to either CD4 or CD8 T cells was seen in the time–course of IL-2 receptor α chain (CD25) cell-surface expression. All γδ T cells expressed the CD25 protein within 8 hr of activation whereas the majority of CD4 or CD8 T cells did not express CD25 until 24 hr post-concanavalin A (Con A) stimulation. This difference in the rate of expression of activation molecules was not restricted to CD25, as a similar trend was seen with cell-surface expression of major histocompatibility complex (MHC) class II molecules. We have used the distinct phenotypic profile of ovine γδ T cells to purify these cells by positive selection via the T19 molecule to assess their *in vitro* proliferative response to various antigens. Routinely, cell populations comprising more than 93% γδ T cells with yields of approximately 55% were obtained. Purified γδ T cells were capable of responding to *Mycobacterium tuberculosis* antigen in a primary and secondary *in vitro* proliferation assay and to ovalbumin in a secondary response. Ovine γδ T cells showed little, if any, proliferative response to allogeneic stimulator cells.

INTRODUCTION

The receptor for antigen on T cells is a heterodimeric structure consisting of either αβ or γδ polypeptide chains. The ligand for the αβ T-cell receptor (TcR) is known to comprise a complex of antigen in association with a presentational molecule.[1,2] In contrast to αβ T cells the ligand for the γδ TcR remains to be defined. Several presentational molecules have been described for γδ T cells which include major histocompatibility complex (MHC) class I, class II,[3–5] *Tl* antigens[6] and CD1.[7] The function of the receptor for antigen on T cells is to bind its ligand and allow transmembrane signal transduction to promote clonal expansion. The signal transduction event in αβ T cells is regulated, in part, by the co-receptor molecules CD4 and CD8 whose cytoplasmic domains interact with the *src*-related tyrosine kinase p56[lck].[8] The co-receptor molecules are believed to associate physically with the TcR and cause tyrosine phosphorylation of the CD3 ζ chain.[9] The majority of peripheral blood γδ T cells do not express the accessory

molecules CD4 or CD8[10,11] and while they do express CD45 the role of p56[lck] in these cells remains to be established. Collectively, this information might suggest that γδ T cells recognize presentational elements other than classical MHC molecules, and that different pathways of activation may occur in these cells compared to αβ T cells.

The majority of these findings have been made using human and mouse γδ T cells which comprise only 1–5% of the total circulating lymphocyte population in these species. Two significant features of ovine γδ T cells exist. Firstly, there is a high level of γδ T cells in the peripheral blood of sheep, especially in young animals where they may represent 50% of all T cells present.[12,13] Secondly, analysis of the expressed V region repertoire of ovine γδ T cells has shown a greater selection and diversity of Vγ and Vδ genes compared to humans and mice.[14] These observations imply a much more prominent role for ovine γδ T cells in immune responses by these animals. Here we report our initial findings on responses to antigen, and activation of γδ T cells from the ovine species which will help broaden our understanding of the role of this T-cell subset in the immune system.

MATERIALS AND METHODS

Animals

Male and female Finnish Landrace × Dorset, Grey Faced × Suffolk and Dorset × Suffolk sheep of various ages were obtained from the Moredun Research Institute, Edinburgh.

One- to 2-year old Finnish Landrace × Dorset sheep and 6-month-old Grey Face × Suffolk lambs were immunized with 1 mg ovalbumin and 1 mg heat-killed *Mycobacterium tuberculosis* injected over two sites intramuscularly in a 1-ml mixture (1:1) of phosphate-buffered saline (PBS) and complete Freund's adjuvant.

Antigens
Ovalbumin (grade V, cat. no. A5503) was obtained from Sigma Chemical Co. (Poole, U.K.). Purified protein derivative of human tuberculin (PPD, batch 298) was obtained from the Central Veterinary Laboratory (Weybridge, U.K.). Heat-killed *M. tuberculosis* strain H37RA was obtained from Difco (Detroit, MI).

Medium
RPMI-1640 (cat. no. 074-1800; Gibco Biocult, Uxbridge, U.K.) was supplemented with 2mM L-glutamine, 100 U/ml benzylpenicillin and 100 U/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol and 2 g/l sodium bicarbonate. Culture medium consisted of RPMI-1640 with supplements and 10% fetal calf serum (FCS; Ato-Tek Ltd, Glasbury, U.K.). Wash medium consisted of RPMI with supplements and 1% FCS.

Isolation of peripheral blood mononuclear cells (PBMC)
PBMC for FACS analysis and proliferation assays were isolated by centrifugation of heparinized venous blood at 800 g for 20 min to obtain buffy coat cells which were subsequently centrifuged at 600 g for 20 min at room temperature over Lymphoprep (Nycomed, Oslo, Norway). Cells collected at the Lymphoprep density medium interface were washed three times in PBS plus 1% FCS prior to any other treatment.

Monoclonal antibodies and immunofluorescence staining
The monoclonal antibodies used are described in detail in Table 1. All the monoclonal antibodies were obtained from their originators as generous gifts except 19.19, SBU-T4 and

Table 1. The monoclonal antibodies used in this investigation are listed with their specificity and isotype

Antibody	Specificity	Isotype	Reference
86D	γδ TcR	IgG1	15
19.19	T19	IgG1	15
ST197	T19	IgG2b	16
CC15	T19	IgG2a	17
ST4	CD4	IgG1	18
SBU T4	CD4	IgG2a	18
ST8	CD8	IgM	18
SBU T8	CD8	IgG2a	18
36F	CD2	IgG2a	19
VPM8	Ig light chain	IgG1	I. McConnell, unpublished data
73B	CD45RA	IgG1	20
SW73.2	MHC class II	IgG2a	21
IL-A111	CD25	IgG1	22

SBU-T8 which were purchased. Cell-surface phenotype w assessed using aliquots of 1×10^{6} PBMC washed with Pl containing 1% FCS and 0·1% sodium azide (PBS–FA). F single-colour fluorescence analysis cells were incubated wi 50 μl of monoclonal antibody at the appropriate dilution f 30 min on ice, washed three times with PBS–FA and the incubated with 50 μl of FITC conjugated F(ab')₂ fragment rabbit anti-mouse immunoglobulin (cat. no. F313; Dakopat Glostrup, Denmark) for 30 min on ice. For two-colo immunofluorescence, cells were stained with two monoclon antibodies of different isotypes then treated with a biotinylat sheep anti-mouse IgG1 (cat. no. PB273; The Binding Sit Birmingham, U.K.) followed by streptavidin–phycoerythr (cat. no. STAR 4A; Serotec, Oxford, U.K.) and either shee anti-mouse IgG2a–FITC (fluorescein isothiocyanate) co jugate (cat. no. PF274; The Binding Site) or goat anti-mou IgG2b–FITC conjugate (cat. no. SBA 1090-02; Sera La Crawley, U.K.) for 30 min on ice. Alternatively, when dua colour fluorescence was carried out with two IgG1 monoclon antibodies, cells were stained with the first antibody an anti-IgG1–FITC as described above and then with 10% normal mouse sera. Cells were reacted with the second IgG antibody, which was biotinylated, followed by streptavidin phycoerythrin. In all cases, cells were finally washed three time and analysed by flow cytometry using a Becton Dickinso FACScan system gating on mononuclear cells (Becto Dickinson, Cowley, U.K.). Ten thousand cells were analyse per sample with dead cells excluded on the basis of forwar light scatter. Background fluorescence, which was routine 1–3%, was established with normal mouse serum in single colour fluorescence analysis and irrelevant isotype-specifi monoclonals in two-colour immunofluorescence analysis.

Activation of PBMC by concanavalin A (Con A)
PBMC prepared as described above were washed once wit PBS and twice with wash medium then resuspended in cultur medium at 2×10^{6}/ml with either 5 μg/ml Con A (cat. n C2010; Sigma) in T25 tissue culture flasks (Life Technologie Ltd, Paisley, U.K.). At the various times indicated, cells wer removed from culture, harvested over lymphoprep an analysed by immunofluorescence analysis for either CD25 c MHC class II expression.

Purification of γδ T cells by positive selection using MACS
Lymphocytes were resuspended in PBS supplemented with 1% FCS at 4×10^{7}/ml and incubated with biotinylated anti-T1 monoclonal antibodies CC15 and ST197, simultaneously, fc 30 min at 4° then washed with PBS supplemented with 5 m EDTA (PBS–EDTA). Cells were further incubated wit avidin-coated microbeads for 15 min on ice followed b washing with loading buffer (PBS–EDTA supplemented wit 1% FCS). The labelled lymphocyte pellet was resuspended i loading buffer and loaded onto a MACS separator (Becto Dickinson, Oxford, U.K.) which consisted of a ferromatri column, precooled by washing with ice-cold loading buffe located within a strong magnetic field. Unlabelled cells wer washed out of the column by a series of back flushes wit loading buffer in the absence of the magnetic field, followed b subsequent washing with 3–4 column volumes of loading buffe in the presence of the magnetic field. Positively selected cell were subsequently recovered by removal of the magnetic fiel

nd eluting the column with loading buffer. γδ T-cell-depleted
mphocytes were obtained by removal of CC15⁺ cells by
1ACS separation of PBMC. The γδ T-cell-depleted popu-
tions are referred to as αβ T cells.

roliferation of purified γδ T cells

Antigen-specific proliferation to soluble protein antigen.
urified γδ T cells were resuspended in culture medium at
× 10⁶/ml and dispensed in 100-μl aliquots in 96-well flat-
ottom microculture plates (Life Technologies Ltd). Triplicate
ultures were stimulated where appropriate with the indicated
oses of antigen and 1 × 10⁵/well irradiated autologous PBMC
s antigen-presenting cells.

Allogeneic mixed lymphocyte cultures. Purified γδ T cells, or
δ T-cell-depleted populations, were resuspended in culture
nedium at 1 × 10⁶/ml and dispensed in 100-μl aliquots in
6-well round-bottom microculture plates (Life Technologies
_td). Triplicate cultures were stimulated with varying concen-
rations of irradiated unfractionated allogeneic PBMC in a
inal volume of 200 μl. Cultures were incubated at 37° in a
umidified atmosphere of 5% CO_2/95% air for 5 days in the
ase of allogeneic responses, and 3 days for responses to soluble
rotein antigen, then pulsed with 1 μCi [³H]thymidine (specific
ctivity 2 Ci/mM; Amersham International, Amersham, U.K.)
ver the last 5 hr. Cells were collected onto glass filter paper
sing a semi-automated harvester and [³H]thymidine incor-
oration was assessed by liquid scintillation counting. The data
re expressed as the geometric mean for the triplicate cultures
nd SD was generally less than 10% of the mean.

X-ray irradiation of antigen-presenting cells

PBMC were resuspended in culture medium to a concentration
f 2 × 10⁶/ml and irradiated with 5000 rads using a cobalt–
aesium source.

Statistical analysis

Statistical analysis was carried out by Student's *t*-test.

RESULTS

ack of CD45RA expression by γδ T cells

Ve have noticed in our analysis of T-cell subsets in sheep that
PBMC of young lambs are characterized by relatively high
evels of γδ T cells compared to those in other non-ruminant
pecies. The large number of γδ T cells, rather than CD4⁺ or
CD8⁺ cells, in peripheral blood of young lambs at a time of
arly antigenic experience may lead to preferential clonal, or
ligoclonal, selection and expansion of γδ T cells. Antigen-
pecific activation of γδ T cells in the periphery may result in an
ltered cell-surface phenotype for these cells. In the human
pecies it has been suggested that naive and memory γδ T cells
an be distinguished on the basis of differential expression of
M_r isoforms of CD45 [or leucocyte common antigen (LCA)].[23]
Ve have used the monoclonal antibody 73B which recognizes a
igh M_r isoform of sheep CD45 (M_r 220,000)[20] and is therefore
egarded as an anti-CD45RA monoclonal antibody, to
nvestigate any differences between sheep peripheral blood γδ
cells and CD4 and CD8 T cells in their expression of this
CD45 isoform. Table 2 shows that the majority of γδ T cells in
oung animals did not express CD45RA when stained with the

Table 2. Percentage (with standard deviation in brackets) of cells in
each lymphocyte subset expressing CD45 p220. PBMC were prepared
from Suffolk × Dorset animals, both fetal lambs (day 130 gestation)
and sheep of various ages (*n* = 6/group). Cells were stained with the
monoclonal antibody 73B in association with either 86D, CC15, SBU-
T4, SBU-T8 or VPM8 which were all biotinylated

Age of animals	Percentage of cells expression CD45 p220 isoform				
	CD4	CD8	γδ	T19	sIg
Fetal	79·2 (11·7)	69·9 (14·6)	11·7 (4·1)	9·1 (2·2)	97·8 (1·4)
6 days	68·5 (12·2)	63·5 (11·6)	23·4 (5·2)	27·8 (3·4)	94·6 (3·8)
4 months	34·3 (11·6)	55·0 (6·7)	11·7 (5·7)	11·1 (6·1)	97·0 (2·5)
>2 years	15·0 (5·0)	37·9 (7·1)	11·4 (3·5)	11·2 (3·4)	97·0 (2·6)

monoclonal antibody 73B, irrespective of the age of the animal.
In contrast, the majority of CD4⁺ and CD8⁺ subsets, in young
animals 6 days old, did express this isoform of CD45. 73B⁺
cells decreased within the CD4⁺ and CD8⁺ subsets in older
sheep.

Expression of activation molecules by γδ T cells

It has been reported that human memory, but not naive, T cells
express IL-2 receptor subunits.[24] As the majority of sheep γδ
but not CD4⁺ and CD8⁺ lack CD45RA expression, and
therefore resemble memory cells as described in other species,
we reasoned that differences may occur in the rate of activation
between these cells types. This was investigated by studying the
time–course of CD25 expression by ovine γδ T cells following
activation by Con A. While PBMC were cultured with Con A,
CD25 expression by γδ, CD4 and CD8 T cells was assessed by
two-colour immunofluorescence analysis. Figure 1 shows that
the majority of γδ T cells expressed the IL-2 receptor within 8 hr
following the start of culture with Con A. In contrast, the data
in Table 3 show that the maximal number of CD4⁺ and CD8⁺
cells which expressed the IL-2 receptor was not seen until 24 hr
after the start of culture with mitogen. It can also be seen that
not all the CD4⁺ and CD8⁺ T cells expressed CD25 after
activation. Figure 1 shows that the rapid expression of IL-2
receptors by γδ T cells was not restricted to this activation
molecule. MHC class II expression by γδ T cells, detected by the
monoclonal antibody SW73.2, showed a rapid rise in
expression similar to that seen for CD25. Higher percentages
of CD4⁺ and CD8⁺ T cells, compared to γδ T cells, expressed
MHC class II molecules before activation with Con A.
Expression of MHC class II molecules by CD4⁺ and CD8⁺
T cells which were negative for these molecules, at the start of
culture, occurred at a slower rate than was seen for γδ T cells.
These observations suggest the rate of activation is more rapid
in γδ T cells than it is in CD4⁺ and CD8⁺ T cells.

Positive selection of γδ T cells via the T19 molecule

Figure 2 shows the phenotype of ovine peripheral blood γδ
TcR⁺ cells with respect to the expression of the cell-surface
molecules CD2, CD4, CD8 and T19. The data show that the
majority of γδ T cells express the T19 molecule, while less than
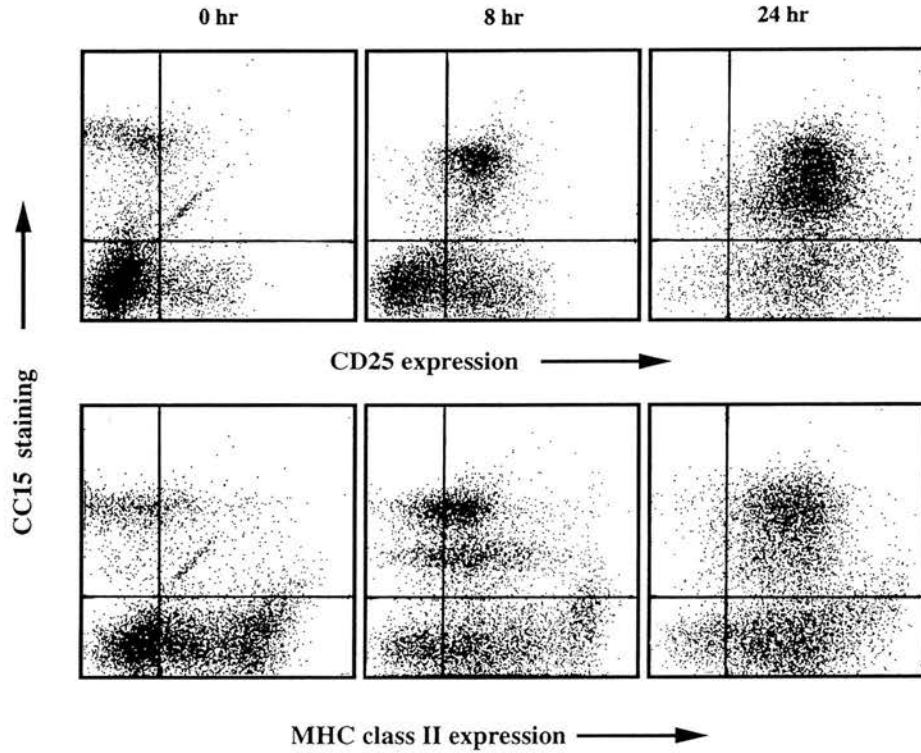5% of γδ T cells express either CD2, CD4 and CD8. The

C. W. *Evans* et al.

Figure 1. PBMC from Grey Faced × Suffolk sheep were cultured with 5 μg/ml Con A for 24 hr. At time 0 and at the times shown viable cells were harvested over Lymphoprep and stained with CC15 (to detect γδ T cells), and either IL-A111 or SW73.2. Monoclonal antibodies were detected by isotype-specific second-layer reagents. Background gates were set with use of irrelevant IgG1 and IgG2a monoclonal antibodies.

expression of the T19 molecule by almost all ovine peripheral blood γδ T cells allowed the purification of these cells to be attempted by negative and positive selection techniques. Of the procedures investigated, positive selection using anti-T19 monoclonal antibodies and MACS resulted in the highest purity and highest recovery of γδ T cells. Figure 3 shows a typical immunofluorescence profile of unfractionated PBMC and purified cells. γδ T cells were routinely purified to > 93% purity from a starting level of approximately 15% in unfractionated PBMC. Less than 3% of the T19 positively selected cells expressed either CD4, CD8 or surface immuno-globulin. Routinely, the recovery of γδ T cells was more than 55% of starting population. Viability of the purified γδ T cells was shown by their ability to proliferate in the presence of

Con A. After 3 days of culture with Con A the purified cell incorporated [^{3}H]thymidine to a level of 100,000 c.p.m compared to a background proliferation of 2000 c.p.m. fo cells cultured in media alone. In the bovine species, purified γ T cells responded poorly to Con A in the absence of exogenou IL-2.[17]

Purified γδ T cells were used in antigen-presentation assay using proliferation of cells as a measure of antigen recognition To show that there was no selective outgrowth of contaminat ing cells within the population of purified γδ T cells, Con A cultured cells were re-examined by immunofluorescence analysis for the presence of different T-cell subsets. As show in Fig. 3 the percentage of cells which expressed the γδ TcR following stimulation of purified populations of γδ T cells with

Table 3. PBMC from Grey Faced × Suffolk sheep were cultured with 5 μg/ml Con A for 24 hr. At time 0 and at the times shown viable cells were harvested over Lymphoprep and stained with CC15 (to detect γδ T cells), SBU-T4 or SBU-T8, and either IL-A111 or SW73.2. The values shown are the mean percentages (*n* = 3) of each T-cell subset co-expressing activation molecules. Standard deviations are shown in brackets

	Time after Con A stimulation (hr)					
	0	8	24	0	8	24
	CD25			MHC class II		
γδ	17·5 (3·6)	95·4 (2·5)	97·2 (1·2)	33·2 (2·2)	87·5 (2·5)	94·5 (2·7)
CD4	32·2 (2·5)	69·9 (4·5)	92·0 (3·3)	60·3 (5·8)	86·2 (6·7)	88·4 (1·9)
CD8	9·6 (1·6)	47·9 (4·3)	78·4 (7·0)	83·6 (5·4)	88·2 (4·1)	95·4 (1·0)

Con A was routinely greater than 95%. Less than 1% of these cultured cells expressed either CD4, CD8 or surface immunoglobulin. This suggests that the purity of the γδ T-cell population is maintained upon culture *in vitro*. To show that the purification technique did not activate γδ T cells, positively selected cells were cultured in media for 3 days and subsequently analysed for CD25 expression. As a control, unfractionated PBMC were cultured in an identical manner. Of the purified γδ T cells 13·8% were CD25$^+$ while 12·8% of γδ T cells within unfractionated PBMC expressed CD25. After
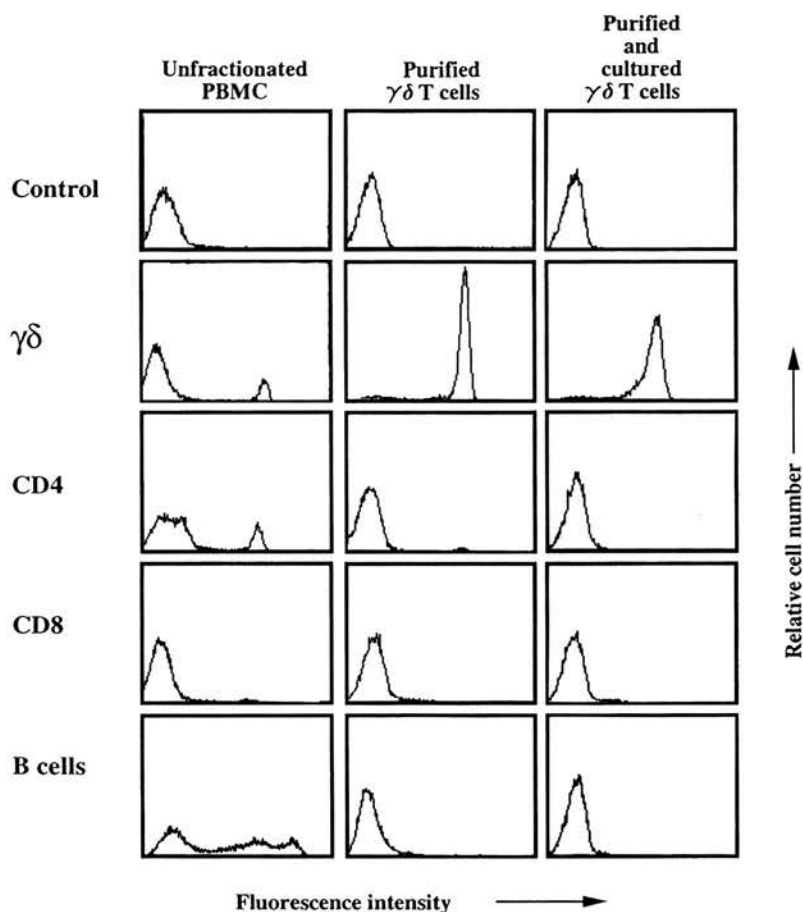

Figure 3. PBMC were prepared from a Grey Face × Suffolk sheep. T19$^+$ cells were immunolabelled with biotinylated ST197 and CC15 followed by streptavidin and finally MACS biotin microbeads. T19$^+$ cells were isolated by MACS separation and subsequently stained with either 86D, ST4, ST8 or VPM8 followed accordingly by either anti-mouse IgG or anti-mouse IgM isotype-specific FITC and analysed by flow cytometry. Some of the purified cells were cultured with 5 µg/ml Con A for 3 days and the phenotype of viable cells subsequently harvested over Lymphoprep determined.
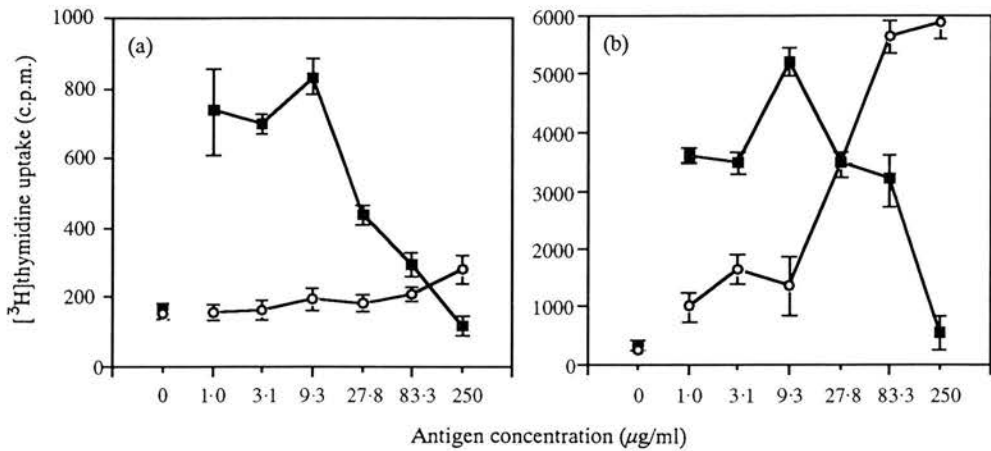
Figure 4. $\gamma\delta$ T cells were purified from peripheral blood of non-primed (a) or antigen-primed (b) Grey Face × Suffolk lambs ($n = 8$) by MACS using T19$^+$ selection as described in the Materials and Methods. 10^5 $\gamma\delta$ T cells/well were cultured with 10^5/well autologous irradiated PBMC and various concentrations of ovalbumin ($\circ$) or *M. tuberculosis* antigen ($\blacksquare$) in flat-bottom plates for 5 days and proliferation was measured by the uptake of [^{3}H]thymidine over the last 5 hr. The results shown are data from one experiment.

culturing isolated $\gamma\delta$ T cells for 3 days the percentage of CD25$^+$ cells rose to 21·7%. A similar rise in the percentage of CD25$^+$ $\gamma\delta$ T cells in cultured unfractionated PBMC was seen. These observations suggest that the increase in CD25 expression observed on the purified population of ovine $\gamma\delta$ T cells was a general consequence of *in vitro* culture as $\gamma\delta$ T cells within unfractionated PBMC showed a similar percentage increase in CD25 expression.

Proliferation by $\gamma\delta$ T cells to *Mycobacterium* and ovalbumin antigen

The recognition and response by $\gamma\delta$ T cells to antigen may be assumed to occur in a manner analogous to that by $\alpha\beta$ T cells; namely the receptors for antigen on these cells bind a complex comprising processed antigen and presentational element promoting clonal expansion of activated cells. Direct evidence that $\gamma\delta$ T cells were capable of proliferating to exogenous antigen was obtained by using MACS-purified populations of

these cells. Purified $\gamma\delta$ T cells were tested for their ability t respond in a primary and secondary *in vitro* proliferativ response to *Mycobacterium* antigen and ovalbumin usin autologous irradiated PBMC as antigen-presenting cells a shown in Fig. 4. Significant proliferative responses ($P \leqslant 0·10$ by purified $\gamma\delta$ T cells were seen in both cases to *Mycobacteriun* antigen. $\gamma\delta$ T cells showed no significant ($P > 0·10$) prolif eration in a primary response to ovalbumin but did sho\ significant ($P \leqslant 0·10$) secondary response to this antigen. Ther was no difference between stimulation indices of culture harvested after 3 or 5 days of culture (data not shown). Whil $\gamma\delta$ T cells showed a response to the antigens tested, in particula during a secondary *in vitro* proliferation assay, the magnitud of proliferation was regarded as low. This may reflect ai inability of $\gamma\delta$ T cells to provide all the lymphokines require for a proliferative response in an autocrine manner, that i without the help of $\alpha\beta$ T cells, or it might reflect ai inappropriate choice of antigen-presenting cell for these γ T-cell responses.

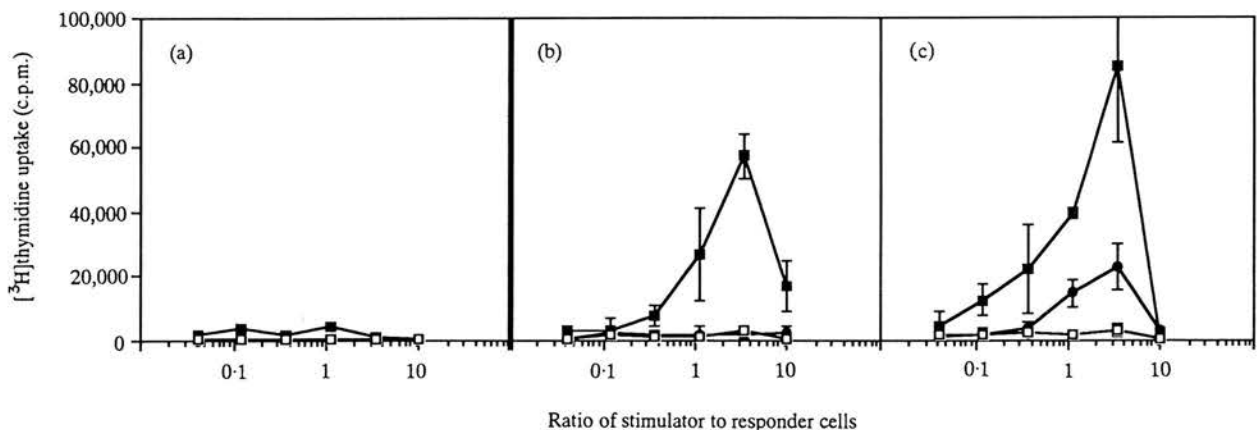**Figure 5.** $\gamma\delta$ T-cell and $\gamma\delta$ T-cell-depleted populations were purified from peripheral blood of Grey Face × Suffolk lambs ($n = 3$) by MACS usin; T19$^+$ selection as described in the Materials and Methods. 10^5 responding cells/well, either unfractionated PBMC ($\bullet$), $\alpha\beta$ T cells ($\blacksquare$) or $\gamma\delta$ T cells ($\square$) were cultured with various numbers of autologous irradiated PBMC (a) or two different allogeneic PBMC (b and c) in round-bottom plates for : days. Proliferation was measured by the uptake of [^{3}H]thymidine over the last 5 hr. The results shown are data from one experiment.

oliferative responses by γδ T cells to allogeneic cells

T cells from mice and humans are reported not to respond to logeneic stimulator cells[6,25] suggesting they do not recognize ssical MHC molecules. Sheep have a higher percentage of γδ cells in their circulation and have a broader expressed pertoire of γ and δ genes compared to these cells in mice and mans.[14] This suggests that ovine γδ T cells as a population n respond to a broader array of presentational elements. gure 5 shows a typical response by purified ovine γδ T cells to logeneic stimulator cells derived from two different sheep. In th experiments the response by fractionated γδ T cells was t significantly ($P > 0.1$) different from response by the me cells to equivalent concentrations of autologous radiated PBMC. The response seen by unfractionated BMC was less than the response seen by αβ T cells. This hanced proliferation by αβ T cells was also seen when lls were cultured in the presence of irradiated autologous BMC alone. The data suggest that the majority of ovine γδ T lls, like those in other species, does not recognize and roliferate to polymorphic molecules presented by allogeneic rget cells.

DISCUSSION

t is valid to assume that cell populations which express omologous molecules show a conservation of function across ide species barriers. With this in mind it is reasonable to xpect a greater understanding of γδ T-cell function by analysis f these cells in species which show a high reliance on this articular T-cell subset. γδ T cells in the peripheral blood of heep, and other ruminants, may comprise up to 50% of all ' cells present which is in marked contrast to humans and mice vhere γδ T cells constitute only 1–5% of T cells. The greater rominence of γδ T cells in young ruminants compared to other pecies may reflect a greater reliance by these animals on he function of γδ rather than CD4 and CD8 T cells at this tage of their development. During the early part of a heep's life γδ T cells must display a sufficiently diverse epertoire of both antigen specificity and effector function to elp deal with the particular pathogen load to which the nimal is subjected. The expressed γδ T-cell repertoire of umans and mice is very small, with T cells expressing distinct γ and Vδ pairings populating particular sites such as skin and ut tissue. In sheep the greater prevalence of γδ T cells in eripheral blood is accompanied by a greater diversity of the xpressed repertoire for these cells compared to humans and nice.[14]

We have utilized the expression of the T19 molecule by vine γδ T cells to purify these lymphocytes from the peripheral lood of sheep. γδ T cells were purified by positive selection rough the binding of immunomagnetic beads to the T19 nolecule and adhering labelled cells to a magnetic ferrous olumn. Routeinly, populations of γδ T cells of more than 93% urity and 55% yield were obtained. Our aim in attempting to urity ovine γδ T cells was to investigate antigen recognition by nese cells. The ability of ovine γδ T cells to respond to xogenous antigen was suggested by the expansion of these cells cultures of PBMC with either *Mycobacterium* antigen or valbumin. The use of polyclonal populations of purified ovine δ T cells confirmed that these cells do indeed respond by

proliferation to these antigens. Ovine γδ T cells were shown to respond to heat-killed *Mycobaterium* antigen in a primary and a secondary *in vitro* proliferative response, and to ovalbumin in only a secondary response. The relatively weak responses made by γδ T cells to these exogenous antigens may reflect an inherent poor ability of these cells to proliferate in the absence of an interaction with αβ T cells, possibly for the supply of interleukins in a paracrine manner. Alternatively, the responses may be a reflection of the choice of antigen-presenting cells in the assays. It is not known what presentational elements are recognized by ovine γδ T cells and therefore which cells act as their principal antigen-presenting cell. In the mixed lymphocyte reactions reported here purified ovine γδ T cells responded less vigorously than did αβ T cells. This suggests that polymorphic MHC molecules may not be the principal presentational elements recognized by the majority of γδ T cells in sheep.

A comparison of the rate of activation *in vitro* reported here has shown that ovine γδ T cells were activated by Con A prior to CD4 or CD8 T cells as assessed by expression of IL-2 receptor α chain, CD25, and expression of MHC class II molecules. The expression of IL-2 receptors by γδ T cells has been described in other species. Human γδ T cells constitutively express the β chain of the IL-2 receptor[26] which is believed to bind IL-2 and initiate the expression of CD25,[27] yielding an IL-2 receptor of high affinity. The constitutive expression of the β chain of the IL-2 receptor would allow a population of cells a faster rate of activation compared to those which require the induction and synthesis of this molecule. Several other explanations may exist for the difference in activation between the two T-cell subsets. Firstly, different signal transduction pathways may exist between the two T-cell lineages as the majority of ovine γδ T cells lack the CD4 and CD8 molecules which in αβ T cells associate with the tyrosine kinase p56^{lck},[8] and fail to express CD2 which is also functionally associated with p56^{lck}.[28] Secondly, the two T-cell subsets may have different transcriptional and translational control of CD25 expression. Ovine γδ T cells differ from their counterparts in other species in that they lack expression of the activation and adhesion molecule CD2. The functions of CD2 may be replaced on ovine γδ T cells by molecules such as T19. The T19 molecule might therefore serve as a co-receptor molecule for ovine γδ T cells, like CD4 and CD8 for αβ T cells.

The putative role of this molecule in the activation/maturation of γδ T cells is supported by the recent cloning and sequencing of three different cDNAs which share approximately 80% homology and encode the bovine T19 molecule.[29] The predicted amino acid sequence of the T19 molecule is that of a type I integral membrane protein with an extracellular domain consisting of 11 scavenger receptor cysteine-rich repeats. Membrane molecules with similar repeat units include the prototypic macrophage scavenger type I receptors. Significantly, the T19 protein has sequence similarity with CD5 and CD6 which have been shown to be involved in T-cell activation.[30]

In humans and rodents the 190,000–200,000 MW CD45RA molecules are found on naive T cells which, when activated, lose expression of this isoform of CD45 and express the low M_r 180,000 isoform CD45RO. The majority of ovine peripheral blood γδ T cells lack expression of CD45RA. In the absence of

a monoclonal antibody to the low M_r isoform of CD45 we cannot confirm that these cells express CD45RO and whether they display a memory phenotype which has been described for T cells in other species. However, the rapid appearance of CD25 on the surface of ovine $\gamma\delta$ T cells reported here is similar to that described for memory T cells in other species.[24] In experiments to be reported elsewhere we have found that while less than 10% of $\gamma\delta$ T cells in ovine peripheral blood express CD25, 50% of $\gamma\delta$ T cells in afferent lymph express the IL-2 receptor. This supports the notion that $\gamma\delta$ T cells mediate a functional role at epithelial sites[31] and as a consequence of recognition of antigen at these sites drain to peripheral lymph nodes in an activated state. As the percentage of CD25$^+$ $\gamma\delta$ T cells in efferent lymph is equal to the percentage found in peripheral blood, and as the lymphocyte composition of efferent lymph is derived mainly from peripheral blood[32] it would appear that activated $\gamma\delta$ T cells which enter the node do not leave this site. The function of $\gamma\delta$ T cells at epithelial sites is proposed to be the elimination of autologous transformed or stressed cells.[33] If this is the case two features of $\gamma\delta$ T cells may be expected. Firstly, activated $\gamma\delta$ T cells should be present in lymph fluid draining epithelial sites. Our unpublished observations that show approximately 50% of $\gamma\delta$ T cells in afferent lymph express CD25 suggests these cells have been activated at peripheral sites. Secondly, $\gamma\delta$ T cells should display a cytotoxic function. Published data by others suggest ovine $\gamma\delta$ T cells are capable of killing allogeneic cells via MHC class I molecules,[34] although the MHC restriction pattern of these cells was subsequently reported to be inconsistent.[12]

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