A Study of Ovine $\gamma\delta$ 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.

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ACKNOWLEDGEMENTS.

The three years I have spent in Edinburgh have been a very rewarding period not only in terms of the science I have learnt but also because of the people I have met and the enjoyment of living in such a scenic City.

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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.

Carys Wyn Evans (February 1993)
As in other species ovine T cells are subdivided on the basis of their TCR and express either a γδ or αβ molecule. In the ovine, the majority of the γδ T cell subset are further defined by the expression of a cell surface glycoprotein termed T19. The T19 molecule is not expressed by ovine αβ T cells which do express CD2, CD4 and CD8.

In this thesis a panel of monoclonal antibodies was utilised to investigate the prevalence of γδ T cells in peripheral blood isolated from animals of different ages and exposed to various antigenic burden. In addition these reagents enabled a characterisation of the cell surface molecules expressed by ovine γδ T cells. These studies demonstrated that ovine γδ T cells lack the expression of the co-receptor molecules CD4 and CD8, as do γδ T cells in most species. CD4 and CD8 are associated with T cell activation pathways. This suggested that aspects of activation in γδ T cells may differ to activation pathways in CD4-positive and CD8-positive T cells.

An investigation of γδ T cell activation, as assessed by CD25 expression, demonstrated two novel aspects of γδ T cell immunobiology. Firstly, the majority of γδ T cells within afferent lymph draining epithelial sites, in the absence of any intentional in vivo antigen challenge, were activated. This was in contrast to γδ T cells within peripheral blood and efferent lymph which were primarily CD25-negative. Secondly, γδ T cells were activated prior to αβ T cells in vitro. Furthermore, ovine γδ T cells respond by proliferation to antigens in vitro presented by antigen presenting cells within peripheral blood mononuclear cells. Ovine γδ T cells appear to recognise their ligand as a complex of antigen in association with MHC class I and MHC class II molecules.
Publications Arising From Work In This Thesis.

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CHAPTER 1

Literature Review.

The hallmark of the vertebrate immune system is its ability to mount a highly specific immune response against virtually any foreign entity. This specificity can be determined by two different types of cells known as B and T lymphocytes. These cells are similar morphologically and derive from common haematopoietic stem cell precursors. In the case of B cells, the molecules responsible for specific recognition are the different classes of antibodies that can be expressed either as cell-surface molecules acting as receptors, or in the secreted forms serving a variety of purposes including the initiation of complement-mediated killing and induction of viral particles by direct binding. The equivalent recognition molecule on T cells is the membrane-bound T cell antigen receptor (TCR). TCRs comprise heterodimeric polypeptide chains that recognise cell-associated fragments of foreign antigens. All TCRs were initially thought to be composed of α and β polypeptide chains. However, the search for the genes encoding these polypeptide chains led to the identification of a third rearranging gene encoding a γ chain (Saito et al 1984, Hayday et al 1985) that was later shown to code for one of the two subunits of another heterodimeric TCR, the γδ TCR (Bennett et al 1986, Brenner et al 1986, Weiss et al 1986).

This thesis is an investigation of ovine γδ T cells, therefore this introduction will focus on aspects of T cell immunology, with particular reference to γδ T cells. The areas covered in this introduction are primarily focused on how the repertoire of TCR is generated, ligand recognition by T cells and functional roles of γδ within the immune system.

1.1 T Cell Receptor Gene Segments.

As in the case of immunoglobulin genes the TCR α, β, γ and δ loci consist of variable (V), joined (J), diverse (D - in the case of the β and δ loci) and constant (C) gene segments which undergo somatic rearrangement to generate functional genes. The diversity of the rearranged genes results from combinatorial diversity arising from the use of different segments present in the germline sequence and somatic rearrangement.
LITERATURE REVIEW.

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1.1. T Cell Receptor Gene Segments.

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appear to be mediated by processes similar to those that control the rearrangement of immunoglobulin gene rearrangements, comprising both looping out and deletion (Fujimoto et al 1987, Okazaki et al 1987, Lai et al 1987) and inversion (Malissen et al 1986). The orientation of the gene segments to be joined determines whether looping out or deletion occurs. Junctional diversity arises from differential trimming of the termini of the recombinig gene segments by an exonuclease, and additional junctional diversification is initiated by insertion of template independent nucleotides by terminal transferase (Tonegawa 1983). Additional, although limited, diversity may be generated by the inclusion of nucleotides (P elements), perhaps by the transfer of nucleotides to the blunt end of the opposite strand by a novel enzyme prior to trimming, insertion of NGE and ligation (Lafaille et al 1989).

1.2. Diversity of the γδ TCR.

In the human and murine species the TCR γ and δ locus structures share an important feature in that, unlike the TCR α and β loci, they contain a limited number of germline V and C segments, limiting their capacity for germline diversity. In humans 14 Vγ genes (of which only 7 or 8 are potentially functional) belonging to four families and 6 Vδ genes have been identified (Moretta et al 1991). The murine γ locus encodes 7 Vγ genes belonging to 5 families, whilst the δ locus encodes 8 Vδ genes, each comprising a family (Raulet 1989).

It has been estimated in the human species that 700 V region combinations are possible for the γδ heterodimer, whilst those of the αβ TCR are thought to number approximately 2500 (Davis and Bjorkman 1988). Despite the limited number of these germline V and C segments, investigations analysing the rearranged TCR γ and δ genes have disclosed extensive junctional diversity. Such variability in the junctional region is due to the imprecision of the joining processes and the addition of short stretches of nucleotides, called the N regions, for both TCR γ and δ. The junctional combinations for the human γδ heterodimer are calculated to be even higher than those of the αβ heterodimer (10^{18} vs 10^{15}) (Brenner et al 1988, Davis and Bjorkman 1988).
This limited V region repertoire of the \( \gamma \) and \( \delta \) loci appears not to be a consensus of all species, as it is apparent from the recent studies of Hein and Dudler (1993) that ovine \( \gamma \delta \) T cells express a broad repertoire of \( \gamma \delta \) T cell antigen receptors, contributed by the increased diversity in both V and C region gene segments of both the \( \gamma \) and \( \delta \) loci. V region diversity in the sheep results mainly from the utilisation of a large family of duplicated V8 genes. 28 V8 regions belonging to 4 families and 10V\( \gamma \) regions belonging to 6 families have been described (Hein and Dudler 1993). In addition the ovine \( \gamma \) and \( \delta \) loci encode 5 and 1 constant gene segments, respectively. Specific splicing of each of the \( C\gamma \) segments to distinct sets of rearranged \( V\gamma \) segments increases the diversity of the ovine \( \gamma \delta \) TCR in comparison to other species as the homologous locus in humans and mice encode 2 \( C\gamma \) and 4 \( C\gamma \) genes, respectively.

A comparison of the nucleotide sequence of the ovine \( \gamma \) and \( \delta \) chain C region gene segments with known human and murine sequences has established that in the ovine species, in contrast to the human and murine species, the hinge region of the \( C\gamma \) sequences contains two additional cysteine residues and a motif of 5 amino acids (Hein et al 1990b). These differences probably arose from the modification of ancestral genes by deletion, duplication or triplication of the short exon encoding this region, as occurred in the human \( C\gamma 2 \) gene (Lefranc and Rabbits 1985, Buresi et al 1989). It has been suggested that structural differences in the C regions of TCRs may modify the signal transducing and / or other functional properties such as tissue localisation. The existence of an expanded V gene repertoire and multiple receptor isotypes of \( \gamma \delta \) T cells in the sheep may imply that this lineage of T cells has a more elaborate functional role than their \( \alpha \beta \) T cell counterparts within this species. Four different \( C\gamma \) transcripts that are similar in structure to the sheep have been detected in cattle (Takeuchi et al 1992) indicating that the repertoire features described for the sheep are conserved in other ruminants.

1.3. Expression of TCR Genes.

It has been established that a single locus contains the genes for both the TCR \( \alpha \) and \( \delta \) chains in humans (Chien et al 1987, Griesser et al 1988, Satyanarayana et al 1988),
mice (Allison and Havran 1991) and sheep (Hein et al 1991). During T cell ontogeny, \( \gamma\delta \) TCRs appear at the cell surface prior of their \( \alpha\beta \) counterparts (Havran and Allison 1988). In view of these observations and the fact that some \( \alpha\beta \) T cells express nonfunctionally rearranged \( \gamma \) gene RNA, it was suggested that T cell precursors may sequentially rearrange their \( \gamma \) and \( \delta \) TCR loci prior to their \( \alpha \) and \( \beta \) loci (Pardoll et al 1987), indicative that \( \gamma\delta \) and \( \alpha\beta \) T cells derive from the same lineage. However examination of the \( \delta \) gene deleted as circular DNA during \( \alpha \) gene rearrangement showed that the \( \delta \) gene was in the germline configuration (Winoto and Baltimore 1989a). This implies that the earliest thymocytes are capable of differentiation either along the \( \gamma\delta \) or the \( \alpha\beta \) T cell lineage and that the two lineages have separate origins.

Regulation of the rearrangement of TCR gene segments at the \( \alpha / \delta \) locus enables the expression of these two genes in different sets of T cells and at different times during T cell development. Winoto and Baltimore (1989b) have previously identified an enhancer of \( \alpha \) chain transcription, 3' of the C\( \alpha \) gene that functions in cells that differentiate into both \( \alpha\beta \) and \( \gamma\delta \) T cells. However, negative elements, known as silencers, 5' of this enhancer act to suppress its enhancer function in \( \gamma\delta \) T cells, providing \( \alpha\beta \) cell specificity to this \( \alpha \) chain enhancer (Winoto and Baltimore 1989c). These silencer elements are spread over several kilobases of DNA and act on other enhancers in a cell specific manner and appear to be active in non-T cells, suggesting that differentiation of \( \alpha\beta \) T cells involves specific relief of silencer activity. Silencers and enhancers are thought to regulate rearrangement of the \( \beta \) and \( \gamma \) loci in a similar manner (Tonegawa et al 1989).

The localisation of the \( \delta \) locus within the \( \alpha \) locus enables the sharing of specific V regions between these loci in humans and mice, hence some V regions contribute to either the \( \alpha\beta \) or \( \gamma\delta \) T cell repertoire (Takihara et al 1989). In contrast, under the experimental conditions tested by Hein and Dudler (1993), within the sheep species, the sharing of V regions between the \( \alpha \) and \( \delta \) loci was not an apparent feature.
1.4. Thymic Development of Early Thymocyte Precursors.

The major site of T cell differentiation in rodents, ruminants, frogs and birds is the thymus. In adults, thymocyte precursors originate from bone marrow and continue to seed the thymus throughout life. They migrate to the thymus under the influence of chemoattractants, such as β₂-microglobulin (Dunon et al 1990) and initially localise at the thymic corticomedullary junction or subcapsule (Penit et al 1988). The exact site of entry is still controversial, probably because these areas overlap, given their trabeculae-linked continuity.

The thymocyte precursor population are phenotypically similar to bone marrow derived stem cells, but express moderate surface levels of CD4 (Wu et al 1991b). Upon further development, these early precursors cease expression of CD4 to become CD4-negative CD8-negative (double negative, DN), and progress through a series of developmental stages, identifiable by their surface phenotype (Wu et al 1991b, Pearse et al 1989). This progression is marked by acquisition of high levels of heat stable antigen (HSA) expression, a reduction in CD44 levels and by transient expression of the IL-2 receptor 55KDa chain, CD25 (Pearse et al 1989, Shimonkevitz et al 1987). This population of cells has the potential to generate mature cells of both the γδ and αβ T cell lineages. Lineage commitment is characterised by the rearrangement of either the γδ or αβ loci (Petrie et al 1992), and is paralleled with the a decreased cell surface expression of CD25. Subsequent to this, it appears that DN thymocytes that have rearranged either the γδ or αβ loci migrate along T cell differentiation pathways distinct to one another (Nikolic-Zugic 1991).

1.4.1. Thymic Development of αβ T Cells.

Thymic development of T cells remains to be fully elucidated. Consequently, various hypothesis have been established describing the generation of T cells from haemopoietic precursors. The following section provides an overview of one such pathway of T cell development.
It is established that the TCR β locus is rearranged prior to the TCR α locus and its rearrangement leads to the expression of TCR β chain homodimers on the surface of pre-T cells, which have the ability to transmit signals across the cell membrane (Groettrup et al 1992). Since the TCR α loci rearranges prior of the TCR- β loci, it is conceivable that the expression of TCR- β homodimer regulates the induction of the rearrangement at the TCR- α locus, which, however, does not show allelic exclusion. Recently Mombaerts et al (1992) showed that TCR- β rearrangement, or expression, is critical for the differentiation of DN thymocytes to double positive (DP) thymocytes, as well as for the expansion of the pool of DP thymocytes, whilst the α chain is irrelevant in these developmental processes.

Thymocytes differentiate to express a DP phenotype via a transient single positive (SP) stage - either CD4-positive CD8-negative or CD4-negative CD8-positive (MacDonald et al 1988). The resulting DP αβ T cells express low levels of CD3. The repertoire of this population of immature αβ T cells is filtered in the thymus in two selection processes, so that, at the end, autoreactive cells recognising self molecules are deleted (negative selection) and potentially useful cells are selected (positive selection). These cells then seed the periphery of secondary lymphoid organs.

Negative selection is effected by thymic cells of haemopoietic origin, which have the capacity to express MHC class I and class II molecules and which can present antigen by these molecules. These cells comprise macrophages, dendritic cells or B cells. If the avidity between the MHC peptide complex and TCR is high enough, this interaction will signal the thymocyte to initiate its own demise, via a process of apoptosis (Jenkinson et al 1989, MacDonald and Lees 1990). Alternatively, if the interaction is of intermediate or low avidity the thymocytes will progress to the next stage of selection. Negative selection of αβ thymocytes occurs at the transition from DN to DP thymocyte development, i.e, as soon as αβ TCRs become detectable on the surface.

It remains to be established whether MHC class I and class II molecules are the only presentational elements which, together with self peptides, eliminate thymocytes with high avidity.
DP thymocytes are subjected to positive selection by MHC class I and class II molecules with self peptides presented on thymic epithelial cells. T cells expressing αβ TCRs with intermediate avidities for either the MHC class I or class II peptide complexes, are positively selected. Subsequently, these cells make a second contact with the thymic epithelial cells. If the TCR made its initial contact by binding MHC class I peptide complexes, the second contact of the T cell is made between CD8 molecules on the surface of the T cell and MHC class I on the surface of the thymic epithelial cell. Alternatively, if the initial contact of the T cell was via binding MHC class II peptide complexes on thymic epithelial cells, the second contact of the T cell is between CD4 molecules expressed by the T cell and MHC class II molecules on the thymic epithelial cell. The second contact induces a decreased cell surface expression of unengaged CD4 or CD8 molecules (Robey et al 1991, Borgulya et al 1991). This generates either CD4-negative CD8-positive, MHC class I restricted T cells with the potential to become cytotoxic lymphocytes. Alternatively, CD4-positive CD8-negative MHC class II restricted T cells develop with the potential to become helper lymphocytes. All T cells expressing TCR with low binding affinities for the MHC peptide complexes on thymic epithelial cells are unable to make the initial contact by binding MHC class I and class II peptide complexes and are destined to die (Huesmann et al 1991).

1.4.2. Thymic Development and Ontogeny of γδ T Cells.

Based on the observation that both γδ and αβ T cells develop from the same precursor cell and that rearrangement of the γ and δ loci occur prior of the αβ loci (Takagaki et al 1989a), it is suggested that the early thymus preferentially induces γδ TCR expression. This is highly likely as the stromal determinants required for αβ TCR repertoire selection have not developed at the time of γδ TCR appearance (Hayes 1984). This suggests that the intrathymic requirements of γδ T cells may not be as stringent as those retired by αβ T cells. Alternatively, γδ T cells may require different stromal elements, relative to αβ T cells, for their maturation which appear earlier during thymic development.

No evidence is available to suggest that DN thymocytes with rearranged γ and δ loci progress to the DP stage observed during the maturation of thymocytes with rearranged
α and β loci. This implies different pathways of γδ and αβ T cell maturation within the thymus. From the studies of Mombaerts et al (1992), it appears that γδ T cell maturation is completely independent of αβ T cells, as the former develop normally in mice with a mutation at the TCR β locus.

Early in ontogeny of the immune system of fetal mice, two waves of monoclonal γδ T cell populations are generated in the thymus. T cells of the first wave have the Vy3 segment rearranged, with no N region diversity, to Jγ1, which is expressed with Cγ1. The δ chain comprises Vδ1Dδ2Jδ2Cδ. In the second wave, Vγ4 is used in the same combinations with the same δ chain, again with no N region diversity (Asarnow et al 1988, Itohara et al 1990). These findings indicate that, in the fetal thymus, there is a strong positive selection of T cells expressing either of the two canonical γδ TCR by self antigen. The first wave populates the skin, in particular its epidermal layer, the second wave the mucosal epithelia of the tongue, the uterus, and the vagina. The two waves are characteristic of mice and have not been found in other species. A recent report on ovine γδ T cells, established that there was no emigration from the fetal thymus of γδ T cells with invariant junctions analogous to those found in the skin and mucosal epithelia of mice, despite an apparent distinct pattern of γδ T cell repertoire development in the sheep (Hein and Dudler 1993).

The two early monoclonal waves of γδ T cells in mice are then followed by the generation of the γδ T cells in the thymus, which are more diverse in the V, D, J segment usage and very diverse in their N region insertions in both γ and δ. γδ T cells of other species exhibiting such diversity are found in the mucosal epithelia of the intestine, in blood and in secondary lymphoid organs of many species (Hein and Dudler 1993).

In accordance with the postnatal shift in the usage of γ and δ gene segments and the receptor diversity, the intrathymic localisation of γδ T cells changes drastically during the first few postnatal days. Fetal and neonatal γδ thymocytes are closely associated with medullary epithelial cells, whereas adult γδ thymocytes are scattered throughout the thymus and are most concentrated in the subcapsular areas (Tonegawa et al 1989). This
may imply that adult γδ thymocytes are subjected to either a different or a broader array of presentational elements in comparison to fetal γδ thymocytes that skew the repertoire of these cells during positive selection.

Self ligands are suspected to play a role in the positive selection of γδ T cells within the thymus (Lafaille et al 1990). Heat shock proteins and other endogenous ligands such as viral sequences may be the selecting ligands. Evidence in favour of the former is from the observation that some γδ T cells recognise endogenous stress proteins (Maki et al 1990). Evidence in favour of the latter comes from the observations that some γδ T cell populations present only in BALB/c mice, are characterised by the expression of an invariant TCR δ chain referred to as BID. It is suggested that Xmmv-60 and Mpmv-30, both of which are viral sequences integrated on chromosome 1 in BALB/c mice, may be the selecting ligands of these particular γδ T cells (Sim and Augustin 1990, Sim and Augustin 1991).

These ligands may be presented as peptides on non-classical class I or class I-like molecules (Tonegawa et al 1989, Houlden et al 1989), such as class Ib and CD1 molecules, respectively. Class Ib molecules are encoded within loci referred to as HLA-E, HLA-F and HLA-G in the human genome and in mice, within the Q and TL loci. The CD1 molecules are encoded outside of the MHC. Both class Ib and CD1 molecules, unlike classical MHC class I and class II molecules which are characterised by extensive allelic polymorphism, are non-polymorphic.

1.5. Extrathymic Origin of γδ T Cells.

For most γδ T cells, rearrangement of the TCR-γ and TCR-δ loci appears to be induced and completed in the thymus. In fetal lambs thymectomy irreversibly abolishes the capacity to generate γδ T cells, whilst αβ T cells recover after birth (Hein et al 1990a). In contrast, athymic nu/nu mice and athymic di George syndrome human patients do not generate normal levels of αβ T cells, but they have γδ T cells, although some populations are missing (Van Dongen et al 1990). These observations imply an extrathymic differentiation pathway of γδ T cells within these species. In addition
thymectomised chickens generate intestinal \( \gamma \delta \) T cells in two waves from precursors in spleen and bone marrow (Bucy et al 1991).

The thymic stroma contains epithelial elements of both ectodermal and endodermal origin important during thymic differentiation of thymocytes (Goldstein and Mackay 1969, von Gaudecker 1986). Recently it has been established that some murine \( \gamma \delta \) T cells are generated extrathymically from bone marrow derived precursors in endoderm-derived organs such as the gut (Guy-Grand et al 1991, Rocha et al 1991), the liver (Abo et al 1991) and lungs (Sim and Augustin 1990, Sim and Augustin 1991, Augustin et al 1989). The thymus-independent nature of these \( \gamma \delta \) T cells was determined by their presence both in nude and \textit{scid} mice and in irradiated and thymectomised mice repopulated by T-depleted bone marrow cells bearing an identifiable marker. In addition, gut intraepithelial \( \gamma \delta \) T cells (Guy-Grand et al 1991) and lung \( \gamma \delta \) T cells (Sim and Augustin 1990, Sim and Augustin 1991), in contrast to mature T cells, contain mRNA for the protein product of the recombinase activating genes (RAG) which are essential for recombination of TCR genes (Oettinger 1992).

The extrathymic expansion of T cells within tissues such as the intestinal epithelia may imply the selective expansion of TCR \( \gamma \delta \) rearrangements by selective pressure from the gut microenvironment. It has been suggested that these cells are selected by antigen presented in the villous microenvironment and may be of major importance as a first line of defence against pathogens at this epithelial site (Rocha et al 1992).

1.6. Gut Intraepithelial Epithelial \( \gamma \delta \) T Cells.

The population of intestinal intraepithelial lymphocytes (iIEL) in all species examined comprise T cells bearing \( \gamma \delta \) and \( \alpha \beta \) TCR. Irrespective of their TCR expression, iIEL consist of two distinct populations, one bearing CD8 molecules comprising an \( \alpha \) and \( \beta \) chain, and the other bearing CD8 molecules comprising \( \alpha \alpha \) homodimers. The former population express \( \alpha \beta \) TCRs and are thymus dependent, whilst the latter express either a \( \gamma \delta \) or \( \alpha \beta \) TCR and are thymo-independent (Bandeira et al 1991, Bonneville et al 1988). \( \gamma \delta \) 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). These lymphocytes are found primarily in the small intestine interspersed between the villous epithelial cells. These cells appear unique to all other γδ T cells described with respect to two observations: (i) murine iIEL preferentially use Vγ7, but in contrast to all other γδ T cells localised at epithelial sites within this species, express extensive junctional diversity (Bonneville et al 1989, Asarnow et al 1989, Takagaki et al 1989b) (sequence data on the iIEL repertoire of γδ T cells within other species is awaited); (ii) a large fraction express CD8 as αα homodimers.

The functional significance of the lack of CD8β chain expression is unclear but CD8 α-chain expression is sufficient for signal transduction (Dembic et al 1987). This may imply that these iIEL γδ T cells have the potential to recognise foreign peptide presented by MHC class I (Taguchi et al 1991). Alternatively, in view of the observation that CD8 αα homodimers are expressed by activated γδ T cells from peripheral tissues (Cron et al 1989) and lectin stimulated thymocytes (MacDonald et al 1988), expression of these homodimers may be indicative of an activated phenotype of the γδ T cell lineage within the gastrointestinal microenvironment (Guy-Grand et al 1991).

CD8 expression in the gut is probably a stochastic event, since it does not require expression of TCR or MHC class I molecules: (i) CD8-positive iIEL are found in scid mice (Guy-Grand et al 1991); (ii) and in γδ TCR-positive iIEL from β2 microglobulin deficient mice (Raulet et al 1991). However, the expression of CD8 in the form of homodimers may increase the affinity of the interaction of the TCR with peptides presented by class I, TL, or CD1 antigens, all expressed by gut epithelial cells and implied to have functional roles as presentational elements in antigen presentation to γδ T cells. (Hershberg et al 1990, Bleicher et al 1990, Wu et al 1991a, Eghesady et al 1992). In favour of the latter it was recently shown that γδ TCR IELs are located in the intestinal epithelium apparently in contact with TL expressing epithelial cells (Bonneville et al 1990) implying that TL region class Ib gene products may have evolved to present antigens to γδ T cells (Tonegawa et al 1989, Janeway 1989, Eghesady et al 1992).
Evidence in favour of the fact that γδ T cells do not use the specificity of their receptors for migration and homing to tissues, but rather undergo peripheral expansion at localised sites, is obtained from studies in transgenic mice. The generation of transgenic mice expressing a γδ TCR characteristic of one given epithelium demonstrated that this transgenic TCR was found in other epithelia (Bonneville et al 1990).

1.7. Antigen Processing and Presentation to T Cells.
Both γδ and αβ TCR comprise transmembrane polypeptide chains with particular V, (D), J and C regions in association with the CD3 complex. Because of the genetic and structural similarities between these two different receptors it is hypothesised that γδ T cells recognise antigen in a manner analogous to αβ T cells. To date, the processing and presentation of antigen to αβ T cells is well characterised (Brodsky and Guagliardi 1991). Consequently, the pathways of antigen processing and presentation described in this section are largely those established for αβ T cells.

1.7.1. Processing Of Endogenously Synthesised Proteins.
Studies by Unanue (1984) and Townsend and Bodmer (1989) have shown that the majority of T cell clones do not recognise native antigen but rather a peptide from a full length polypeptide chain derived by proteolytic cleavage. This processed peptide is presented to T cells in association with presentational elements on the surface of an appropriate presenting cell. To date, two major cellular routes of antigen processing and presentation have been identified for the generation of immunogenic peptides. One of these routes generates peptides derived from endogenously synthesised proteins, generally presented in association with MHC class I molecules (Townsend and Bodmer 1989, Morrison et al 1988b). The second pathway yields peptides derived from exogenously acquired protein material, the majority of which is presented in association with MHC class II molecules (Unanue and Allen 1987). Consequently, antigenic peptides destined to bind either MHC class I or class II are processed by different mechanisms intracellularly and in addition associate with the appropriate MHC molecule within different cellular compartments.
1.7.1. Processing Of Endogenously Derived Antigen And Its Presentation In Association With MHC class I Molecules.

1.7.1.1. Synthesis and Assembly of MHC Class I Molecules.
Class I molecules are formed by the association of β2-microglobulin with an α chain of 45KDa which comprise a single transmembrane segment and a carboxy terminus cytoplasmic domain (Bjorkman et al 1987a, Bjorkman and Parham 1990). Both class I subunits are targeted to the endoplasmic reticulum (ER) by classical amino terminal signal sequences that are cleaved off once the molecules have been co-translationally translocated into the ER (Ploegh et al 1979). The conformation and stability of the functional class I molecule is effected by the combination of the α chain and β2-microglobulin (Myers et al 1989, Williams et al 1989). As yet no evidence supports the fact that an intracellular protein mediates chaperonin effects to help the α and β2-microglobulin of class I fold and assemble in the correct manner.

1.7.1.2. Processing Of Endogenously Synthesised Proteins.
A major biochemical pathway for the intracellular degradation of endogenously derived proteins occurs via a ubiquitin-dependent or independent process which is effected by a multi-enzyme complex referred to as a proteosome. This proteolytic complex which exists in the cytoplasm of the cell degrades incorrectly folded or unwanted proteins to short peptides and eventually individual amino acids.

A proteosome of 700KDa, the 20S proteosome, comprises 30 subunits of similar size (21-31KDa) (Orlowski 1990), stacked into a barrel shaped complex of four layers. On cross section these layers are ring-shaped, 160 Å in diameter, with a central hole of 10-20 Å which may be a site of entry for protein subunits (Tanaka et al 1988). Recent studies have established that the 20S proteosome is an essential component of a larger structure referred to as the 26S proteosome complex (Goldberg and Rock 1992), made of three smaller components, CF-1, CF-2 and CF-3, the latter corresponds to the 20S proteosome which is the proteolytic core of the complex. Components CF-1 and CF-2 are complexes of 10-20 polypeptides of between 40-110KDa.
An initial step in the hydrolysis of proteins is their covalent association with ubiquitin (Rechsteiner 1987, Hough et al 1987). In this reaction, the carboxy terminus of ubiquitin becomes attached by an isopeptide bond to e-amino groups on lysine residues on the protein substrate. The process requires ATP and is mediated by the CF-3 region of the 26S proteosome complex. CF-1 binds ATP and activates the proteosomes numerous peptidase activities, whilst CF-2 regulates the proteolytic function of the complex by inhibition.

The 20S proteosome resembles in size and subunit structure to the MHC linked low molecular weight protein (LMP), thought to process endogenous protein into antigenic peptides for association with MHC class I. The LMP and proteosome are not identical, but recent reports have provided evidence that the LMP is a special type of 20S proteosome (Brown et al 1991, Glynne et al 1991, Martinez and Monaco 1991, Ortiz-Navarrete et al 1991). It is not clear whether the MHC encoded proteosome subunits are found as part of the 26S complex as the conditions used during the investigation favoured the dissociation of the CF-1 and CF-2 components of this complex (Brown et al 1991, Ortiz-Navarrete et al 1991).

1.7.1.3. Immunological Relationship Between The Proteosome and The LMP.

A hypothesis of the relationship between the LMP and the proteosome is that the two structures differ solely in the presence or absence of the two MHC linked subunits. The immune system, during the course of evolution, may have recruited a previously existing structure required for the normal cellular protein turnover by attaching two extra subunits that serve to direct the output of peptides to the endoplasmic reticulum where they associate with class I molecules. Alternatively, or additionally, the two MHC linked subunits may serve to modify the proteolytic action of the complex to produce peptides better suited for MHC class I binding (Monaco 1992).
1.7.1.4. **Tap Genes.**

The MHC regions of the mouse (Monaco et al 1990), rat (Deverson et al 1990) and human (Trowsdale et al 1990, Spies et al 1990) contain two genes that belong to a family of related transporter genes. These genes are referred to as *Tap-1* and *Tap-2* for transporter associated with antigen processing, encoding TAP-1 and TAP-2 proteins respectively. These products associate as a heterodimer, and are responsible for transporting fragments of antigen, produced in the cytoplasm by the LMP (Monaco 1992), into the lumen of the ER where they associate with class I molecules. The functional implication of these proteins is indicated by studies of antigen presentation in TAP1 mutant mice (Van Keer et al 1992) and the TAP-2 mutant cell line RMA-S (Powis et al 1991, Attaya et al 1992), where antigen presentation associated with MHC class I was significantly affected in both situations. In the TAP1 mutant mice this resulted in the absence of CD8-positive T cells within their immune system.

Genes encoding the TAP transporter and the LMP complex are closely linked in the genome, and are transcribed in synchrony (Cho et al 1991). As yet, no physical association has been shown to occur between the two protein complexes. Therefore, delivery of peptides from the LMP complex to the transporters, which resides in the ER, may be mediated by chaperonin like peptide carrier proteins, or diffusion.

1.7.1.5. **Polymorphism of LMP and Tap Genes.**

It has been suggested that polymorphism occurs in the LMP genes (Monaco and McDevitt 1982). In addition, the work of Powis et al (1992) established a specific relationship between the TAP transporter proteins and the resulting peptides found within the groove of MHC class I molecules. This phenomenon was referred to as class I modifier (*dm*) and occurs as a consequence of polymorphism in *Tap-1* and *Tap-2*.

Polymorphism of the LMP genes may result in the production of different sets of peptides in different individuals, whereas polymorphism in the transporter genes may result in the transport of different subsets of peptides into the ER. The end result in both cases could be the presentation of different epitopes of the same antigen to the T cell repertoire in different individuals.
1.7.1.6. The Structure of MHC Class I Molecules.
The peptide binding portion of MHC class I has been established at atomic resolution by X-ray crystallographic studies (Bjorkman et al 1987a, Bjorkman et al 1987b). The peptide binding groove of these molecules is formed by two α helices lying parallel on a β pleated sheet. In this model, polymorphic regions of both the α and β chains cluster in and around the peptide binding groove. The groove of MHC class I is closed at both ends (Madden et al 1991), as one end of the MHC class I molecule is occluded by a tyrosine residue, the other has a salt bridge. This feature presumably dictates the binding of peptides of restricted size, of 8-10 amino acids, to MHC class I molecules (Van Bleek and Nathenson 1990, Schumacher et al 1991, Falk et al 1991).

1.7.1.7. Peptides Bound to MHC Class I Molecules.
Studies of peptides eluted from class I molecules has shown that nonamers of amino acids bind to class I molecules with 100-1000-fold higher affinity than longer and shorter versions (Van Bleek and Nathenson 1990, Falk et al 1991, Hunt et al 1992). In addition, data suggests that peptide comprising nonamers of amino acids are generated as a consequence of the proteolytic effects of the LMP complex rather than the preferential binding and transport of nonamers by the TAP transporter proteins. Peptides isolated from each MHC allele have a distinct sequence motif where amino acids at positions 2 of the peptide chain and the carboxy-terminal residue are conserved (Guo et al 1992). These conserved amino acids are responsible for anchoring the peptide to conserved residues of the MHC molecule and in addition upon binding to class I molecules, stabilise its structure (Townsend and Bodmer 1989, Townsend et al 1989).

The functional significance of MHC class I stabilisation upon peptide binding is implicated by investigations utilising the RMA-S cell line, mutated in its Tap genes. These genes encode TAP proteins which transport fragments of antigen from the cytoplasm into the ER lumen (Monaco 1992). These mutant cell lines have a reduced number of MHC class I molecules inserted within their plasma membrane, suggested to be the consequence of the instability of the MHC class I molecules in the absence of bound peptide (Powis et al 1991, Attaya et al 1992).
The anchoring residues at both ends of the peptide are substantially buried in the peptide binding site of the class I molecule. The amino acid residues in the middle of the peptide are predominantly exposed and could be recognised directly by TCR (Silver et al. 1992), in particular the CDR3 region of the receptor molecule.

1.7.2. Processing Of Exogenously Derived Antigen And Its Presentation In Association With MHC class II Molecules.

1.7.2.1. Assembly and Intracellular Transport of MHC Class II molecules.

MHC class II molecules are assembled in the endoplasmic reticulum (ER) (Kvist et al. 1982), and expressed at the cell surface as αβ heterodimers. Only correctly assembled αβ heterodimers leave the ER, free α and β chains are retained by the ER-resident immunoglobulin heavy chain binding protein (BIP). During the biosynthesis of MHC class II molecules, a third chain, the invariant or γ chain, associates transiently with the class II αβ dimer (Claesson-Welsh and Peterson 1985). The γ chain is responsible for targeting the αβ dimers to the endocytic pathway and prevents the binding of peptides to the dimer in the ER.

Some γ chains leave the ER in the absence of the class II dimer, but the γ chain is more efficiently transported when associated with the αβ dimer. Similarly, association of the αβ dimer with the γ chain is not an absolute requirement for assembly and surface expression (Miller and Germain 1986, Sekaly et al. 1986), rather, it increases the efficiency of the process (Claesson-Welsh and Peterson 1985). In the ER, the γ chain forms a homotrimer (Marks et al. 1990). Class II molecules are transported from the ER in a complex consisting of a scaffold of three γ chains, onto which three αβ dimers assemble (Roche et al. 1991).

On arrival in the trans-Golgi reticulum (TGR), MHC class II molecules are sorted to the endocytic route (Cresswell 1985, Neefjes et al. 1990), directed by a targeting signal for delivery to endosomes which is located in the cytoplasmic tail of the γ chain (Bakke and...
Dobberstein 1990, Lotteau et al 1990). While in transit from the TGR to the endocytic route, the γ chain is degraded by endosomal proteases (Blum and Cresswell 1988, Pieters et al 1991), and the rate of this degradation determines the rate at which class II molecules transport through the endocytic pathway to the cell surface. γ chain degradation is required for the class II molecules to bind exogenous antigen, processed in the endocytic pathway (Roche and Cresswell 1990). The exact location in the endocytic route where class II molecules bind peptides has not been established and may differ between cells.

1.7.2.2. Internalisation of Exogenous Derived Material.

Complexes of MHC class II and peptides must reach a critical threshold for T cell activation to occur. The concentration of these complexes on the surface is determined primarily by two factors: (i) the efficiency of antigen capture by antigen presenting cells; and (ii) the concentration of class II molecules on the antigen presenting cells. These two factors vary among different cell types and determines to a large extent the antigen-presenting capacity of a given cell (Lanzavecchia 1990).

Macrophages internalise exogenous material by nonspecific adsorption or fluid phase pinocytosis, but the internalisation of exogenous material is significantly increased when it is bound and internalised via a specific cell surface receptor. In particular, different isoforms of the FcγRII receptor found on the surface of macrophages facilitate the internalisation of exogenous material associated with antibodies in the form of immune complexes.

B cells, unlike macrophages, have clonally distributed immunoglobulin receptors within their membrane that specifically bind antigen and therefore facilitate its capture. Antigen specific B cells have the ability to capture small amounts of antigen due to the high affinity of their immunoglobulin receptors for antigen, and provide an important pathway for the internalisation of antigen when limiting amounts are available. In contrast to macrophages, B cells have a 100-1000 fold lower capacity to pinocytose and a 10-50 fold lower capacity to absorb antigen non-specifically (Chesnut et al 1982).
Finally, dendritic cells are potent antigen presenting cell, they are not very active in endocytosis (Austyn 1987), but evidence suggests that they internalise complexes of antigen and antibody via Fc receptors (Bujdoso et al 1990, Harkiss et al 1990). Alternatively, dendritic cells may acquire processed antigen from nearby macrophages or process antigen using surface proteases (King and Katz 1990). However, dendritic cells express significantly higher levels of MHC class II molecules on their cell surface in comparison to both macrophages and B cells (Steinman 1991), increasing their ability to present peptides to T cells.

1.7.2.3. Endosomal Degradation of Exogenously Derived Material.
Endocytosed antigen first enters early endosomes, then late endosomes and finally lysosomes. The concentration of proteolytic enzymes required for the breakdown of endocytosed antigen may be achieved towards the late endosomal/lysosomal portion of the endocytic pathway. Whether the proteases involved in the breakdown of the \( \gamma \) chain are also involved in the generation of presentable peptides remains to be established. The acidic pH in endosomes/lysosomes contributes to both an increase in antigen degradation and to the efficiency of peptide binding by MHC class II molecules (Neefjes and Ploegh 1992).

The complex of MHC class II and antigenic peptide may be transported to the cell surface by the reverse route taken by the endocytosed antigen, hence travelling from lysosomes, via late and early endosomes to the cell membrane (Neefjes and Ploegh 1992).

1.7.2.4. Peptides Bound to MHC Class II Molecules.
Processed peptide fragments bound to MHC class II molecules are peptides of 13-17 amino acids in length (Rudensky et al 1991a). This finding is in keeping with the observation that, whereas CD4-positive T cells can be stimulated by peptides of a minimum length of 8 amino acids, longer peptides are more potent in their activation. Structural analysis of MHC class II molecules has established that, in contrast to MHC class I molecules, these molecules have a salt bridge at one end of the groove but the other end lacks a tyrosine or other bulky side chain residue, suggesting that the class II
peptide binding groove is open at this end (Rudensky et al 1991a). Consequently, the size of peptides bound to Class II molecules are not as restricted as peptides bound to MHC class I molecules.

Contrary to recent reports of sequence motifs in MHC class I associated peptides, no analogous motifs have been identified in peptides bound to MHC class II molecules (Rudensky et al 1991a). The peptides bound to MHC class II molecules have precise N-terminal cleavages but varying C-terminal cleavages, suggesting that the N terminus is protected in the peptide binding groove of the MHC class II molecule, the C terminus being accessible and truncated to a minimum of 13 amino acids by the action of carboxypeptidases.

1.7.3. A Model for the Generation and Presentation of Self Peptides in MHC Molecules.

The synthesis of MHC molecules and their association with peptides is an ongoing process. It is likely that self-peptides are generated to allow MHC molecules to be continually synthesised and expressed at the cell membrane. In the event of a cell becoming infected or transformed the peptides generated from these foreign proteins are rapidly presented by MHC molecules at the cell surface and are recognised by the hosts immune system.

Evidence in favour of such a theory is from the observation that some peptides isolated from the peptide binding groove of MHC class I (Van Bleek and Nathenson 1990, Falk et al 1991) and class II (Rudensky et al 1991b) molecules comprise self peptides. The association of self peptides with MHC molecules not only stabilises the complex (Townsend and Bodmer 1989), but prevents the binding of peptides derived from the external milieu to MHC molecules, subsequently leading to non-specific killing by cytotoxic T cells of MHC class I expressing cells.
1.7.4. Presentational Elements Other Than MHC Class I and Class II Molecules.

Recent studies of antigen recognition by γδ T cells has determined that some γδ T cells recognise presentational elements other than the MHC linked determinants recognised by their αβ counterparts, such as class Iα and CD1 molecules.

MHC class I molecules comprise an α chain and β₂m microglobulin. The α chain is folded to form α₁, α₂ and a α₃ domains. The α₃ domain is in close proximity with the cell membrane and is disulphide linked to the β chain, β₂m microglobulin. Similarly, both the class Iα and CD1 molecules comprise an α chain thought to form α₁, α₂ and a α₃ domains similar to class I, and associate with β₂m microglobulin (Terhorst et al 1981).

Significant homology of the corresponding α₃ domains of CD1 and class I has been reported at the DNA level (Calabi and Milstein 1986, Balk et al 1989).

To date, it is unknown if presentational elements recognised by γδ T cells are complexed with peptides. If γδ T cells recognise such a complex it remains to be established if the presentational elements acquire peptides in a manner similar to the pathways described for either MHC class I or class II molecules. The homology at the DNA level of CD1 and class Iα molecules with MHC class I molecules (Calabi and Milstein 1986, Balk et al 1989), and similar tissue distribution of class Iα and MHC class I molecules (Eghtesady et al 1992), may be indicative that these molecules present immunogenic peptides to γδ T cells in a manner analogous to MHC class I molecules. If this is the case, then class Iα and CD1 molecules may associate with immunogenic peptides in the endoplasmic reticulum.

1.8. Antigen Recognition By αβ T Cells.

As yet, no X-ray crystallographic studies of TCR-MHC-peptide crystals are available, but considerable experimental evidence from mutational and transfection studies, suggests that the structure of the TCR molecule is orientated such that it interacts with peptide fragments (Hong et al 1992, Kaye and Hedrick 1988, Malissen et al 1988). In addition, based on the high degree of structural similarity between antibody and TCR
genes, it has been proposed that the antigen-binding sites of these two molecules will be comparable and that the TCR contains complementarity determining regions (CDR) as shown for immunoglobulins.

The putative CDR1 and CDR2 loops of the TCR comprise regions of the polypeptide chains that attribute to the V gene segments of the germline sequences encoding TCR chains. Similarly, the TCR CDR3 regions are formed from the V(D)J gene segments of the germline sequence (Jorgensen et al 1992). It has been suggested that the CDR3 loops (Hong et al 1992), the most diverse regions of the TCR, are involved in recognising peptide fragments of the antigen, while the less variable CDR1 and CDR2 regions make direct contact with the MHC molecules binding these fragments (Claverie et al 1989, Davis and Bjorkman 1988).

1.9. Antigen Recognition By γδ T Cells.

It has been shown that CDR3 region of the TCR has a critical role in the antigen recognition by αβ T cells (Hong et al 1992). The studies of Rellahan et al (1991) have demonstrated that junctionally encoded sequences corresponding to the putative CDR3 also influence the antigen specificity of TCR γδ T cells, further implying that γδ T cells recognise antigens in a manner analogous to αβ T cells. In addition, based on reports that γδ T cells generate the majority of their diversity by the addition of nucleotides at junctional regions between V, D and C gene segments, (regions of the TCR that form the CDR3), it is possible that γδ T cells may have the ability to generate a broader repertoire within the CDR3 region of their TCR than seen with αβ T cells (Davis and Bjorkman 1988).

To date, a heterogeneous group of γδ T cell ligands have been identified, including bacterial antigens, autologous stress proteins and classical and non-classical MHC molecules.
1.9.1. Recognition of Microbial Antigens by $\gamma\delta$ T cells.

It has been suggested that selection by two powerful antigens account for the presence of the majority of $\gamma\delta$ T cells expressing $V\gamma 9$ gene segments in human peripheral blood (De Libero et al 1991), and that these cells do not comprise a single population but actually two nested sets. The larger set express $V\gamma 9$ gene segments but different $V\delta$ gene segments. It is suggested that this distinct population may be expanded in the periphery by cellular superantigens identical or related to the Molt-4 (De Libero et al 1991), and microbial superantigens like Staphylococcus enterotoxin A (SEA) (Rust et al 1990, Duan and Kabelitz 1991), or Daudi superantigens (Fisch et al 1990a).

The smaller set express $V\gamma 9$ gene segments in association with $V\delta 2$. This $V\gamma 9 V\delta 2$ subset comprise 50-70% of total $\gamma\delta$ T cells within human peripheral blood but umbilical cord blood $\gamma\delta$ T cells primarily express the $V\gamma 1 V\delta 1$ gene segments with only a minor population expressing $V\gamma 9 V\delta 2$ gene segments (Parker et al 1990, Faure et al 1990). It is reported that the majority of the $V\gamma 9 V\delta 2$ subset of cells within human peripheral blood respond to Mycobacterium tuberculosis in vitro (De Libero et al 1991, Kabelitz et al 1990). In addition, the stimulation of umbilical cord blood lymphocytes with Mycobacterium tuberculosis selectively expanded the $V\gamma 9 V\delta 2$ subset of $\gamma\delta$ T cells. Further analysis of the TCR sequences of these $V\gamma 9 V\delta 2$ cells revealed extensive junctional diversity, indicating that the cellular response to this antigen results in the polyclonal expansion of $\gamma\delta$ T cells expressing $V\gamma 9 V\delta 2$ gene products.

Some conclude that a component within Mycobacterium tuberculosis activates these $\gamma\delta$ T cells in a manner similar to superantigens (Pfeffer et al 1992). The components within Mycobacterium tuberculosis appear to interact with the variable regions of both the $\gamma$ and $\delta$ TCR chains, as both $V\gamma 9$ and $V\delta 2$ are a prerequisite for the response. This is in contrast to the staphylococcal superantigen responses by $\alpha\beta$ T cells described by Kappler et al (1989), where it has been shown that only a particular $\beta$ chain of the receptor is required for interaction with the ligand.

The components within Mycobacterium tuberculosis responsible for the activation of a large fraction of $\gamma\delta$ T cells remain to be defined. However, Pfeffer et al (1990) reported
that the majority of freshly isolated human $\gamma\delta$ T cells were selectively triggered by *Mycobacterial* components in the molecular weight range of 1 to 3 KDa. Furthermore, these components proved to be highly resistant to protease digestion, implying that non-proteinaceous constituents of *Mycobacterial* antigens were responsible for the activation of $\gamma\delta$ T cells. This provided evidence for a previously stated hypothesis which suggested that $\gamma\delta$ T cells recognise carbohydrate rather than protein ligands (Strominger 1989).

Accessory cells are required to support the proliferation of $\gamma\delta$ T cells specific for *Mycobacterium tuberculosis* (De Libero et al 1991, Kabelitz et al 1990). Some workers report that the response of $\gamma\delta$ T cells to *Mycobacterium tuberculosis* components required autologous MHC molecules (Haregewoin et al 1989, Modlin et al 1989), whilst others report that allogeneic mononuclear cells are equally efficient in this respect (Kabelitz et al 1990, Holoshitz et al 1989, Janis et al 1989, De Libero et al 1991). The data may imply that $\gamma\delta$ T cells respond to a ligand comprising non-polymorphic presentational elements in association with an immunogen presented on the surface of accessory cells.

A substantial fraction of peripheral blood $\gamma\delta$ T cells are activated by the parasite responsible for malaria, *Plasmodium falciparum* (Goodier et al 1992a, Goerlich et al 1991). The responding cells express a TCR comprising $\text{V}_{\gamma}9$ gene segments, the majority of which, but not all, are associated with $\text{V}_{\delta}2$ chains (Goodier et al 1992b). The response of $\gamma\delta$ T cells to *Plasmodium falciparum* required the expression of autologous MHC molecules on the surface of the antigen presenting cells, implying a response characteristic of an antigen specific MHC-restricted response.

1.9.2. Recognition of Stress Proteins by $\gamma\delta$ T cells.

Whilst it is apparent that some $\gamma\delta$ T cells recognise *Mycobacterium tuberculosis*, some report the isolation of human peripheral blood $\gamma\delta$ T cells specific for the *Mycobacterial* stress protein hsp65 (Holoshitz et al 1989, Haregewoin et al 1989). However it has been estimated that the frequency of cells recognising the hsp65 component of
1.9.3. Recognition of Peptides Derived From Stress Protein by $\gamma\delta$ T cells.

As described earlier, it is well established that $\alpha\beta$ T cells recognise peptides in association with presentational elements on the surface of antigen presenting cells. As $\gamma\delta$ T cells express antigen receptors on their cell surface comprising transmembrane polypeptide chains homologous to those of $\alpha\beta$ T cells, it is hypothesised that $\gamma\delta$ T cells also recognise peptide fragments of antigen rather than native protein. Evidence to support this is from the work of O’Brien et al. (1991), who reported that a large subset of $\gamma\delta$ T cells, present in both murine newborn thymus and in adult spleen, respond to a synthetic peptide of 16 amino acids in length of the stress protein, hsp65. This peptide represented amino acids 180-196 of Mycobacterial hsp65.

These observations may imply that $\gamma\delta$ T cells recognise these peptides in association with presentational elements on the surface of antigen presenting cells. The $\gamma\delta$ T cells that respond to this peptide bear very similar receptors, all expressing $V_{\gamma}1$ and most expressing $V_{\delta}6$. The junctional variations of the TCR of these cells indicate that they are not clonal.

1.9.4. Recognition of Endogenous Stress Proteins and Peptides by $\gamma\delta$ T cells

A response to autologous proteins would be predicted for $\gamma\delta$ T cells if their functions include the whole or part of a mechanism for immune surveillance that eliminates transformed and stressed cells from the host. In favour of this hypothesis, it has been reported that autologous stress proteins, such as hsp58, on the surface of human cell lines function as target molecules for $\gamma\delta$ T cells (Maki et al. 1990, Fisch et al. 1990b). hsp58 is the human homologue of the Mycobacterial hsp65, and the E. coli GroEL protein.
In addition, evidence suggests that murine γδ T cells respond *in vivo* to endogenous stress proteins on the surface of macrophages during infection with both *Salmonella* and influenza A virus (Emoto et al 1992, Carding et al 1990).

Finally, O'Brien et al (1991) demonstrated that murine γδ T cell hybridomas were stimulated by a synthetic peptide representing the region of the murine stress protein equivalent to the stimulatory region in the *Mycobacterial* stress proteins hsp65, albeit yielding a slightly weaker response. In addition these γδ T cell hybridomas consistently showed high constitutive secretion of IL-2, suggesting that their TCRs may be autoreactive and cross-react with the murine homologue of *Mycobacterial* hsp65 that is synthesised by these γδ TCR hybridomas.

1.9.5. Recognition of MHC Molecules by γδ T Cells.

The evidence that γδ T cells recognise MHC molecules as presentational elements includes the following observations. Murine fetal cytotoxic T cells expressing a γδ TCR that lyse maternal T cells via MHC class I molecules have been isolated (Miyagawa et al 1992). In addition, murine γδ T cell clones reactive with allogeneic MHC class I and II have been reported (Bluestone et al 1988) but the frequency of these clones appears to be low. Finally, only a limited amount of data is available reporting the recognition of immunogens derived from *Mycobacterium tuberculosis* in association with the classical MHC presentational elements (Haregwoin et al 1989, Modlin et al 1989).

The rather limiting amount of data available on the recognition of MHC molecules as presentational elements for γδ T cells may not be surprising when the phenotype of these cells is considered. γδ T cells are generally CD4-negative CD8-negative (with the exception of a minor population of γδ T cells that express either CD4 or CD8). As CD4 and CD8 act as co-receptor molecules for MHC class II and class I molecules, respectively, on αβ T cells it is possible that different molecules will act as presentational elements for these two subsets.
1.9.6. Recognition of Other Presentational Elements By γδ T Cells.

It is strongly suggested that the V gene segments form the CDR1 and CDR2 regions of the TCR and that these regions that associate with presentational elements on the surface of antigen presenting cells. γδ T cells have only a limited number of V gene segments encoded within their γ and δ loci, relative to the α and β loci which have considerably more. Collectively, with respect to this and the previously discussed features of the selection of the γδ T cell repertoire, these observations may imply that γδ T cells recognise non-polymorphic presentational elements, such as class Ib and CD1 molecules.

Recent data in the human and murine species supports the notion that γδ T cells recognise nonclassical MHC products, such as the class Ib and CD1 antigens. Such examples are (i) a human γδ T cell clone that lysed target cells subsequent to the recognition of the CD1c antigen (Porcelli et al 1989), (ii) a murine γδ T cell hybridoma lysed target cells upon recognition of the molecule termed T22b encoded within the Tla region of the genome (Ito et al 1990), and (iii) a murine γδ TCR positive hybridoma specific for a GT copolymer which was presented by the Qa-1 molecule encoded within the Q locus (Vidovic et al 1989).

1.9.7. Other Potential Ligands For γδ T Cells.

Human γδ T cell clones isolated from patients with B cell lymphomas have been reported to show specific recognition of surface immunoglobulin expressed by the lymphoma cells (Wright et al 1989). In addition, the T Cell Target 1 (TCT.1) molecule has been described as a ligand of γδ T cells (Mami-Chouaib et al 1990). This molecule of 43KDa is: broadly expressed on haemopoietic tissues and cell lines; belongs to the immunoglobulin gene superfamily and it is located in the same band as the CD1 gene cluster on human chromosome 1 (Del Porto et al 1991). The overall degree of protein sequence similarity between the two molecules is low and the distal domain of CD1 is not of the immunoglobulin type (Calabi et al 1989). TCT.1 is believed to be distinct from conventional MHC class I molecules since it appears not to be associated with
\( \beta_2 \) microglobulin and it is expressed on the class I-negative Daudi cell line. However, the relationship of TCT.1 to other known T cell ligands is currently unknown.

1.10. Have TCR and Presentational Elements Co-evolved?

It has been proposed that nonclassical class I restriction elements and \( \gamma \delta \) T cells have co-evolved for the recognition of certain endogenous ligands which are common to microbial antigens (Ito et al 1989). Whilst the classical restriction elements for the \( \alpha \beta \) T cells show extensive allotypic polymorphism (Watts et al 1989) which presumably allows the recognition of antigenic peptides by the species as a whole via \( \alpha \beta \) T cells, the nonclassical restriction elements implicated for \( \gamma \delta \) T cells show mainly isotypic polymorphism, perhaps to ensure the recognition of evolutionary conserved antigens by each member of a species.

Interestingly, in the membrane-distal domains (\( \alpha 1 \) and \( \alpha 2 \) domains) the various TL molecules have diverged from each other much more than the classical MHC molecules (Flaherty et al 1990). These domains of CD1 are quite distinct from those of TL molecules (Bradbury et al 1988). Since these domains are likely to contain the binding sites not only for antigens but for the TCR, the diverse \( \alpha 1 \) and \( \alpha 2 \) domains of TL and CD1 proteins might be specialised for the presentation of different sets of antigens and for the recognition by different \( \gamma \delta \) TCR subsets. In accordance of this model, the expression of distinct nonclassical class I molecules at particular sites may coincide with the presence of particular \( \gamma \delta \) T cells. However an investigation by Eghtesady et al (1992) has shown that this does not appear to be the case, as they reported that both the skin and gut express T3d/T1818 gene transcripts, despite having \( \gamma \delta \) T cells that express different V region gene segments. Although these results argue against a role for \( Tla \) region gene products in the determination of the tissue-specific pattern of \( \gamma \delta \) TCR V gene segment expression, the presence of one or more of these molecules in most of the tested tissues enriched for \( \gamma \delta \) T lymphocytes suggests some interaction between the two.
1.11. T Cell Activation.

The physiological trigger for T cell activation is the engagement of the TCR with a complex of antigen and presentational elements on the surface of antigen presenting cells. Subsequent to this interaction, T cells undergo a programme of gene expression that culminates in the altered or de novo synthesis of various transmembrane molecules and secreted proteins. A variety of cell surface receptor molecules are expressed whose combinations with their specific ligand allows the T cell to progress through the cell cycle. These receptors expressed by activated T cells bind molecules including lymphokines such as IL-2 which is secreted by activated T cells in a paracrine or autocrine manner.

1.11.1. Molecules Associated With The TCR.

The TCR associates non-covalently with five polypeptide chains involved in signal transduction, namely the \( \gamma \), \( \delta \), \( \epsilon \), \( \zeta \) and \( \eta \) chains (Clevers et al 1988). These proteins are referred to collectively as the CD3 complex, and couple antigen recognition to intracellular signal transduction pathways. The CD3 chains assemble in the same combination on T cells irrespective of whether the cell expresses a TCR of \( \alpha \beta \) or \( \gamma \delta \) polypeptide chains (Van Neerven et al 1990). The genes encoding CD3 \( \gamma \), \( \delta \) and \( \epsilon \) are structurally homologous (Gold et al 1987) and are predicted to have arisen from an ancestral precursor by gene duplication. The \( \zeta \) chain of the CD3 complex shares no homology to the \( \gamma \), \( \delta \) and \( \epsilon \) chains, whilst the \( \eta \) chain is a different splice product of the \( \zeta \) gene product (Jin et al 1990b, Weissman et al 1988). The \( \zeta \) chain exists on the surface of cells as a disulphide linked homodimer (Weissman et al 1986), or as a heterodimer with the \( \eta \) chain (Jin et al 1990a).

Different components of the TCR associate with different CD3 proteins. The TCR\( \beta \) chain associates with the CD3\( \epsilon \gamma \) dimer, whilst the TCR\( \alpha \) chain associates with the CD3\( \epsilon \delta \) dimer (Koning et al 1990). These dimers associate with the TCR during intracellular assembly, as the CD3\( \epsilon \gamma \) and CD3\( \epsilon \delta \) pairs are required for efficient TCR assembly in the ER (Blumberg et al 1990), stabilised in particular by the presence of the CD3\( \epsilon \) chain (Hall et al 1991). The cytoplasmic region of the CD3\( \epsilon \) chain contains a ten
amino acid motif that functions as an endoplasmic reticulum retention signal (Mallabiabarrena et al 1992). After assembly of the TCR and the CD3 $\gamma$, $\delta$ and $\varepsilon$ proteins in the endoplasmic reticulum, the retention signal is assumed to be subsequently buried within the molecule thereby allowing surface expression of the TCR. The $\zeta$ chain homodimer or $\zeta\eta$ heterodimer have no role in assembly of the TCR / CD3 complex but their association is a key step in permitting cell surface expression of the TCR / CD3 complex. Receptor complexes which fail to assemble correctly are retained in the ER and degraded (Minami et al 1987).

1.11.2. A Role For Tyrosine Protein Kinases During T Cell Activation.

The primary mechanism used by the TCR to transduce signals across the cell membrane involves the activation of various protein tyrosine kinase (PTK) pathways. Proteins are activated by phosphorylation of specific tyrosine residues (Izquierdo and Cantrell 1992). To date, no direct evidence is available showing that a PTK is activated directly as a result of the TCR binding its ligand. However, the cytoplasmic domains of the CD3 are non-covalently complexed to the TCR and associate with one or more PTK (Chan et al 1991). Therefore, conformational changes in the TCR / CD3 complex as a consequence of ligand binding may activate the PTK (Chan et al 1992). Alternatively, a guanine-binding protein (G protein) may mediate a signal from the TCR to the PTK associated with CD3, inducing its activation (Harnett and Rigley 1992). PTK are members of the src family of proteins. Examples of src proteins are p56lck, and two forms of fyn, namely p59fyn and p72fyn (Rudd 1990).

Co-precipitation studies have shown that p59fyn is associated with the TCR / CD3 complex (Samelson et al 1990). The $\zeta$ chain of the CD3 complex associates with a PTK suggested to be p72fyn (Rudd 1990). The cytoplasmic domain of the $\varepsilon$ chain of the CD3 complex associates with an undefined PTK. In view of these observations it is thought that the TCR complex comprises at least two autonomous signal transducing units where both $\zeta$ and $\varepsilon$ chain couple to PTK pathways (Wegener et al 1992, Letourneur and Klausner 1992, Irving and Weiss 1991). Transfection studies with chimeric TCR
molecules indicate that stimulation of T cells via either the ζ or ε chains results in distinct patterns of tyrosine phosphorylation, suggesting activation of different PTK pathways (Letourneur and Klausner 1992).

p56^leuk is found in association with both CD4 and CD8. Both of these co-receptors possess in the cytoplasmic tail of their α chains a specific sequence that binds noncovalently to an N terminal region of the tyrosine kinase p56^leuk. Therefore, the CD4 and CD8 glycoproteins not only enable T cells to bind MHC class II or class I molecules, respectively, but also introduce a potent tyrosine kinase activity into the vicinity of the TCR complex. It has been shown that the majority of the CD3ζ chain is found in association with the co-receptor structures CD4 and CD8 on resting T cells (Suzuki et al 1992). T cell activation may involve either changes in the positions of individual members in the CD3 / CD4 or CD3 / CD8 complex relative to each other. Alternatively, or additionally, T cell activation may induce conformational changes in these complexes (Suzuki et al 1992).

Substrates of the CD3 associated PTK include the CD3 ζ chain and the γ1 subunit of Phospholipase C (PLC) (Beyers et al 1992). Once PLC is activated by this phosphorylation it hydrolyses phosphatidyl inositol into inositol triphosphate and diacylglycerol (Izquierdo and Cantrell 1992). These products increase intracellular calcium levels and activate a variety of enzymes including protein kinase C (PKC), which further increases intracellular calcium levels. Subsequently, signals are transmitted to the nucleus and mobilise DNA binding proteins which induce expression of a distinct set of T cell genes involved in cellular activation and proliferation. Such genes are those encoding IL-2 and IL2-R (Waldman 1989).

1.1.1.3. Regulation Of PTK Activity.
The catalytic activity of the src family of PTK is negatively controlled by a C-terminus tyrosine phosphorylation (Tyr505 in p56^leuk and Tyr528 in p59^fyn). Dephosphorylation enhances their activity and is mediated by the phosphatase activity of Leucocyte Leucocyte 32
Common Antigen (CD45). The cytoplasmic domain of CD45 has two protein tyrosine phosphatase domains. The importance of CD45 phosphatase activity is implied in its obligatory surface expression enabling TCR stimulation of the PTK pathways. In addition, it is thought that CD45 dephosphorylates CD3 after its activation (Alexander et al 1992).

1.11.4. Other Cell Surface Glycoproteins that May Have A Role in Lymphocyte Signal Transduction.

CD2 and CD5 are transmembrane glycoproteins, of 50 and 67KDa respectively, and expressed by the majority of T cells. Monoclonal antibodies generated to CD2 molecules have the ability to activate T cells in vitro by a pathway distinct from that of the TCR/CD3 complex (Wesselborg et al 1991). As yet, the physiological implication of this pathway remains to be fully defined. A better characterised role of CD2 is in mediating adhesion of T cells and antigen presenting cells during immune responses by binding its ligand, CD58 (LFA-3), expressed by all cells of haemopoietic origin.

CD5 is physically associated to the TCR/CD3 complex of T cells (Osman et al 1992) and represents a substrate of p56<sup>lck</sup>. This implies a role for CD5 during T cell activation (Mcateer et al 1988). In addition, it has been suggested that CD6 mediates a role in T cell activation (Rieber et al 1986, Baldwin et al 1988), but the implication of this molecule in T cell activation remains to be fully established.

1.11.5. The Expression of Activation Markers By T Cells.

T cells recognise their ligand and transmit signals to the nucleus that modulate gene expression and protein synthesis. The insertion of these proteins within the cell membrane distinguishes activated T cells from resting cells. The best characterised receptor molecule which may be used to define activated T cells is the IL-2 receptor.
The IL-2 receptor is a complex of at least three different transmembrane proteins referred to as α-chain (CD25 or p55 KDa), the β-chain (p75 KDa) and γ-chain (Takeshita et al. 1992, Lowenthal and Greene 1987, Robb et al. 1984). The α and β chains may bind IL-2 on their own with a binding constant (Kd) of $10^{-8}$M and $10^{-9}$M, respectively, or as an αβ heterodimer in association with the γ chain with a Kd of $10^{-11}$M (Wang and Smith 1987).

The β-chain of the IL-2 receptor is the transducing structure involved in the generation of the proliferative signal (Tsudo et al. 1987), whereas the α-chain, although of prime importance in the formation of the high affinity heterodimer, seems to be devoid of signalling capacity (Greene et al. 1985). It is reported that the β-chain of the IL-2 receptor is expressed constitutively on the surface of either resting γδ T cells (Aparicio et al. 1989) or resting CD8-positive T cells (Taga et al. 1991). In contrast, resting CD4-positive T cells do not express detectable levels of the IL-2 receptor β-chain (Taga et al. 1991). In addition, it appears that natural killer (NK) cells have the ability to express the β-chain of the IL-2 receptor independent of the α-chain (Bich-Thuy et al. 1987, Tsudo et al. 1987).

The α-chain is the inducible subunit of the IL-2 receptor (Bich-Thuy et al. 1987). Full expression of the functional high affinity IL-2 receptor can be induced by exposure of T cells to ligands that cross-link the TCR on the cell surface (Hemler et al. 1984, Meuer et al. 1984). In addition, it appears that IL-2 has the ability to induce the expression of the α-chain (Proust et al. 1991), suggested to occur as the consequence of IL-2 interacting with the β-chain of the IL-2 receptor (Tsudo et al. 1987). This consequently yields T cells with the ability to respond to pM concentrations of IL-2.

IL-2 is a lymphokine produced and secreted by activated T cells, which binds to the IL2-R in an autocrine and paracrine manner and subsequently triggers the growth and differentiation of lymphocytes. The binding of IL-2 to the high affinity form of the receptor results in signal transduction mediated by the 75KDa protein, internalisation of the ligand receptor complex, and the progression of the antigen-specific activated T cell
through the S phase of the cell cycle and onto cell division.

1.12. Naive and Memory T Cells.
Memory within the immune system is thought to reside among long lived lymphocytes which (Horgan et al 1992), through clonal expansion, are present at higher frequencies compared with naive precursors. These memory cells distribute and recirculate throughout the body and so provide a systemic immune protection, in that an immune response at one site will lead to a memory type response upon challenging in another tissue.

Physiologically, naive and memory T cells can be defined according to their immunological response. Naive T cells are characterised by their ability to mediate graft versus host reactions (Spickett et al 1983), respond to alloantigens in mixed lymphocyte cultures (Spickett et al 1983, Arthur and Mason 1986), produce and respond to IL-2 in vitro (Arthur and Mason 1986, Bottomly 1989) and react strongly to lethcin stimulation (Spickett et al 1983, Arthur and Mason 1986), but are ineffective in helping primed B cells to produce antibody (Spickett et al 1983, Arthur and Mason 1986, Clement et al 1988). In contrast, memory T cells help B cells synthesise antibody (Spickett et al 1983, Arthur and Mason 1986, Clement et al 1988), and have a rapid and increased proliferative response to recall antigens (Merkenschlager et al 1989). These have the ability to respond more readily to antigen in the absence TCR of a higher affinity by the increased expression of adhesion molecules that influence cell-cell interactions during antigen presentation, characterised by an increase in the cell surface expression of CD2, CD58 and CD11a (Makgoba et al 1989), or alternatively via the expression of different isoforms of some cell surface glycoproteins which may alter intracellular signalling pathways.

To date, the best cell surface marker that delineates naive and memory T cells are the variant forms of CD45 (Merkenschlager et al 1989). CD45 is a major cell surface component of lymphocytes, where 10% of the lymphocyte surface protein comprise one or more isoform of CD45 (Thomas 1989). The CD45 gene is composed of 34 exons
and the various isoforms of CD45 are generated by alternative splicing of exons 4, 5 and 6. Potentially eight different isoforms are generated, but to date, only 6 of these have been isolated. Exons 4, 5 and 6 encode extracellular domains of CD45, therefore all isoforms expressed have identical intracytoplasmic domains, which as discussed above, have phosphatase activity, crucial for the regulation of T cell activation.

CD45 isoforms differ in molecular weights, in the region of 180-220KDa (Thomas 1989). The high molecular weight isoforms are found on cells of the naive phenotype, and are referred to as CD45RA-positive cells, and the low molecular weight isoforms on cells of the memory phenotype, referred to as CD45RO-positive cells. Studies in the ovine species have demonstrated that naive and memory lymphocytes have a distinct distribution within the immune system (Mackay et al 1990, Mackay 1991, Mackay et al 1992). The majority of lymphocytes of a naive phenotype are present in both peripheral blood and efferent lymph, whilst lymphocytes with a memory locate primarily within afferent lymph.

1.13. Lymphokine Production By γδ T Cells.

T cell derived lymphokines execute the cellular and molecular reactions that ultimately are recognised as cellular immunity: T cell proliferation; T cell help for B cell proliferation; and antibody secretion, as well as lymphocyte and macrophage recruitment. The capacity to express genes encoding different lymphokines may imply functional differences between lymphocytes. In the murine species, CD4-positive T cells can be further divided into Th1 and Th2 subsets based on their lymphokine profiles.

It appears that γδ T cells have the ability to generate a similar array of lymphokines as their αβ T cell counterparts. The ability of human γδ T cells clones to produce lymphokines appears to correlate with their surface phenotype rather than with Vγ or Vδ gene segment utilisation (Morita et al 1991). CD4-positive γδ T cell clones, relative to CD8-positive and CD4-negative CD8-negative γδ T cell clones, produced significantly higher amounts of IL-2 and granulocyte-monocyte colony-stimulating factor (GM-
CSF). γδ TCR-positive thymocytes also have the ability to produce lymphokines and are shown to produce IL-4, IL-5, IFNγ and GM-CSF. Postnatal thymocytes produce only IL-4 and IL-5 which suggests they may play a role in early αβ T cell development (Krangel et al 1990).

The finding that murine iIEL γδ T cells have the ability to produce IL-5, a cytokine normally associated with the Th2 subset, was implied to be indicative that these cells exhibit helper functions and are important for induction of surface IgA-positive B cells to differentiate into IgA producing plasma cells within the gut (Taguchi et al 1991). In addition, these cells produced IFNγ, a lymphokine associated with the Th1 subset. This implies either that no differentiation of murine γδ T cells into Th1 and Th2 subsets is possible, or alternatively, these cells may be precursors for γδ T cells that produce either IFNγ or IL-5, analogous to the postulated Th0 cells which can develop into either Th1 and Th2 cells (Mossmann and Coffman 1989). No evidence exists that human γδ T cells can be differentiated into subsets analogous to Th1 and Th2 of the murine species (Morita et al 1991).


1.14.1. The In Vitro Cytotoxic Ability of γδ T Cells.

γδ T cells isolated from humans (Koide et al 1989, Spits et al 1989, Paliard et al 1989, Rivas et al 1989), mice (Goodman and Lefrancois 1988, Klein 1986) and sheep (Mackay et al 1988a) are reported to mediate killing of target cells in vitro, implying that this T cell lineage has the ability to mediate cytotoxic functions. Some of these cells mediate their cytotoxicity independently of classical MHC molecules (Klein 1986), whilst other cells expressing γδ TCR are reported to exhibit MHC restricted cytotoxicity (Mackay et al 1988a). In the case of the latter the MHC-restriction pattern was found to be inconsistent (Hein and Mackay 1991).

In view of these reports, it is highly feasible that γδ T cells mediate target cell lysis by recognition of molecules, other than classical MHC molecules, such as
non-polymorphic class I molecules, including class Ib and CD1 molecules. Direct evidence that γδ T cells mediate cytotoxic functions by recognising CD1 molecules on the surface of their targets comes from the work of Porcelli et al (1989) who reported that a γδ T cell line lysed the thymic leukemia-cell line, MOLT-4 via recognition of CD1c molecules.

An apparent correlation exists between the cytotoxicity of human γδ T cells and the receptor they use. It appears that most γδ T cells expressing a disulphide-linked form of the γδ TCR are cytotoxic, whereas most γδ T cells expressing the non-disulphide do not lyse cells (Christmas 1989, Dastost et al 1990, Jitsukawa et al 1988). The disulphide form of the human γδ receptor occurs through use of the Cγ1 gene segment (Porcelli et al 1991). No correlation has been observed between cytotoxic potential and particular V and J gene segment use (Christmas 1991). In addition it has been suggested that cytotoxicity of human γδ T cells is associated with surface phenotype (Morita et al 1991). CD8-positive and CD4-negative CD8-negative γδ T cell clones appear to mediate cytotoxic functions, whilst CD4-positive γδ T cell clones do not. Hence, cytotoxicity of γδ T cells may be associated with a particular subset of γδ T cells rather than a feature of γδ T cells collectively as a population. It is noteworthy that the majority of human γδ express a γδ TCR utilising the Cγ1 gene segment (Sturm et al 1989) and only 0-5% of the total γδ population within human peripheral blood express CD4 (Morita et al 1991).

1.14.2. In Vivo Implication of γδ T Cell Cytotoxicity.

The expression of class Ib is well documented at epithelial sites (Eghtesady et al 1992), and the role of these molecules as presentational elements for γδ T cells during ligand recognition has been implicated (Ito et al 1989, Vidovic et al 1989). In some species γδ T cells exhibit a tropism for epithelial tissues (Vroom et al 1991). It has been suggested that γδ T cells recognise infected or transformed cells via the expression of an immunogen in association with class Ib molecules on the surface of these cells (Janeway et al 1988), and the cytotoxic ability of γδ T cells may enable the destruction of such cells. Infection or transformation of epithelial cells may be complemented by an increased expression of class Ib molecules or alternatively may induce the expression of
different class Iib molecules, more potent for γδ T cell recognition of target cells (Janeway et al 1988).

1.14.3. Target Cell Lysis By Cytotoxic Lymphocytes.
Cytotoxic T lymphocytes recognise target cells and induce their lysis by the secretion of various mediators, such as perforin (Lu et al 1992), numerous serine esterases and tumour necrosis factor (TNF) (Cairns et al 1992). The pore forming protein perforin, binds to target cell membranes in the presence of calcium and polymerises to form transmembrane channels, responsible for the osmotic lysis of the target cell. Cytotoxic T cells store perforin in cytoplasmic electron dense granules, visible by electron microscopy.

Further evidence that indirectly shows that murine γδ T cells mediate cytotoxic functions is via the localisation of electron dense granules within their cytoplasm (Koizumi et al 1991). It is suggested that such granules contain mediators capable of lysing the target cell during cytolysis. In addition, the ability of murine γδ T cells to produce IFNγ is consistent with cytotoxic T cell functions (Taguchi et al 1991).

1.15. γδ T Cells and Diseases.
Increased percentages of γδ T cells have been observed in several immunodeficient patients: Wiskott-Aldrich syndrome, severe combined immune deficiency and DiGeorge syndrome patients (Van Dongen et al 1990). It remains to be established if the increased levels of γδ T cells in the peripheral blood of these patients may be related to the defects in development and/or maturation of αβ T cells.

Indicative that γδ T cells mediate an immune response to pathogens is provided by the observation of the apparent increased number of these cells either within peripheral blood or at localised sites during infection with various pathogens including, *Mycobacterium leprae* (Modlin et al 1989), *Mycobacterium tuberculosis* (Janis et al

Increased numbers of γδ T cells are observed within peripheral blood and at particular tissue sites during autoimmune disease such as Rheumatoid arthritis (Olive et al 1992a, Olive et al 1992b) and Polymyositis (Pluschke et al 1992). In accordance with this observation it is proposed that autoreactive γδ T cells are activated systemically by molecular mimicry, involving epitopes of bacteria or viruses that are shared by host self antigens, or by superantigens (Wucherpfennig et al 1991). Once activated, autoreactive T cells may undergo clonal expansion and initiate an autoimmune response, after exposure to self antigen(s) in the target tissue.

Collectively the data suggests that γδ T cells have a defined function within the immune system during various disease states. The ability of particular antigens to select γδ T cells of particular V gene usage implies a preferential expansion of γδ T cells subsets to particular ligands. Despite extensive research, the role of γδ T cells in host defence mechanisms against invading microbes remains elusive and requires further investigation.

Studies of the sheep immune system have been advanced with the development in recent years of monoclonal antibodies specific for both γδ and αβ T cells. These studies have shown that the level of γδ T cells in the peripheral blood of sheep is significantly higher than in the human or mouse species. However, the role of γδ T cells in the immune system remains elusive and requires further investigation.
1.16. Introduction To This Thesis.

One of the principal research animals investigated within the Department of Veterinary Pathology is the sheep. The use of this relatively large experimental animal allows the cannulation of individual lymphatic vessels running to (afferent lymph) and from (efferent lymph) peripheral lymph nodes. The ability to access these different lymphoid compartments \textit{in vivo} allows the collection of lymphocytes and antigen presenting cells for a study of the immune system \textit{in vitro}. As this technique is not easily carried out in either humans or rodents a study of the sheep immune system should provide novel information which will support or modify current hypothesis on the function of this unique defence mechanism.

Studies of the sheep immune system have been advanced with the development in recent years of a panel of monoclonal antibodies specific for ovine cell surface glycoproteins. Work carried out by others prior to the start of this thesis established that sheep peripheral blood T cells comprise three major subsets namely, CD4-positive, CD8-positive and CD4-negative CD8-negative cells. The CD4-negative CD8-negative T cells expressed a novel glycoprotein termed T19 and were subsequently shown to express a \(\gamma\delta\) TCR molecule as defined by the monoclonal antibody 86D. These early studies on \(\gamma\delta\) T cells in the sheep also suggested that the level of these cells in peripheral blood of ruminants might be significantly higher than in the human or mouse species. If these findings could be verified and substantiated these observations would provide a valid argument for an extensive study of \(\gamma\delta\) T cells in a species whose immune system was far more dependent upon these cells than the human or rodent species from which the majority of the information of these cells has so far been derived.

With these points in mind the initial objectives of this thesis were to investigate the prevalence and phenotype of sheep \(\gamma\delta\) T cells. These studies were intended to establish a base of information on the expression of various cell surface molecules by sheep \(\gamma\delta\) T cells, isolated from animals of different ages and antigenic burden. A second objective was to establish a purification technique for the isolation of \(\gamma\delta\) T cells from sheep peripheral blood for the assessment of antigen recognition and analysis of the
presentational elements used by these cells. A third objective of this thesis was to investigate the activation of sheep $\gamma\delta$ T cells.

A third objective of this thesis was to investigate the phenotype of ovine $\gamma\delta$ T cells.

$\gamma\delta$ T cells in most species lack the expression of the co-receptor molecules CD4 and CD8 which are intimately associated with T cell activation pathways. This suggests that aspects of activation in $\gamma\delta$ T cells may be different to activation in CD4-positive and CD8-positive T cells. This objective was first approached with the monoclonal antibody Ki67 which recognises nuclear antigens expressed solely in proliferating cells. During the course of this thesis monoclonal antibodies to the bovine IL-2 receptor $\alpha$-chain were made available and subsequently used to meet this objective. Information from these first three objectives, namely phenotype, activation and antigen responsiveness, of $\gamma\delta$ T cells were then applied to a fourth objective which was to study these cells in vivo by the technique of lymphatic cannulation.

At the start of this thesis no monoclonal antibody was available which defined the ovine $\alpha\beta$ TCR molecule or any protein of the CD3 complex. During the studies on activation of $\gamma\delta$ T cells it became apparent that monoclonal antibodies to these particular cell surface proteins would greatly enhance the comparison of $\alpha\beta$ and $\gamma\delta$ T cell activation via crosslinking of their respective antigen receptor molecules.

An attempt was made to generate monoclonal antibodies to the sheep $\alpha\beta$ TCR / CD3 complex by two strategies. Firstly, immunoprecipitation of sheep $\alpha\beta$ TCR / CD3 proteins was investigated using a monoclonal antibody HMT3.1 which recognises an internal epitope of human CD3$\epsilon$ protein and cross-reacts with sheep CD3. The aim was to purify these for use in the immunisation of mice for monoclonal antibody production. Secondly, an attempt to generate sheep CD3$\delta$ chain by expression in yeast of DNA encoding this molecule was made, also with the aim of generating sufficient protein for monoclonal antibody production.
In summary, the objectives of this thesis were:

(i) to investigate the phenotype of ovine $\gamma\delta$ T cells.
(ii) establish a protocol for the purification of ovine T cell subsets.
(iii) compare the activation of ovine $\gamma\delta$ and $\alpha\beta$ T cells.
(iv) investigate the response to antigen of ovine $\gamma\delta$ T cells \textit{in vivo} and \textit{in vitro}.
(v) attempt to generate monoclonal antibodies to the ovine CD3 complex.
CHAPTER 2

Materials And Methods.

2.1. Antigens.

Guinea pig foot pad protein (Charlton, 1965) was obtained from Sigma Chemical Company Ltd. Paris. Pasteur Pasteur purified protein derivative of human tuberculin (PPD, batch 298) was obtained from the Central Veterinary Laboratory, Weybridge. Heat killed Mycobacterium tuberculosis strain H37RA was obtained from Difco (Detroit, USA).

2.2. Animals.

2.2.1. Sheep.

Male and female Finnish Landrace x Shropshire and Dorset x Suffolk sheep of various ages were obtained from the Scottish Agricultural Research Institutes, Edinburgh. Animals 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 PBS and complete Freund's adjuvant containing H37RA Mycobacterium tuberculosis.

2.2.2. Mice.

Balb/c mice bred in the Department of Veterinary Pathology animal house, University of Edinburgh were used for monospecific antibody production. Mice were primed with either purified ovine lymphocyte antigens or yeast-derived fusion proteins emulsified in Freund's incomplete adjuvant. Mice were immunized by subcutaneous injection at several sites. Subsequent immunizations were given by intraperitoneal injection of antigen in PBS at 2-3 week intervals. After immunising mice three times animals were tested by removal of a portion of the tail. 100-200μl of blood was collected and left at room temperature for one hour at 37°C. The blood sample was microfuged for 5 minutes, the serum removed and stored for its reactivity towards ovine lymphocytes.

2.3. Media.

2.3.1. Medium For Lymphocyte Cultures.

Lymphocyte cultures were maintained in RPMI 1640 (Cat. no. 074-1800, Gibco, Biscuit, Uxbridge) supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin, 5 x 10^-5 M 2-mercaptoethanol and 2 g litre^-1 sodium bicarbonate. Culture
2.1. Antigens.

Ovalbumin (Grade V, Cat. no. A5503) was obtained from Sigma Chemical Company Ltd. (Poole, Dorset). Purified protein derivative of human tuberculin, (PPD, batch 298) was obtained from the Central Veterinary Laboratory, Weybridge. Heat killed *Mycobacterium tuberculosis* strain H37RA was obtained from Difco (Detroit, USA).

2.2. Animals.

2.2.1. Sheep.

Male and female Finnish Landrace x Dorset, Grey Faced x Suffolk and Dorset x Suffolk sheep of various ages were obtained from the Moredun Research Institute, Edinburgh. Animals were immunised with 1 mg ovalbumin and 1 mg heat-killed *Mycobacterium tuberculosis* injected over two sites intramuscularly in a 1 ml mixture (1:1) of PBS and complete Freund's adjuvant containing H37RA *Mycobacterium tuberculosis*.

2.2.2. Mice.

Balb/c mice bred in the Department of Veterinary Pathology animal house, University of Edinburgh were used for monoclonal antibody production. Mice were primed with either purified ovine lymphocyte antigens or yeast derived fusion proteins emulsified in Freund's complete adjuvant. Mice were immunised by subcutaneous injection at several sites. Subsequent immunisations were given by intraperitoneal injection of antigen in PBS at 2-3 week intervals. After immunising mice three times animals were test bled by removal of a portion of the tail. 100-200μl of blood was collected and left at room temperature for one hour to clot. The blood sample was microfuged for 5 minutes, the serum removed and tested for its reactivity towards ovine lymphocytes.

2.3. Media.

2.3.1. Medium For Lymphocyte Cultures.

Lymphocyte cultures were maintained in RPMI 1640 (Cat. no. 074-1800, Gibco Biocult, Uxbridge) supplemented with 2 mM L-glutamine, 100 U/ml benzylpenicillin and 100 U/ml streptomycin, 5 x 10⁻⁵ M 2-mercaptoethanol and 2 g litre⁻¹ sodium bicarbonate. 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.

2.3.2. Medium For Hybridoma Cultures.
Hybridoma cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 1mM sodium pyruvate.

2.3.3. Medium For Bacterial Cultures.
The basic growth medium was LB (Luria-Betani) medium (1% tryptone, 0.5% yeast extract, 1% NaCl made to pH 7.0 with NaOH). Selective medium contained 150μg ml⁻¹ of ampicillin as all the plasmids used in this thesis carried ampicillin resistance genes.

2.3.4. Medium For Yeast Cultures.
Non-transformed yeast were grown in YEPD medium (2% peptone, 1% yeast extract, 2% glucose). Yeast transformants were grown in selective medium of either Sc-glc medium (0.67% yeast nitrogen base (YNB) without amino-acids (Difco Ltd.), with 1% glucose) or Sc-gal medium (0.67% YNB, with 0.3% glucose and 1% galactose).

Ovine lymphocytes were isolated from either peripheral blood, cannulated lymphatic vessels or isolated lymph nodes.

2.4.1. Preparation of Peripheral Blood Mononuclear Cells (PBMC).
Ovine peripheral blood was obtained by venipuncture and collected into preservative free heparin at a concentration of 10 units ml⁻¹ of blood. White blood cells were buffy coated by centrifugation of heparinised whole blood at 1300g for 20 minutes at 20°C which were then diluted 1:3 with sterile PBS and centrifuged over Lymphoprep (Nyegaard, Oslo, Norway) at 850g for 20 minutes at 20°C. Mononuclear cells were isolated from the Lymphoprep-PBS interphase and washed once with fresh PBS and twice with wash media prior to any further
treatment. Cells were harvested during each wash procedure by centrifugation at 250g for 4 minutes at 4°C. Throughout this thesis when cells were washed and harvested by centrifugation the conditions were 250g for 4 minutes at 4°C unless otherwise stated.

2.4.2. Isolation of Afferent and Efferent Lymphocytes.

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 duct remaining after removal of the node. 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 2.5x10^3 units of heparin and 2.5x10^4 units each of penicillin and streptomycin. A two hour time-window was used to collect lymphocytes on each day. Lymphocytes were washed once with PBS and twice with wash media and subsequently used for in vitro proliferation assays, alternatively cells were washed twice in PBA (PBS supplemented with 1% FCS and 0.1% sodium azide) and used for immunofluorescence analysis.

2.4.3. Isolation of Lymphocytes From Lymph Nodes.

Lymph nodes were removed from animals by standard surgical techniques. Lymph nodes were processed in sterile PBS supplemented with benzylpenicillin and streptomycin at a final concentration of 100 units ml⁻¹ by teasing the node 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 viable lymphocytes isolated by centrifugation over Lymphoprep at 850g for 20 minutes at 20°C. Lymphocytes were collected from the lymphoprep interphase and washed once with PBS and twice with wash media prior to any further treatment.
2.4.4. Preparation of Single Cell Suspension of Mouse Splenocytes.
Mice were killed by cervical dislocation. The spleen was removed aseptically and teased apart in PBS. Single cell suspensions were prepared by sedimentation of debris at 1g for 5 minutes. The cell suspension was washed three times in PBA.

2.5. Monoclonal Antibodies And Immunofluorescence Staining.

2.5.1. Monoclonal Antibodies.
The monoclonal antibodies used in this thesis are shown in Table 1. Included is their isotype and specificity.

2.5.2. Immunolabelling of Cell Surface Antigens and Fluorochrome Reagents.
Cell surface phenotype was assessed using aliquots of $1 \times 10^6$ cells washed with PBA. Cells were incubated with 50 $\mu$l of the primary monoclonal antibody at the appropriate dilution for 30 minutes on ice. Unbound antibody was removed by washing three times with PBA. For single colour immunofluorescence 50 $\mu$l of FITC conjugated F(ab)$_2$ fragment of rabbit anti-mouse immunoglobulin (Cat. no. F313, Dakopatts, Denmark) was added for 30 minutes on ice. Dual colour immunofluorescence analysis requires that each monoclonal antibody is detected by a different fluorochrome. One method used to achieve this, and used routinely throughout this thesis was to biotinylate one monoclonal antibody (as described in section 2.5.3.) which was subsequently detected by the addition of phycoerytherin conjugated to streptavidin (Cat. no. STAR 4A, Serotec, Oxford). The other primary monoclonal antibody was detected with an isotype specific FITC-conjugated second antibody for 30 minutes on ice. The conjugates used were either sheep anti-mouse IgG1-FITC conjugate (Cat. no. PF273, The Binding Site, Birmingham), sheep anti-mouse IgG2a-FITC conjugate (Cat. no. PF274, The Binding Site, Birmingham), goat anti mouse IgG2b-FITC conjugate (Cat. no. SBA 1090-02, Sera Lab, Crawley, Sussex) or sheep anti-mouse IgM-FITC conjugate (Cat. no. PF278, The Binding Site, Birmingham), the reagent selected being dependent on the isotype of the primary antibody. Subsequently, cells were washed three times and analysed by flow cytometry using a Becton Dickenson FACScan system. Ten thousand cells were
Table 1.

Table 1 lists the monoclonal antibodies used in this investigation and shows their specificity and isotype.
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<th>Antibody</th>
<th>Specificity</th>
<th>Isotype</th>
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</table>
analysed per sample with dead cells excluded on the basis of forward light scatter.

2.5.3. Permeabilisation of Lymphocytes Prior to Internal Immunofluorescence Staining.

Monoclonal antibodies HMT3.1 and Ki67 recognise internally expressed antigens. The former is specific for the ε chain of the CD3 complex and recognises an epitope of the intracellular region of the polypeptide, whilst the latter is specific for nuclear antigens expressed by proliferating lymphocytes. To enable immunofluorescence analysis using these monoclonal antibodies the lymphocytes were permeabilised prior to their immunofluorescent labelling. PBMC were obtained as described in Section 2.4.1. and washed 3 times in PBA. On the final wash cells were resuspended in 0.1% solution of paraformaldehyde for 20 minutes on ice. Excess paraformaldehyde was removed by washing 3 times in PBS and cells were resuspended at 5x10^6 cells in a solution of digitonin at a final concentration of 20µg/ml for 5 minutes on ice then washed 3 times in PBA. Permeabilised lymphocytes were immunofluorescently labelled with either HMT3.1 or Ki67. For dual colour immunofluorescence analysis to detect surface antigens as well as those expressed internally cells, were incubated with the relevant monoclonal antibodies and second layer reagents prior to the addition of the paraformaldehyde.

2.5.4. Immunofluorescence Analysis.

All the fluorescence analysis described in this thesis was carried out using a Becton Dickinson FACScan. Use of this machine allowed fluorescence measurements to be made on homogeneous populations of cells by setting electronic gates, thus gating upon cells with particular forward and side scatter profiles (FSC and SSC, respectively). Hence cells only of particular forward and side scatter profiles were analysed. The fluorescence profile of a population of cells is displayed as a histogram of fluorescence intensity versus relative cell number. Positive fluorescence was established in comparison to background fluorescence. A marker was set to the right hand side of the background fluorescence peak, and any fluorescence of greater intensity than this marker (i.e. to the right of the marker) is judged as positive, or more accurately, as specific fluorescence due to the binding of monoclonal...
antibody to its specific ligand.

2.5.5. Biotinylation of Monoclonal Antibodies.

Immunoglobulin was purified from ascitic fluid by precipitation of unwanted proteins with 1.0M acetic acid and 99-100% caprylic acid. Precipitated proteins were removed by microfuging for 15-30 minutes and the supernatant was dialysed extensively into PBS over 72 hours. To the purified immunoglobulin, a 1mg ml⁻¹ solution of biotin-o-succinimide ester in DMSO and 0.1M NaHCO₃ pH 8.3 was added in the ratio of 75μg biotin : 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.

2.6. In vitro Lymphocyte Culture and Proliferation Assays.

2.6.1. In vitro proliferation Assays With Ovine PBMC.
PBMC prepared from antigen-primed and non-primed sheep were resuspended in culture medium and 1 x 10⁵ cells were cultured in 96-well flat-bottom micro-culture plates (Nunculon, Denmark) with various concentrations of antigen. Cultures were made to a final volume of 200 μl and established in triplicate. Positive and negative controls were established by substituting antigen with either Con A or culture media, respectively. The proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air for 5 days and pulsed with 1 μCi ³H-thymidine (specific activity 2Ci/mMole, Amersham) over the last 7 hours. Cells were collected onto glass filter paper using a semi-automated harvester and ³H-thymidine incorporation was assessed by liquid scintillation counting. The data is expressed as the geometric mean for the triplicate cultures and the standard deviation was generally less than 10% of the mean.

2.6.2. In vitro proliferation of Ovine PBMC and Subsequent Expansion of Activated Cells with IL2.
PBMC prepared from antigen-primed or non-primed sheep were resuspended in culture medium at 2 x 10⁶ ml⁻¹ in 24-well plates (Cel Cult, Sterilin limited) with an optimum
concentration of antigen. After 5 days incubation at 37°C viable cells were harvested over Lymphoprep as previously described and subsequently cultured at 1 x 10^5 ml⁻¹ in medium containing 100 pM recombinant human IL-2 (a generous gift from Biogen SA, Switzerland). Cells were cultured for 14 days with fresh additions of IL-2 every 3-4 days. The phenotype of cultured PBMC at the various time points was assayed by monoclonal antibody staining and immunofluorescence analysis.

2.6.3. Irradiation of Antigen Presenting Cells.

PBMC prepared from ovine peripheral blood as described above were resuspended in culture medium at a concentration of 2 x 10^6 ml⁻¹ and irradiated using a cobalt-caesium source. The amount of radiation that prevented subsequent proliferation of PBMC when cultured with Con A was determined. A titration of irradiation showed this to be 3000 rads (data not shown) which was the amount of radiation used for treatment of antigen presenting cells in subsequent proliferation assays.

2.6.4. Antigen-Specific Proliferation of Purified γδ T Cells.

Sheep γδ T cells were purified from peripheral blood of young lambs by positive selection using anti-T19 monoclonal antibodies and magnetic activated cell sorting (MACS) as described in detail in Section 2.7.4. (Evans et al, in preparation). Briefly, PBMC isolated from whole blood by centrifugation over Lymphoprep were reacted with biotinylated monoclonal antibodies ST197 and CC15 followed by Streptavidin-conjugated magnetic microsphere beads. Washed cells were applied to a sterile ferromatrix wool column under the influence of a strong magnetic field. Unbound cells were removed from the column matrix by washing through with medium. Bound cells were eluted with buffer after removal of the column from the magnetic field. These positively selected cells were routinely >93% γδ T cells as judged by immunofluorescence staining with monoclonal antibody 86D. γδ T cells purified from antigen-primed or non-primed sheep were resuspended in medium and 1 x 10^5 cells were cultured in 96-well flat-bottom micro-culture plates (Nunculon, Denmark) with various concentrations of antigen. 1 x 10^5 of autologous irradiated PBMC as antigen presenting cells were added to each of the wells. Cultures were made to a final volume of
200μl and established in triplicate. Positive and negative controls were established by substituting antigen with either Con A or culture media, respectively. The proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air for 3 or 5 days and subsequently pulsed with 1 μCi ³H-thymidine and harvested as previously described.

2.6.5. Concanavalin A Activation of PBMC.

PBMC prepared from ovine peripheral blood, were resuspended in culture medium at 2 x 10⁶ ml⁻¹ in either T25 (Cel Cult, Sterelin limited) or T75 (Nunculon, Denmark) tissue culture flasks and cultured in the presence of Con A at a final concentration of 20μgml⁻¹. At various time intervals viable blast lymphocytes were harvested by density centrifugation over lymphoprep as previously described and analysed by single or dual colour immunofluorescence.

2.6.6. Proliferation of Activated Lymphocytes to Exogenous IL-2.

Lymphocytes were obtained from ovine peripheral blood (as either unfractionated or fractionated γδ T cells), and afferent or efferent lymphatic vessels. Cells were resuspended in culture medium and 1 x 10⁵ cells were cultured in 96-well flat-bottom micro-culture plates (Nunculon, Denmark) with various concentrations of recombinant human IL-2. Cultures were made up to a final volume of 200 μl and established in triplicate. Positive and negative controls were established by substituting IL-2 with either Con A or culture media, respectively. The proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air for 3 days and subsequently pulsed and harvested with ³H-thymidine as previously described.

2.6.7. Inhibition of Antigen Specific Proliferation By Monoclonal Antibodies.

In each assay the cells to which the monoclonal antibody was targeted were cultured in its presence at 37°C in a humidified atmosphere of 5% CO₂/95% air for 30 minutes prior to the addition of any other cells or reagents. γδ T cells purified from antigen-primed sheep, resuspended in culture medium and 1 x 10⁵ cells aliquoted into 96-well flat-bottom
micro-culture plates (Nunculon, Denmark) with optimum concentrations of antigen and 1 \times 10^5 autologous irradiated PBMC as antigen presenting cells. In addition, 50\mu l of the appropriate antibody at varying concentrations was added to each of the wells. Cultures were made up to a final volume of 200 \mu l with culture medium and established in triplicate. Positive and negative controls were established by substituting antigen with either Con A or culture media, respectively. The proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}/95% air for 3 days and subsequently pulsed with \textsuperscript{3}H-thymidine and harvested as previously described.

2.7. Purification of \(\gamma\delta\) T cells.

Techniques for the purification of cells routinely employ either positive or negative selection of the desired cell type. This section describes various protocols established for the purification of ovine \(\gamma\delta\) T cells from ovine PBMC.

2.7.1. Isolation Of \(\gamma\delta\) T Cells By Negative Selection Utilising Adherence, Complement Mediated Lysis and Dynabeads.

Ovine PBMC were depleted of plastic adherent cells by incubation in either T25 or T75 tissue culture flasks in culture medium at 37°C in 5% CO\textsubscript{2} for 60 minutes. Nonadherent cells were removed by washing with media and harvested by centrifugation. Surface immunoglobulin-positive cells and \(\alpha\beta\) T cells were depleted by complement mediated lysis. Briefly, cells were incubated with monoclonal antibodies SBUT4 (anti-CD4), SBUT8 (anti-CD8), 36F (anti-CD2) and rabbit polyclonal antisera specific for sheep immunoglobulin for 30 minutes at 4°C. Unbound antibody was removed and the lymphocytes were incubated in the presence of normal rabbit sera immunoabsorbed against ovine lymphocytes. Sera was used at a dilution of 1/5 and incubated with cells for 45 minutes at 37°C, with periodical mixing. Optimum concentration of the rabbit sera was determined by the dilution that yielded greatest lysis of immunolabelled lymphocytes without depleting unlabelled lymphocytes. Viable lymphocytes were harvested and washed three times in washing media. Cells were resuspended at 10^7 ml\textsuperscript{-1} and incubated with Dynabeads coated with goat anti-mouse immunoglobulins at a bead to cell ratio of 40:1. Lymphocytes and beads were brought into
close proximity by centrifugation at 150g for 5 minutes at 4°C and incubation for 5 minutes on ice, then gently resuspended for a further incubation of 10 minutes on ice. The cell/ bead mixture was diluted in 5mls of washing media and the immunomagnetic lymphocyte rosettes and free Dynabeads removed using a magnet. Unrosetted lymphocytes were harvested and exposed to a second round of Dynabead depletion using a bead to cell ratio of 10:1. An aliquot of isolated cells was analysed to determine the percentage of γδ T cells and contaminating cells within this final population. Cells were immunofluorescently labelled with monoclonal antibodies specific for different epitopes to those used for the negative selection (which would be blocked) and analysed by flow cytometry.

2.7.2. Isolation Of γδ T Cells By Negative Selection Utilising Adherence And MACS.
Plastic adherent cells were removed by incubating the PBMC either in T25 or T75 tissue culture flasks in culture medium at 37°C in 5% CO₂ for 60 minutes. Nonadherent cells were removed by washing with media consisting of PBS supplemented with 1% FCS (MACS buffer 1) and harvested by centrifugation. Surface immunoglobulin-positive cells and αβ T cells were labelled with monoclonal antibodies as stated above in Section 2.7.1. Unbound antibody was removed and the lymphocytes were further incubated in the presence of goat anti-mouse IgG (whole molecule) biotin conjugate and goat anti-rabbit immunoglobulin (whole molecule) biotin conjugate for 30 minutes on ice. Excess conjugates were removed by washing with MACS buffer 2 (PBS and 0.1M EDTA) and the lymphocytes were re-suspended in 1ml of this buffer and further incubated with streptavidin for 15 minutes on ice. Unbound streptavidin was removed by washing in MACS buffer 2. The cells were resuspended in 1ml of this buffer then finally incubated with 1μl of biotinylated microbeads per 5x10⁶ immunolabelled cells for 5 minutes on ice, following manufacturers instructions. Excess beads were removed by washing in MACS buffer 3 (PBS, 1% FCS and 0.1M EDTA) and cells were re-suspended in 1ml of MACS buffer 3. The MACS separator column was cooled by flushing through with ice cold MACS buffer 3. The labelled lymphocyte suspension was loaded onto the MACS separator and the magnetic fraction was retained on the column, whilst unlabelled cells were collected as column eluate. An aliquot of the eluted
cells were analysed by immunofluorescence staining and flow cytometry to determine the percentage of γδ T cells within the sample.

2.7.3. Enrichment Of γδ T Cells By Positive Selection Using Dynabead And Detachabead Via The T19 Molecule.

PBMC were resuspended in wash medium at 10^7 ml^-1 and incubated with Dynabeads coated with mouse immunoglobulins specific for the ovine T19 molecule (monoclonal antibody ST197 - IgG2b isotype) at a bead to cell ratio of 10:1 by the same procedure as described in section 2.7.1. After the attachment of Dynabeads to the lymphocytes, the cell/ bead mixture was diluted up to 5mls with washing media and the immunomagnetic lymphocyte rosettes and free Dynabeads were removed by placing the sample on a magnet. The supernatant was discarded and the lymphocyte rosettes were resuspended in 5mls of washing media and subsequently placed on the magnet to remove residual contaminating immunomagnetic lymphocyte rosettes. This step was repeated twice to minimise contamination of nonmagnetic lymphocytes trapped by the cell / Dynabead complexes. The positively selected lymphocytes were finally resuspended at 10^7 ml^-1 and incubated with 5 units of Detachabead / 5 x 10^6 cells attached to Dynabeads for 60 minutes at 4°C with periodical mixing. The immunomagnetic fraction was removed by placing the sample on a magnet. The supernatant was collected and lymphocytes were harvested by centrifugation. Cells were immunofluorescently labelled with monoclonal antibodies specific for various subset markers of isotopes other than IgG2b, followed by isotype specific second layer reagents and analysed by flow cytometry.

2.7.4. Enrichment Of γδ T Cells By Positive Selection Using MACS via The T19 Molecule.

Lymphocytes were resuspended in MACS buffer 1 at 4 x 10^7 ml^-1 and incubated with biotinylated anti-T19 monoclonal antibodies, namely CC15 and ST197, for 30 minutes at 4°C. Unbound antibody was removed by washing with MACS buffer 2 and the lymphocytes were re-suspended in 1ml and incubated with streptavidin for 15 minutes on ice. The remainder of the protocol is as described in section 2.7.2, with the exception that the magnetic fraction contained the enriched γδ TCR-positive population of cells. The magnetic
fraction was harvested by removing the column from the magnet and flushing through with MACS buffer 3. Lymphocytes were harvested by centrifugation. Cells were immunofluorescently labelled with monoclonal antibodies specific for various subset markers of isotopes other than IgG2a and IgG2b followed by isotype specific second layer reagents. Cells were analysed by flow cytometry.

2.8. Protein Immunobiochemistry.

2.8.1. 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 Hanks Basic Salt Solution (HBSS). Lymphocytes were harvested by centrifugation at 250g for 4 minutes at 4°C. Cells were lysed by resuspending at a concentration of 1 x 10^8 cells ml^-1 in 2% NP40/PBS buffer containing 0.2 mM PMSF, for 30 minutes on ice. Cellular debris was removed by centrifugation in a Microfuge for 10 minutes. The supernatant was collected and diluted 1 : 1 with sample buffer as described in section 2.8.2.

2.8.2. SDS PAGE of Proteins.

Protein separation was by discontinuous polyacrylamide gel electrophoresis with a Tris-glycine buffer containing SDS (384 mM glycine, 0.1% SDS and 50 mM Tris HCl). Gels were 5-20% linear gradient acrylamide gels (30% acrylamide : 0.8% bisacrylamide) made with 375 mM Tris HCl pH 8.7 and 0.1% SDS. Stacking gels consisted of 3% acrylamide, 0.15% N,N'-methylenebisacrylamide made with 125 mM Tris HCl pH 6.8 and 0.1% SDS. Samples were prepared as described in Section 2.8.1. and mixed with an equal volume of sample buffer (10% (v/v) β-mercaptoethanol, 10% (w/v) SDS, 125 mM Tris HCl pH 6.8, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue), were boiled for 3 minutes before electrophoresis through vertical slab gels using Bio-Rad Mini-protean II gel equipment. Gels were run at 200 V for 30-45 minutes.

2.8.3. Staining of SDS-PAGE.

Gels were stained by either of two methods:

a) Coomassie Blue staining: Coomassie Brilliant Blue G-250 was dissolved in methanol and
then made to 20% methanol, 5% acetic acid and filtered through Whatman No. 1 filter paper. Gels were stained in Coomassie blue for 15 minutes at room temperature and then destained in several changes of 20% methanol, 5% acetic acid.

b) Silver staining: Gels were fixed in 50% methanol, 10% acetic acid for 15 minutes, 5% methanol, 7% acetic acid for 30 minutes and then 10% glutaraldehyde for 30 minutes. Gels were then washed thoroughly in several changes of distilled water over several hours. After washing, gels were stained with 0.1% silver nitrate solution for 15 minutes, rinsed briefly in water and then developed with a 3% Na$_2$CO$_3$, 0.02% formaldehyde solution. Development was halted by addition of solid citric acid and the gels were washed in several changes of distilled water. Gels were then fixed in 10% 'Ilfofix' for 1 minute and washed in three changes of water. Gels were dried at 80°C under vacuum.

2.8.4. Western Blot Analysis.

After electrophoretic separation on SDS-PAGE, proteins were transferred to nitrocellulose membranes (Hybond C, Amersham) using a semi-dry electrophot blotter (Ancos, Denmark) according to Khyse-Andersen (1984). 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 consisting of PBS/1% non-fat dried milk supplemented with 0.05% Tween 20 (Sigma) over 60 minutes at room temperature. Bound primary antibody was detected by incubating for 60 minutes at room temperature with either an anti-mouse (Cat.No. B-7264, Sigma) or anti-rabbit (Cat.No. B-7389, Sigma) IgG whole molecule biotin conjugate followed by alkaline phosphatase conjugated to streptavidin (Boehringer Mannheim Chemica, Germany). Excess reagents were removed by washing in several changes of buffer over 60 minutes at room temperature. The immunoblots were developed with nitro-blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 0.1M Tris HCl pH 9.5 and MgCl$_2$ as described by Pluzek and Ramlau (1988). Development of blots was halted by washing in water.
2.8.5. Preparation of Spleen Lysates.

Splenocytes were removed from the spleen via dissection of the enclosing connective tissue capsule with a scalpel and forceps and subsequently homogenised with cold Hanks Basic Salt Solution (HBSS) for 1 minute. Cells were harvested 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) containing a final concentration of 0.2mM PMSF. 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. Residual cellular debris was removed by filtering through millipore prefilters of 0.6μm pore size (Cat.No. AP 1504700, Millipore, Ireland).

2.8.6. Affinity Chromatography.

2.8.6.1. Affinity Column For the Immunopurification of Immunoglobulin.

Immunoglobulin from culture supernatant of HMT3.1 hybridoma was isolated by affinity chromatography on a GammaBind - PLUS affinity column (Cat No GBA 030005, BDH, Poole) previously washed with 10 volumes of the fresh culture medium. Culture supernatant was applied to the column at a flow rate of 10ml hr⁻¹ which was subsequently washed extensively with washing buffer (consisting of 0.01M sodium phosphate pH 7.0, 0.15M sodium chloride and 0.01M EDTA) until no protein was detectable in the flow through. The bound material was eluted with elution buffer (consisting of 0.05M Acetic acid adjusted to pH 3.0 with ammonium hydroxide). 2ml fractions were collected into 1.0M Tris HCl 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.

2.8.6.2. Affinity Columns For the Immunopurification of Ovine CD3.

Immunoglobulin (HMT3.1) was coupled to cyanogen bromide (CNBr) activated Sepharose 4B (Pharmacia, Bucks, UK) 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 sintered glass filter with 1mM HCl, the final wash being with the coupling buffer, a sodium bicarbonate buffer pH 8.3 containing 0.5M NaCl. Immunoglobulin 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 into 1.0M ethanolamine pH 8.0 and mixed for 16 hrs at 4°C. Excess absorbed 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, 0.5M NaCl and 0.5% sodium deoxycholate at pH 11.3) and re-equilibrated with neutralising buffer (0.2M phosphate buffer pH 8.5).

Pre-columns were made as described above for the HMT3.1 columns, with the exception that normal mouse sera was used rather than HMT3.1 immunoglobulin.

Spleen lysate prepared as described in section 2.4.4. of this thesis was applied to the column and allowed to flow through at a rate of 10ml hr⁻¹. The column was washed extensively with washing buffer consisting 15mM triethanolamine and 0.5% sodium deoxycholate at pH 8.0 until no protein was detectable in the flow through. The bound material was eluted with eluting buffer. 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 and washed with 10 volumes of washing buffer.

2.8.7. Preparation of Antigen Obtained By Immunopurification For The Immunisation Of Mice To Generate Monoclonal Antibodies.

Antigen was prepared by affinity chromatography as described above and precipitated from the elution buffer with acetone. Five volumes of acetone pre-chilled to -20°C was added to one volume of immunopurified putative ovine CD3 antigen and stored at -20°C overnight.
The antigen was pelleted by centrifugation at 4750g for 15 minutes at 4°C and after removal of the supernatant was resuspended in PBS before being aliquoted and stored at -70°C.

2.9. Immunocytochemistry and Electron Microscopy.

2.9.1. Immunohistology of Lymph nodes.
Lymph nodes were removed by standard surgical techniques from animals which had been immunised with *Mycobacterium tuberculosis* and ovalbumin as described in Section 2.2.1. and were snap frozen in dry ice/isopentane. Cryosections of tissue cut at 5μm thickness were air-dried onto glass slides and fixed for 2 minutes in acetone at 4°C. Expression of various ovine lymphocyte surface antigens was detected by staining with some of the monoclonal antibodies listed in section 2.5.1. using standard indirect immunofluorescence or avidin-biotin phosphate staining using a commercially available kit (Imm-MWKTM, ICN Biomedicals Inc, USA). Positive staining was visualised by conventional or fluorescence microscopy.

2.9.2. Cytospins.
Lymphocytes were obtained by one of the techniques described in Section 2.4. and washed three times in PBA. 1 x 10⁵ lymphocytes were applied to each glass slide by centrifugation at 300g for 5 minutes at room temperature. Lymphocytes were fixed in cold acetone for 3 minutes followed by a 4% paraformaldehyde solution for 1 minute. Cells were washed in PBS for 5 minutes. Endogenous peroxidase activity was inhibited by treatment of the cells with 0.5% solution of hydrogen periodate in water for 10 minutes, washing for 5 minutes in PBS followed by a wash in a 0.3% solution of hydrogen peroxide in methanol for 15 minutes. Cells were washed in PBS for 5 minutes and subsequently blocked with a 2% solution of normal rabbit sera for 30 minutes. The primary antibody was added at the appropriate concentration for 60 minutes, excess reagent was removed by washing in PBS for 15 minutes. Bound primary antibody was detected by incubating for 60 minutes at room temperature with an anti-rat biotin conjugate followed by a further 30 minutes at room temperature with Horseradish Peroxidase conjugated to streptavidin (both reagents were obtained from the StreptA-B Universal Kit, Immustain, Diagnostic Product Corporation).
Excess reagents were removed by washing with PBS after which the enzyme substrate and the chromogen, diaminobenzidine (DAB) in sterile distilled water (SDW) were added to the slide and left to develop for 10 minutes. The reaction was halted by rinsing the slide with water.

The slides were counter stained with Haematoxylin followed by lithium carbonate, then washed with excess water and dehydrated by passing through alcohol and subsequently histoclear gradients prior to mounting with DPX. The slides were visualised by conventional microscopy.

2.9.3. Transmission Electron Microscopy.
Ovine T lymphocytes obtained from peripheral blood or cannulated lymphatic vessels were fractionated into either γδ TCR positive, CD4 positive or CD8 positive subsets by MACS separation as described in Section 2.4. Cells were fixed overnight in buffer consisting of 0.1M sodium cocodylate pH 7.3 and 3% glutaraldehyde. Cells were washed three times in 0.1M sodium cocodylate pH 7.3 and post fixed treated with 1% osmium tetroxide made in 0.1M sodium cocodylate buffer pH 7.3. Samples were dehydrated through graded acetone solutions and embedded in araldite. Ultrathin sections were cut, stained with 50% uranyl acetate (in ethanol) and Reynold’s lead citrate and examined in a Phillips TEM 400 operating at 100kV.

2.10. Molecular Techniques.
2.10.1. Bacterial Strains.
*E. coli.* strain JM101 (supE, thi, Δ(lac-proAB), [F’, traD 36, proAB, lacI q Z ΔM15]) (Yanisch-Perron 1985) was used for bacterial transformations involving pTZ based plasmids, while the *E. coli.* strain JM83 (ara, Δ(lac-proAB), rpsL(=strA), Φ80, lac Z ΔM15) (Yanisch-Perron 1985) was used for transformations with pOGS 40 plasmids.
2.10.2. Media and Growth Conditions For Bacteria.

Bacteria were grown in Luria-Bertani (LB) medium, as listed below. Single colonies of untransformed *E. coli* were grown by streaking an inoculum from a frozen stock onto LB agar plates.

**LB**

- tryptone (Oxoid) 10.0g
- sodium chloride 10.0g
- yeast extract (Difco) 5.0g
- distilled water 1000ml

The pH was adjusted to 7.0 with 5N sodium hydroxide.

LB agar comprised the above with 15g of agar.

Transformed JM101 were grown on LB agar supplemented with 150μg ml⁻¹ ampicillin (LBAp). Blue-white selection of bacteria transformed with pTZ derived plasmids was achieved by the addition of 40μl of 5-bromo-4-chloro-3-indolyl-b-D-galactoside (x-gal) (20mg ml⁻¹ in dimethylformide) and 20μl of isopropylthio-b-D-galactoside (IPTG) (40mg ml⁻¹ in SDW) to the LBAp agar. A thin overlay of the LBAp / x-gal / IPTG was poured over LBAp plates and allowed to set before plating out bacteria. Stocks of transformed bacteria were stored in glycerol at -70°C (Maniatis 1982).

2.10.3. Transformation of *E. coli*.

*E. coli* cells competent for transformation by plasmid DNA were prepared from 200μl of a fresh overnight culture of *E. coli* and diluted 1:100 into *psi* broth.

*psi* broth

- 2% tryptone
- 0.5% yeast extract
- 20mM magnesium sulphate
- 10mM sodium chloride
- 5mM potassium chloride
Cells were grown with vigorous shaking (230rpm) at 37°C, until the OD$_{550\text{nm}}$ reached 0.3. Five ml of the culture was diluted 1:20 in psi broth and grown until OD$_{550\text{nm}}$ reached 0.48. The culture was chilled briefly on ice, and centrifuged at 2000g for 5 minutes at 4°C. The cell pellet was resuspended in 33ml ice-cold Transforming Buffer I (TfBI) and held on ice for 10-15 minutes before centrifuging at 2000g for 5 minutes at 4°C. The pellet was resuspended in 4ml of Transforming Buffer II (TfBII) and kept on ice for a further 20 minutes. 200µl aliquots were snap-frozen in liquid nitrogen and stored at -70°C. TfBI and TfBII comprised the following:

**TfBI**

35mM sodium acetate,
100mM calcium chloride,
100 mM rubidium chloride,
50mM manganese chloride,
15% glycerol,
Made to pH 5.9 with acetic acid.

**TfBII**

10mM morpholinopropane sulfonic acid
10mM rubidium chloride
75mM calcium chloride
15% glycerol
Made to pH 6.8 with potassium hydroxide.

For bacterial transformation, aliquots of competent *E. coli* were thawed on ice and 100-200ng of DNA added (2-5µl of the ligation reaction mixture). 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 (containing no antibiotics) and incubated, with shaking, at 37°C for 1 hour. 200µl of the suspended cell culture were plated onto LBAp agar plates. Ampicillin resistant colonies were picked and investigated for the required plasmid by restriction enzyme analysis.
2.10.4. Preparation of DNA and Molecular Cloning Techniques.

Many of the methods used in manipulation and cloning 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 (1989).

2.10.4.1. Vectors Used in Cloning, Sequencing and Expression.

The phagemid vectors pTZ 18 and pTZ 19 were purchased from Pharmacia. The pOGS 40 expression vector was a gift from Dr. S.E. Adams, (British Bio Technology Ltd., Oxford).

2.10.4.2. DNA Agarose Gel Electrophoresis.

DNA was analysed by horizontal gel electrophoresis using 0.8-1.5% agarose gels. The gels were made using a buffer comprising 40mM Tris HCl, 8mM sodium acetate, 0.4mM EDTA, pH 7.85, to which ethidium bromide (Sigma) at a final concentration of 0.1µg ml⁻¹ was added when the agarose had cooled. DNA was loaded into the wells in 5% glycerol, 12.5mM EDTA pH 7.9, 0.01% SDS, with bromophenol blue. Gels were run at 70 volts for 60 minutes and the DNA was visualised on an ultra-violet (U.V.) transilluminator.

2.10.4.3. Polymerase Chain Reaction.

Polymerase chain reaction (PCR) was carried out according to Saiki (1989). The substrate for the generation of ovine CD3γchain was cDNA prepared from ovine PBMC RNA as described by Sambrook (1989). The PCR reaction tubes contained 50mM potassium chloride, 10mM Tris.HCl (pH 8.4), 100µg ml⁻¹ bovine serum albumin (BSA), 0.25µM of each primer, 200µM of deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) referred to collectively as dNTP, 2.5 units of Taq polymerase, and 1-5mM magnesium chloride. The final reaction volume was 100µl and the reaction mixture was overlayed with an equal volume of sterile mineral oil.

PCR was carried out in a thermal cycler for 35 cycles, where one cycle comprised denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1.5 minutes. The final extension was for 10 minutes. PCR products were analysed by
2.10.4.4. Preparation of DNA Fragments For Blunt-Ended Ligation.

Protruding ends of the PCR product were converted to blunt ends using the Klenow fragment of E. coli DNA Polymerase 1 (Sambrook, 1989). The PCR product was phenol:chloroform extracted and the final pellet resuspended in 39 µl of SDW. 5 µl of 10 x dNTP and 5 µl of the T4 kinase buffer supplied by the manufacturer were added to the pellet. 1U of the Klenow enzyme was added and the reaction incubated at 37°C for 30 minutes. Fragment ends were phosphorylated by treatment with T4 polynucleotide kinase (Sambrook 1989), where 3U of the enzyme was added directly to the above reaction mixture and incubated at 37°C for 60 minutes.

2.10.4.5. Restriction Endonuclease Digestion of DNA.

Digestions were carried out in the incubation buffer supplied by the manufacturer with the enzyme in a final volume of 20-50µl with 10U of enzyme per 1µg of purified DNA. The DNA was digested for 2-3 hours at 37°C.

2.10.4.6. Preparation of Cut Vector.

In preparation for ligation the purified plasmid DNA was digested with the required restriction endonuclease at 37°C. Two hours into the digest, DNA was analysed on an agarose gel to ensure that linearisation of the plasmid had taken place. Subsequent to this, the 5' phosphate group of the linearised plasmid was removed to prevent self ligation (Sambrook 1989). This was achieved by adding 10U of calf intestinal alkaline phosphatase (CIP) for a further 30 minutes to the digestion. The reaction mixture was heated to 75°C for 10 minutes to inactivate the CIP and the DNA purified by phenol-chloroform extraction (Sambrook 1989).

2.10.4.7. Ligation of DNA.

Ligation reactions were carried out in a final volume of 20µl. 50-100ng of vector DNA were used in each ligation, at a molar ratio of insert : vector of 5 : 1. Insert and vector DNA were
mixed and heated to 65°C. The mix was allowed to cool to room temperature for 10 minutes and buffer supplied by the manufacturer and 10U of T4 DNA ligase enzyme were added for the ligation of DNA fragments. The ligation reactions were incubated for 16-20 hours at 15°C. Ligation mixtures were used to transform E. coli without purification.

2.10.4.8. Miniprep of Plasmid DNA.
Transformed E. coli were grown to saturation in LBAp broth and 1.5 ml of this culture was used for the preparation of plasmid DNA isolated by the alkaline / SDS lysis method of Ish-Horowicz (1981). The bacteria were pelleted, digested with lysosyme and then lysed in Sodium Laurel Sulphate (SDS) buffer (0.2N sodium hydroxide, 1% SDS). Following the addition of salt (3M sodium acetate, pH 4.8) precipitated denatured chromosomal DNA and proteins were recovered by centrifugation. Plasmid DNA was recovered from the supernatant by ethanol precipitation, resuspended and phenol:chloroform extracted. The final DNA pellet was resuspended in 20μl of SDW containing a final concentration of 50μg ml⁻¹ RNAase A. 2-5μl of miniprep DNA was used for each restriction enzyme digest. The presence of plasmid insert was verified by agarose gel electrophoresis.

2.10.5. DNA sequencing.

2.10.5.1. Preparation of Single Stranded DNA.
Single stranded DNA for use as template in sequencing reactions was prepared by a modification of the protocol obtained from the supplier of the pTZ phagemid (Pharmacia Literature 1983). JM101 cells transformed with recombinant pTZ were grown in 2x YT (where 1x YT is 1.6% tryptone, 1% yeast extract, 86mM NaCl, made to pH7.4 with potassium hydrogen phosphate) supplemented with ampicillin (150μg ml⁻¹) until OD₆₆₀nm reached 0.5. 400μl of this culture was infected with helper phage M13K07, at a multiplicity of infection of 10, and shaken vigorously for 1 hour at 37°C. The culture was then made to 10mls with 2x YT supplemented with 150μg ml⁻¹ ampicillin. Kanamycin was added to 70μg ml⁻¹ and the culture grown at 37°C for 16-18 hours with vigorous shaking. The supernatant was then centrifuged at 4000g for 15 minutes at 4°C to remove bacterial cells and phage particles were precipitated from the supernatant by the addition of polyethyleneglycol (PEG) /
sodium chloride (to a final concentration of 4% PEG, 700mM NaCl). After 30 minutes on ice, the phage precipitate was recovered by centrifugation at 11000g for 40 minutes at 4°C, resuspended in 400μl of TEN buffer (20mM Tris HCl pH 7.5, 20mM NaCl, 1mM EDTA) and extracted four times with phenol-chloroform pH8.0, three times with chloroform and ethanol precipitated. The DNA pellet was resuspended in 22μl of SDW and 2μl of this suspension was electrophoresed through 1% agarose and examined under UV to determine the yield of DNA.

2.10.5.2. Sequencing Reactions.
DNA was sequenced using the dideoxynucleotide chain termination method (Sanger 1977) and [α-35S]-dATP (Biggin 1983) with a Sequenase kit (USB corporation) and M13 reverse sequencing primer (Pharmacia). Sequencing reactions were carried out according to the protocol supplied with the Sequenase kit.

2.10.5.3. Sequencing Gel Electrophoresis.
DNA sequencing samples were electrophoresed through 6% acrylamide (19 : 1 acrylamide : bisacrylamide, 7.67M urea in 45mM Tris HCl, 45mM Boric acid, 5mM Na_2EDTA, pH 8.3). Prior to casting the gel acrylamide solutions were de-gassed under vacuum and filtered through a 0.45μm filter. Slab gels were cast in a Bio-Rad Sequi-Gen nucleic acid sequencing cell (38 x 50 cm). Wells were formed using a sharkstooth comb. The running buffer in the electrode tanks comprised 45mM Tris HCl, 45mM hydrogen borate, 5mM EDTA, pH 8.3. Gels were pre-run at 50 W until the gel temperature reached 50°C, then loaded with 3μl of samples that were denatured by heating to 80°C for 3 minutes and quenched on ice. Sequencing gels were run for 2-6 hours at 50°C (40-50 W). Gels were dried under vacuum at 80°C and exposed to Kodak XAR-5 film for 1-3 days at room temperature.
CHAPTER 3

Characterisation And Surface Phenotype Of Ovine \( \gamma \delta \) T Cells.

INTRODUCTION.

Lymphocytes can be divided into distinct subsets based on the expression of cell surface glycoproteins which may be detected by conformation antibodies and immunofluorescence staining. The cluster determinant (CD) nomenclature used to distinguish human cell surface glycoproteins is adapted for other species, including the sheep, when molecules are conserved with respect to those distribution, function and structure. CD molecules mediate a variety of functions associated with lymphocyte adhesion (Mackay et al. 1986), signalling (Janeway 1992) and activation (Alexander et al. 1992, Tsudo et al. 1987). The expression of CD molecules is likely to be dependent on both the lymphocyte lineage and the activation status of the cell.

Ligand recognition by T cells is facilitated by the expression of various adhesion molecules, increasing the cell avidity with an appropriate antigen presenting cell (Makgoba et al. 1989). The result of this cellular interaction is the transmission of signalling to the nucleus of the lymphocyte which initiates the expression of various genes de novo (Rich-Thuy et al. 1987). The translation of these gene products yields proteins expressed exclusively by activated or proliferating lymphocytes. In addition, T cell activation may result in the expression of different isoforms of a particular CD molecule which arise as a consequence of alternative exon splicing at the messenger RNA level (Thomas 1989). These characteristics of cell surface glycoproteins enable a detailed phenotype characterisation of lymphocytes within the immune system and will provide information of the function of these cells.

Three major populations of mature ovine peripheral blood T cells exist which are defined by their expression of either the T19 molecule, CD4 or CD8 (Mackay et al. 1986). Those cells which express the T19 molecule react with the rabbit antiseraum W5 which is specific for a conserved region of human and mouse C\( \alpha \) TCR chain (Mackay et al. 1989). The proteins immunoprecipitated by W5 from ovine T19-positive cells are probed by a mouse anti-ovine monoclonal antibody termed 1363 which itself immunoprecipitates proteins from these cells of 75Kd when run under non-reducing conditions, and of 41-44KDa under reducing condition. Collectively, this data strongly suggests that the
INTRODUCTION.

Lymphocytes can be divided into distinct subsets based on the expression of cell surface glycoproteins which may be detected by monoclonal antibodies and immunohistochemical staining. The cluster determinant (CD) nomenclature used to distinguish human cell surface glycoproteins is adopted for other species, including the sheep, where molecules are conserved with respect to tissue distribution, function and structure. CD molecules mediate a variety of functions associated with lymphocyte adhesion (Mackay et al. 1980), signalling (Janeway 1992) and activation (Albrechtsen et al. 1992, Bode et al. 1987). The expression of CD molecules is developmentally dependent on both the lymphocyte lineage and the activation stage of the lymphocytes.

Ligand recognition by T-cells is facilitated by the expression of various adhesion molecules, increasing the cell avidity with an appropriate antigen presenting cell (Makgoba et al. 1989). The result of this cellular interaction is the transmission of signals to the nucleus of the lymphocyte which initiates the expression of various genes de novos (Rich-Thuy et al. 1987). The translation of these gene products yields proteins expressed exclusively by activated or proliferating lymphocytes. In addition, T-cell activation may result in the expression of different isoforms of a particular CD molecule, which arise as a consequence of alternative exons splicing in the messenger RNA level (Thomas 1989). These characteristics of cell surface glycoproteins enable a detailed phenotypic characterisation of lymphocytes within the immune system and will provide information of the function of those cells.

Three major populations of mature ovine peripheral blood T-cells exist which are defined by their expression of either the CD4 molecule, CD8 or CD2 (Mackay et al. 1986). Those cells which express the CD4 molecule react with the rabbit antiseraum W6 which is specific for a conserved region of human and mouse C3 TCK chain (Mackay et al. 1989). The proteins immunoprecipitated by W6 from ovine T4-positive cells are precipitated by a mouse anti-ovine monoclonal antibody termed BD which itself immunoprecipitates proteins from these cells of 75Kd which run under non-reducing conditions, and of 41-44Kd under reducing condition. Collectively, this data strongly suggests that the

CHAPTER 3

Characterisation And Surface Phenotype Of Ovine γδ T Cells.

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INTRODUCTION.

Lymphocytes can be divided into distinct subsets based on the expression of cell surface glycoproteins which may be detected by monoclonal antibodies and immunofluorescence staining. The cluster determinant (CD) nomenclature used to distinguish human cell surface glycoproteins is adopted for other species, including the sheep, when molecules are conserved with respect to tissue distribution, function and structure. CD molecules mediate a variety of functions associated with lymphocyte adhesion (Mackay et al 1990), signalling (Janeway 1992) and activation (Alexander et al 1992, Tsudo et al 1987). The expression of CD molecules is highly regulated, dependent on both the lymphocyte lineage and the activational status of the cell.

Ligand recognition by T cells is facilitated by the expression of various adhesion molecules, increasing the cells avidity with an appropriate antigen presenting cell (Makgoba et al 1989). The result of this cellular interaction is the transmission of signals to the nucleus of the lymphocyte which initiates the expression of various genes de novo (Bich-Thuy et al 1987). The translation of these gene products yields proteins expressed exclusively by activated or proliferating lymphocytes. In addition, T cell activation may result in the expression of different isoforms of a particular CD molecule which arise as a consequence of alternative exon splicing at the messenger RNA level (Thomas 1989). These characteristics of cell surface glycoproteins enable a detailed phenotypic characterisation of lymphocytes within the immune system and will provide information of the function of these cells.

Three major populations of mature ovine peripheral blood T cells exist which are defined by their expression of either the T19 molecule, CD4 or CD8 (Mackay et al 1986). Those cells which express the T19 molecule react with the rabbit antiserum W6 which is specific for a conserved region of human and mouse C8 TCR chain (Mackay et al 1989). The proteins immunoprecipitated by W6 from ovine T19-positive cells are precleared by a mouse anti-ovine monoclonal antibody termed 86D which itself immunoprecipitates proteins from these cells of 75Kd when run under non-reducing conditions, and of 41-44KDa under reducing condition. Collectively, this data strongly suggests that the
monoclonal antibody 86D identifies the ovine $\gamma\delta$ TCR molecule expressed by T19-positive cells. Subsequent studies using ovine cDNA probes encoding TCR genes have confirmed the expression of $\gamma$ and $\delta$ genes by T19-positive cells (Mackay et al 1989).

To date, no monoclonal antibody specific for the ovine $\alpha\beta$ TCR exists. It has been established that the majority of human (Brenner et al 1986) and murine (Bluestone et al 1991) $\alpha\beta$, but not $\gamma\delta$ T cells express the co-receptor molecules CD4 and CD8, and it is assumed that an analogous situation exists in the sheep. The presence of T cells expressing an $\alpha\beta$ TCR in the sheep can be estimated by the summation of the percentage of CD4-positive and CD8-positive T cells and it has been suggested (Mackay et al 1986), but not thoroughly investigated that the peripheral blood of the young sheep is characterised by the predominance of $\gamma\delta$ T cells, rather than CD4-positive or CD8-positive T cells. The exposure of these young animals to antigens which are recognised by $\gamma\delta$ T cells may lead to the activation and preferential clonal, or oligoclonal, selection of $\gamma\delta$, rather than $\alpha\beta$, T cells. Antigen-specific activation of $\gamma\delta$ T cell subsets in the periphery may then lead to the subsequent expression of a memory phenotype by these cells (Horgan et al 1992, Vitetta et al 1991). In the human species it has been suggested that naive and memory $\gamma\delta$, and $\alpha\beta$, T cells can be distinguished on the basis of the expression of different molecular weight isoforms of leucocyte common antigen (CD45) (Braakman et al 1991).

In this chapter populations of T cells within ovine PBMC were investigated using a panel of monoclonal antibodies generated to various cell surface antigens. The phenotype and activational status of T cells was examined with particular reference to $\gamma\delta$ T cells. With the base line information obtained the percentages and phenotype of $\gamma\delta$ T cells in animals of various ages and experiencing different antigenic loads was investigated.
RESULTS.

3.1. Immunofluorescence Analysis.

Figure 1 shows the scatter profiles (forward vs side scatter) of ovine peripheral blood lymphocytes. Distinct populations of cells have characteristic scatter profiles which are assigned particular P numbers and which can be defined by electronic gates on the FACScan machine. By comparison with published literature, sheep peripheral blood can be divided into lymphocytes and monocytes (P1), granulocytes (P2) and mononuclear cells (P3). Routinely, gate P1 was used on unfractionated PBMC to analyse the lymphocyte population. Gate P1 was also used for gating on lymphocytes isolated from afferent and efferent lymph.

The fluorescence profile of a population of cells is displayed as a histogram of fluorescence intensity versus relative cell number. The mean channel number (MCN) of a particular fluorescence profile provides an indication of the intensity of fluorescence either on, or within, the cells under examination. The MCN is a consequence of at least three variables (i) the level of expression of lymphocyte surface antigen, (ii) the affinity of the monoclonal antibody for its epitope, (iii) the affinity of the second layer reagent for the primary antibody. The MCNs are of particular value in studying the changes in the level of expression of a specific surface antigen, where the first and second layer reagents are the same. An increase in MCN denotes an elevated expression of the antigen under investigation by the population of cells as a whole.

Included in Table 1 are the irrelevant monoclonals used to establish background fluorescence level. The choice of normal mouse sera as the reagent to be used routinely to establish background fluorescence was made after a comparison of this reagent with irrelevant monoclonal antibodies of different isotypes. The immunofluorescent profiles shown in Figure 2 show that there was no significant difference in the percentage of background staining when normal mouse sera was used compared to irrelevant monoclonal antibodies of different isotypes. In each situation less than 2% of the cells were immunofluorescent with any of the reagents. In dual colour immunofluorescence, the assessment of background staining required the use of irrelevant monoclonal
Figure 1.
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk sheep and analysed by flow cytometry for their forward and side light scatter profiles.
FIGURE 1. Scatter profiles Of Ovine Peripheral Blood Lymphocytes.

$P1$ - Lymphocytes and Monocytes

$P2$ - Granulocytes

$P3$ - Mononuclear Cells
Table 1.

Table 1 lists the monoclonal antibodies used routinely in this investigation and shows their specificity and isotype.
TABLE 1. Monoclonal Antibodies Used For The Immunofluorescence Analysis Of Ovine Cell Surface Antigens.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>86D</td>
<td>anti-ovine γ8 TCR</td>
<td>IgG1</td>
<td>Mackay et al 1989</td>
</tr>
<tr>
<td>19.19</td>
<td>anti-ovine T19</td>
<td>IgG1</td>
<td>Maddox et al 1986</td>
</tr>
<tr>
<td>ST197</td>
<td>anti-ovine T19</td>
<td>IgG2b</td>
<td>Maddox et al 1986</td>
</tr>
<tr>
<td>CC15</td>
<td>anti-ovine T19</td>
<td>IgG2a</td>
<td>Clevers et al 1990</td>
</tr>
<tr>
<td>ST4</td>
<td>anti-ovine CD4</td>
<td>IgG1</td>
<td>Maddox et al 1985</td>
</tr>
<tr>
<td>SBU T4</td>
<td>anti-ovine CD4</td>
<td>IgG2a</td>
<td>Maddox et al 1985</td>
</tr>
<tr>
<td>ST8</td>
<td>anti-ovine CD8</td>
<td>IgM</td>
<td>Maddox et al 1985</td>
</tr>
<tr>
<td>SBU T8</td>
<td>anti-ovine CD8</td>
<td>IgG2a</td>
<td>Maddox et al 1985</td>
</tr>
<tr>
<td>I35A</td>
<td>anti-ovine CD2</td>
<td>IgG1</td>
<td>Giegerich et al 1989</td>
</tr>
<tr>
<td>36F</td>
<td>anti-ovine CD2</td>
<td>IgG2a</td>
<td>Mackay et al 1988a</td>
</tr>
<tr>
<td>VPM8</td>
<td>anti-ovine Ig light chain</td>
<td>IgG1</td>
<td>McConnell et al, unpublished</td>
</tr>
<tr>
<td>SBU1</td>
<td>anti-ovine MHC class I</td>
<td>IgG1</td>
<td>Gogolin-Ewens et al 1985</td>
</tr>
<tr>
<td>HMT3.1</td>
<td>anti-human CD3</td>
<td>IgG1</td>
<td>Kubo et al, unpublished</td>
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<tr>
<td>ZG1</td>
<td>irrelevant monoclonal</td>
<td>IgG1</td>
<td>unpublished</td>
</tr>
<tr>
<td>UPC10</td>
<td>irrelevant monoclonal</td>
<td>IgG2a</td>
<td>unpublished</td>
</tr>
<tr>
<td>2F7</td>
<td>irrelevant monoclonal</td>
<td>IgM</td>
<td>unpublished</td>
</tr>
</tbody>
</table>
Figure 2.
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk sheep and stained with either normal mouse sera or the irrelevant monoclonal antibodies ZG1 (IgG1), UPC10 (IgG2a) and 2F7 (IgGM) followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry.
FIGURE 2. Comparison of Background Immunofluorescence Staining With Irrelevant Monoclonal Antibodies of Various Isotypes.

Normal Mouse Sera

ZG1 (IgG1)

UPC 10 (IgG2a)

2F7 (IgGM)

Fluorescence intensity
antibodies which were of the same isotype as the specific monoclonal antibodies under test. Dual colour immunofluorescence requires the use of different fluorochromes. In this thesis isotype specific immunoglobulin FITC-conjugated reagents were used for analysis on the FL1 channel which measures green fluorescence, whilst biotinylated monoclonal antibodies followed by phycoerytherin-streptavidin were used for analysis on the FL2 channel which measures red fluorescence. Background fluorescence on the FL1 channel during dual colour immunofluorescence was established with irrelevant monoclonal antibodies followed by isotype specific FITC reagents. Biotinylated isotype matched irrelevant monoclonal antibodies followed by phycoerytherin-streptavidin were used to assess background fluorescence on the FL2 channel. Figure 3 shows an example of background immunofluorescence staining obtained by dual fluorescence analysis using biotinylated UPC10 (IgG2a) and ZG1 (IgGl) which were used in some of the fluorescence staining assays described later.

3.2. Monoclonal Antibodies to Ovine Lymphocyte Molecules.

Table 1 lists the monoclonal antibodies used within this investigation, and shows their isotype and specificity. The majority of the monoclonal antibodies used in this study were derived from fusions following immunisation of mice with ovine lymphocytes. Other monoclonal antibodies were generated to lymphocyte antigens of other species and have been found to fortuitously cross react with ovine lymphocytes. The cross reactivity of these particular monoclonal antibodies indicates that certain epitopes expressed within regions of the specific proteins are conserved between some species. The availability of monoclonal antibodies of different isotypes, and each specific for a different molecule, allowed two colour immunofluorescence analysis without direct conjugation of fluorochrome to the monoclonal antibodies. This was achieved by immunofluorescently labelling one monoclonal antibody with FITC conjugated isotype specific second layer reagents whilst the other monoclonal antibody was biotinylated and subsequently labelled with a fluorescent layer by the addition of phycoerytherin conjugated to streptavidin.
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk sheep and stained with the irrelevant monoclonal antibodies ZG1 (IgG1) and biotinylated UPC10 (IgG2a) followed by anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Cells were analysed by flow cytometry.
FIGURE 3. Background Immunofluorescence Of Dual Colour Immunofluorescence Analysis With Irrelevant Monoclonal Antibodies of Various Isotypes.
3.3. Major Subsets of Ovine Peripheral Blood Lymphocytes.
A representative set of immunofluorescence profiles of ovine lymphocytes from one animal stained with a panel of monoclonal antibodies specific for various cell surface antigens is shown in Figure 4. All animals of similar age investigated showed similar profiles and percentages as those shown here. All of the cells stained with the monoclonal antibody SBU-1 (Gogolin-Ewens et al 1985) which reacts with all ovine Major Histocompatibility Complex Class I molecules (MHC Class I).

3.3.1. Lymphocytes.
Peripheral blood lymphocytes of all species so far examined, by others, consist of two mutually exclusive populations of cells, namely T and B cells. B cells are classically defined by the expression of surface immunoglobulin. Staining with the anti-ovine light chain-specific monoclonal antibody, VPM8 (McConnell et al unpublished), shows that 35.8% of PBMC of the animal under investigation were B cells (Figure 4). The immunofluorescence profile demonstrates that B cells as a population have a varied expression of surface immunoglobulin, some cells express a high level whilst others express low levels of surface immunoglobulin.

3.3.1.1. \( \gamma \delta \) TCR And T19-Positive Lymphocytes.
Ovine peripheral T cells are divided into two major populations based on their TCR usage or expression of T19 (Mackay et al 1986). One subpopulation is defined by a monoclonal antibody, termed 86D, generated to a surface antigen on ovine lymphocytes. The monoclonal antibody has been shown to be specific for an epitope of the \( \gamma \delta \) TCR (Mackay et al 1989). Figure 4 demonstrates that 22.2% of the cells within ovine peripheral blood were \( \gamma \delta \) TCR-positive.

The \( \gamma \delta \) TCR subset in ruminants is further characterised by the expression of an unique cell surface glycoprotein of molecular weight 215KDa (Mackay et al 1986), termed T19. Three monoclonal antibody specific for ruminant T19 exist, namely 19.19. (Mackay et al 1986), ST197 (McClure et al 1989) and CC15 (Morrison et al 1988a, Goddeeris et al 1986). The immunofluorescence profiles obtained with these monoclonal antibodies are...
Figure 4.
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal and stained with either 86D, 19.19, ST197, CC15, ST4, SBU-T4, ST8, SBU-T8, I35A, 36F, VPM8 or SBU1 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 4. Immunofluorescence Analysis With Monoclonal Antibodies Specific For Cell Surface Molecules On Ovine Lymphocytes.

Relative cell number

Fluorescence intensity
shown in Figure 4 and demonstrate that different monoclonal antibodies specific for different epitopes of the same antigen yield similar but not identical percentages of immunofluorescence positive cells. Monoclonal antibodies 19.19 and ST197 have similar immunofluorescence profiles that consisted of a population of cells with low and high intensity of immunofluorescence, staining 17.3% and 18.7% respectively. In contrast the monoclonal antibody CC15 generated to bovine T19, labelled 21.9% of cells and these were predominantly of high immunofluorescence intensity.

The immunofluorescence profiles obtained by dual colour analysis of T19 expression by ovine γδ T cells are shown in Figure 5. The epitopes recognised by the monoclonal antibodies 19.19 and ST197 were expressed on 65.0% and 80.0% of γδ TCR-positive cells, respectively. A minor population of cells (<3%) were γδ TCR-negative, T19-positive. The dual colour immunofluorescence profiles obtained with monoclonal antibodies CC15 and 86D demonstrate that the majority (95.8%) of γδ TCR-positive lymphocytes co-expressed the epitope recognised by CC15. A minor population of cells existed that were negative for the γδ TCR but expressed the epitope recognised by CC15. As yet, the function of the T19 molecule remains to be determined and no homologue of the T19 molecule on γδ T cells of other non-ruminant species has been reported.

3.3.1.2. The CD4 and CD8 Cell Surface Molecules Define The Ovine αβ T Cell Subset.

The other major ovine T cell subset is negative for the γδ TCR and T19 molecule and is defined by monoclonal antibodies which recognise the T cell co-receptor molecules, CD4 and CD8. In other species, it is well established that the majority of the CD4-positive and CD8-positive T cells express a TCR of αβ polypeptide chains. To date, no monoclonal antibody has been generated to ovine αβ TCR, but it is assumed in analogy with other species that these cells in the sheep will express an αβ TCR.

Currently, the ovine CD4 and CD8 co-receptor molecules are each defined by two monoclonal antibodies. Figure 4 shows that the anti-CD4 monoclonal antibodies, ST4 (Mackay et al 1988a) and SBU-T4 (Maddox et al 1985), immunolabelled 27.2% and
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal and analysed by two colour immunofluorescence for their co-expression of the γδ TCR and T19. Cells were stained with biotinylated monoclonal antibody 86D (IgG1) in association with either 19.19 (IgG1), ST197 (IgG2b) or CC15 (IgG2a). When monoclonal antibodies of different isotypes were used cells were treated with phycoerytherin-streptavidin and anti-mouse IgG2b-FITC or anti-mouse IgG2a-FITC accordingly. Alternatively, when 86D and 19.19 were used cells were treated with 19.19 alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D, followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the dual colour immunofluorescence profiles are shown.
FIGURE 5. Dual Colour Immunofluorescence Analysis of T19 Expression By Ovine γδ Cells.

The dual colour immunofluorescence analysis of ovine lymphocytes is shown in Figure 5 and demonstrates that in contrast to the predominant expression of CD4 and CD8, expression of CD2 is expressed by a minority of γδ T cells. The T19 expression was confirmed by the greater MCN of CD2 expressing γδ T cells as shown by the greater MCN of γδ T cells expressing CD4 and CD8. However, it has been reported in humans that some γδ T cells express either CD4 or CD8 (Mills et al. 1994) or CD8 (DeAngelis et al. 1991). Human γδ T cells, in particular the subset expressing γδ TCR, are of particular interest as they are thought to be analogous to the majority of CD4-negative and CD8-negative γδ T cells in the human and mouse species. The antibody used in the present study was a murine monoclonal antibody specific for the γδ TCR and C19 (OvM and CD19). The data shows that the majority of γδ T cells do not express the T19 antigen, whilst very few γδ T cells express C19. In contrast, the T19 antigen was detected in a minority of γδ T cells.
27.3% of PBMC, respectively, yielding identical immunofluorescence profiles and MCN of 118. The anti-CD8 monoclonal antibodies ST8 (Maddox et al 1985) and SBU-T8 (Maddox et al 1985) stained 10.8% and 14.5%, respectively, of ovine peripheral blood. In contrast, the immunofluorescence intensity of positive cells was higher with the SBU-T8 monoclonal antibody, compared to ST8 as shown by the greater MCN of 55 for ST8 and 95 for SBU-T8.

Figure 6 shows the dual colour immunofluorescence profiles obtained using monoclonal antibodies specific for the γδ TCR, T19, CD4 and CD8. The data shows that the majority of γδ T cells co express the T19 molecule, whilst very few γδ T cells co-express CD4 and CD8. However, it has been reported in humans that some γδ T cells do express either CD4 (Morita et al 1991) or CD8 (Deusch et al 1991). Human CD8-positive γδ T cells are localised primarily at epithelial sites, in particular the intestinal epithelium rather than peripheral blood. This data confirms that the majority but not all, of ovine γδ T cells do not overlap with the αβ T cell subset, as defined by the expression of CD4 and CD8.

3.3.1.3. Ovine γδ T Cells Do Not Express CD2.

In the human and mouse species, all mature peripheral T cells can be defined by their expression of CD2. In contrast, a report has suggested that CD2 does not define all ovine T cells (Mackay et al 1988a). Two monoclonal antibodies specific for ovine CD2, namely 135A (Giegerich et al 1989) and 36F (Mackay et al 1988a), have been generated and the immunofluorescence profiles obtained with each is shown in Figure 4. Monoclonal antibodies 135A and 36F, give identical immunofluorescence profiles and approximately 42.0% of ovine peripheral blood cells were positive for CD2 expression, which is approximately equal to the combined percentages of CD4 and CD8, suggesting that CD2 is expressed exclusively by ovine αβ T cells.

The dual colour immunofluorescence analysis of ovine lymphocytes shown in Figure 7 and demonstrates that in contrast to other non-ruminant species, >90% of ovine γδ T cells do not express CD2. Virtually all of the ovine CD4-positive and CD8-positive T cells express CD2. The immunofluorescence intensity of CD2-positive staining by CD4-
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal and analysed by two colour immunofluorescence for co-expression of the γδ TCR with either T19, CD4 or CD8. Cells were stained with biotinylated monoclonal antibody 86D (IgGl) in association with either CC15 (IgG2a), SBU-T4 (IgG2a) or SBU-T8 (IgG2a). Cells were treated with phycoerytherin-streptavidin and anti-mouse IgG2a-FITC and analysed by flow cytometry. The dual colour immunofluorescence profiles are shown.
FIGURE 6. Dual Colour Immunofluorescence Analysis of \( \gamma \delta \) TCR Expression By Ovine PBMC.
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal and analysed by two colour immunofluorescence for co-expression of CD2 with either γδ TCR, CD4 or CD8. Cells were stained with monoclonal antibody I35A (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when I35A and 86D were used cells were treated with I35A alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the dual colour immunofluorescence profiles are shown.
FIGURE 7. Dual Colour Immunofluorescence Analysis of CD2 Expression by Ovine PBMC.
positive, CD8-positive and the small percentage of γδ-positive lymphocytes were of low but equal intensity. Furthermore, it has been reported in the literature that ovine T cells have a lower level of expression of CD2 than human T cells (Mackay et al 1988a).

These findings may imply that ovine αβ T cells have a decreased dependence on CD2 for cellular adhesion and activation during an immune response, whilst the population of γδ T cells that are CD2-negative may rely on other cellular antigens, such as T19, to mediate these effects.

3.3.1.4. CD3 Is Expressed By All Ovine T Cells.

In non-ruminant species T cells have been defined by monoclonal antibodies to CD3, a molecular complex of 5 different proteins non-covalently associated with either the γδ or the αβ TCR molecules (Clevers et al 1988). No monoclonal antibody specific for any ovine CD3 molecule exists, at present, but some anti-human CD3 reagents do crossreact with ovine T cells. One monoclonal antibody in particular, HMT3.1, a hamster monoclonal antibody generated to human CD3, cross reacts with an epitope of ovine CD3 (Kubo et al unpublished). Immunofluorescence analysis with HMT3.1 required the permeabilisation of lymphocytes prior to their immunostaining, as this monoclonal antibody reacts with an internally expressed epitope of the CD3ε chain. Figure 8 shows the internal immunofluorescence profile of ovine PBMC stained with HMT3.1. Following permeabilisation the percentage of ovine CD3-positive cells was found to be 59.4%. The percentage of T cells estimated by immunofluorescence obtained with monoclonal antibodies specific for γδ TCR and CD2 was 66.7%. The percentage of T cells estimated by immunofluorescence using monoclonal antibodies specific for γδ TCR, CD4 and CD8 was 60.3%. The close agreement of these figures for the percentage of T cells in PBMC indicates that monoclonal antibodies to either the γδ TCR and the CD2 molecule, or monoclonal antibodies to the γδ TCR together with monoclonal antibodies to CD4 and CD8, define the three T cell subpopulations within ovine peripheral blood. Throughout this thesis, αβ T cells infers CD4-positive and CD8-positive T cells, or CD2-positive cells.
Figure 8.
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal. Some of the cells were stained with either monoclonal antibodies 86D, ST4, ST8 or I35A followed by anti-mouse immunoglobulin-FITC. The remainder of the cells were permeabilised as described in Materials and Methods and subsequently stained with monoclonal antibody HMT3.1 followed by anti-mouse immunoglobulin-FITC. All cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 8. Identification of Ovine CD3-Positive Cells by FACS Analysis With HMT3.1 Monoclonal Antibody.

Positive staining with HMT3.1 = 59.4%

T cells estimated by:

CD2 + γδ = 66.7%

CD4 + CD8 + γδ = 60.3%

KEY:

HMT3.1 monoclonal antibody

normal hamster sera
3.3.2. Monocytes and Macrophages.

It is known that monocytes and macrophages have a slightly higher FSC and SSC than small lymphocytes, thus the P1 gate may contain a few contaminating monocytes and macrophages. As yet, no anti-macrophage or anti-monocyte monoclonal antibody has been fully characterised in the sheep, and in their absence the percentage of these cells can not be fully assessed. In other species, it has been established that approximately 1-5% of PBMC consist of monocytes and macrophages. If an analogous situation occurs in the sheep, then the percentage of monocytes and macrophages within the P1 gate will represent an even smaller percentage of the small lymphocytes, but may account for the residual population when the percentage positive T cells and the percentage positive B cells does not equal 100%. It has been suggested that monocytes and macrophages express CD8 and/or CD4 (Kazazi et al 1989) at a very low intensity, thus when the percentage of positive T cells and the percentage of positive B cells are greater than 100% it may be due to CD8 and/or CD4 expression by monocytes and macrophages. Generally, the summation of the percentage of T cells and the percentage of surface immunoglobulin cells was equal to 100% ± 5%.

3.4. The Use of Monoclonal Antibodies to Define Adhesion Molecules, Activation and Proliferation Markers on Sheep Lymphocytes.

3.4.1. Adhesion Molecules.

Adhesion molecules play a critical role in establishing antigen independent contact between T cells and antigen presenting or target cells (Makgoba et al 1989). It is believed that the binding of pairs of adhesion molecules on opposing cells allows sufficient time for molecules to diffuse within the membrane enabling TCRs to come into close contact with antigen-MHC complexes. Once a threshold level of TCRs are occupied with their specific ligand, the T cell will subsequently progress to proliferation and clonal expansion.

Monoclonal antibodies specific for various ovine adhesion molecules have been generated. Table 2 lists the monoclonal antibodies used in this thesis to investigate some ovine adhesion molecules. The monoclonal antibodies used were specific for
Table 2.

Table 2 lists the monoclonal antibodies used to analyse adhesion, activation and proliferation markers in this investigation. Included is their specificity and isotype.
TABLE 2. Monoclonal Antibodies Used To Define Markers of Adhesion, Activation, Proliferation and CD45 on Ovine Lymphocytes.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F10/150/39</td>
<td>anti-ovine LFA1</td>
<td>IgGl</td>
<td>Mackay et al 1990</td>
</tr>
<tr>
<td>L180/1</td>
<td>anti-ovine LFA3</td>
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<td>Hunig et al 1985</td>
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<td>IL-A111</td>
<td>anti-bovine CD25</td>
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<td>Naessens et al 1992</td>
</tr>
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<td>anti-Ki67 antigens</td>
<td>IgGl</td>
<td>Gerdes et al 1991</td>
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</tr>
<tr>
<td>73B</td>
<td>anti-ovine CD45RA</td>
<td>IgGl</td>
<td>Mackay et al 1990</td>
</tr>
</tbody>
</table>
lymphocyte function associated antigen 1 (LFA-1 or CD11A) (Mackay et al 1990) and lymphocyte function associated antigen 3 (LFA-3 or CD58) (Hunig 1985). Figure 9 shows the immunofluorescence profiles obtained with these two monoclonal antibodies. The total population of ovine lymphocytes reacted with both monoclonal antibodies. The majority of cells had low intensity fluorescence level of LFA-1 and LFA-3 expression compared to background immunofluorescence, whilst the remaining minor population expressed both antigens with increased intensity. An increased surface expression of LFA-1 and LFA-3 on a small population of lymphocytes within ovine peripheral blood may imply that these cells were recently activated as it has been reported that activation increase the surface of adhesion molecules (Makgoba et al 1989).

3.4.2. Activation Markers.

Ligand recognition by antigen specific T cell receptors triggers signals across the membrane which are transmitted to the nucleus. These events control the expression of genes within T cells which encode proteins involved in T cell activation and subsequently induce the proliferation of these cells. The expression of some proteins is increased upon T cell ligand recognition, whilst others are synthesised de novo (Waldman 1989). Table 2 includes two monoclonal antibodies that enabled an investigation of the percentage of activated and proliferating T cells, namely IL-A111 and Ki67, respectively. Monoclonal antibody IL-A111 is specific for the bovine IL-2 receptor α chain (CD25) (Naessens et al 1992) but crossreacts with ovine CD25. The immunofluorescence profile shown in Figure 9 demonstrates that 9.1% of unstimulated PBMC were CD25-positive. Alternatively monoclonal antibody Ki67 is specific for human nuclear antigens of 395kD and 345kD referred to as the Ki67 antigens (Gerdes et al 1991), present in proliferating cells but absent in quiescent cells (Falini et al 1989, Gerdes et al 1983). This investigation has established that monoclonal antibody Ki67 crossreacts with antigens expressed by ovine lymphocytes and the immunofluorescence profile of permeabilised ovine PBMC stained with Ki67 is shown in Figure 9. 16.7% of cells stained positive for Ki67 antigens. Unpermeabilised cells were Ki67-negative (data not shown).
Figure 9.
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal. Some of the cells were stained with either monoclonal antibodies L180/1, F10-150, VPM18, 73B or IL-A111 followed by anti-mouse immunoglobulin-FITC. The remainder of the cells were permeabilised as described in Materials and Methods and subsequently stained with FITC conjugated monoclonal antibody Ki67. All cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 9. Monoclonal Antibodies Used to Define Markers of Adhesion, Activation, Proliferation and CD45 on Ovine Lymphocytes.

Adhesion Molecules

Markers of activation and Proliferation

CD45
3.4.3. Leucocyte Common Antigen.

Leucocyte Common Antigen (LCA or CD45) consists of a family of glycoproteins expressed on the surface of all lymphoid and myeloid cells. CD45 represents a major component of lymphocytes as it has been estimated that 10% of the lymphocyte surface protein comprises one or more of the CD45 isoforms (Thomas and Lefrancois 1988). The extracellular portion of the molecule varies in size which is generated by alternative RNA splicing of three individuals exons, namely A, B, and C, potentially allowing eight different molecules (Thomas 1989). In the human species the CD45RA and CD45RO nomenclature has been introduced to characterise different CD45 isoforms that occur as a consequence of variable exon expression. CD45RA denotes a high molecular weight isoform of CD45 (Mr 220Kd) while CD45RO denotes a low molecular isoform of CD45 (Mr 180Kd). It has been suggested in the human species that differential expression of CD45RA and CD45RO can be used to distinguish naive and memory T cells, respectively (Horgan et al 1992).

CD45 isoform expression by ovine lymphocytes was investigated using VPM18, an anti-ovine CD45 monoclonal antibody specific for an epitope expressed on all CD45 molecules (Hopkins and Dutia 1990). As shown in Figure 9, all ovine PBMC stained positive with VPM18. Monoclonal antibody 73B (Mackay et al 1990), which recognises the high molecular weight isoform of ovine CD45 (Mr 220Kd), and may therefore be regarded as an anti-CD45RA monoclonal antibody, enabled an investigation of the percentage of naive lymphocytes within ovine peripheral blood. The immunofluorescence profile shown in Figure 9 demonstrates that 65.0% of ovine peripheral blood lymphocytes were 73B-positive.

3.5. CD45 p220 Isoform Expression By Ovine T Cells.

The lower percentage of γδ T cells within ovine fetal blood in comparison to that of young lambs blood, and the predominance of γδ T cells rather than αβ T cells in the peripheral blood of lambs, may imply that higher levels of γδ T cells occur in sheep as a consequence of clonal expansion of this subset in the periphery. If this is the case, γδ T cells in the ovine peripheral blood may express a memory phenotype. This hypothesis
was investigated using the monoclonal antibodies VPM18 and 73B, specific for ovine CD45 and CD45RA, respectively.

3.5.1. CD45 Expression By Animals Of Various Ages.
PBMC were obtained from animals between 6 days and greater than 2 years of age and analysed by dual colour immunofluorescence for their expression of CD45RA. The results are shown in Table 3. Less than 25% of γδ T cells expressed CD45RA irrespective of animal age. A small decrease in this phenotype occurred with an increase in the age of the animals investigated. In contrast, the majority of the CD4-positive and CD8-positive T cells in lambs six days of age expressed CD45RA but this decreased significantly with an increase in animal age, especially within the CD4-positive subset. Greater than 95% of surface immunoglobulin-positive lymphocytes were of the naive phenotype irrespective of the age of the animals under investigation.

3.5.2. CD45 Expression By Animals Exposed To Various Antigenic Burdens.
The above data implies that irrespective of animal age the majority of γδ T cells in ovine peripheral blood were of the memory phenotype. This is in contrast to the αβ T cell subset, where the cells acquire a memory phenotype with age. Ovine γδ T cells may express a memory phenotype in the periphery as a consequence of recognition of self antigens prior to birth. Alternatively, γδ T cells may undergo an unique selection procedure in the thymus, distinct from that followed by the αβ T cell subset, which induces a memory phenotype on these cells. To investigate these possibilities differential CD45 isoform expression was studied on peripheral blood lymphocytes of lambs maintained under gnototobiotic conditions and lambs exposed to various antigenic burdens. Peripheral blood was obtained from all animals at six days of age. For comparison fetal blood (day 130 gestation) was included in the investigation.

The results are shown in Table 4. In all animals investigated approximately 25% or less of the γδ T cell subset expressed CD45RA, whilst greater than 60% of αβ T cells
Table 3.
Peripheral blood mononuclear cells were prepared from Suffolk x Dorset animals of various ages (n=6 per group) and analysed by two colour immunofluorescence for their co-expression of CD45 p220 with either CD4, CD8, γδ TCR or T19. Cells were stained with the monoclonal antibody 73B (IgG1) in association with either SBU-T4 (IgG2a), SBU-T8 (IgG2a), 86D (IgG1), CC15 (IgG2a) or VPM8 (IgG1) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were subsequently treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when two IgG1 monoclonal antibodies were used cells were treated with 73B alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were subsequently treated with biotinylated monoclonal antibodies and finally with phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the percentage of each lymphocyte subset co-expressing CD45 p220 are tabulated. Standard deviations are included in brackets.
TABLE 3. CD45 Isoform Expression By Lymphocytes From Animals Of Various Ages.

<table>
<thead>
<tr>
<th>Age of Animals (n=6)</th>
<th>% Of Cells Co-Expressing CD45 p220 Isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4</td>
</tr>
<tr>
<td>6 Days</td>
<td>68.5 (12.2)</td>
</tr>
<tr>
<td>4 Months</td>
<td>34.3 (11.6)</td>
</tr>
<tr>
<td>&gt; 2 Years</td>
<td>15.0 (5.0)</td>
</tr>
</tbody>
</table>
Table 4.

Peripheral blood mononuclear cells were prepared from Suffolk x Dorset lambs aged six days and housed under gnotobiotic, SPF and normal conditions (n=6 per group). For comparison fetal blood (day 130 gestation) from breed matched animals was included in the investigation (n=6). Cells were analysed by two colour immunofluorescence for their co-expression of CD45 p220 with either CD4, CD8, γδ TCR or T19. Cells were stained with the monoclonal antibody 73B (IgG1) in association with either SBU-T4 (IgG2a), SBU-T8 (IgG2a), 86D (IgG1), CC15 (IgG2a) or VPM8 (IgG1) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when two IgG1 monoclonal antibodies were used cells were treated with 73B alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were subsequently treated with biotinylated monoclonal antibody and finally with phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the percentage of each lymphocyte subset co-expressing CD45 p220 are tabulated. Standard deviations are included in brackets.
TABLE 4. CD45 Isoform Expression By Lymphocytes From Animals Exposed To Various Antigenic Burden (n=6).

<table>
<thead>
<tr>
<th>Type of lambs</th>
<th>% Of Cells Co-Expressing CD45 p220 Isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4</td>
</tr>
<tr>
<td>normal lambs</td>
<td>68.5 (12.2)</td>
</tr>
<tr>
<td>SPF lambs</td>
<td>68.4 (10.7)</td>
</tr>
<tr>
<td>gnotobiotic lambs</td>
<td>81.0 (4.3)</td>
</tr>
<tr>
<td>fetal</td>
<td>79.2 (11.7)</td>
</tr>
</tbody>
</table>
expressed CD45RA. The data suggests that ovine γδ T cells acquire a memory phenotype prior to birth and exposure to environmental antigens. A recent report has shown that the predominant γδ population within human peripheral blood which expresses the Vγ9 Vδ2 TCR all express CD45RO, and are regarded to be of the memory phenotype (Braakman et al 1991).

Dual colour immunofluorescence profiles obtained with the anti-CD45RA and various monoclonal antibodies which define the ovine T cell subsets are shown in Figure 10, which shows the immunofluorescence analysis of peripheral blood obtained from a gnotobiotic animal. These immunofluorescence profiles clearly demonstrate a distinct difference in the expression of CD45RA between the γδ and αβ T cell subsets in the sheep. As previously discussed, only a small percentage of the γδ subset express the CD45RA molecule, and did so at a low level of immunofluorescence intensity relative to background immunofluorescence. The αβ T cell subset expressed CD45RA with a heterogeneous expression of immunofluorescence intensity. All surface immunoglobulin positive cells co-expressed CD45RA, and in contrast to CD45RA expression on T cells, did so at a higher level of intensity (data not shown).

3.6. The Percentage Of Sheep Peripheral Blood γδ T Cells Decreases With Age.

It has been noticed that peripheral blood of young sheep is characterised by the presence of relatively high numbers of γδ T cells compared to peripheral blood of other non-ruminants. To determine if this high percentage of γδ T cells remained throughout the life of the animal, an investigation of the percentage of sheep peripheral blood γδ T cells in animals of various ages was carried out. The following results are from an investigation into the percentage of γδ T cells in the peripheral blood of sheep of various ages.

3.6.1. Lambs Age 1 - 6 Weeks.

The data in Table 5 shows the weekly changes that occurred with respect to the level of peripheral blood γδ T cells in a group of 8 Grey Faced x Suffolk lambs between the age
Figure 10.
Peripheral blood mononuclear cells were prepared from a six day old Suffolk x Dorset animal born by suzerain and housed under gnotobiotic conditions. Cells were analysed by two colour immunofluorescence for their co-expression of CD45 p220 with either γδ TCR, CD4 or CD8. Cells were stained with the monoclonal antibody 73B (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were subsequently treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when 73B and 86D were used cells were treated with 73B alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry for the co-expressing CD45 p220. The dual colour immunofluorescence profiles are shown.
FIGURE 10. Dual Colour Immunofluorescence Analysis Of CD45 p220 Expression By Lymphocytes Obtained From Gnotobiotic Lambs.

CD45 p220 (green fluorescence)

γδ TCR

subset marker (red fluorescence)

CD4

CD8
Table 5.
Peripheral blood mononuclear cells were prepared from Grey Face x Suffolk lambs (n=8) of various ages then stained with either monoclonal antibodies ST4, ST8, 86D, 19.19 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry. Standard deviations are included in brackets. Statistical analysis was carried out using a Mann Whitney test from the Minitab software package.
TABLE 5. Lymphocyte Composition Of Peripheral Blood From Young Lambs (n=8).

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>CD4</th>
<th>CD8</th>
<th>γδ</th>
<th>T19</th>
<th>sIg</th>
<th>CD4 + CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41.2 (8.4)</td>
<td>16.8 (3.2)</td>
<td>30.5 (9.5)</td>
<td>19.3 (6.4)</td>
<td>10.9 (4.6)</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>38.1 (9.3)</td>
<td>20.4 (3.5)</td>
<td>23.7 (11.1)</td>
<td>16.2 (6.9)</td>
<td>29.8 (13.7)</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>33.3 (6.0)</td>
<td>20.3 (2.7)</td>
<td>19.7 (4.9)*</td>
<td>18.3 (3.6)</td>
<td>30.9 (4.4)</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>27.0 (6.9)</td>
<td>19.9 (5.1)</td>
<td>19.5 (5.0)*</td>
<td>18.0 (4.4)</td>
<td>47.8 (8.9)</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>26.4 (11.2)</td>
<td>18.8 (6.6)</td>
<td>20.2 (5.6)*</td>
<td>18.5 (5.5)</td>
<td>43.6 (10.0)</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>17.9 (5.2)</td>
<td>14.9 (4.1)</td>
<td>17.6 (4.4)*</td>
<td>16.3 (5.1)</td>
<td>59.0 (8.2)</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* p < 0.01 relative to 1 week of age.
of one and six weeks. The highest level of $\gamma \delta$ T cells were recorded in 1 week old lambs when 30.5% of PBMC stained with the monoclonal antibody 86D. This level decreased with time and at six weeks of age the percentage of $\gamma \delta$ T cells had fallen to 17.6%.

Statistical analysis showed that there was no significant statistical change in the percentage of $\gamma \delta$ T cells ($p > 0.05$) between one and two weeks of age. In contrast, there was a significant decrease ($p < 0.01$) in the percentage of ovine $\gamma \delta$ T cells between one week and all other time points analysed. This decrease in the number of $\gamma \delta$ T cells was not accompanied by a decrease in the percentage of cells expressing the T19 molecule, T19-positive cells remained at a similar level for the first 6 weeks of age. It has been suggested that T19 is a maturation marker for $\gamma \delta$ T cells during their progression through the thymus (Mackay et al 1989). Alternatively, $\gamma \delta$ TCR-positive, T19-negative lymphocytes may be a distinct population of cells. The ratio of $\alpha \beta / \gamma \delta$ was determined to enable a comparison to be made of the changes in the level of the $\gamma \delta$ T cells relative to the total T cell population. During the first six weeks of lamb life this ratio fluctuated between 1.9 and 2.7.

Over the same 6 week period the percentage of CD4-positive T cells decreased, whilst the percentage of CD8-positive cells remained similar. At one week of age lambs were B cell lymphopenic with only 10.9% of lamb peripheral lymphocytes expressing surface immunoglobulin, confirming a previous observation (Hein et al 1990a). A significant increase ($p < 0.01$) in the level of B cells occurred over the next six weeks and at 6 weeks of age 59.0% of cell were surface immunoglobulin-positive.

3.6.2. Lambs Age 2 - 26 Weeks.

Lambs analysed in Section 3.6.1. were further analysed beyond 6 weeks of age, at 4 week intervals. The results are shown in Table 6, and for comparison the data at 2 and 6 weeks of age from Table 5 are included. Between the age of 2 and 26 weeks a decrease in the level of $\gamma \delta$ T cells was observed but this was not statistically significant ($p > 0.05$). At 6 weeks of age, and beyond, the percentage of T19-positive cells was similar to the percentage of $\gamma \delta$ TCR-positive cells, and on no occasion greater than the percentage of $\gamma \delta$ TCR-positive cells. The percentage of CD4-positive cells decreased
Table 6.
Peripheral blood mononuclear cells were prepared from Grey Face x Suffolk lambs (n=8) of various ages then stained with either monoclonal antibodies ST4, ST8, 86D, 19.19 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry. Standard deviations are included in brackets. Statistical analysis was carried out using a Mann Whitney test from the Minitab software package.
To show that the above observations were not peculiar to the Grey Faced x Suffolk sheep, a similar experiment using PBMC of Dorset x Suffolk lambs aged between 2 and 26 weeks was performed. The data shows the same trends as were observed with the Grey Faced x Suffolk sheep with no statistically significant changes in the level of γδ T cells between the same points investigated (p > 0.05).

Table 6 shows the percentage of γδ T cells in PBMC of Grey face x Suffolk sheep of 2 weeks to 26 weeks of age. Table 6 shows a significant decrease (p < 0.01) in the percentage of γδ T cells beyond 6 weeks of age. No further significant decrease was observed (p > 0.05), 6-9 months of age. This decrease was maintained in the older age groups. The decrease in the percentage of γδ T cells relative to the total T cell population is reflected in the number of γδ T cells in the peripheral blood of young animals relative to older animals. During the course of this thesis a report by Huse et al (1990) showed that the absolute number of γδ T cells in lamb peripheral blood increases during the first 20 weeks of neonatal life. Collectively, therefore, if the absolute number of γδ T cells increases over the time period when the percentage of γδ T cells begins to decrease (i.e., the first 20 weeks of neonatal life) then the decrease in percentage is likely to be due to an increase in the absolute number of other lymphocytes.

* p > 0.05 relative to 2 weeks of age.

### Table 6. Lymphocyte Composition Of Peripheral Blood From Lambs Between The Age Of 2 - 26 Weeks (n=8).

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>γδ (%)</th>
<th>T19 (%)</th>
<th>sIg (%)</th>
<th>CD4 + CD8 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>38.1(9.3)</td>
<td>20.4(3.5)</td>
<td>23.7(11.1)</td>
<td>16.2(6.9)</td>
<td>29.8(13.7)</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>17.9(5.2)</td>
<td>14.9(4.1)</td>
<td>17.6(4.4)*</td>
<td>16.3(5.1)</td>
<td>59.0(8.2)</td>
<td>1.9</td>
</tr>
<tr>
<td>10</td>
<td>12.8(3.0)</td>
<td>17.6(5.6)</td>
<td>22.7(5.1)*</td>
<td>20.4(4.6)</td>
<td>53.3(7.5)</td>
<td>1.4</td>
</tr>
<tr>
<td>14</td>
<td>21.6(8.2)</td>
<td>19.7(8.2)</td>
<td>18.0(3.1)*</td>
<td>17.4(4.1)</td>
<td>51.4(9.5)</td>
<td>2.3</td>
</tr>
<tr>
<td>18</td>
<td>20.1(6.7)</td>
<td>24.9(12.1)</td>
<td>19.0(6.2)*</td>
<td>18.1(6.4)</td>
<td>46.1(9.1)</td>
<td>2.4</td>
</tr>
<tr>
<td>22</td>
<td>26.0(6.8)</td>
<td>20.5(5.7)</td>
<td>21.8(3.4)*</td>
<td>18.4(3.9)</td>
<td>41.7(11.3)</td>
<td>2.1</td>
</tr>
<tr>
<td>26</td>
<td>25.3(7.6)</td>
<td>23.7(5.1)</td>
<td>15.0(5.3)*</td>
<td>13.8(5.3)</td>
<td>45.2(13.3)</td>
<td>3.3</td>
</tr>
</tbody>
</table>
between the age of 2-10 weeks, and then increased. The level of B cells increased between 2 and 6 weeks of age, and thereafter decreased. Over the first ten weeks of life the ratio of \( \alpha \beta / \gamma \delta \) T cells decreased and at older ages increased.

To show that the above observations were not peculiar to the Grey Faced x Suffolk breed of sheep, a similar experiment was performed on Dorset x Suffolk sheep. Table 7 shows a similar experiment using PBMC of Dorset x Suffolk lambs aged between 2 and 26 weeks. The data shows the same trends as were observed with the Grey Faced x Suffolk sheep with no statistically significant changes in the level of \( \gamma \delta \) T cells between the time points investigated \((p > 0.05)\).

### 3.6.3. Animals Of Age 2 Weeks To > 3 Years.

Table 8 shows the percentage of \( \gamma \delta \) T cells in PBMC of Grey face x Suffolk sheep of 2 weeks to > 3 years of age. The results show that between the age of 2 weeks and 6-9 months there was a significant decrease \((p < 0.01)\) in the percentage of \( \gamma \delta \) T cells. Beyond this age, no further significant decrease was observed \((p > 0.05)\). At 6-9 months of age the percentage of \( \gamma \delta \) TCR cells and the percentage of T19-positive cells were similar and this trend was maintained in the older age groups. The decrease in the percentage of \( \gamma \delta \) T cells relative to the total T cell population is reflected in the \( \alpha \beta / \gamma \delta \) ratio which rises from 2.5 in animals aged 2 weeks, to 6.8 and 6.9 in animals aged 1-3 years and greater than 3 years, respectively.

Collectively, the above data shows a higher percentage of \( \gamma \delta \) T cells in the peripheral blood of young animals relative to older animals. During the course of this thesis a report by Hein et al (1990) showed that the absolute number of \( \gamma \delta \) T cells in lamb peripheral blood increases during the first 20 weeks of neonatal life. Collectively, therefore, if the absolute number of \( \gamma \delta \) T cells increases over the time period when the percentage of \( \gamma \delta \) T cells begins to decrease (i.e. the first 20 weeks of neonatal life) then the decrease in percentage is likely to be due to an increase in the absolute number of other lymphocytes.
Table 7.
Peripheral blood mononuclear cells were prepared from Grey Face x Dorset lambs (n=7) of various ages then stained with either monoclonal antibodies ST4, ST8, 86D, 19.19 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry. Standard deviations are included in brackets. Statistical analysis was carried out using a Mann Whitney test from the Minitab software package.
TABLE 7. Lymphocyte Composition Of Peripheral Blood From Dorset / Grey Faced Lambs Between The Age Of 2 - 26 Weeks (n=7).

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>CD4</th>
<th>CD8</th>
<th>γδ</th>
<th>T19</th>
<th>sIg</th>
<th>CD4 + CD8 γδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>33.2 (8.2)</td>
<td>14.4 (3.0)</td>
<td>29.8 (8.4)</td>
<td>20.5 (7.1)</td>
<td>17.4 (4.5)</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>20.2 (5.7)</td>
<td>18.6 (5.3)</td>
<td>18.7 (4.2)*</td>
<td>12.4 (2.9)</td>
<td>45.1 (9.8)</td>
<td>2.1</td>
</tr>
<tr>
<td>10</td>
<td>21.6 (5.1)</td>
<td>15.3 (4.2)</td>
<td>14.6 (2.4)*</td>
<td>9.9 (1.4)</td>
<td>50.2 (8.9)</td>
<td>2.5</td>
</tr>
<tr>
<td>14</td>
<td>13.9 (4.8)</td>
<td>15.4 (3.5)</td>
<td>12.7 (3.4)*</td>
<td>9.0 (2.4)</td>
<td>63.2 (9.6)</td>
<td>2.3</td>
</tr>
<tr>
<td>18</td>
<td>11.9 (5.3)</td>
<td>12.4 (2.8)</td>
<td>15.4 (2.8)*</td>
<td>11.1 (1.8)</td>
<td>63.0 (7.0)</td>
<td>1.6</td>
</tr>
<tr>
<td>22</td>
<td>14.3 (7.2)</td>
<td>11.2 (2.8)</td>
<td>16.5 (3.6)*</td>
<td>13.2 (1.6)</td>
<td>64.4 (6.6)</td>
<td>1.5</td>
</tr>
<tr>
<td>26</td>
<td>11.1 (1.9)</td>
<td>11.7 (2.8)</td>
<td>30.7 (6.2)*</td>
<td>25.4 (0.4)</td>
<td>49.4 (2.1)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* p > 0.05 relative to 2 weeks of age.
Peripheral blood mononuclear cells were prepared from Grey Face x Suffolk animals (n=5) of various ages then stained with either monoclonal antibodies ST4, ST8, 86D, 19.19 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry. Standard deviations are included in brackets. Statistical analysis was carried out using a Mann Whitney test from the Minitab software package.
TABLE 8. Lymphocyte Composition Of Peripheral Blood From Sheep Of Various Ages (n=5).

<table>
<thead>
<tr>
<th>Age</th>
<th>CD4</th>
<th>CD8</th>
<th>γδ</th>
<th>T19</th>
<th>slg</th>
<th>γδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>38.1 (9.3)</td>
<td>20.4 (3.5)</td>
<td>23.7 (11.1)</td>
<td>16.2 (6.9)</td>
<td>29.8 (13.7)</td>
<td>2.5</td>
</tr>
<tr>
<td>6-9 months</td>
<td>21.4 (7.0)</td>
<td>10.1 (3.3)</td>
<td>9.2 (5.1)*</td>
<td>9.0 (4.7)</td>
<td>59.9 (11.0)</td>
<td>3.4</td>
</tr>
<tr>
<td>1-3 years</td>
<td>23.3 (4.0)</td>
<td>13.4 (6.1)</td>
<td>6.9 (2.3)*</td>
<td>6.3 (2.5)</td>
<td>63.7 (7.9)</td>
<td>6.8</td>
</tr>
<tr>
<td>&gt;3 years</td>
<td>32.1 (6.8)</td>
<td>18.6 (6.2)</td>
<td>7.4 (3.0)*</td>
<td>6.2 (2.7)</td>
<td>45.0 (14.1)</td>
<td>6.9</td>
</tr>
</tbody>
</table>

* p < 0.01 relative to 2 weeks of age.
3.7. Lower Levels Of $\gamma\delta$ T Cells In Gnotobiotic Animals.

Ruminants are hosts for a large number of pathogenic protozoa and metazoan parasites which may infect mucosal surfaces and replicate within intestinal cells (Hein and Mackay 1991). The higher level of circulating $\gamma\delta$ T cells in ruminants, including sheep, may reflect a greater reliance by these animals on $\gamma\delta$ T cell function, rather than $\alpha\beta$ T cells, for protection against these particular pathogens. Table 9 shows the results of a comparison that was made of the level of $\gamma\delta$ T cells in lambs maintained under gnotobiotic conditions to the level of $\gamma\delta$ T cells in animals exposed to various antigenic burdens. Peripheral blood was obtained from all animals at six days of age. For comparison, fetal blood (day 130 gestation) was included in the investigation.

Fetal lambs, and lambs born by caesarean section and subsequently maintained in gnotobiotic conditions had a reduced percentage of $\gamma\delta$ T cells compared to lambs born and housed under normal conditions. The $\alpha\beta$ / $\gamma\delta$ ratio of the fetal and gnotobiotic lambs was 2.27 and 2.25, respectively, compared to 1.03 in the normal lambs. In comparison to fetal and animals housed under normal conditions, PBMC of lambs maintained under specific pathogen free (SPF) conditions had both an intermediate percentage and ratio of $\gamma\delta$ T cells. Animals housed under SPF conditions, but intentionally infected within the first 24 hours of life with the metazoan parasite Cryptosporidium parvum had levels of $\gamma\delta$ T cells, and an $\alpha\beta$ / $\gamma\delta$ ratio, that was intermediate to the values obtained for normal and SPF lambs. Statistical analysis demonstrated that there was no significant difference ($p > 0.05$) in both (i) the percentage of $\gamma\delta$ T cells within fetal and gnotobiotic animals, and (ii) the percentage of $\gamma\delta$ T cells within normal, Cryptosporidium parvum infected and SPF animals. In contrast it was established that a significant difference ($p < 0.05$) was apparent in the percentage of ovine $\gamma\delta$ T cells within the fetal and gnotobiotic animals relative to all other animals.

These changes in the $\alpha\beta$ / $\gamma\delta$ ratio reflect that the percentage of CD4-positive and CD8-positive cells decreased with an increase in the pathogen load of the animals. The data suggests that the greater the antigenic load in sheep the greater the reliance on $\gamma\delta$ T cells, at least in young lambs.
TABLE 9. Lymphocyte Composition Of Peripheral Blood From Animals Exposed To Various Antigenic Burden (n=6).

<table>
<thead>
<tr>
<th>Type of lambs</th>
<th>CD4</th>
<th>CD8</th>
<th>γδ</th>
<th>T19</th>
<th>sIg</th>
<th>CD4 + CD8 γδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>31.9  (9.5)</td>
<td>12.1  (5.9)</td>
<td>42.7  (7.4)*</td>
<td>35.1  (6.6)</td>
<td>10.3  (7.6)</td>
<td>1.03</td>
</tr>
<tr>
<td>pathogen infected</td>
<td>34.9  (8.3)</td>
<td>13.5  (2.9)</td>
<td>38.8  (4.9)*</td>
<td>30.4  (5.5)</td>
<td>13.0  (2.2)</td>
<td>1.25</td>
</tr>
<tr>
<td>SPF</td>
<td>31.5  (5.2)</td>
<td>20.1  (9.8)</td>
<td>33.3  (12.9)*</td>
<td>26.5  (9.9)</td>
<td>10.3  (6.0)</td>
<td>1.55</td>
</tr>
<tr>
<td>gnotobiotic</td>
<td>42.1  (4.4)</td>
<td>24.8  (5.9)</td>
<td>30.0  (6.1)</td>
<td>21.1  (3.5)</td>
<td>9.4   (2.8)</td>
<td>2.25</td>
</tr>
<tr>
<td>fetal</td>
<td>36.9  (7.4)</td>
<td>21.8  (1.8)</td>
<td>25.9  (5.5)</td>
<td>19.1  (5.7)</td>
<td>10.9  (5.4)</td>
<td>2.27</td>
</tr>
</tbody>
</table>

* p < 0.05 relative to fetal and gnotobiotic animals.
Suffolk x Dorset lambs were born by caesarean or normal birth (n=6 per group) and housed under different conditions of sterility. One group of normal born lambs which were housed under specific pathogen free conditions were infected with the pathogen Cryptosporidium parvum within 24hrs of birth. Peripheral blood mononuclear cells were prepared from 6 day old animals of each group. For comparison fetal blood (day 130 gestation) from breed matched animals was included in the investigation (n=6). Cells stained with either monoclonal antibodies ST4, ST8, 86D, 19.19 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry. Standard deviations are included in brackets.
DISCUSSION.

In this study a panel of monoclonal antibodies that define ovine cell surface molecules has been used to investigate the T cell composition of sheep peripheral blood with particular reference to \(\gamma\delta\) T cells and their phenotype.

Immunofluorescence analysis of ovine PBMC demonstrated that its composition consisted primarily of lymphocytes with a small percentage of monocytes and macrophages. Ovine lymphocytes, as is the case for all species so far examined, consist of two mutually exclusive populations of cells, namely T and B cells. The peripheral blood of lambs at 1 week of age is B cell lymphopenic consisting of approximately 10% B cells and 90% T cells, whilst B lymphocytes were approximately 60% of peripheral blood lymphocytes in older sheep. These age related changes in the percentage of T and B lymphocytes within ovine PBMC indicate a greater requirement of the antibody producing lineage in older sheep relative to lambs.

The receptor for antigen on T cells is a heterodimeric structure consisting of either \(\alpha\beta\) or \(\gamma\delta\) polypeptide chains (Brenner et al 1986). It has been shown that human and murine TCRs are associated non-covalently with the signal transducing CD3 complex (Clevers et al 1988). It is assumed that T cells in all species will have their receptors for antigen associated with CD3. This study has demonstrated that all ovine T cells express CD3.

This chapter has shown that peripheral blood of young sheep is characterised by the presence of relatively high numbers of \(\gamma\delta\) T cells. In lambs of one week of age, up to 45% of the lymphocyte pool express a \(\gamma\delta\) TCR. However, this high level of \(\gamma\delta\) T cells in the sheep is not maintained, and decreases with age. In comparison, only 1-5% of human peripheral blood lymphocytes express a \(\gamma\delta\) TCR with a peak in the level of \(\gamma\delta\) T cells observed between 3-7 years of age (Parker et al 1990). Considerable homology exists between the \(\alpha\beta\) and \(\gamma\delta\) TCR structure suggesting that the two T cell lineages function in an analogous manner in terms of the ligands they may recognise (Davis and Bjorkman 1988). Here it is shown that the majority of ovine \(\gamma\delta\) T cells, like those from other species, fail to express CD4 and CD8. In humans and mice, it has been reported
that some γδ T cells do express these molecules. Human γδ T cells expressing CD4 have been found in peripheral blood (Morita et al. 1991), whilst those expressing CD8 localise primarily at epithelial sites, such as the intestinal epithelium and lamina propria of the large intestine (Deusch et al. 1991). Murine γδ T cells expressing CD4 have been found in peripheral blood (Morita et al. 1991), whilst those expressing CD8 localise primarily at epithelial sites, such as the intestinal epithelium and lamina propria of the large intestine (Deusch et al. 1991). Murine γδ T cells expressing CD8 predominate in skin epithelia but these cells display a unique feature in that they are of a dendritic appearance (Havran et al. 1989, Asarnow et al. 1988). However, it is noteworthy that γδ T cells within humans and mice comprise a very minor population of PBMC making an analysis of the significance of these CD4 and CD8 expressing γδ T cells difficult (Parker et al. 1990, Allison and Havran 1991).

The lack of CD4 and CD8 expression by the majority of γδ T cells suggests that they do not recognise their ligand in association with classical MHC Class I or Class II molecules in the same manner as do their αβ counterparts. Some data is available where γδ T cell clones recognise their ligand in association with either classical MHC Class I or Class II molecules (Bluestone et al. 1988, Matias et al. 1989) but is limited. In contrast, some reports imply ligand recognition by γδ T cells involves non classical MHC like molecules, such as CD1 (Porcelli et al. 1989), TL antigens (Ito et al. 1989) and Qa antigens (Vidovic et al. 1989). This implies that recognition by the γδ TCR of these presentational elements occurs either without the assistance of a co-receptor molecule, or alternatively, to date, by the presence of an undefined co-receptor structure. The results in this chapter also demonstrated that in contrast to γδ T cells in all other non-ruminant species examined the majority of ovine γδ T cells lack CD2 expression. CD2 is known to be a cell surface molecule involved in T cell adhesion (Shaw et al. 1986), activation (Springer et al. 1987) and possibly lymphocyte recirculation (Mackay et al. 1988a). Lack of CD2 expression by the majority of ovine γδ T cells may indicate that these cells rely on other cellular antigens to mediate adhesion and activation.

A molecule that is expressed by ovine γδ T cells but not αβ T cells is the T19 molecule. It is observed by dual colour immunofluorescence analysis that monoclonal antibody 19.19 stains a smaller population of γδ TCR-positive lymphocytes than monoclonal antibodies ST197 and CC15. Recent immunoprecipitation studies of T19 have shown that the T19 molecule may exist in molecular weight isoforms of either 140, 220 or
300KDa (Metzelaar et al. 1992). These studies show that monoclonal antibody 19.19 is specific for the 220 and 300KDa isoforms of T19, whilst the other two monoclonal antibodies recognise all three isoforms. Collectively, the data suggests that all three isoforms are not always simultaneously expressed on the same γδ T cells.

T19 is a likely candidate on ovine γδ T cells to substitute for some of the functions mediated by either CD2, CD4 or CD8 expressed by the αβ T cells. The exact function of T19 remains to be defined. It has been proposed that T19 represents a marker of γδ T cell activation based on the observation that T19 is absent on cortical thymocytes and expressed only on medullary thymocytes which are MHC Class I-positive (Mackay et al. 1989). This putative role in activation/maturation of γδ T cells is supported by its recent cloning and sequencing (Metzelaar et al. 1992) which shows the molecule to have homology to CD5 and CD6, known to be involved in T cell activation (Geppert et al. 1990). These studies have shown that the T19 molecule is encoded by at least three different genes which share approximately 80% homology at the nucleotide level.

It has been suggested from studies in other species that differential expression of CD45 can to be used to distinguish naive and memory T cells (Vitetta et al. 1991). This investigation has shown that the majority of ovine γδ T cells appear to be of the memory phenotype, with respect to CD45 expression, irrespective of animal age or antigenic burden. In contrast, the majority of ovine αβ T cells in young animals are of the naive phenotype, but would appear to lose expression of this molecule with age. The data from fetal animals suggests that ovine γδ T cells acquire a memory phenotype prior to birth and exposure to environmental antigens. A recent report demonstrated that human fetal cytotoxic γδ T cell clones were generated that were specific for maternal alloantigens (Miyagawa et al. 1992). If ovine γδ T cells were exposed to maternal alloantigens prior to birth and subsequently induced to mediate an immune response to these antigens, as a means of fetal protection, then this may explain the memory phenotype of this lineage. However, this is an unlikely event in the sheep as the ovine placenta is unique to that of other species in that it is impermeable to the majority of substances, including maternal immunoglobulin (Brambell 1970). Alternatively, ovine γδ T cells may be expanded in the periphery by an endogenous superantigen(s) and in
the process acquire a memory phenotype as is suggested in the human species (Braakman et al. 1991).

The higher level of circulating γδ T cells in ruminants, including sheep, may be indicative of an enhanced dependence on γδ T cell function, rather than that by αβ T cells, for protection against particular pathogens that sheep may encounter. This is supported by the observations made here that as the antigenic burden of young lambs is increased the ratio of αβ/γδ T cells decreases. In addition, the apparent memory phenotype of ovine γδ T cells relative to a naive phenotype of αβ T cells at the birth of the animal, may imply that the latter show a degree of functional immaturity in that a population of potent memory T cells is not established. A large population of memory γδ T cells could confer a strong evolutionary advantage by providing early immunity by recognising either classical MHC molecules or non-polymorphic MHC like molecules until a more mature αβ T cell system becomes established.
INTRODUCTION.

The antigen receptor on T cells recognise fragments of foreign antigen on the surface of antigen-presenting cells held in association with presentation elements (Kronenberg et al 1996). The TCR structure consists of a heterodimer of polypeptide chains of either α or γδ chains (Drennan et al 1986). Associated non-covalently with the TCR are five polypeptide chains involved in signal transduction, namely the γ, δ, ε, η and ζ chains (Clevers et al 1985). These proteins are referred to collectively as the CD3 complex. The CD3 chains are invariant in sequence and assemble in the same orientation on T cells irrespective of the cell expression of the TCR (Van Noerven et al 1990).

CHAPTER 4

Differences In The Rate Of Activation By Ovine γδ and αβ T Cells.

When the TCR/CD3 complex binds a target antigen on an APC, it activates the T cell tyrosine kinases p56lck and p52p56fyn (Abboud et al 1992). These enzymes are closely associated with the TCR/CD3 complex, and phosphorylate the CD3 ε chain and a subunit of Phosphatidylinositol Phospholipase C (PI/PLC) (Beyers et al 1992). Once PI/PLC is activated by this phosphorylation it hydrolyses phosphatidylinositol linkage intoinositol trisphosphate and diacylglycerol (Izquierdo and Cattorelli 1992). These products activate a variety of enzymes including protein kinase C (PKC), and cause an increase in intracellular calcium levels. Subsequently, signals are transmitted to the nucleus and mobilise DNA binding proteins which induce expression of a distinct set of T cell genes involved in cellular activation and proliferation. Such genes are those encoding IL-2 and IL-2 R (Waldman 1993). In addition, proliferating cells express nuclear proteins of non-histone origin such as the Ki67 antigen (Gerdes et al 1991). The role of Ki67 antigen in cellular proliferation remains to be established.

The IL-2 receptor is a complex of several transmembrane proteins comprising α, β and γ chains (Takeyoshi et al 1992, Lowenthal and Gao 1987, Kohl et al 1984). This receptor exists in a low, intermediate or high affinity form on the surface of activated T cells. The low affinity receptor is a 55kDa protein (α chain), designated CD25, whilst the intermediate affinity receptor is a 75kDa protein (γ chain). The high affinity form is a non-covalent complex of at least the 55kDa and the 75kDa protein chains (Wang and
INTRODUCTION.

The antigen receptor on T cells recognise fragments of foreign antigens on the surface of antigen presenting cells held in association with presentational elements (Kronenberg et al 1986). The TCR structure consists of a heterodimer of polypeptide chains of either αβ or γδ chains (Brenner et al 1986). Associated non-covalently with the TCR are five polypeptide chains involved in signal transduction, namely the γ, δ, ε, ζ and η chains (Clevers et al 1988). These proteins are referred to collectively as the CD3 complex. The CD3 chains are invariant in sequence and assemble in the same combination on T cells irrespective of the cell expressing an αβ or γδ TCR (Van Neerven et al 1990).

When the TCR/CD3 complex binds a target antigen on an APC, it activates the T cell tyrosine kinases p56^lek and p59^fyn (Abraham et al 1992). These enzymes are closely associated with the TCR/CD3 complex, and phosphorylate the CD3 ζ chain and a subunit of Phosphatidylinositol Phosphlipase C (PIPLC) (Beyers et al 1992). Once PIPLC is activated by this phosphorylation it hydrolyses phosphatidyl inositol into inositol triphosphate and diacylglycerol (Izquierdo and Cantrell 1992). These products activate a variety of enzymes including protein kinase C (PKC), and cause an increase in intracellular calcium levels. Subsequently, signals are transmitted to the nucleus and mobilise DNA binding proteins which induce expression of a distinct set of T cell genes involved in cellular activation and proliferation. Such genes are those encoding IL-2 and IL2-R (Waldman 1989). In addition, proliferating cells express nuclear proteins of non-histone origin such as the Ki67 antigens (Gerdes et al 1991). The role of Ki67 antigens in cellular proliferation remains to be established.

The IL-2 receptor is a complex of several transmembrane proteins comprising α, β and γ chains (Takeshita et al 1992, Lowenthal and Greene 1987, Robb et al 1984). The receptor exists in a low, intermediate or high affinity form on the surface of activated T cells. The low affinity receptor is a 55KDa protein (α chain), designated CD25, whilst the intermediate affinity receptor is a 75KDa protein (β chain). The high affinity form is a non covalent complex of at least the 55KDa and the 75KDa protein chains (Wang and
Smith 1987). IL-2 is a lymphokine produced and secreted by activated T cells, which binds to the IL2-R in an autocrine and paracrine manner and subsequently triggers the growth and differentiation of lymphocytes. The binding of IL2 to the high affinity form of the receptor results in signal transduction mediated by the 75KDa protein, internalisation of the ligand receptor complex, and the progression of the antigen-specific activated T cell through the S phase of the cell cycle and onto cell division.

T cell proliferation subsequent to activation yields clones of T cells with repertoires identical to that expressed by the parent cell. This occurs as T cells do not undergo affinity maturation by somatic hypermutation as described for B cells. These T cell clones will be of a memory rather than naive phenotype (Horgan et al 1992), and will have an ability to respond more readily to antigen probably through expression of different isoforms of some cell surface glycoproteins relative to their naive counterparts. To date, the best studied markers that delineate naive and memory T cells are the variant forms of CD45 (Merkenschlager et al 1989). The isoforms of CD45 arise as a consequence of differential expression of exons encoding the extracellular domain of the molecule, yielding cell surface glycoproteins of molecular weight 180-220KDa (Thomas et al 1989). The high molecular weight isoforms are found on cells of the naive phenotype and the low molecular weight isoforms on cells of the memory phenotype (Horgan et al 1992).

The data in Chapter 3 indicated that γδ T cells are present in high numbers in the peripheral blood of young lambs, and that elevated levels of these cells are found in animals undergoing immune responses. Because of these findings it is likely that γδ T cells play an important role in the immune function of these animals. As γδ T cells generally lack expression of the co-receptor molecules CD4 and CD8 which are involved in T cell activation (Janeway 1992), differences in the mechanism and hence rate of activation between these cells and αβ T cells may occur. This study has investigated CD25 and Ki67 expression by ovine γδ T cells. In addition, the expression of different CD45 isoforms by γδ T cells was established to determine any correlation between their CD45 isoform expression and their activation. Finally, in view of the suggestion that the
cell surface glycoprotein T19 is lost upon activation (Hein and Mackay 1991), the expression of this molecule after Con A stimulation was studied on the surface of the γδ T cell subset.

**Expression By Ovine Lymphocytes.**

During the course of this thesis monoclonal antibodies specific for bovine CD25 were made available (Naassens et al. 1992). Nucleotide sequence analysis has shown that 94% homology exists between cDNA encoding ovine and bovine CD25 (Bajdor et al. 1992). On this basis it was predicted that anti-bovine CD25 monoclonal antibodies would cross-react with ovine CD25. This hypothesis was tested and the results are shown in Figure 1a. The data demonstrates that the anti-bovine CD25 monoclonal antibody, IL- A111, cross-reacts with greater than 80% of 3 day Con A activated ovine lymphocytes. Control immunofluorescent staining was the reactivity of the monoclonal antibody with unstimulated ovine PBMC (Figure 1b) where approximately 10% of PBMC were labelled by IL-A111, implying the presence of activated lymphocytes within unstimulated ovine PBMC.

Two colour immunofluorescence staining on unstimulated PBMC from young lambs blood was carried out to determine which T cell subset in resting peripheral blood expressed the IL-2 receptor α chain. Table 1 shows that less than 10% of the γδ T cells or CD8-positive cells expressed CD25 on their surface. In contrast, 35.1% of the CD3-positive lymphocytes co-expressed CD25, indicating that these cells were responsible for the majority of IL-2 receptor α chain expression in unstimulated ovine peripheral blood.

### 4.2. CD25 Expression on Ovine γδ T Cells After Con A Treatment.

The above data has shown that the majority of γδ T cells within ovine peripheral blood do not express CD25. This may occur because of one, or more, of the following: (i) activated γδ T cells do not occur in ovine peripheral blood, (ii) γδ T cells lack the ability to express CD25 when activated, (iii) monoclonal antibody IL-A111 does not react with the CD25 molecule expressed by γδ T cells. To test these alternatives ovine PBMC were cultured with Con A for eight days during which time the IL-2 receptor expression by lymphocytes was assessed by two colour immunofluorescence analysis. The results
RESULTS.

4.1. CD25 Expression By Ovine Lymphocytes.

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Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal and cultured with 20\mu g ml\(^{-1}\) of Con A for 3 days. Viable cells were harvested by density centrifugation and stained with monoclonal antibody IL-A111 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry.
FIGURE 1. Immunofluorescence Analysis of CD25 Expression By Con A Activated Ovine Lymphocytes.

(a) Con A activated PBMC

(b) Resting PBMC

Fluorescence intensity

Relative cell number

KEY:  
--- anti-CD25 monoclonal antibody IL-A111

.. normal mouse sera
Table 1.
Peripheral blood mononuclear cells were prepared from 6 month old Grey Face x Suffolk animals (n=3) and analysed by two colour immunofluorescence for co-expression of CD25 with either γδ TCR, CD4 or CD8. Cells were stained with monoclonal antibody IL-A111 (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were subsequently treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when IL-A111 and 86D were used cells were treated with IL-A111 alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the percentage of cells within each subset co-expressing CD25 are tabulated. Standard deviations are included in brackets.
TABLE 1. CD25 Expression By Unstimulated Ovine Lymphocytes (n=3).

<table>
<thead>
<tr>
<th>Lymphocyte Subset</th>
<th>% Of Cells Co-Expressing CD25</th>
</tr>
</thead>
<tbody>
<tr>
<td>γδ</td>
<td>8.2 (1.5)</td>
</tr>
<tr>
<td>CD4</td>
<td>35.1 (2.8)</td>
</tr>
<tr>
<td>CD8</td>
<td>6.2 (1.9)</td>
</tr>
</tbody>
</table>

The data reported here clearly defines three important aspects of CD25 expression by ovine lymphocytes. Firstly, all ovine lymphocytes have the ability to express the IL-2 receptor in tetramer when activated; secondly, the acquisition and the loss of cell surface expression of this receptor protein appears to occur more rapidly with γδ T cells as compared to the αβ T cell subset, and finally that ovine γδ T cells fail to express CD25 at the immunofluorescence intensity exhibited by the αβ T cell subset.
are shown in Table 2. The same PBMC populations were used in this experiment as were used to generate the results of Table 1.

Twenty four hours after activation 93.9% of γδ T cells were CD25-positive and remained CD25-positive for 3 days after activation. On day five 44.4% of γδ T cells had lost their expression of CD25 and by day 8 the level of γδ T cells expressing CD25 had returned to the control level. The T19-positive population of lymphocytes showed the same trends as the γδ T cell subset. In contrast, the majority of the αβ T cell population were not CD25-positive until 72 hours post Con A stimulation, and remained so for a further 2 days. B cells expressed CD25 with a similar trend to that observed by the αβ T cell subset. It is noteworthy that eight days after Con A treatment, B cells represented less than 2% of the total lymphocyte population, making an accurate analysis of CD25 on this population difficult.

The intensity of cell surface expression of CD25 on ovine lymphocytes at the time points investigated was recorded and is shown in Figure 2. All the lymphocyte subsets expressed CD25 with maximum immunofluorescence intensity 48 hours after treatment with Con A, shown above to be a time point where all γδ T cells but not all αβ T cells were CD25-positive. From the data it is apparent that the γδ T cell subset express CD25 at a level of intensity lower than that seen by the αβ subset. The γδ subset showed a sharp decrease in MCN of CD25 expression between day 2 and 3 followed by a gradual decrease between day 3 and 5, whilst the αβ subset showed a gradual decrease in the MCN of CD25 expression between days 2 and 5. By day 8 the MCN of CD25 expression by all subsets had returned to levels observed on unstimulated PBMC.

The data reported here clearly defines three important aspects of CD25 expression by ovine lymphocytes. Firstly, all ovine lymphocytes have the ability to express the IL-2 receptor α chain when activated, secondly, the acquisition and the loss of cell surface expression of this receptor protein appears to occur more rapidly with γδ T cells compared to the αβ T cell subset, and finally that ovine γδ T cells fail to express CD25 at the immunofluorescence intensity exhibited by the αβ T cell subset.
Peripheral blood mononuclear cells were prepared from 6 month old Grey Face x Suffolk animals (n=3) and cultured with 20µg ml\(^{-1}\) of Con A for the time points indicated. Viable cells were harvested by density centrifugation and analysed by two colour immunofluorescence for co-expression of CD25 with either \(\gamma\delta\) TCR, CD4, CD8 or surface immunoglobulin. Cells were stained with monoclonal antibody IL-A111 (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a), SBU-T8 (IgG2a) or VPM8 (IgG1) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when two IgG1 monoclonal antibodies were used cells were treated with IL-A111 alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Subsequently, cells were treated with either biotinylated 86D or VPM8 and finally with phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the percentage of cells within each subset co-expressing CD25 are tabulated. Standard deviations are included in brackets.
TABLE 2. CD25 Expression By Ovine Lymphocytes After Con A Stimulation (n=3).

<table>
<thead>
<tr>
<th>Days After Con A Stimulation</th>
<th>γδ</th>
<th>T19</th>
<th>CD4</th>
<th>CD8</th>
<th>sIg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>8.2 (1.5)</td>
<td>8.3 (1.7)</td>
<td>35.1 (2.8)</td>
<td>6.2 (1.9)</td>
<td>4.5 (4.5)</td>
</tr>
<tr>
<td>Day 1</td>
<td>93.9 (4.4)</td>
<td>93.9 (4.2)</td>
<td>82.8 (3.4)</td>
<td>59.2 (7.1)</td>
<td>46.7 (9.7)</td>
</tr>
<tr>
<td>Day 2</td>
<td>94.6 (2.6)</td>
<td>94.1 (2.6)</td>
<td>95.5 (1.5)</td>
<td>81.8 (1.9)</td>
<td>76.9 (2.3)</td>
</tr>
<tr>
<td>Day 3</td>
<td>92.8 (3.1)</td>
<td>93.1 (2.9)</td>
<td>98.0 (1.5)</td>
<td>89.9 (2.6)</td>
<td>87.0 (4.1)</td>
</tr>
<tr>
<td>Day 5</td>
<td>55.6 (1.8)</td>
<td>54.9 (2.5)</td>
<td>92.6 (2.6)</td>
<td>83.0 (5.3)</td>
<td>73.5 (7.2)</td>
</tr>
<tr>
<td>Day 8</td>
<td>4.3 (0.8)</td>
<td>4.2 (0.7)</td>
<td>19.4 (0.9)</td>
<td>6.8 (1.4)</td>
<td>37.4 (2.2)</td>
</tr>
</tbody>
</table>
Peripheral blood mononuclear cells were prepared from 6 month old Grey Face x Suffolk animals (n=3) and cultured with 20μg ml⁻¹ of Con A for the time points indicated. Viable cells were harvested and analysed by two colour immunofluorescence for co-expression of CD25 with either γδ TCR, CD4 or CD8. Cells were stained with monoclonal antibody IL-A111 (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when IL-A111 and 86D were used cells were treated with IL-A111 alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the MCN of CD25 expression by each subset is shown.
FIGURE 2. MCN of CD25 Expression By T Cells After Con A Stimulation (n=3).

Days After Con A Stimulation

**KEY:**
- ○ γδ T Cells
- ▲ CD4 T Cells
- ■ CD8 T Cells
4.3. Ki67 Expression by ovine lymphocytes

Monoclonal antibody Ki67 recognises nuclear antigens expressed by proliferating human cells (Gerdes et al 1991). The data presented in Figure 3a shows that this monoclonal antibody cross reacts with antigen(s) expressed by activated ovine cells. 96.1% of three day Con A activated ovine lymphocytes were immunofluorescently labelled by the reagent. Control immunofluorescent staining was the reactivity of the monoclonal antibody with unstimulated PBMC (Figure 3b) where 15.9% of cells reacted with Ki67.

4.4. Expression of Ki67 by γδ T Cells After Con A Treatment.

The above data shows that 96.1% of three day Con A blasts express the Ki67 antigens, however it provides no information on the time course of expression of this marker of cellular proliferation. The time course of Ki67 antigen expression was investigated on Con A activated PBMC by two colour immunofluorescence analysis. Figure 4 illustrates the percentage of each lymphocyte subset expressing Ki67 antigens at various time points following Con A activation. 24 hours after Con A activation approximately 15% of lymphocytes were Ki67-positive, of which 5.8% were γδ T cells, 14.8% CD4-positive T cells and 9.9% of CD8-positive T cells. At 48 and 72 hours after Con A activation, greater than 90% of all lymphocytes irrespective of whether they expressed a γδ or αβ TCR were Ki67-positive. Dual colour immunofluorescence analysis showed that 5 days after treatment with Con A, 54.1% of γδ T cells expressed Ki67 antigens. In contrast, approximately 90% of the αβ subset expressed Ki67 antigens at this time point. By day 8 only a minimal population of all subsets remained Ki67-positive.

The MCN of Ki67 expression by the various ovine lymphocyte subsets is shown in Figure 5. A gradual increase in the MCN of Ki67 expression by all subsets was observed between 24 and 72 hours post Con A treatment. The highest MCN value of Ki67 expression was observed by all subsets at 72 hours. At this time point the γδ subset had a MCN value of approximately 140, whilst all the other lymphocytes had a MCN of approximately 200. Five days after activation, Ki67 MCN had decreased significantly on all subsets and by day 8 was within the range observed on unstimulated
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal and cultured with 20μg ml⁻¹ of Con A for 3 days. Viable cells were harvested by density centrifugation, permeabilised and stained with the FITC-conjugated monoclonal antibody Ki67 as described in Materials and Methods. Background immunofluorescence was established with FITC-conjugated anti-human CD23. Cells were analysed by flow cytometry.
FIGURE 3. Ki67 Expression By Permeabilised Con A Activated Ovine Lymphocytes.

(a) Con A activated PBMC

(b) Resting PBMC

KEY:

--- anti-Ki67 monoclonal antibody

---------------------- Control monoclonal antibody
Figure 4.
Peripheral blood mononuclear cells were prepared from 6 month old Grey Face x Suffolk animals (n=3) and cultured with 20μg ml⁻¹ of Con A for the time points indicated. Viable cells were harvested by density centrifugation and analysed by two colour immunofluorescence for co-expression of Ki67 with either γδ TCR, CD4 or CD8. Cells were stained with either monoclonal antibodies 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated, followed by phycoerytherin-streptavidin. Subsequently, cells were permeabilised as described in Materials and Methods and finally reacted with FITC-conjugated monoclonal antibody Ki67. Cells were analysed by flow cytometry and the percentage of cells within each subset co-expressing Ki67 are shown.
FIGURE 4. Ki67 Expression By T Cells After Con A Stimulation (n=3).

KEY:

- γδ T Cells
- CD4 T Cells
- CD8 T Cells
Peripheral blood mononuclear cells were prepared from 6 month old Grey Face x Suffolk animals (n=3) and cultured with 20μgml⁻¹ of Con A for the time points indicated. Viable cells were harvested by density centrifugation and analysed by two colour immunofluorescence for co-expression of Ki67 with either γδ TCR, CD4 or CD8. Cells were stained with either monoclonal antibodies 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated, followed by phycoerytherin-streptavidin. Subsequently, cells were permeabilised as described in Materials and Methods and finally reacted with FITC-conjugated monoclonal antibody Ki67. Cells were analysed by flow cytometry and the MCN of Ki67 expression by each subset is shown.
FIGURE 5. MCN of Ki67 Expression By T Cells After Con A Stimulation (n=3).

KEY:

- □ - CD4 T Cells
- ○ - CD8 T Cells
- △ - γδ T Cells
PBMC.

The data show that over the time points analysed, the γδ and the αβ subsets express the Ki67 antigens at equivalent times within their nucleus, but the former appear to become Ki67-negative prior to their αβ counterparts. In addition ovine γδ T cells did not express the Ki67 antigens at the intensity exhibited by the αβ T cell subset.


The data in the previous sections is indicative of similarities between CD25 and Ki67 expression on in vitro activated ovine γδ T cells. Firstly, approximately 50% of γδ T cells do not express CD25 or Ki67 antigens 5 days after Con A activation and secondly, CD25 protein and the Ki67 antigens are expressed at lower intensities compared to αβ T cells. The experiments in this section have attempted to correlate CD25 and Ki67 antigen expression by γδ T cells by dual colour immunofluorescence. The results of the analysis are shown in Figure 6.

Prior to activation with Con A the majority of cells did not express either CD25 or Ki67 antigens. 24 hours post Con A treatment a population of cells which were CD25-positive and Ki67-negative was observed (data not shown). 3 days post Con A treatment the majority of CD25-positive cells expressed Ki67 antigens. At 5 days post Con A treatment a population of cells were negative for expression of CD25 and Ki67 antigens.

It should be remembered from Sections 4.2 and 4.4 that at 24 hours post Con A activation whilst all γδ T cells expressed CD25 only 5.8% of γδ T cells expressed Ki67 antigens. Secondly, at 5 days post Con A treatment approximately 50% of γδ T cells had lost expression of CD25 and Ki67 antigens. Indirectly, this suggests that the 24 hour post Con A population of cells that were CD25-positive and Ki67-negative and the 5 day post Con A cells that were CD25-negative Ki67-negative were γδ T cells.
Figure 6.
Peripheral blood mononuclear cells were prepared from a 6 month old Grey Face x Suffolk animal and cultured with 20μg ml⁻¹ of Con A for the time points indicated. Viable cells were harvested by density centrifugation and analysed by two colour immunofluorescence for co-expression of CD25 and Ki67. Cells were stained with monoclonal antibody IL-A111 followed by anti-mouse IgG1 biotinylated monoclonal antibody and finally phycoerytherin-streptavidin. Subsequently, cells were permeabilised as described in Materials and Methods and finally treated with the FITC-conjugated monoclonal antibody Ki67. Cells were analysed by flow cytometry and the dual colour immunofluorescence profiles are shown.
FIGURE 6. CD25 and Ki67 Expression By Con A Activated Lymphocytes.
Collectively, the CD25 and Ki67 data suggests four salient features of γδ activation in the ovine species: (i) that ovine γδ T cells have the ability to express both the IL-2 receptor α chain protein and Ki67 antigens; (ii) CD25 expression by γδ T cells occurs prior to that observed by αβ T cells, whilst the appearance of nuclear expression of Ki67 antigens occurs at an equivocal time point by both γδ and αβ T cells; (iii) ovine γδ T cells express both CD25 and Ki67 antigens with a reduced immunofluorescence intensity compared to other lymphocytes, (iv) ovine γδ T cells lose their expression of both CD25 and Ki67 antigens prior to αβ T cells. These observations highlight features of cellular activation that would appear to be unique to γδ T cells, at least in the ovine species, and are likely to provide a basis for a distinct function(s) by these cells during immune responses.

4.6. Expansion Of γδ T Cells In Culture.

It appears that following Con A activation the γδ T cell subset express CD25 prior to αβ T cells, even though there appeared to be no apparent difference in the early expression of Ki67 by the two T cell lineages. The former observation may suggest that IL-2 produced in cultures of activated cells will, initially, selectively expand the γδ subset. Single colour immunofluorescence analysis was utilised to determine if a preferential expansion of the γδ T cell subset did occur when PBMC were cultured with Con A. The results are shown in Table 3.

Within unstimulated PBMC, the γδ subset represented 20.5% of the lymphocytes but after five days of culture with Con A 71.3% of the lymphocytes expressed a γδ TCR. This value decreased to 66.7% by day eight. In contrast, over the same time period the percentage of αβ T cells within the culture remained very similar to the percentage observed within unstimulated PBMC. The increase in the percentage of γδ T cells relative to the αβ subset is reflected by the ratio of the two T cell subsets shown in Table 3. Prior to culturing the αβ / γδ ratio was 1.4 but decreased and remained below 0.7 at all post Con A treatment times analysed. The data demonstrates that the γδ subset was highly enriched relative to the αβ TCR subset by in vitro Con A stimulation.
Table 3.
Peripheral blood mononuclear cells were prepared from 6 month old Grey Face x Suffolk animals (n=3) and cultured with 20μg ml⁻¹ of Con A for the time points indicated. Viable cells were harvested by density centrifugation and stained with either monoclonal antibodies 86D, ST4, ST8 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the percentage of each subset is tabulated. Standard deviations are included in brackets.
TABLE 3. Subset Composition of Ovine Lymphocytes After Con A Stimulation (n=3).

<table>
<thead>
<tr>
<th>Days After Con A Stimulation</th>
<th>Percentage Positive Cells</th>
<th>CD4 + CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γδ</td>
<td>CD4</td>
</tr>
<tr>
<td>Day 0</td>
<td>20.5 (7.5)</td>
<td>19.5 (7.6)</td>
</tr>
<tr>
<td>Day 1</td>
<td>55.0 (10.1)</td>
<td>21.7 (7.2)</td>
</tr>
<tr>
<td>Day 2</td>
<td>50.6 (3.0)</td>
<td>24.5 (7.3)</td>
</tr>
<tr>
<td>Day 3</td>
<td>55.7 (3.9)</td>
<td>27.6 (5.7)</td>
</tr>
<tr>
<td>Day 5</td>
<td>71.3 (6.9)</td>
<td>20.1 (3.7)</td>
</tr>
<tr>
<td>Day 8</td>
<td>66.7 (2.7)</td>
<td>22.0 (3.2)</td>
</tr>
</tbody>
</table>

4.7. Loss of CD45RA expression by Con A Activated γδ T Cells.

The availability of a monoclonal antibody 53-6.7 (Reynolds and Davis 1990) in the high molecular weight isofrom of ovine CD45 (monoclonal antibody 731) (Mackay et al 1990) enabled an investigation of different CD45 isoforms expressed by ovine lymphocytes. It has been suggested that a memory phenotype is defined by the lack of expression of the high molecular weight isofrom of CD45. The aim of this study was to determine if the minor population of γδ T cells exhibiting a naïve phenotype, i.e. the γδβ-negative population, could be induced to convert to a memory phenotype, i.e. to the γδβ-negative population, upon Con A stimulation for 48 hours in vitro. γδ T cells lose CD45RA expression at a faster rate than γδβ T cells.


To date, common fluorescence staining of ovine lymphocytes has shown that T19 is a molecule expressed exclusively by γδ T cellison these. Mackay et al 1989). The exact function of T19 remains undetermined but its role as a maturation marker of γδ T cells (Mackay et al 1989), or as an accessory molecule of γδ T cells which is lost from
4.7. Loss of CD45RA Isoform by Con A Activated γδ T Cells.

The availability of a monoclonal antibody specific for all isoforms of ovine CD45 [monoclonal antibody VPM18 (Hopkins and Dutia 1990)] or the high molecular weight isoform of ovine CD45 [monoclonal antibody 73B (Mackay et al 1990)] enabled an investigation of different CD45 isoforms expressed by ovine lymphocytes. It has been established in Section 3.5.1. and 3.5.2. of this thesis that the majority of γδ T cells are of a memory phenotype as defined by the lack of expression of the high molecular weight isoform of CD45. The aim of this study was to determine if the minor population of γδ T cells exhibiting a naive phenotype, that is the 73B-positive population, could be induced to convert to a memory phenotype, that is to the 73B-negative population, upon in vitro activation, and if so at a rate comparable to that of αβ T cells. Dual colour immunofluorescence was used to determine at which time point during an eight day in vitro culture with Con A ovine γδ T cells lose their expression of CD45RA. The results of the analysis are shown in Figure 7.

22.0% of γδ T cells within unstimulated PBMC expressed CD45RA, but 24 hours after Con A treatment less than 7% of this subset were CD45RA-positive, a percentage that failed to decrease further even after culturing with Con A for 8 days. In contrast the αβ TCR subset required an 8 day exposure to Con A before CD45RA isoform expression had decreased to approximately 7%.

The data indicates that CD45RA was expressed by a smaller percentage of γδ relative to αβ T cells within unstimulated PBMC, and that γδ T cells lose CD45RA expression at a faster rate than αβ T cells.

4.8. Changes In The Expression Of The T19 Molecule By Activated γδ T Cells.

To date, immunofluorescence staining of ovine lymphocytes has shown that T19 is a molecule expressed exclusively by γδ T cells (this thesis, Mackay et al 1989). The exact function of T19 remains undetermined but its role as a maturation marker of γδ T cells (Mackay et al 1989), or as an accessory molecule of γδ T cells which is lost from
Figure 7.
Peripheral blood mononuclear cells were prepared from 6 month old Grey Face x Suffolk animals (n=3) and cultured with 20μg ml⁻¹ of Con A for the time points indicated. Viable cells were harvested by density centrifugation and analysed by two colour immunofluorescence for their co-expression of CD45 p220 with either γδ TCR, CD4 or CD8. Cells were stained with the monoclonal antibody 73B (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when 73B and 86D were used cells were treated with 73B alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the percentages shown represent the fraction of each lymphocyte subset co-expressing CD45 p220.
FIGURE 7. Loss Of CD45RA Expression By Activated T Cells (n=3).

The loss of T19 on Con A activated γδ T cells was investigated by single colour immunofluorescence microscopy. This utilized 3 different monoclonal antibodies that reacted with the same T19 molecule, namely T19, STI97, and CC15. The percentage of cells expressing T19 was evaluated by immunofluorescence analysis with the anti-γδ TCR monoclonal antibody, 86D. A comparison was made of particular T19 epitopes expressed on purified PBMC and after Con A treatment. The ratio of T19/γδ TCR was calculated for the individual and T19 monoclonal antibodies. The ratio of T19/γδ TCR cells in freshly isolated PBMC varied depending on the monoclonal antibody used to analyse T19 expression. The ratios were 0.58, 0.77 and 1.05 for monoclonal antibody STI97 respectively. This indicates that monoclonal antibody CC15 labelled a percentage of cells in number to those labelled by the anti-γδ TCR monoclonal antibody 86D. The data also implies that the epitopes recognised by the monoclonal antibodies T19.19 and STI97 are expressed by a limited population of γδ T cells. These results were investigated over the eight day period of culture with Con A, the ratio of T19/γδ TCR was obtained with monoclonal antibody CC15 remained at approximately 0.58, the ratio of T19/γδ TCR obtained with the other monoclonal antibodies decreased slightly. Statistical analysis demonstrated that the changes observed in the ratio of T19/γδ with monoclonal antibody 19.19 on days 5 and 8 of the investigation were significantly different (p < 0.05) in comparison to the ratio obtained before Con A activation.

KEY:
- γδ
- CD4
- CD8
activated cells has been suggested (Hein and Mackay 1991). All published data investigating T19 expression to date, has analysed expression of this protein by cell-surface immunofluorescence analysis. Loss of T19 cell surface expression, if it does occur, may result from a consequence of decreased T19 gene(s) transcription and translation, or removal of the molecule from the cell surface, or alternatively, the molecule may undergo a conformational change such that particular epitopes normally recognised during immunofluorescence analysis are lost.

The loss of T19 from Con A activated γδ T cells was investigated by single colour immunofluorescence. This utilised 3 different monoclonal antibodies that react with the ovine T19 molecule, namely 19.19, ST197 and CC15. The percentage of cells expressing a γδ TCR was determined by immunofluorescence analysis with the anti γδ TCR monoclonal antibody, 86D. A comparison was made of particular T19 epitope expression on unstimulated PBMC and after Con A treatment. The ratio of T19/γδ TCR was calculated for the individual anti-T19 monoclonal antibodies.

Table 4 shows the ratios of T19/γδ TCR calculated for the three anti-T19 monoclonal antibodies. The ratio of T19/γδ T cells in freshly isolated PBMC varied depending on the monoclonal antibody used to analyse T19 expression. The ratios were 0.55, 0.77 and 1.05 for monoclonal antibodies 19.19, ST197 and CC15, respectively. This indicates that monoclonal antibody CC15 labelled a percentage of cells equal in number to those labelled by the anti-γδ TCR monoclonal antibody 86D. The data also implies that the epitopes recognised by monoclonal antibodies 19.19 and ST197 are expressed by a limited population of γδ T cells. At all time points investigated over the eight day period of culture with Con A, the ratio of T19/γδ TCR obtained with monoclonal antibody CC15 remained at approximately 1.0. In contrast, the ratio of T19/γδ TCR obtained with the other monoclonal antibodies decreased slightly. Statistical analysis demonstrated that the changes observed in the ratio of T19/γδ with monoclonal antibody 19.19 on days 5 and 8 of the investigation were significantly different (p < 0.05) in comparison to the ratio obtained before Con A activation.
Peripheral blood mononuclear cells were prepared from 6 month old Grey Face x Suffolk animals (n=3) and cultured with 20µg ml\(^{-1}\) of Con A for the time points indicated. Viable cells were harvested by density centrifugation and stained with either monoclonal antibodies 86D, 19.19, ST197 or CC15 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the ratios of T19 / γδ are tabulated. Statistical analysis was carried out using a Two Sample t test from the Minitab software package.
TABLE 4. Expression Of The T19 Molecule By Activated γδ T Cells (n=3).

<table>
<thead>
<tr>
<th>Days After Con A Stimulation</th>
<th>19.19 γδ</th>
<th>197 γδ</th>
<th>CC15 γδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>0.58</td>
<td>0.77</td>
<td>1.05</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.60</td>
<td>0.75</td>
<td>1.06</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.53</td>
<td>0.66</td>
<td>1.02</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.49</td>
<td>0.49</td>
<td>0.96</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.45*</td>
<td>0.57</td>
<td>0.97</td>
</tr>
<tr>
<td>Day 8</td>
<td>0.45*</td>
<td>0.58</td>
<td>0.99</td>
</tr>
</tbody>
</table>

* p < 0.05 relative to day 0.
The data indicates that the T19 protein remains on the cell surface after activation of γδ T cells and that T19 epitope expression may vary upon activation as the epitope recognised by monoclonal antibody 19.19 appears to have a more restricted expression after mitogenic stimulation.
The analysis of CD25 and Ki67 expression by ovine lymphocytes has demonstrated that the majority of unstimulated lymphocytes are negative for expression of these particular molecules. The limited expression of these activation markers is not due to the inability of cells to express the molecules, as all ovine lymphocyte subsets express both CD25 and Ki67 upon activation with Con A. The majority of ovine γδ T cells express CD25 24 hours after Con A activation, whilst the majority of the αβ T cell subset are only CD25-positive 72 hours after activation. In contrast, all lymphocyte subsets expressed the Ki67 antigens 48 hours after activation. The γδ subset lose CD25 and Ki67 expression prior of the αβ subset, suggesting that the γδ lineage are not only activated but also return to a resting phase prior of αβ T cells. A feature distinguishing the γδ subset is that these cells fail to express both CD25 and Ki67 at the immunofluorescence intensity shown by the αβ subset. If the intensity of expression of these molecules correlates with the level of activation induced within the lymphocyte then this data may imply that γδ T cells do not reach a state of activation comparable to that of the αβ subset as measured by these markers. Alternatively, the data might imply that γδ T cells have a lower threshold for activation and consequently a more rapid response time than αβ T cells. These features of γδ T cell activation will dictate the functions provided by this T cell lineage within the immune system. Alternatively, molecules other than CD25 and Ki67 may play a more significant role in regulation of activation and proliferation by γδ T cells.

With respect to the data reported above showing that γδ T cells express CD25 prior of αβ T cells, it would be predicted that expansion of the former will occur when the cells are cultured in vitro. Analysis of the relative percentages of each ovine lymphocyte subsets in an in vitro culture of Con A activated PBMC showed that there is a significant percentage increase in the number of γδ T cells within the culture over eight days relative to the other lymphocyte subsets. This implies that the γδ and αβ T cell subsets differ not only in their rates of activation but also their time of proliferation. The apparent preferential expansion of the γδ subset during the first five days of culturing with Con A may occur as γδ T cells express the CD25 protein before αβ T cells which
enables γδ T cells to bind endogenously produced IL-2 prior to the αβ subset. Further evidence to confirm this hypothesis is reported in section 6.1. of this thesis, where the γδ lineage are preferentially expanded over the first five days of the culture with antigen in vitro. The addition of exogenous IL-2 to 5 day old cultures of T cells preferentially expands αβ TCR-positive cells, which may be explained by the observation that five days after mitogenic activation the majority of the αβ lineage were CD25-positive, whereas only 55.6% of the γδ T cells expressed CD25.

Several explanations may exist for this difference in CD25 expression between γδ and αβ T cells. Firstly, γδ T cells maybe already pre-activated in contrast to αβ T cells as suggested by CD45 isoform expression, indicating that the majority of ovine γδ T cells are of the memory phenotype. Secondly, different signal transduction pathways may exist between the two T cell lineages as γδ T cells lack the CD4 and CD8 molecules which associate with the tyrosine kinase p56^ck (Janeway 1992, Vega et al 1990). This enzyme is known to play an important part in T cell signal transduction via the phosphorylation and subsequent control of expression of various cellular proteins. In addition, the majority of ovine γδ T cells fail to express CD2, a molecule shown recently to be also functionally associated with p56^ck (Danielian et al 1992). Significant consequences on γδ T cell activation, relative to the αβ lineage, may occur as cellular proteins associated with activation are likely to be regulated by different mechanisms in absence of CD4, CD8 and CD2. Thirdly, the two T cell subsets may have different transcriptional and translational control of CD25 expression.

A recent publication has reported that human γδ T cells exhibit differences in their TCR associated signal transducing element, the CD3 complex, relative to αβ T cells (Brenner et al 1986). These differences have been assigned to the δ chain of the CD3 complex and are based on distinct differences in their mobility by SDS-PAGE. Since Con A induces its mitogenic effects on T cells via the CD3 complex, variability in CD3 structure expressed by γδ and αβ T cells may either (i) transmit activation signals to the nucleus at different rates or (ii) produce a stimulus differing in magnitude, that induces a difference in the expansion of the two T cell lineages. The observation that the majority of both γδ
and αβ T cells only appear to express Ki67 antigens 48 hours after Con A stimulation argues against the former hypothesis as the data implies no difference between the rate at which signals are transduced to the nucleus. The observation that the populations of cells within unstimulated PBMC expressing either CD25 or Ki67 appear to be mutually exclusive, suggests that the expression of CD25 and Ki67 are not coordinately regulated in some cells. Alternatively γδ T cells require a lower threshold of Ki67 expression relative to αβ T cells for the initiation of proliferation and at levels not detected by immunofluorescence analysis. However, this or any other sequence of events associated with Ki67 remains to be established.

The results in this chapter have highlighted important aspects of CD45 expression which distinguish γδ and αβ T cells. It has been demonstrated here that γδ T cells and αβ T cells differ in their expression of the external domain of CD45. From studies in other species it has been shown that this domain can be divided into four subdomains (Pingel and Thomas 1989). The region at the amino terminus contains O-linked carbohydrate sites, followed by two separate cysteine clusters and then a short spacer region before the membrane spanning region. The differential use of the three variable exons encoding the external domain of CD45 results in changes in the O-linked carbohydrate region which may alter its ligand specificity. If different ligands bind the various isoforms of CD45, then expression of different epitopes may have significant implications on lymphocyte cell signalling. It is known that CD45 mediates key functions within these cascades of events as its cytoplasmic region contains tyrosine phosphatase activity (Alexander et al 1992). Known substrates for this phosphatase enzyme are tyrosine kinase p56lck and p59fyn which have increased activity upon removal of a phosphate group at tyrosine residue 505 and 528, respectively (Shiroo et al 1992). In addition Schraven et al (1990) have shown that CD45 and CD2 are physically associated on the surface of T cells which may imply that these molecules provide a means of regulation over one another.

Not only do a smaller proportion of ovine γδ T cells express the CD45RA isoform compared to αβ T cells, but upon activation ovine γδ T cells cease to express CD45RA
within 24 hours, whilst not all their αβ equivalents were CD45RA-negative until 8 days after activation by Con A. This implies different kinetics of CD45 isoform switching by the two T cell lineages. In accordance with published observations (Braakman et al 1991) it is likely that the previously CD45RA-positive population switched to express the CD45RO isoform but in the absence of a monoclonal antibody specific for ovine CD45RO this can not be shown conclusively. It has been suggested that different isoforms of CD45 can be used as markers to delineate naive and memory T cells (Merkenschlager et al 1989), based on the observation that lymphocytes expressing the low molecular weight isoform of CD45 (CD45RO) have an increased ability to respond to recall antigens relative to lymphocytes expressing the high molecular weight isoforms (CD45RA) (Thomas 1989). If this is the case then an increased rate of activation is expected of a population of cells expressing CD45RO. Data reported here provides evidence in favour of this hypothesis in that γδ T cells have a memory cell phenotype with respect to their response time for cellular activation and proliferation.

It is reported that γδ T cells comprise the major T cell subset in the peritoneal cavity of mice three days after infection with Listeria monocytogenes (Ohga et al 1990), where approximately 40% of lymphocytes express a γδ TCR, relative to 1-5% in control mice. Consequently it was proposed that γδ T cells are the mediators of early immune recognition and provide a first line of defence until the αβ subset are fully engaged in the immune response (Hiromatsu et al 1992). The observations made in this report indicates that ovine γδ T cells are activated and proliferate in vitro prior of the αβ lineage, hence providing indirect evidence for this theory.

The availability of three anti-T19 monoclonal antibodies enabled a detailed investigation of differential T19 epitope expression. Analysis of cell surface expression of T19 subsequent of T cell activation in this study has demonstrated that this molecule is not removed or cleaved from the surface of γδ T cells. It is apparent from the data obtained that the epitope recognised by monoclonal antibody CC15 is expressed on the surface of resting and activated γδ T cells. Other epitopes, recognised by monoclonal antibodies 19.19 and ST197 are expressed not only on a smaller percentage of T cells, but in the case of the former, a significant reduction of cells expressing the epitope recognised by
the monoclonal antibody was apparent after activation. Recently three cDNAs encoding the T19 molecule have been isolated. Based on the observation that only 80% homology exists between these cDNAs it has been implied these cDNAs were derived from three separate genes encoding the T19 protein (Metzelaar et al 1992). Activation may then induce the expression of different genes encoding T19 proteins or lead to alternative splicing within mRNA encoding the T19 protein.
CHAPTER 5.

Purification of γδ T Cells And Their Subsequent Use In Functional Assays.
INTRODUCTION.

The function of γδ T cells remains to be established despite extensive research over the past six years into the ontogeny and role of this subset of T cells within the immune system. To date, the majority of γδ research has focused on cells from the human and murine species. In these species the γδ T cell lineage represents only a minor population. The observations of this thesis, and other published data have shown that ruminants (Mackay et al 1986, Clevers et al 1990, Hirt et al 1990) and chickens (Sowder et al 1988) have large numbers of γδ T cells within their peripheral blood, consequently offering greater potential for studying γδ T cell function relative to humans and mice. In addition, the predominance of γδ T cells within the immune system of ruminants may be indicative of additional functions of γδ T cells unique to these species.

A detailed analysis of γδ T cell phenotype and function ideally requires these cells to be purified to homogeneity. Techniques for the purification of cells routinely employ either positive or negative selection of the desired cell type. Numerous techniques are available for isolating homogeneous populations of cells, each technique differing in its limitations. Some conventional immunological techniques such as panning (Mage et al 1977, Wyosaki and Saito 1978), complement mediated lysis and rosetting (Pellegrino et al 1976) have been used successfully in the past for isolating populations of lymphocytes. However, these techniques have various disadvantages, including low sensitivity, poor quality of separation, loss of the labelled cells, and amount of reagents and time required.

The development of fluorescence activated cell sorting (FACS) as a method for the isolation of subpopulations of lymphocytes from a mixed population of cells has greatly enhanced lymphocyte separation (Loken and Herzenberg 1975). However, its application is limited by the relatively small separation capacity of the technique and financial aspects involved. Despite these factors FACS has the potential to yield a population of cells of very high purity, irrespective of the percentage representation within the starting population of the cells to be selected. Until recently FACS provided one of the most satisfactory lymphocyte separation techniques, but the development of
Immunomagnetic procedures as a means of isolating homogeneous populations of cells has provided a technique capable of yielding populations of cells of comparable purity to FACS, but without the limitations of obtaining small numbers of the purified cells in realistic time periods.

Immunomagnetic separation is a procedure that involves attachment of magnetic particles to lymphocytes via the use of antibody specific for a particular lymphocyte surface antigen. Two different methods of immunomagnetic separation are available, namely, Dynabead affinity extraction (Funderud et al 1987, Brinchmann et al 1988) and magnetic cell sorting (MACS) (Miltenyi et al 1991). In the former, Dynabeads are the source of immunomagnetic particles whilst the latter uses MACS microbeads. Dynabeads are paramagnetic monodisperse polymer particles. Each particle is coated with a thin polystyrene shell providing a defined surface area and chemical coating, allowing physical absorption and covalent binding of monoclonal antibodies. Each particle has a diameter of 4.5 microns, being approximately half the size of a resting lymphocyte (10 microns). Dynabeads are not biodegradable in cell culture. Alternatively, MACS microbeads consist of superparamagnetic ferric complexes. The microbeads have a diameter of 0.02 microns, 500 times smaller than the size of a eukaryotic lymphocytic cell. Microbeads are biodegradable and decompose upon culture of positively selected MACS sorted cells.

The establishment of techniques where distinct populations of ovine lymphocytes can be purified will enable the investigation of some of the functional aspects of these cells. Of particular interest is the analysis of the cytotoxic potential of ovine γδ T cells as recent data has shown that human (Fisch et al 1990a, Nakata et al 1990), mouse (Goodman and Lefrancois 1988, Klein 1986) and rat (Ericsson et al 1991) γδ T cells derived from various lymphoid compartments may exhibit cytotoxicity in vitro. In particular, it has been suggested that γδ T cells resident within epithelial sites mediate cytolytic functions at these sites by eliminating transformed or virally infected epithelial cells (Janeway et al 1988, Tonegawa et al 1989). Target cell lysis is induced by effector cells which release mediators, such as perforin (Tschopp 1990), serine esterases (Masson and Tschopp 1987) and tumor necrosis factor (TNF) (Cairns et al 1992). It has been shown that
human and murine cytotoxic lymphocytes store perforin and serine esterases in cytoplasmic electron dense granules (Tschopp 1990), and it is thought that TNF is stored in cytoplasmic secretory vesicles not visible by EM. Preliminary results by other groups on the cytotoxic potential of ovine γδ T cells suggested that these cells were capable of the cytotoxic killing of allogeneic target cells but their MHC restricted pattern was inconsistent (Hein and Mackay 1991). The purification of a homogeneous population of ovine γδ T cells by MACS will enable an investigation of the cytotoxic potential of these cells.

This chapter describes and compares various techniques that were investigated to yield a pure population of γδ T cells starting from a mixed population of PBMC. Each protocol made use of immunomagnetic separation, either by itself or in combination with other classical techniques of cell fractionation, such as complement mediated lysis. Subsequently, a protocol was selected and utilised to purify populations of ovine γδ T cells and other lymphocytes populations to homogeneity. The purified populations of cells were then used to investigate the cytotoxic potential of ovine lymphocyte subsets. Electron microscopy (EM) studies were utilised to determine if ovine lymphocytes expressed cytoplasmic electron dense granules. In addition, the availability of various monoclonal and polyclonal reagents generated to murine perforin enabled an investigation of perforin expression in ovine lymphocytes. Various monoclonal and polyclonal reagents have been generated to murine perforin, such as the monoclonal antibody, Pl-8 (Kawasaki et al 1990) and a rabbit polyclonal antisera, 275 (Koizumi et al 1991). The reagents have been shown by immunoprecipitation and Western blot analysis to react with a protein of 70 KDa expressed exclusively by cytotoxic cells. These reagents were tested for their ability to cross react with protein present in ovine lymphocytes, either by immunocytochemistry or via Western Blot analysis.
RESULTS.

5.1. Comparison Of Purification Techniques For γδ T Cells.

Techniques for the purification of cells routinely employ either positive or negative selection of the desired cell type. In this chapter various protocols of either type are described which have been investigated for their use in the isolation of ovine γδ T cells. The protocols are summarised in Figure 1. All of the protocols make use of immunomagnetic separation for either the depletion or enrichment of lymphocyte subsets.

The negative selection techniques employed the removal of unwanted cells by (a) adherence followed by complement mediated lysis and then immunomagnetic Dynabead affinity extraction, or (b) adherence followed by magnetic cell sorting using a Magnetic Cell Sorter (MACS). The techniques involving positive selection focussed on isolating cells expressing the T19 molecule. This is expressed exclusively, as far is known, by γδ T cells of ovine and other ruminants species (Mackay et al 1989, Clevers et al 1990). The protocols involved (c) positive selection of T19 positive cells using immunomagnetic Dynabead and their subsequent removal from the selected cells with Detachabead, or (d) positive selection of T19 positive cells by MACS.

The starting population of cells for each of the fractionation protocols was peripheral blood obtained by venepuncture. PBMC were prepared by density gradient centrifugation over lymphoprep as described in section 2.4.1, and at appropriate stages, aliquots of cells set aside for analysis by flow cytometry. The results of each purification procedure are reported below and Table 1 shows the percentage purity of the isolated population in terms of γδ TCR expression as determined by immunofluorescence staining, and percentage yield of γδ T cells relative to the number of γδ T cells present in the starting population.
Figure 1.

An outline of the purification protocols investigated are shown.
FIGURE 1. Protocols For The Purification Of Ovine γδ T Cells.

**NEGATIVE SELECTION**

Removal of plastic adherent cells by incubating for 60 minutes at 37°C followed by depletion of CD4+, CD8+ and sIg+ cells via complement mediated lysis and two cycles of dynalbeading.

Removal of plastic adherent cells by incubating for 60 minutes at 37°C followed by depletion of CD4+, CD8+ and sIg+ cells by MACS.

**POSITIVE SELECTION**

Selection by the T19 molecule with dynalbeads and detachabeads.

Selection by the T19 molecule using the MACS system.
Table 1.

Table 1 summarises the percentage yield, purity and response to Con A (where tested) of populations of ovine γδ T cells isolated by the various protocols described in this chapter. Standard deviations are included in brackets.
5.1.3. Negative Selection: Incorporating Complement Mediated Lysis and Dynabeads

In this protocol the response was assessed upon the starting population by a classical method of analysis. In our experiments, a classical method of assessing the response of the T cell and B cell populations, complement mediated lysis and Dynabeads were employed. A classical method of analysis was performed using a suspension of lymphocytes that were separated by complement mediated lysis and Dynabead affinity extraction. This protocol was determined to be effective in removing T cells.

### Table 1. Summary of Purification Techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>% Yield</th>
<th>% Purity</th>
<th>Response To Con A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Negative Selection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>complement mediated lysis and Dynabeads (n=9)</td>
<td>41.9 (5.8)</td>
<td>67.1 (23.5)</td>
<td>no response</td>
</tr>
<tr>
<td>MACS (n=8)</td>
<td>28.8 (7.2)</td>
<td>80.2 (5.8)</td>
<td>not tested</td>
</tr>
<tr>
<td><strong>Positive Selection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynabeads and Detachabeads (n=7)</td>
<td>8.6 (1.5)</td>
<td>84.6 (4.2)</td>
<td>not tested</td>
</tr>
<tr>
<td>MACS (n=12)</td>
<td>56.9 (5.7)</td>
<td>92.6 (1.8)</td>
<td>50-100 fold over background</td>
</tr>
</tbody>
</table>
5.1.1. Negative Selection Incorporating Complement Mediated Lysis And Dynabeads.

In this protocol monocytes were depleted from the starting population by a classical method of adherence to plastic. This was followed by depletion of the γδ TCR-negative lymphocytes using complement mediated lysis and Dynabead affinity extraction. This protocol is described in detail in section 2.7.1.

In the absence of monoclonal antibodies which define ovine macrophages and monocytes the presence of these cells was evaluated by FSC and SSC profiles. The dot plot profile of PBMC cells obtained by density centrifugation consisted of lymphocytes and a population of cells with a slightly higher FSC and SSC which have been suggested to be cells of the monocyte lineage. Figure 2 shows that after adherence, the majority of cells with the higher FSC and SSC, compared to that of small resting lymphocytes, were depleted. The immunofluorescence staining profiles in Figure 3 show that the percentage of surface immunoglobulin-positive cells had decreased suggesting that B cells were removed, to some extent, which resulted in an increased percentage of T cells.

The T cell enriched population was subjected to complement mediated lysis using monoclonal antibodies specific for CD4 and CD8 and rabbit polyclonal antisera specific for sheep immunoglobulin. This removed a proportion of the immunolabelled lymphocytes but failed to lyse them all, as shown by the FACS profile obtained when post-complement mediated lysed cells were incubated with an anti mouse Ig FITC reagent. 34.2% of the post-complement mediated lysed cells stained positive, indicating that a significant number of antibody labelled CD4, CD8 or surface immunoglobulin-positive cells remained in the fractionated pool. Further incubation of the lymphocytes with monoclonal antibodies specific for different epitopes of CD4, CD8 and surface immunoglobulin, followed by anti Ig FITC, failed to yield an increase in the percentage staining to that observed when normal mouse sera was used as a first layer. This suggested that the remaining αβ T cells and B cells were successfully immunolabelled after the first antibody incubation.
Figure 2.
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep and plastic adherent cells were removed by incubating for 60 minutes at 37°C. The non-adherent cells were analysed by flow cytometry for their forward and side light scatter profiles. Gate 1 indicates the forward and side scatter profiles of the monocytes and macrophages.
FIGURE 2. Scatter profiles of ovine peripheral blood lymphocytes before and after adherence to plastic.
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep and plastic adherent cells were removed by incubating for 60 minutes at 37°C. γδ TCR-negative cells were depleted by incubating with monoclonal antibodies SBU-T4 and SBU-T8 and rabbit polyclonal antisera specific for sheep immunoglobulin followed by rabbit complement and Dynabeads, as described in Materials and Methods. Cells were stained at the end of each step with either monoclonal antibodies 86D, ST4, ST8 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 3. Immunofluorescence Analysis of Cells Purified By Negative Selection Incorporating Complement Mediated Lysis And Dynabeads.

(i) Fresh PBMC

(ii) Mononuclear cells after removal of plastic adherent cells

(iii) Mononuclear cells after removal of plastic adherent cells and removal of γδ-negative cells by complement mediated lysis

(iv) Mononuclear cells after removal of plastic adherent cells and removal of γδ-negative cells by complement mediated lysis and Dynabeads
To attempt further removal of these unwanted immunolabelled cells, Dynabead affinity extraction using Dynabead coated with goat anti mouse immunoglobulin were used. The percentage of immunolabelled cells remaining after extraction was determined by immunofluorescence analysis. This was achieved by further incubating the cells with an anti-mouse Ig FITC reagent. Two rounds of Dynabead extraction reduced the percentage of immunolabelled cells to 15.4% but failed to completely deplete the γδ TCR-negative lymphocytes.

Of this final population of isolated cells, approximately 80% were immunofluorescent when incubated with the anti-γδ TCR monoclonal antibody. However, this overestimated the percentage purity of γδ T cells as some of this staining was due to the second layer reagent binding the remaining contaminating surface labelled CD4, CD8 or surface immunoglobulin-positive lymphocytes, estimated to be 15.4%. As shown in Table 1, the average percentage purity of the γδ population isolated by this protocol was 67.1% (n=7), with a recovery of 41.9% of γδ T cells from the original starting population. This protocol failed to produce a population of γδ T cells of sufficient purity for antigen presentation experiments and the number of cells isolated at the end of the protocol was relatively low. A large number of cells was lost during the adherence step and an equally large number was lost during each of the numerous washes which were required because of the various antibody treatments to cells. The whole protocol was very time consuming, exposed the cells to a great deal of stress and left them prone to fragment as judged by the large amount of cell debris in the final recovery tube. No proliferative response was seen by γδ T cells purified by this protocol when cultured with the T cell mitogen Con A (data not shown).

5.1.2. Negative Selection Incorporating MACS.
An alternative technique for the isolation of ovine γδ T cells based on negative selection was the removal of monocytes and macrophages by adherence to plastic, followed by removal of γδ TCR-negative cells by MACS. This protocol is described in detail in section 2.7.2.
As already described above, the adherence step decreased the percentage of monocytes and macrophages and surface immunoglobulin-positive lymphocytes resulting in an increase in the percentage of T cells. For MACS separation, \( \gamma \delta \) TCR-negative lymphocytes were surface labelled with monoclonal antibodies specific for CD4 and CD8 and rabbit anti-sheep immunoglobulins. Cells were then treated with polyclonal goat anti mouse or polyclonal goat anti-rabbit biotinylated antibody, followed by streptavidin. The lymphocyte population was applied to the MACS separator located within a permanent magnet and allowed to flow through the column at a regulated flow rate. Only the column eluate which flowed through the column after the lymphocyte suspension had been applied to the top of the separator was harvested. No buffer was applied to wash the column as this may have increased the percentage of \( \gamma \delta \) TCR-negative lymphocytes within the non-magnetic eluate fraction by dislodging weakly bound cells from the separator.

The percentage expression of either \( \gamma \delta \) TCR, CD4, CD8 or surface immunoglobulin was assessed at each step and is shown in Figure 4. Subsequent to the negative depletion of \( \alpha \beta \) T cells and surface immunoglobulin-positive cells by MACS, 79.7% of the cells were 86D-positive when detected by mouse anti-immunoglobulin second layer. However, this was an overestimation of the purity of the isolated population because 9.8% of the column eluate were positive for either CD4, CD8 or surface immunoglobulin, as determined by incubating cells with mouse anti-Ig FITC.

The purity of \( \gamma \delta \) T cells isolated by this protocol was, on average of 80.2% (\( n=8 \)). However, only 28.8% of the \( \gamma \delta \) TCR-positive lymphocytes within the starting PBMC population, were retrieved. Negative selection of \( \gamma \delta \) T cells by this protocol was more time consuming than the previous protocol, as the immunolabelling of the lymphocytes for MACS required four layers of reagents, whilst use of Dynabead only required two layers. The lower recovery of cells with this protocol was likely to be the result of increased cell loss during washes used to remove excess reagents after each incubation. Some \( \gamma \delta \) T cells may have been retained on the ferromagnetic column as no wash buffer was applied to the column to remove lightly bound cells. The ability of these purified \( \gamma \delta \) T cells to respond to Con A was not tested.
Figure 4.
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep and plastic adherent cells were removed by incubating for 60 minutes at 37°C. γδ TCR-negative cells were immunolabelled by incubating with monoclonal antibodies SBU-T4 and SBU-T8 and rabbit polyclonal antisera specific for sheep immunoglobulin, followed by a goat anti-mouse IgG biotin conjugate, then streptavidin and finally MACS biotin microbeads. The γδ TCR-negative cells were depleted by loading the cells onto the MACS separator and collecting the eluate as described in Materials and Methods. Cells were stained at the end of each step with either monoclonal antibodies 86D, ST4, ST8 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 4. Immunofluorescence Analysis of Cells Purified By Negative Selection Incorporating MACS.

(i) Fresh PBMC
(ii) Mononuclear cells after removal of plastic adherent cells
(iii) Mononuclear cells after removal of plastic adherent cells the removal of γδ-negative cells by MACS
5.1.3. Positive Selection of γδ T Cells Via The T19 Molecule Using Dynabeads and Detachabeads.

Ovine (Mackay et al. 1989), and other ruminant (Clevers et al. 1990) γδ T cells express a protein, known as T19, which is expressed on the majority of γδ TCR-positive lymphocytes and a very minor population of γδ TCR-negative cells. Studies have shown that cross linking of the T19 molecule with monoclonal antibodies fails to activate γδ T cells (section 5.2.2. of this thesis). These two salient features of the T19 molecule make it a suitable target molecule for the positive selection of ovine γδ T cells.

Ovine γδ T cells were positively selected by magnetic affinity extraction using Dynabead coated with the T19-specific monoclonal antibody ST197. As purified γδ T cells were to be used in functional assays where proliferation of the cells was taken as an indication of their activation, Dynabead removal was required from the surface of the positively selected lymphocytes by a technique that would not involve the loss of the T19 antigen. Attempts were made to remove these magnetic particles using a reagent known as Detachabead, polyclonal goat anti-mouse F(ab)₂, which should bind to the F(ab)₂ regions of the cell surface specific monoclonal antibodies attached to the Dynabeads and prevent subsequent binding to lymphocytes. This protocol is described in detail in section 2.7.3.

The percentage purity of the isolated population was determined with subset specific monoclonal antibodies of isotypes other than IgG2b that define ovine lymphocyte subsets. Figure 5 shows that positive selection of ovine lymphocytes via the T19 molecule isolated a population of cells of which approximately 88% stained with the anti γδ TCR monoclonal antibody, 86D. Less than 4% of the lymphocytes were positive for either CD4 or CD8, while 9.7% were surface immunoglobulin-positive. Less than 1% of cells were stained by normal mouse sera, followed by either anti IgG1 FITC or anti IgM FITC reagents (data only shown with anti-IgG1 FITC). At this point, a population of relatively pure γδ T cells had been isolated with Dynabeads coated with anti-T19 covalently linked to the magnetic Dynabead particles. After the addition of Detachabead the percentage purity of the γδ T cells and the percentage of γδ TCR-negative cells was similar to those obtained prior to the addition of Detachabead.
Figure 5.
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep and T19-positive cells were isolated with Dynabeads coated with mouse anti-T19 immunoglobulins (ST 197 - IgG2a) and Detachabeads as described in Materials and Methods. Cells were stained at the end of each step with either monoclonal antibodies 86D (IgG1), ST4 (IgG1), ST8 (IgM) or VPM8 (IgG1) followed accordingly by either anti-mouse IgG1-FITC or anti-mouse IgM-FITC. Cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 5. Immunofluorescence Analysis of Cells Purified By Positive Selection Using Dynabeads and Detachabeads.

(i) Fresh PBMC

(ii) Lymphocytes positively selected via the T19 molecule using Dynabeads

(iii) Lymphocytes positively selected via the T19 molecule using Dynabeads followed by treatment with Detachabeads
This procedure provided a population of γδ T cells with a purity of 84.6% on average (n=7). The cell yield was low with only 8.6% of γδ T cells recovered from the starting population. This was probably a consequence of the Detachabead reagent failing to remove the Dynabeads from the majority of the lymphocytes, as rosettes of lymphocytes and Dynabeads could be seen by light microscopy. Subsequent additions of fresh Detachabead and incubation at either 4°C, room temperature or at 37°C failed to increase the percentage of γδ T cells devoid of Dynabead (data not shown). An advantage of the technique was the time scale involved. T19-positive cells were obtained approximately 90 minutes after the start of the procedure. The response of the isolated population to Con A was not tested.

5.1.4. Positive Selection Via The T19 Molecule Using MACS.
An alternative method of positive selection of ovine γδ T cells from PBMC was by MACS. Ovine γδ T cells were labelled with monoclonal antibodies ST197 and CC15 which were attached to magnetic microbeads via a biotin-avidin bridge. The lymphocyte sample was applied to the MACS separator located within a permanent magnet and positively selected cells isolated by removal of the magnetic field after washing through contaminating non-bound cells. This protocol is described in detail in section 2.7.4.

Figure 6 shows the purity of γδ T cells isolated by this procedure. Contaminating cells were assessed using subset specific monoclonal antibodies of different isotypes to those used for the positive selection, followed by isotype specific FITC-conjugated immunoglobulin. γδ T cells of >90% purity were obtained. Less than 3% of cells were positive for either CD4, CD8 or surface immunoglobulin. Greater than 55% of γδ T cells within the starting PBMC population were recovered. With this protocol the majority of cell losses occurred in washes to remove excess reagents. Viability of the purified γδ T cells was shown by their ability to proliferate in the presence of Con A. Cells cultured with Con A gave 3H thymidine cpm of around 100,000 with a background proliferation of 2,000 cpm. Isolation of γδ T cells by positive selection using MACS was relatively efficient in terms of time, with a purified population of γδ T
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep. T19-positive cells were immunolabelled with ST197 (IgG2b) and CC15 (IgG2a) which were both biotinylated, followed by streptavidin and finally MACS biotin microbeads as described in Materials and Methods. T19-positive cells were isolated by loading the cells onto the MACS separator. Cells were stained at the end of each step with either monoclonal antibodies 86D (IgG1), ST4 (IgG1), ST8 (IgM) or VPM8 (IgG1) followed accordingly by either anti-mouse IgG1-FITC or anti-mouse IgM-FITC. Cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 6. Immunofluorescence Analysis of Cells Purified By Positive Selection Using MACS.

6.1.5. Evaluation Of γδ T Cell Purification Protocols. Several procedures for the isolation of ovine γδ T cells have been investigated and are shown in Table 1. Positive selection via the T19 molecule using MACS resulted in the highest purity and highest yield of γδ T cells in a cell population. Of the four different isolation procedures, those tested, positive selection by MACS was the only protocol that resulted in a purified population of γδ T cells capable of responding to Cox A. Cells were isolated from nylon wool columns with monoclonal antibodies specific for CD4, CD8, or the immunoglobulin. This protocol was established using peripheral blood mononuclear cells as a source of lymphocytes, but the technique can be applied to isolate γδ T cells or other ovine γδ T cells, or other ovine γδ T cells, or other

6.2. Characterization Of Purity And Activation Of Isolated γδ T Cells. Positive selection was used to purify lymphocytes positively selected via the T19 molecule using MACS.

(i) Fresh PBMC

(ii) Lymphocytes positively selected via the T19 molecule using MACS
cells obtained 90 minutes from the start of the procedure.

5.1.5. Evaluation Of γδ T Cell Purification Protocols.
Several procedures for the isolation of ovine γδ T cells have been investigated and are shown in Table 1. Positive selection via the T19 molecule using MACS resulted in the highest purity and highest recovery of γδ T cells in a comparison of the four different isolation procedures. Where tested, positive selection by MACS was the only protocol that resulted in a purified population of γδ T cells capable of responding to Con A. Cells were routinely purified to >90% purity with less than 3% of the isolated population reacting with monoclonal antibodies specific for CD4, CD8 or surface immunoglobulin. This protocol was established using peripheral blood mononuclear cells as a source of lymphocytes, but the technique can be applied to isolate cells from any lymphoid compartment. In all subsequent experiments involving purified ovine γδ T cells, or other ovine T cell subsets, purification was achieved by positive selection via subset-specific molecules by MACS.

5.2. Validation Of Purity And Activational Status Of Isolated γδ T Cells.
Purified γδ T cells were to be used in antigen presentation assays involving proliferation of cells as a measure of antigen recognition. Firstly, it was required to establish that during culture of the isolated γδ T cells there was no outgrowth of a contaminating population which may invalidate any statements made about the contribution to the response by γδ T cells. Secondly, it was necessary to show that the isolation procedure did not activate γδ T cells, resulting in a population of hyperresponsive cells. Described below are the results obtained from various analyses to establish the above statements.

5.2.1. Maintenance of Purity of Isolated γδ T Cells.
To show that there was no selective outgrowth of contaminating cells from a population of γδ T cells purified to greater than 90% purity, isolated cells were cultured in vitro with either Con A, human recombinant IL-2 or media alone, for five days. Viable
lymphocytes were harvested by density centrifugation and analysed by flow cytometry for FSC and SSC profiles which are shown in Figure 7. The cell surface expression of either \( \gamma \delta \) TCR, CD4, CD8 and surface immunoglobulin was determined by immunofluorescence staining and analysed by flow cytometry. The results are shown in Figure 8.

Con A treatment of cells resulted in lymphocytes with an increased FSC and SSC compared to cells cultured in media alone, indicating the presence of activated cells [Figure 7(i)]. Immunofluorescent staining of cells following culture with Con A showed that the percentage of \( \gamma \delta \) TCR bearing cells had increased from 90% to greater than 96% [Figure 8(ii)]. Less than 1% of Con A cultured cells expressed either CD4, CD8 or surface immunoglobulin compared to 3% prior to Con A treatment. This suggests that when ovine \( \gamma \delta \) T cells are purified to greater than 90% homogeneity and subsequently cultured in the presence of Con A, the percentage of cells expressing a \( \gamma \delta \) TCR is further increased, with no outgrowth of \( \gamma \delta \) TCR-negative lymphocytes.

The forward and side scatter profiles of the majority of isolated cells were not altered by culturing in the presence of IL-2 [Figure 7(ii)]. The majority of cells remained as small lymphocytes, typical of those in the resting phase [Figure 7(iii)]. However, some cells were of increased forward and side scatter, and probably represented cells that were IL-2 receptor-positive upon isolation which responded by proliferation to exogenous IL-2. Immunofluorescence staining of cells obtained after culture with IL-2 and analysed by flow cytometry showed no difference in the percentage expression of \( \gamma \delta \) TCR, CD4, CD8 or surface immunoglobulin, compared to the freshly isolated cells, as shown by Figure 8(iii).

Isolated cells cultured in media alone remained as small resting cells, as shown by Figure 7(iii). The percentage expression of \( \gamma \delta \) TCR, CD4 and CD8 by this population of cells cultured in media for five days was similar to those obtained on the freshly isolated population of cells, with greater than 90% of cells remaining \( \gamma \delta \) TCR-positive [Figure 8(iv)].
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep. γδ T cells were purified by MACS using T19-positive selection as described in Materials and Methods and cultured for 5 days in the presence of either 20μg/ml-1 of Con A, 100 pM of IL-2 or culture media alone. Viable lymphocytes were harvested by density centrifugation and analysed by flow cytometry for their forward and side light scatter profiles.
FIGURE 7. Scatter profiles of purified ovine γδ T cells cultured in Con A or IL-2.

(iii) Culture Media Alone

(ii) IL-2

(i) Con A
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep. γδ T cells were purified by MACS using T19-positive selection as described in Materials and Methods and cultured for 5 days in the presence of either 20μg/ml-1 of Con A, 100 pM of IL-2 or culture media alone. Viable lymphocytes were harvested by density centrifugation stained with either monoclonal antibodies 86D (IgG1), ST4 (IgG1), ST8 (IgM) or VPM8 (IgG1) followed accordingly by either anti-mouse IgG1-FITC or anti-mouse IgM-FITC. Cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 8. Immunofluorescence Analysis of γδ T Cells Purified by MACS and Cultured in Either Con A or IL-2.

(i) purified γδ T cells
(ii) purified γδ T cells cultured with Con A
(iii) purified γδ T cells cultured with IL-2
(iv) purified γδ T cells cultured in media alone
The data demonstrates that γδ T cells can be routinely purified by MACS, and in the
culture conditions tested there was no measurable expansion of contaminating
lymphocytes.

5.2.2. Positive Selection By MACS Did Not Activate γδ T Cells.
The T19 may function as a co-receptor molecule of γδ T cells which provides an
alternative pathway of T cell activation for ovine γδ T cells, distinct from the TCR ligand
occupancy pathway. It was important to determine if the purification technique adopted
causd activation of γδ T cells as a consequence of T19 perturbation. CD25 expression
by purified γδ T cells was assessed to determine if this was the case.

Ovine γδ T cells were positively selected as previously described, and cultured in media
for three days. At the end of three days viable lymphocytes were harvested over
lymphoprep and analysed for CD25 expression. As a control unfractionated PBMC
were treated in an identical manner. The immunofluorescence analysis of CD25
expression by purified γδ T cells and PBMC is shown in Figures 9 and 10, respectively.

Unfractionated PBMC were analysed by dual colour immunofluorescence analysis to
determine the percentage of γδ T cells co-expressing CD25. It was established that
12.8% of γδ T cells were CD25-positive (data not shown), whilst following their
purification by MACS positive selection 13.8% of γδ T cells expressed CD25 as shown
in Figure 9. After culturing for 3 days the percentage of CD25-positive γδ T cells had
risen to 21.7%. Similarly, the percentage of CD25-positive unfractionated PBMC
cultured in media alone for 3 days had risen from 14.6% to 23.3% (Figure 10).

These observations suggest that the increase in CD25 expression observed on the
purified population of ovine γδ T cells cultured for three days was a consequence of in
vitro culture conditions as γδ T cells within unfractionated PBMC showed a similar
percentage increase in CD25 expression. It may be argued that cellular activation by
positively selecting via the T19 molecule may require at least three days for CD25 to be
expressed on the cell surface. From data reported in Section 4.2. of this thesis this
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep. γδ T cells were purified by MACS using T19-positive selection as described in Materials and Methods and cultured for 3 days in media alone. Viable lymphocytes were harvested by density centrifugation stained with either monoclonal antibodies 86D (IgG1) or IL-111 (IgG1) followed by anti-mouse IgG1-FITC. Cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 9. CD25 Expression by MACS Purified γδ T Cells

After Culture in Media Alone.
Figure 10.

Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep and cultured for 3 days in media alone. Viable lymphocytes were harvested by density centrifugation and stained with the anti-CD25 monoclonal antibody, IL-A111, followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the immunofluorescence profiles are shown.
FIGURE 10. CD25 Expression by PBMC Before and After Culture in Media Alone.

Before Culture

After Culture

Background Immunofluorescence

CD25-positive cells

Fluorescence intensity

Relative cell number
seems an unlikely event as the data shows that following Con A activation of ovine γδ T cells, which involves T cell receptor crosslinking, the signal transduction event and subsequent IL-2 receptor mRNA transcription and translation occurs rapidly, as almost all γδ T cells express this protein by 24 hours. In addition, Lund et al (1993) have recently reported that ovine γδ T cells express CD25 within 8 hours of Con A activation.

A comparison was made of the proliferative response of MACS fractionated γδ T cells and unfractionated PBMC to human recombinant IL-2. Cells were cultured for three days in the presence of various concentrations of human recombinant IL-2. The dose response curves shown in Figure 11 demonstrate that with increasing IL-2 concentrations the proliferative response of the fractionated γδ T cells increased, with maximal proliferation obtained at 200pM. A similar trend was observed with the unfractionated population. Unfractionated PBMC gave a higher proliferation than fractionated γδ T cells. A possible explanation for the increased proliferation of unfractionated PBMC relative to the fractionated population was due to the fact that 34.8% of the CD4-positive population within unfractionated PBMC expressed CD25 (data not shown).

The data suggests that the positive selection of γδ T cells via the T19 molecule by MACS did not cause an increased expression of CD25 by these cells. Secondly, purified cells were capable of responding to IL-2 suggesting selection via T19 did not inactivate γδ T cells.

In summary the data demonstrates that positive selection of a population of ovine γδ T cells utilising MACS yields a population of cells that are not activated or in a state of unresponsiveness. These cells could subsequently be used for functional studies, in particular to study the response of γδ T cells to various antigens in vitro.

5.3. Investigation Of The Cytotoxic Potential Of The γδ Lineage.
Despite the availability of information on the localisation, genetical and biochemical properties of γδ T cells, their biological role still remains enigmatic and remains to be
Peripheral blood mononuclear cells were prepared from a 2 month old Grey Face x Suffolk sheep. γδ T cells were purified by MACS using T19-positive selection as described in Materials and Methods. 10^5 γδ T cells / well were cultured with various concentrations of IL-2 in flat-bottom plates. For comparison, 10^5 unfractionated PBMC / well were cultured in the same manner. The assays were incubated for 3 days and proliferation was measured by the uptake of 3H-thymidine over the last seven hours of culture.
FIGURE 11. Proliferative Response Of MACS Purified γδ T Cells To IL-2.

KEY:

- Purified γδ T Cells
- Unfractionated PBMs

The results shown in Figure 13 and demonstrate that antisera 375 reacted with a protein of approximately 30 KDa present in both the ovine lymphocytes and the murine CTL-Lyse.
fully clarified. This section has investigated the cytotoxic potential of ovine γδ T cells.

5.3.1. Expression Of Perforin By Ovine γδ T Cells.

Purified perforin has an apparent molecular mass of 70 KDa and has been shown to exist in human (Koizumi et al 1991, Nakata et al 1990) and murine (Youn et al 1991) cytotoxic T cells. P1-8 is a rat monoclonal antibody specific for murine perforin (Kawasaki et al 1990). The ability of the monoclonal antibody to react with ovine perforin was tested by immunocytochemistry. Figure 12a and 12b shows the results of staining cytopsins of ovine lymphocytes with monoclonal antibody P1-8 and normal rat sera as a negative control respectively. The data shows that the monoclonal antibody P1-8 failed to stain ovine lymphocytes in a manner that was significantly different to that obtained with normal rat sera. Figures 12c demonstrates the staining obtained with murine spleen lymphocytes as a positive control for P1-8 reactivity. Approximately 30% of the murine lymphocytes were stained by monoclonal antibody P1-8 and the staining was localised in cytoplasmic regions just below the cell membrane. Control staining of murine spleen lymphocytes with normal rat sera is shown in Figure 12d.

The absence of histochemical staining of ovine lymphocytes with monoclonal antibody P1-8 has at least two implications: (i) perforin is not sufficiently conserved between the murine and ovine species to obtain crossreactivity with the monoclonal antibody P1-8; or alternatively (ii) ovine lymphocytes do not express perforin. The former implication was tested by Western Blot analysis using rabbit polyclonal antisera, 275, which is specific for the native form of murine perforin. The positive control of the investigation was the ability of antisera 275 to react with a protein within a lysate prepared from an established murine cytotoxic cell line, CTL-L.

The results shown in Figure 13 and demonstrate that antisera 275 reacted with a protein of approximately 70 KDa present in both the ovine lymphocyte lysate and the murine CTL-L lysate.
Peripheral blood mononuclear cells were prepared from an 18 month old Finnish Landrace sheep and used for cytospins as described in Materials and Methods. Lymphocytes were stained with either: (a) monoclonal antibody P1-8; or (b) normal rat sera (b); followed by an anti-rat biotin-conjugated immunoglobulin and finally reacted with Horseradish peroxidase conjugated to streptavidin. Positive staining was achieved by developing with the chromogen, diaminobenzidin. A positive control was established with murine spleen lymphocytes, stained with either: (c) monoclonal antibody P1-8; or (d) normal rat sera. Staining was visualised by conventional microscopy.
FIGURE 12. Analysis of Perforin Expression in Ovine Lymphocytes Via Immunocytochemistry.

(a) Ovine Lymphocytes - PI- 8

(b) Ovine Lymphocytes - normal rat sera

(c) Murine Lymphocytes - PI- 8

(d) Murine Lymphocytes - normal rat sera
An ovine lymphocyte lysate was prepared from peripheral blood mononuclear cells of an 18 month old Finnish Landrace sheep, whilst a murine lymphocyte lysate was prepared from the cytotoxic cell line CTL-L as described in Materials and Methods. The presence of perforin in ovine lymphocytes was analysed by SDS-PAGE and Western blot analysis using rabbit polyclonal antisera, 275, specific for murine perforin. Background staining was established with normal rabbit sera.
FIGURE 13. Identification Of Perforin in Ovine Lymphocytes By Western Blot Analysis.

KEY:

(i) Mouse CTL-L lysate
(ii) Ovine Lymphocyte lysate
Figure 14.

γδ TCR-positive, CD4-positive and CD8-positive T cells were purified from the peripheral blood of an 18 month old Finnish Landrace animal by MACS positive selection, via the γδ TCR, CD4 and CD8 molecules, respectively. Lysate were prepared of each as described in Materials and Methods and analysed by SDS-PAGE and Western blot analysis for the presence of perforin using rabbit polyclonal antisera, 275, specific for murine perforin. Background staining was established with normal rabbit sera.

275 Antisera Normal Rabbit Sera

(i) (ii) (iii) (i) (ii) (iii)

KEY:
(i) Ovine CD8 Lysate
(ii) Ovine γδ Lysate
(iii) Ovine CD4 Lysate
To determine which subset(s) of ovine lymphocytes expressed perforin, positive selection by MACS was used to isolate populations of ovine T cell subsets which were subsequently analysed by Western Blot Analysis for perforin expression. The results shown in Figure 14 demonstrate that antisera 275 reacted with perforin present in a lysate of CD8 lymphocytes. In contrast, the 275 antisera did not show the presence of perforin in either the γδ T cell or the CD4 lysate. Sera obtained from an unimmunised animal, used at an equivalent dilution to antisera 275 (1/4000), failed to detect protein of molecular weight 70 KDa in any of the lysates.

The above data suggests that polyclonal antisera generated to murine perforin crossreacts with ovine perforin, and that perforin expression by ovine lymphocytes is restricted to the CD8-positive population.

5.3.2. Lack of Electron Dense Granules In Ovine Peripheral Blood γδ T Cells.

The above data suggests that ovine γδ T cells do not express perforin as do ovine CD8-positive T cells. This suggests that, either ovine γδ T cells do not mediate cytotoxic functions within the sheep, or their cytotoxicity is mediated by other proteins such as tumour necrosis factor (TNF) (Cairns et al 1992). It is known in human and murine cytotoxic lymphocytes that some mediators of target cell lysis are stored in electron dense granules within the cytoplasm which may be visualised by electron microscopy (Koizumi et al 1991).

Ovine γδ TCR, CD4 and CD8-positive lymphocytes were isolated from either PBMC or afferent lymph by positive selection using MACS and analysed by electron microscopy for the presence of electron dense granules.

Figure 15a shows an electron micrograph of an ovine γδ T cell and demonstrates that the cell was predominantly occupied by a regular nucleus rich in chromatin whilst the cytoplasm possessed well developed mitochondria. In contrast, electron micrograph sections of an ovine CD8-positive lymphocyte revealed features similar to those of the
Figure 15.

γδ TCR-positive, CD4-positive and CD8-positive T cells were purified from the peripheral blood of an 18 month old Finnish Landrace animal by MACS positive selection, via the γδ TCR, CD4 and CD8 molecules, respectively. The T cells were prepared for electron microscopy as described in Materials and Methods and visualised with a Phillips TEM 400 for the presence of cytoplasmic electron dense granules.
FIGURE 15. Detection of Electron Dense Granules Within the Cytoplasm of Ovine Lymphocytes.

(a) Cytoplasmic Electron Dense Granule
(b) CD8-Positive Cell
(c) TCR-Positive Cell

(c) Cytoplasmic Electron Dense Granule
γδ T cell but with the addition of cytoplasmic electron dense granules, as shown in Figure 15b. Furthermore, the granules were of a slightly smaller size than mitochondria and were in the range of 300 to 400 nm. A higher magnification of one of the electron dense granules and a mitochondria is shown in Figure 15c, and clearly shows that the two organelles are quite distinct. In all other species examined, electron dense granules were much smaller than those reported here for ovine CD8 cells, being approximately 100-160 nm in size. In addition, electron dense granules observed in ovine lymphocytes were less numerous than those reported in other species (Koizumi et al 1991), predominantly only one or two granules were apparent per cell, but in rare cases three or four granules were observed.

Four hundred cells within each purified T cell subset were examined and the percentage of cells expressing one or more electron dense granule(s) recorded. The data is shown in Table 2. Only 2.2% and 0.8% of ovine γδ T cells and CD4-positive T cells, respectively, had granules within their cytoplasm. In contrast, 46.1% of ovine CD8 lymphocytes contained cytoplasmic electron dense granule(s). The small percentage of γδ T cells and CD4-positive T cells having cytoplasmic electron dense granule(s) may be a consequence of contamination of CD8-positive lymphocytes within the purified populations. The absence of cytoplasmic electron dense granules from some CD8-positive lymphocytes may be interpreted to suggest that (i) the presence of granules defines distinct subsets of ovine CD8-positive lymphocytes, (ii) that electron dense granules may only be present in recently activated lymphocytes as suggested for CD8-positive cells from other species (Jose et al 1992) or (iii) the plane of the electron micrograph section failed to reveal electron dense granule(s).

5.3.3. Lack of Electron Dense Granule Expression By Ovine Afferent Lymph γδ T Cells.

It is suggested that T cells express electron dense granules after their activation (Nagler-Anderson et al 1989, Muller et al 1989, Young et al 1989). The majority of γδ T cells in afferent lymph express CD25 whilst less than 10% of peripheral blood γδ T cells are CD25-positive (section 7.2), implying that the majority of the former are activated. γδ T
Table 2.

γδ TCR-positive, CD4-positive and CD8-positive T cells were purified from the peripheral blood of an 18 month old Finnish Landrace animal by MACS positive selection, via the γδ TCR, CD4 and CD8 molecules, respectively. The T cells were prepared for electron microscopy as described in Materials and Methods and visualised with a Phillips TEM 400 for the presence of cytoplasmic electron dense granules. The percentage of cells expressing cytoplasmic electron dense granules is shown.
The above observations suggest that ovine +T cells in contrast to human and murine +T cells, do not express peroxidase or have cytoplasmic electron dense granules. However, peroxidase was found in lysates from CD8-positive T cells. This subset also expressed electron dense granules which seem larger and less numerous than those observed in the human species (Kozlowski et al., 1980). The granules in the ovine CD8 subset does not seem to correlate with activation of the cell as indeed by CD25 expression. An alternative explanation is that the presence of these granules is restricted to particular CD8 subsets which are less peripheral blood lymphocytes.

<table>
<thead>
<tr>
<th>lymphocyte subset</th>
<th>γ8</th>
<th>CD8</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of cells with granules</td>
<td>2.2%</td>
<td>46.1%</td>
<td>0.8%</td>
</tr>
<tr>
<td>% purity</td>
<td>90.2%</td>
<td>92.8%</td>
<td>91.9%</td>
</tr>
</tbody>
</table>
cells were purified by MACS positive selection from afferent lymph and examined by electron microscopy for electron dense granules.

The results in Table 3 show that ovine afferent lymph γδ T cells fail to express cytoplasmic electron dense granules even after in vivo activation. Only 1.1% of these cells were found to contain cytoplasmic granules. In contrast to 13.7% of afferent lymph CD8-positive lymphocytes contained electron dense granules. Less than 1% of afferent lymph CD4-positive lymphocytes were positive for these granules.

The above observations suggests that ovine γδ T cells in contrast to human and murine γδ T cells, do not express perforin or have cytoplasmic electron dense granules. However, perforin was found in lysates from CD8-positive T cells. This subset also contained electron dense granules which were larger and less numerous than those reported in the human species (Koizumi et al 1991). The presence of these electron dense granules in the ovine CD8 T cell subset does not seem to correlate with activation of the cell as judged by CD25 expression. An alternative explanation is that the expression of these granules is restricted to particular CD8 subsets which are less prevalent in afferent lymph compared to peripheral blood.
Afferent lymphocytes were collected from a cannulated afferent lymphatic vessel of an 18 month old Finnish Landrace animal. \(\gamma\delta\) TCR-positive, CD4-positive and CD8-positive T cells were purified from afferent lymph cells by MACS positive selection, via the \(\gamma\delta\) TCR, CD4 and CD8 molecules, respectively. The T cells were prepared for electron microscopy as described in Materials and Methods and visualised with a Phillips TEM 400 for the presence of cytoplasmic electron dense granules. The percentage of cells expressing cytoplasmic electron dense granules is shown.
TABLE 3. The Expression Of Cytoplasmic Electron Dense Granules By Ovine Afferent Lymph Lymphocytes (n = 600).

<table>
<thead>
<tr>
<th>lymphocyte subset</th>
<th>γδ</th>
<th>CD8</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of cells with granules</td>
<td>1.1%</td>
<td>13.7%</td>
<td>0.5%</td>
</tr>
<tr>
<td>% purity</td>
<td>95.3%</td>
<td>90.8%</td>
<td>92.9%</td>
</tr>
</tbody>
</table>
DISCUSSION.

Studies of lymphocyte function and cell/cell interaction are enhanced by the purification of lymphocyte subsets to homogeneity. Alternatively lymphocyte clones could be generated but the significance of any functional properties assigned to such clones is highly questionable as it is possible the cloning strategy modifies the functional and phenotypic characteristics of the original lymphocyte. This chapter has investigated various protocols that were used in an attempt to purify a homogeneous population of ovine γδ T cells. The salient features important in selecting a protocol that subsequently enabled the use of these cells in functional assays were: (i) ability to isolate a population of lymphocytes in a viable state; (ii) cell purity in terms of γδ TCR expression; (iii) the yield of purified cells.

An advantage of negative selection over positive selection of lymphocytes via cell surface molecules is that the isolated population is obtained without the binding of an antibody to a receptor molecule on the lymphocyte surface. However, despite this, during the evaluation of the various protocols investigated here it became apparent that the techniques employing negative selection yielded a final population of insufficient purity and low yield. This was likely to be the consequence of the requirement of numerous procedures necessary to deplete each distinct population of unwanted cells. Numerous washes were required because of the various antibody treatments and large numbers of cells were lost during each wash. In addition, the adherent step intended to deplete monocytes led also to excessive depletion of lymphocytes. A recent report has shown that human γδ T cells adhere to plastic upon culture by the formation of pseudopods (Arancia et al 1991). If ovine γδ T cells display analogous features upon culturing, then negative selection involving adherence would seriously deplete the recovery of ovine γδ T cells and may account for some of the large decrease in cell numbers observed above. In terms of the percentage purity and yield of the isolated population, positive selection via the T19 molecule incorporating MACS proved more successful than positive selection via the T19 molecule incorporating Dynabeads and Detachabeads and the negative selection techniques.
The purified population of γδ T cells were eventually to be used in functional studies to investigate the ligand that ovine γδ T cells may recognise. It was necessary that each protocol yielded a viable population of cells as antigen presentation to this population was to be assessed by lymphoproliferation. Any protocol failing to yield viable lymphocytes (where it was tested) was excluded on this basis. Considering all aspects, positive selection by MACS was by far the most satisfactory technique of obtaining a homogeneous population of viable ovine γδ T cells of >92% purity which were then used in functional assays.

Prior to the functional analysis of these purified γδ T cells it was established that: (i) upon culturing there was no outgrowth of contaminating lymphocytes; and (ii) three days after their isolation the percentage of γδ T cells expressing CD25 had not increased relative to the percentage within cultured PBMC population; (iii) purified cells were not inactivated as they were able to proliferate in the presence of IL-2. Collectively, this implied that in subsequent assays any observations made with this purified population of cells could be confidently assigned to γδ T cells.

In light of recent suggestions that γδ T cells provide immunity at epithelial sites eliminating autologous transformed or stressed cells (Janeway et al 1988, Tonegawa et al 1989), a cytotoxic potential may be expected for by this T cell subset. The cytotoxic potential of ovine γδ T cells was investigated. The data suggests that perforin does not play a role in ovine γδ T cells cytotoxicity, if they are truly cytotoxic. This is based on two observations. Firstly, that Western blot analysis with polyclonal rabbit antisera generated to mouse perforin was unable to detect this protein in a lysate of γδ T cells, but did so in a CD8 T cell derived lysate. Secondly, γδ T cells were characterised by the absence of cytoplasmic electron dense granules which were present in approximately 50% of CD8-positive peripheral blood lymphocytes. With regards to the latter it could be argued that electron dense granules containing proteins associated with target cell lysis are only found in activated γδ T cells. This argument may be eliminated by the observation that γδ T cells isolated from an afferent lymphatic vessel, where approximately 50% of this population express CD25, are also devoid of cytoplasmic electron dense granules. It is noteworthy that section 4.2. of this thesis has demonstrated
the appearance of CD25 expression on the surface of γδ T cells 24 hours after in vitro Con A activation, but if the formation of cytoplasmic electron dense granules containing mediators of target cell lysis require longer to appear, then the γδ T cells are likely to have entered the lymph node prior to their appearance. If the γδ lineage have the ability to mediate cytotoxicity they may do so via candidate molecules other than perforin, such as TNF (Cairns et al 1992), or alternatively, by as yet uncharacterised mediators of cytotoxicity which are not located within cytoplasmic granules visible by electron microscopy.

Published data by others suggests ovine γδ T cells are capable of killing allogeneic cells via MHC class I molecules, but it is noteworthy that the MHC restriction pattern of these cells was inconsistent (Hein and Mackay 1991). Despite no definitive conclusion generated within this investigation on the cytotoxic nature of ovine γδ T cells the study has emphasised two features that distinguish ovine γδ T cells from human and murine γδ T cells, in that, irrespective of their cytotoxic potential ovine γδ T cells (i) do not appear to express perforin, (ii) cytoplasmic electron dense granules are absent from ovine γδ T cells. These aspects highlight salient features of ovine γδ T cells that further distinguish them from γδ T cells of other species and suggests that the sheep is not only host to a larger percentage of γδ T cells than found in other species, but may also provide a modified form of immunity to their host relative to that provided by γδ T cells in humans and mice.
CHAPTER 6

In Vitro Ligand Recognition By Ovine γδ T Cells.

A diverse array of antigens have been identified as ligands for the γδ T cell receptor. These include tetanus toxoid (Kozbor et al. 1989), a synthetic polypeptide polyglutamate tyrosine (GAT) (Vidovsek et al. 1989) and bacterial superantigens such as staphylococcal enterotoxin A (Russ et al. 1990). In addition both humans and mice have a large number of γδ T cells that recognise mycobacterial antigens (Klabitz et al. 1990, Jats et al. 1989) including mycobacterial heat shock proteins (O'Brien et al. 1989). Finally some reports propose that γδ T cells respond to various self-antigens, such as cell surface immunoglobulin (Wright et al. 1990) and more recently autoantigens stress proteins (O'Brien et al. 1989).

The mechanisms by which γδ T cells recognize antigen remain undefined. It is noteworthy that despite the differences in the number of circulating γδ T cells in different species, the majority of mice, as well as human and murine, γδ T cells do not express CD4 or CD8 molecules. This suggests that either recognition of MHC class I and class II molecules by these cells does not involve the CD4 or CD8 accessory molecules in the manner as described for αβ T cells. Alternatively, these cells do not recognize MHC class I and class II molecules and may use other cell surface molecules as presensational elements during antigen recognition.

Considerable evidence indicates that γδ T cells recognize presensational elements which are relatively non-polymorphic (Tongawa et al. 1989, Jineway et al. 1988). Some of these are similar but distinct from the MHC class I molecules and are termed class Ib molecules. Molecular analysis of the genomic organization of the human and murine genomic has demonstrated that these class Ib molecules are encoded within the MHC. In humans these molecules are encoded within loci referred to as HLA-E, HLA-F and HLA-G and in mice (Rothbard et al. 1989), the Q and TL loci (Flaherty et al. 1989).

Evidence to support γδ T cell recognition of class Ib molecules is from the mouse where γδ T cell lines and clones, independently derived, recognise a nonpolymorphic MHC-linked molecule mapping within the TL region (Bo et al. 1989, Vidovsek et al. 1989). In addition, other molecules implied to function as presensational structures for human γδ T
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cells are the CD1 molecules (Porcelli et al 1991). These comprise a set of glycoproteins evolutionary related to MHC class I molecules but are encoded by genes outside of the MHC (Calabi and Milstein 1986). CD1 antigens associate with β2-microglobulin in an analogous manner to the MHC encoded class I molecules (Terhost et al 1981).

If γδ T cells do recognise class Ib or non-MHC encoded molecules it remains to be established whether these molecules present peptides to γδ T cells as do classical MHC molecules to αβ T cells. Although no direct evidence is currently available, two pieces of evidence imply this is the case. Firstly, the structural similarities between the γδ and the αβ TCR, and between the MHC encoded and related presentational molecules recognised by the two types of TCR, suggests that γδ T cells recognise peptides as described for the αβ lineage (Jorgensen et al 1992). Secondly, Porcelli et al (1992) have shown that CD1b molecules present immunogens to an αβ TCR expressing T cell line in the form of processed peptides located within a putative peptide binding groove of these molecules. In view of this data it is likely that the γδ T cell line reported by Porcelli et al (1989), which lysed Molt-4 cells did so by recognition of specific peptide associated with CD1c.

To establish that ovine γδ T cells could indeed respond to antigens, initial experiments investigated the response by these cells in unfractionated PBMC using Mycobacterium tuberculosis, PPD and ovalbumin. Purified populations of MACS separated peripheral blood γδ T cells were then used to show that ovine γδ T cells could proliferate to exogenously added antigen. Data reported in section 4.2 of this thesis has shown that subsequent to in vitro Con A activation, ovine γδ T cells express CD25 prior of their αβ counterparts, implying that γδ T cells are activated before the αβ lineage. This initiated a study to determine the optimum in vitro culture period of a population of purified γδ T cells in the presence of antigen. Finally, the presentational elements that γδ T cells may recognise in association with antigen were investigated. This study focused on the classical MHC molecules, MHC class I and II, for three reasons: (i) some data reports that γδ T cells recognise classical MHC molecules (Matis et al 1989, Bluestone et al 1988); (ii) homologues of the human and murine CD1 antigens have been described in the sheep (Dutia and Hopkins 1991) but the generation of defined reagents specific for
these molecules is awaited; (iii) no data is available on MHC class Ib molecules encoded within the ovine genome.

6.1 Response Of γδ T Cells Within PBMC To Antigen.

In the absence of polyclonal or monoclonal ovine γδ T cell lines, PBMC were used to investigate the response of ovine γδ T cells to antigen in vivo. PBMC were isolated from non-primed animals (for use in a primary in vitro proliferative response) and animals primed with Mycobacterium tuberculosis (for use in a secondary in vitro proliferative response). PBMC were cultured in the presence of antigen for 5 days after which live viable lymphocytes were isolated and expanded by the addition of exogenous IL-2 over a period of 14 days. At various stages of these cultures, cells were analyzed by single colour immunofluorescence staining to determine the percentage of each T cell subset present, and the ratio of αβ/γδ T cells.

Table 1 shows the percentage of T cell subsets, and the αβ/γδ ratio, of PBMC cultured in a primary and secondary proliferative response to Mycobacterium tuberculosis. Four animals were investigated and a representative set of data from one animal is shown. In both responses to this antigen γδ T cells appear to proliferate as the percentage of these cells was increased after 5 days of culture compared to the percentage of γδ T cells present at the start of culture. In the primary culture the percentage of CD4-positive cells also increased, whilst the percentage of the CD8 and surface immunoglobulin-positive populations decreased. In the secondary culture the percentage of both the CD4-positive and CD8-positive populations increased, whilst only the surface immunoglobulin-positive population showed a decrease. These changes are reflected in the decrease of the αβ/γδ ratio after 5 days culture compared to the control values. In the primary response the ratio decreased from 3.1 to 1.8, whilst in the secondary response the ratio decreased from 3.3 to 2.4.

When these Mycobacterium tuberculosis antigen activated cells were expanded by the addition of exogenous IL-2 there appeared to be a greater expansion of αβ rather than γδ T cells as shown by the data in Table 1. This was seen in both the primary and secondary cultures as shown by the increase in the ratio of αβ/γδ T cells from 1.8 to 4.0, and from 2.4 to 18.4, respectively. Similar results were obtained with PPD and
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Table 1.
Peripheral blood mononuclear cells were prepared from an antigen-primed or non-primed 18 month old Finnish Landrace sheep. $10^5$ cells / well were cultured with 10$\mu$g/ml$^{-1}$ of heat-killed *Mycobacterium tuberculosis*. After 5 days incubation viable cells were harvested by density centrifugation and cultured in medium supplemented with 100 pM of IL-2 for 14 days with fresh additions of IL-2 every 3-4 days. At the various time points indicated viable cells were harvested by density centrifugation and stained with either monoclonal antibodies ST4, ST8, 86D or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry. The data is a representative set of data from a group of 4 animals. Included in brackets is the range of the data from the 4 animals.
TABLE 1. Expansion Of γδ T Cells In Vitro to Mycobacterium Antigen.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage positive cells</th>
<th>CD4+CD8 γδ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>28.4 (23.0-28.4)</td>
<td>48.0 (48.0-67.8)</td>
</tr>
<tr>
<td>5 days post antigen stimulation</td>
<td>30.6 (17.5-30.6)</td>
<td>40.5 (40.5-56.9)</td>
</tr>
<tr>
<td>7 days post rIL-2</td>
<td>39.4 (20.3-39.4)</td>
<td>4.6 (4.6-9.7)</td>
</tr>
<tr>
<td>14 days post rIL-2</td>
<td>40.3 (15.3-40.3)</td>
<td>5.4 (2.90-5.9)</td>
</tr>
<tr>
<td><strong>Secondary Response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>21.2 (21.2-31.6)</td>
<td>66.6 (49.6-66.6)</td>
</tr>
<tr>
<td>5 days post antigen stimulation</td>
<td>23.2 (22.4-29.4)</td>
<td>57.1 (57.1-68.0)</td>
</tr>
<tr>
<td>7 days post rIL-2</td>
<td>46.4 (46.4-77.3)</td>
<td>20.3 (16.3-36.0)</td>
</tr>
<tr>
<td>14 days post rIL-2</td>
<td>49.0 (49.0-83.1)</td>
<td>5.5 (4.3-8.9)</td>
</tr>
</tbody>
</table>

Note: Values in parentheses indicate ranges.
ovalbumin in secondary cultures (data not shown).

Whilst the data appears to indicate an increase in γδ T cells during the first 5 days of the culture an investigation into the number of γδ T cells would be necessary to show conclusively that this was the case. Such an analysis would eliminate ambiguity that a percentage increase of γδ T cells was observed as a consequence of selective cell death. However, these preliminary experiments were used as only a basis for further investigation into antigen recognition by ovine γδ T cells.

6.2. Comparison Of γδ T Cell Response In Three And Five Day Proliferation Assays.

The above data indicates that during the initial response to antigen there appears to be a preferential expansion of ovine γδ T cells not only in a primary and secondary in vitro response with Mycobacterium antigens but also in a secondary response to ovalbumin and PPD. However, there is a greater expansion of the αβ T cell subset in the cultures upon the addition of exogenous IL-2. In addition, the data reported in sections 4.2 of this thesis suggested that ovine γδ T cells were not only activated at a quicker rate than αβ T cells but also lose the expression of various activation markers prior of the αβ lineage. If this is the case, then collectively, the data suggests that at least during in vitro culture, optimum response by γδ T cells may be seen before the end of 5 days of culture. Lymphocyte proliferation assays using human, mouse and sheep lymphocytes generally use a 5 day culture period to assess the response of T cells to presented antigen but as the intention of the experiments in this chapter was to investigate the in vitro proliferation of γδ T cells in the presence of various antigens, it was necessary to establish the optimum culture period for the proliferation of these cells. 3 and 5 day proliferation assays using purified γδ T cells were investigated.
Peripheral blood mononuclear cells were prepared from an antigen-primed 6 month old Grey Faced x Suffolk sheep. $10^5$ cells/well were cultured in the presence of 10$\mu$gml$^{-1}$ of heat-killed *Mycobacterium tuberculosis*, 40$\mu$gml$^{-1}$ of PPD or 250$\mu$gml$^{-1}$ of ovalbumin in flat-bottom plates. Cultures were incubated for either 3 or 5 days and proliferation was measured by the uptake of $^3$H-thymidine over the last 7 hours of culture.
FIGURE 1. Responses By PBMC To Antigen In Three and Five Day Proliferation Assays.

SHEEP 2104

Day 3  Day 5

SHEEP 2119

Day 3  Day 5

KEY:
- Stimulating Antigen
  - Mycobacterium tuberculosis
  - PPD
  - Ovalbumin
  - Control

The data in sections 6.3 and 6.4 show the relationships and implications of antigen recognition by ovine γδ T cells. Firstly, that these cells are capable of...
6.2.1. Responses By \( \gamma \delta \) T Cells To Antigen In Three And Five Day Proliferation Assays.

To show that ovine lymphocytes could show a proliferative response to antigen in a 3 day assay, PBMC from young animals primed with *Mycobacterium tuberculosis* and ovalbumin were cultured for various times with these antigens. Figure 1 shows the proliferative response by these cells. In the case of PBMC from sheep 2104 the same magnitude of response to each antigen was the seen following 3 or 5 days of culture. In addition, similar background proliferative responses were observed, which resulted in similar stimulation indices at both time points. With PBMC from sheep 2119 not only was an increased magnitude of proliferation observed on day 3, compared to day 5, but the background proliferation was lower on day 3 relative to day 5 resulting in greater stimulation indices on day 3 relative to day 5.

This data indicates that responses to antigen by unfractionated ovine PBMC can be measured after 3 days of culture. As these populations of cells contain relatively high numbers of \( \gamma \delta \) T cells, and as \( \gamma \delta \) T cells appear to be activated prior to \( \alpha \beta \) T cells, a 3 day culture period may be optimum for antigen specific proliferative responses by these cells. To confirm this was the case ovine \( \gamma \delta \) T cells were purified from peripheral blood of antigen primed animals and cultured with autologous irradiated PBMC in the presence of various concentrations of different antigens for 3 and 5 days.

The results in Figure 2 show that a purified population of \( \gamma \delta \) T cells proliferate in an *in vitro* secondary response to *Mycobacterium tuberculosis*, PPD and ovalbumin, with a greater magnitude of response on day 3 compared to day 5. In addition, background proliferation of the cells in the presence of antigen presenting cells and culture medium alone was higher on day 3 yielding stimulation indices on day 3 similar to those of day 5. \( \gamma \delta \) T cells were purified from two other animals and assayed in the same manner as described above. The results of these assays showed the same trends as shown in Figure 2 (data not shown).

The data in sections 6.1 and 6.2 shows three fundamental and important aspects of antigen recognition by ovine \( \gamma \delta \) T cells. Firstly, that these cells are capable of
Figure 2.

γδ T cells were purified from the peripheral blood of an antigen-primed 6 month old Grey Face x Suffolk sheep by MACS using T19-positive selection as described in Materials and Methods. 10^5 γδ T cells/well were cultured with 10^5 autologous irradiated PBMC/well in the presence of either 10μg/ml^-1 of heat-killed *Mycobacterium tuberculosis*, 40μg/ml^-1 of PPD or 250μg/ml^-1 of ovalbumin in flat-bottom plates. Cultures were incubated for either 3 or 5 days and proliferation was measured by the uptake of ^3^H-thymidine over the last 7 hours of culture.
FIGURE 2. Responses By γδ T Cells To Antigen In Three and Five Day Proliferation Assays.

Day 3

Day 5

KEY:

Stimulating Antigen

- Mycobacterium tuberculosis
- PPD
- Ovalbumin
- Control

FIGURE 3 shows the results obtained in a comparison of the proliferative response by γδ T cells to antigen. Ovine γδ T cells yielded stimulation indices in the range of 2-7 in the...
responding to various antigens, secondly that antigen presenting cells and the presentational elements used for presentation to ovine γδ T cells are present within the PBMC population, and thirdly that a 3 day in vitro proliferation assay can be used to observe responses to antigen by these cells. The majority of subsequent assays involving purified populations of γδ T cells were cultured for 3 days in vitro, except where assays were established prior to this comparison.

6.3. Response Of Purified Populations Of γδ T Cells To Mycobacterium tuberculosis And Ovalbumin

It has been established above that ovine γδ T cells have the ability to respond to antigens in secondary in vitro proliferation assays. It has been shown in other species that γδ T cells can respond, in the absence of prior immunisations, to Mycobacterial antigens (Kabelitz et al 1991) and in some cases to stress proteins (O'Brien et al 1991). Because of data in this thesis implying a memory phenotype of ovine γδ T cells, the response of these cells in primary in vitro responses was investigated. Populations of ovine γδ T cells were purified from the PBMC of unprimed animals and their primary response to selected antigens investigated. The animals were subsequently primed with antigen and the in vitro proliferative responses repeated with purified γδ T cells to observe the secondary response mediated by these cells.

6.3.1. Primary Response Of Ovine γδ T Cells To Antigen In Vitro.

γδ T cells were purified from 8 animals and the primary in vitro proliferation of these cells were investigated. Stimulation indices (calculated as the numerical value of proliferation over background at a final concentration of 9.3 μg/ml⁻¹ for Mycobacterium tuberculosis and 250 μg/ml⁻¹ for ovalbumin) are presented which allowed a comparison of the proliferative responses between different animals.

Figure 3 shows the results obtained in a primary in vitro proliferative response by γδ T cells to antigen. Ovine γδ T cells yielded stimulation indices in the range of 2-7 in the
**Figure 3.**

γδ T cells were purified from peripheral blood of non-primed 1 month old Grey Face x Suffolk lambs (n=8) by MACS using T19-positive selection as described in Materials and Methods. 10⁵ γδ T cells / well were cultured with 10⁵ autologous irradiated PBMC / well in the presence of either 9.3μgml⁻¹ of heat-killed *Mycobacterium tuberculosis* or 250μgml⁻¹ ovalbumin in flat-bottom plates. For comparison the proliferative response of 10⁵ unfractionated PBMC / well are included. Cultures were incubated for 5 days and proliferation was measured by the uptake of ³H-thymidine over the last 7 hours of culture. Stimulation indices were calculated as proliferation over background and are shown. Each symbol represents data from one animal. Statistical analysis was carried out using a Two Sample T test from the Minitab software package.
FIGURE 3. Stimulation Indices Of γδ T Cells to Antigen In A Primary In Vitro Proliferation Assay.

The stimulation indices of the unstimulated PBMC to Mycobacterium tuberculosis were in the range of 1-8, whilst stimulation indices were in the range of 1-8. The stimulation indices obtained to PPD are not shown but were either slightly lower than those obtained for Mycobacterium tuberculosis. The data indicates that ovine γδ T cells are capable of proliferating, albeit weakly, to Mycobacterium tuberculosis but are unable to respond to the soluble protein antigen, ovalbumin, in a primary in vitro response.

The results of the investigation are shown in Figure 3. In a secondary in vitro proliferative response to Mycobacterium tuberculosis, the γδ T cells yielded stimulation indices in the range of 5-8, and to ovalbumin in the range of 3-15. On most occasions the stimulation indices obtained for PPD were equivalent to those observed to
presence of *Mycobacterium tuberculosis*, whilst in contrast, negligible stimulation indices were obtained with ovalbumin. Statistical analysis confirmed that 7/8 animals showed significant proliferation to *Mycobacterium tuberculosis* (*p* < 0.05). For comparison the stimulation indices obtained with unfractionated PBMC from the same animals are included. The stimulation indices of the unfractionated PBMC to *Mycobacterium tuberculosis* were in the range of 1-8, whilst stimulation indices were to ovalbumin in the range of 1-6. The stimulation indices obtained to PPD are not shown but were similar or slightly lower than those obtained for *Mycobacterium tuberculosis*. The data implies that ovine γδ T cells are capable of proliferating, albeit weakly, to *Mycobacterium tuberculosis* but are unable to respond to the soluble protein antigen, ovalbumin in a primary *in vitro* response.

Figure 4 shows a dose response curve obtained for γδ T cells cultured *in vitro* in a primary response to *Mycobacterium tuberculosis* and ovalbumin. From Figure 4 it can be seen that maximum proliferation to *Mycobacterium tuberculosis* was obtained at a concentration of 9.3 µgml⁻¹ and to ovalbumin at a concentration of 250 µgml⁻¹. Statistical analysis confirmed that significant proliferation to *Mycobacterium tuberculosis* was obtained within a concentration range of 3.1 µgml⁻¹ - 83.3 µgml⁻¹ (*p* < 0.05).

6.3.2. Secondary Response Of Ovine γδ T Cells To Antigen In Vitro.

After the completion of the primary *in vitro* responses the eight animals assayed above were antigen primed. Subsequently, γδ T cells were purified from PBMC of these animals and assayed for their secondary response to *Mycobacterium tuberculosis*, PPD, and ovalbumin *in vitro*.

The results of the investigation are shown in Figure 5. In a secondary *in vitro* proliferative response to *Mycobacterium tuberculosis* the γδ T cells yielded stimulation indices in the range of 5-8, and to ovalbumin in the range of 3-15. On most occasions the stimulation indices obtained for PPD were equivalent to those observed to
Figure 4.

γδ T cells were purified from the peripheral blood of a non-primed 1 month old Grey Face x Suffolk lamb by MACS using T19-positive selection as described in Materials and Methods. $10^5$ γδ T cells / well were cultured with $10^5$ autologous irradiated PBMC / well with various concentrations of either heat-killed *Mycobacterium tuberculosis* or ovalbumin in flat-bottom plates. Cultures were incubated for 5 days and proliferation was measured by the uptake of $^3$H-thymidine over the last 7 hours of culture. Statistical analysis was carried out using a Two Sample T test from the Minitab software package.
FIGURE 4. Primary In Vitro Proliferative Response of Ovine γδ T Cells To Antigen.

KEY:
- □—Mycobacterium tuberculosis
- ◯—Ovalbumin
- □—Control

* p < 0.05 relative to control
Figure 5.

γδ T cells were purified from peripheral blood of antigen-primed 3 month old Grey Face x Suffolk lambs (n=8) by MACS using T19-positive selection as described in Materials and Methods. 10^5 γδ T cells / well were cultured with 10^5 autologous irradiated PBMC / well in the presence of either 9.3μgml⁻¹ of heat-killed Mycobacterium tuberculosis or 250μgml⁻¹ ovalbumin in flat-bottom plates. For comparison the proliferative response of 10^5 unfractionated PBMC / well are included. Cultures were incubated for 5 days and proliferation was measured by the uptake of ^3H-thymidine over the last 7 hours of culture. Stimulation indices were calculated as proliferation over background and are shown. Each symbol represents data from one animal. Statistical analysis was carried out using a Two Sample T test from the Minitab software package.
FIGURE 5. Stimulation Indices Of γδ T Cells to Antigen In A Secondary In Vitro Proliferation Assay.
Mycobacterium tuberculosis, whilst on the other occasions they were higher. Statistical analysis confirmed that 6/8 animals showed significant proliferation to Mycobacterium tuberculosis \((p < 0.05)\), whilst 5/8 animals showed significant proliferation to ovalbumin \((p < 0.05)\). The stimulation indices obtained with the unfractionated PBMC to both Mycobacterium tuberculosis and ovalbumin are included for comparison and show the increased response by these cells to both antigens relative to the stimulation indices observed in the primary proliferation assays.

A typical dose response curve of the proliferation of a purified population of \(\gamma 8\) T cells to varying concentrations of Mycobacterium tuberculosis and ovalbumin is shown in Figure 6. Statistical analysis confirmed that significant proliferation was observed with Mycobacterium tuberculosis within a concentration range of 1.0 \(\mu\)gml\(^{-1}\) - 9.3 \(\mu\)gml\(^{-1}\) \((p < 0.05)\), whilst significant proliferation to ovalbumin was observed within a concentration range of 27.8 \(\mu\)gml\(^{-1}\) - 250 \(\mu\)gml\(^{-1}\) \((p < 0.05)\). Furthermore it appears that the proliferative responses were relatively low in terms of cpm values but this appears to be a general phenomenon characteristic of \(\gamma 8\) T cells (Kabelitz et al 1990). The concentration of Mycobacterium tuberculosis that stimulates maximum proliferation was at 9.3 \(\mu\)gml\(^{-1}\) and of ovalbumin at 250 \(\mu\)gml\(^{-1}\).

The above data indicates that the stimulation indices of \(\gamma 8\) T cells to Mycobacterium tuberculosis in a primary and secondary in vitro culture were similar, whilst those obtained to ovalbumin were different, with no proliferation observed in a primary proliferation assay but a response seen in the secondary proliferation assay. Based on statistical analysis it appears that ovine \(\gamma 8\) T cells are capable of responding, albeit weakly to Mycobacterium tuberculosis in both primary and secondary in vitro assays as greater that 50% of the animals showed significant proliferation \((p < 0.05)\). In contrast, it appears that \(\gamma 8\) T cells are only capable of responding to ovalbumin in a secondary in vitro assay.
Figure 6.
γδ T cells were purified from the peripheral blood of an antigen-primed 3 month old Grey Face x Suffolk lamb by MACS using T19-positive selection as described in Materials and Methods. 10^5 γδ T cells/well were cultured with 10^5 autologous irradiated PBMC/well with various concentrations of either heat-killed *Mycobacterium tuberculosis* or ovalbumin in flat-bottom plates. Cultures were incubated for 5 days and proliferation was measured by the uptake of ^3^H-thymidine over the last 7 hours of culture. Statistical analysis was carried out using a Two Sample T test from the Minitab software package.

It is clear from the above data that ovine γδ T cells can respond in *vitro* by proliferation to various antigen presentations, indicating cells within the PBMC population. The recognition by γδ T cells of antigen is assumed to occur in a manner analogous to that by αβ T cells, namely the receptors for antigen on these cells bind a complex composed of an antigenic determinant and antibodies to the antigen. MHC class I molecules may be used to investigate the recognition of immunological cells, as used in the present study, by ovine γδ T cells.

The responses were established with γδ T cells from the different animals were retested to confirm the preliminary data obtained.

6.4.1. Interaction Of γδ T Cell Response To Antigen By Anti-MHC Class I Monoclonal Antibody.

To date, the only data available on γδ T cell interaction with MHC class I molecules are fragmentary. The monoclonal antibody VPM19 is specific for ovine MHC class I (Hopp and Delia 1987). This study was formulated to investigate if the proliferative response of ovine γδ T cells to antigen was a consequence of the recognition of antigen in association with MHC class I molecules. The monoclonal antibody, IC3, The inducting antigenic PBMV used as a source of antigen presenting cells were incubated in the presence of the monoclonal antibodies for 30 minutes at 37°C, prior to the addition of the other cells. Figure 7 shows the results of the investigation.

**KEY:**
- □ *Mycobacterium tuberculosis*
- ♦ Ovalbumin
- • Control

*p < 0.05 relative to control*
6.4. Monoclonal Antibody Blocking Experiments Of γδ T Cell Responses To Antigen.

It is clear from the above data that ovine γδ T cells can respond in vitro by proliferation to various antigens presented by autologous antigen presenting cells within the PBMC population. The recognition by γδ T cells of antigen is assumed to occur in a manner analogous to that by αβ T cells, namely the receptors for antigen on these cells bind a complex comprising antigen and presentational element. If this is the case then inhibition studies by monoclonal antibodies to ovine γδ TCR, MHC class I and class II molecules may be used to investigate the recognition of presentational elements used in the response to antigen by ovine γδ T cells.

Ovine γδ T cells were purified from PBMC of antigen primed animals by MACS using T19-positive selection as described in section 2.7.4. and cultured as described in section 2.6.7. The assays were established with γδ T cells isolated from three different animals and were repeated to confirm the preliminary data obtained.

6.4.1. Inhibition Of γδ T Cell Response To Antigen By Anti-MHC Class I Monoclonal Antibody.

To date, the only data available on MHC restriction of ovine γδ T cells suggests they recognise allogeneic target cells in an MHC class I restricted manner (Mackay et al 1988a). This study was formulated to investigate if the proliferative response of ovine γδ T cells to antigen was a consequence of the recognition of antigen in association with MHC class I molecules. Monoclonal antibody VPM19 is specific for ovine MHC class I (Hopkins and Dutia 1990), and was used at final concentrations indicated. Control plates were established with equivalent concentrations of an isotype matched irrelevant monoclonal antibody, IC3. The irradiated autologous PBMC used as a source of antigen presenting cells were incubated in the presence of the monoclonal antibodies for 30 minutes at 37°C, prior to the addition of any other cells. Figure 7 shows the results of the investigation.
Figure 7.

γδ T cells were purified from the peripheral blood of an antigen-primed 12 month old antigen-primed Grey Face x Suffolk lamb by MACS using T19-positive selection as described in Materials and Methods. 10^5 autologous irradiated PBMC / well were incubated in the presence of either 9.3μgml^-1 of heat-killed *Mycobacterium tuberculosis* or 250μgml^-1 ovalbumin and various concentrations of the anti-MHC class I monoclonal antibody, VPM19, for 30 minutes at 37°C prior to the addition of γδ T cells. Subsequently, 10^5 γδ T cells / well were added to these cultures. Control wells were established with an equivalent concentration of an irrelevant isotype matched antibody. The cultures were established in flat-bottom plates and incubated for 3 days. Proliferation was measured by the uptake of ^3^H-thymidine over the last 7 hours of culture. Statistical analysis was carried out using a Two Sample T test from the Minitab software package.
FIGURE 7. Inhibition Of γδ T Cells Response To Antigen By Anti-MHC Class I Monoclonal Antibody.

**KEY:**
- VPM 19 (anti MHC Class I)
- IC3 (irrelevant mAb)
- γδ T cells + APCs

* p < 0.05 relative to control
The data clearly implies that in the presence of an anti-MHC class I monoclonal antibody, significant inhibition of the secondary proliferative responses of ovine γδ T cells to *Mycobacterium tuberculosis* and ovalbumin was observed within an antibody concentration range of 3.1 μg ml⁻¹ - 30 μg ml⁻¹ (p < 0.05). In addition, the proliferative response to PPD was inhibited (data not shown). The extent of the inhibition was dependent on the antigen and the animal under investigation but in no situation was 100% inhibition observed suggesting that not all γδ T cells recognise antigen in association with MHC class I molecules.

6.4.2. Inhibition Of γδ T Cell Response To Antigen By Anti-MHC Class II Monoclonal Antibody.

The presentation of antigen in association with MHC class II to ovine γδ T cells was investigated since data is available showing that γδ T cells of other species recognise exogenous antigen in association with MHC class II molecules (Matis et al 1989, Bluestone et al 1988) and the above data implies that not all ovine γδ T cells respond to antigen in association with MHC class I molecules. Monoclonal antibody SW73.2 (Hopkins et al 1986), which recognises the β chain of all known ovine MHC class II molecules was used at the indicated final concentrations in a manner identical to that described above. Figure 8 shows the results of the investigation.

In the presence of the anti-MHC class II monoclonal antibody SW73.2, the secondary *in vitro* proliferative response to both *Mycobacterium tuberculosis* and ovalbumin were reduced, as was the response to PPD (data not shown). Significant inhibition was observed within an antibody concentration range of 0.37 μg ml⁻¹ - 3.3 μg ml⁻¹ (p < 0.05). The inhibition appeared to be dependent on both the animal and antigen under investigation. A greater inhibition of the response to ovalbumin was observed in the presence of anti-MHC class II monoclonal antibodies compared to the inhibition of the response to *Mycobacterium tuberculosis*. The absence of complete inhibition implies the presence of γδ T cells within the cultures that were proliferating to antigen in


Figure 8.

γδ T cells were purified from the peripheral blood of an antigen-primed 12 month old antigen-primed Grey Face x Suffolk lamb by MACS using T19-positive selection as described in Materials and Methods. 10^5 autologous irradiated PBMC/well were incubated in the presence of either 9.3µgml^{-1} of heat-killed *Mycobacterium tuberculosis* or 250µgml^{-1} ovalbumin and various concentrations of the anti-MHC class II monoclonal antibody, SW73.2, for 30 minutes at 37°C prior to the addition of γδ T cells. Subsequently, 10^5 γδ T cells / well were added to these cultures. Control wells were established with an equivalent concentration of an irrelevant isotype matched antibody. The cultures were established in flat-bottom plates and incubated for 3 days. Proliferation was measured by the uptake of ^3^H-thymidine over the last 7 hours of culture. Statistical analysis was carried out using a Two Sample T test from the Minitab software package.
FIGURE 8. Inhibition Of γδ T Cells Response To Antigen By Anti-MHC Class II Monoclonal Antibody.

**6.6.3. Response Of γδ T Cells To Antigen In The Presence Of An Anti-γδ TCR Monoclonal Antibody.**

Myobacterium tuberculosis

Ovalbumin

Concentration of Antibody μg ml⁻¹

**KEY:**

- SW73.2 (anti MHC Class II)
- MS121 (irrelevant mAb)
- γδ T cells + APCs

* p < 0.05 relative to control
association with other presentational elements than MHC class II.

6.4.3. Response Of γδ T Cells To Antigen In The Presence Of An Anti-
γδ TCR Monoclonal Antibody.

86D is a monoclonal antibody which recognise the ovine γδ T cell receptor complex (Mackay et al 1989). As this monoclonal antibody binds all γδ TCR bearing cells the monoclonal antibody presumably binds a framework or constant region of the TCR molecule. The ability of 86D to inhibit the response by purified γδ T cells to antigen presented by autologous PBMC was investigated. The results obtained are shown in Figure 9. Monoclonal antibody 86D was unable to inhibit proliferation to either Mycobacterium tuberculosis, PPD (data not shown) or ovalbumin in these assays. Statistical analysis confirmed that there was no significant difference in the proliferative response in the presence of 86D (p > 0.05). Immunofluorescence analysis showed that incubation of γδ T cells with 86D for 30 minutes followed by anti-mouse Ig-FITC, was sufficient time for the monoclonal antibody to label these cells (data not shown), indicating that 86D had reacted with its epitope.
Figure 9.

γδ T cells were purified from the peripheral blood of an antigen-primed 12 month old antigen-primed Grey Face x Suffolk lamb by MACS using T19-positive selection as described in Materials and Methods. 10^5 γδ T cells / well were incubated with various concentrations of the anti-γδ TCR monoclonal antibody, 86D, for 30 minutes at 37°C. Subsequently, 10^5 autologous irradiated PBMC / well and either 9.3μg/ml of heat-killed *Mycobacterium tuberculosis* or 250μg/ml ovalbumin was added to the cultures. Control wells were established with an equivalent concentration of an irrelevant isotype matched antibody. The cultures were established in flat-bottom plates and incubated for 3 days. Proliferation was measured by the uptake of ³H-thymidine over the last 7 hours of culture. Statistical analysis was carried out using a Two Sample T test from the Minitab software package.
FIGURE 9. Inhibition Of γδ T Cells Response To Antigen By Anti-γδ TCR Monoclonal Antibody.

Mycobacterium tuberculosis

Ovalbumin

Concentration of Antibody μg ml⁻¹

KEY:

- 86D (γδ TCR)
- IC3 (irrelevant mAb)
- γδ T cells + APCs
DISCUSSION.

To elucidate the biological role of γδ T cells, it is essential to obtain information on the ligands and presentational elements that govern antigen recognition by these cells. Numerous reports on antigen recognition by human peripheral blood γδ T cells show that a significant number of these cells appear to respond to mycobacterial antigens and evolutionary conserved stress proteins. It is noteworthy that γδ T cells constitute only a minor population of human PBMC and have limited variable region usage within their TCR repertoire. In contrast, the high proportion of γδ T cells within sheep PBMC and the extensive diversity within the variable regions of ovine γδ TCR (Hein and Dudler 1993), relative to the human (Kabelitz 1992) and murine (Allison and Havran 1991) species, suggests that this lineage of T cells has developed different levels of genetic and perhaps functional complexity within various species. This may imply that ovine γδ T cells recognise a more varied set of antigens than the mycobacterial related antigens implicated to stimulate approximately 5-50% of human γδ T cells (Kabelitz et al 1990) and some murine γδ T cell clones (O'Brien et al 1989).

This Chapter has investigated the ability of ovine γδ T cells to proliferate in response to both Mycobacterial antigens and a soluble protein antigen, namely ovalbumin. The main aspects of the study were focussed on investigating: (i) the in vitro response of γδ T cells within unfractionated PBMC to antigens; (ii) establishing the optimum culture in vitro period of γδ T cells; (iii) a comparison of the primary and secondary in vitro proliferation by γδ T cells; and finally (iv) the presentational elements that govern antigen recognition by ovine γδ T cells. The large percentage of γδ T cells within ovine PBMC provide a favourable population of cells for functional analysis of this T cell lineage and hence were used for this study.

Ovine γδ T cells have the ability to proliferate in vitro to exogenous antigens. In a comparison of the primary and secondary proliferation of purified populations of ovine γδ T cells it was established that ovine γδ T cells respond, albeit weakly, to heat-killed Mycobacterium antigen irrespective of whether it was a primary or secondary in vitro assay. In contrast a response to ovalbumin was only observed in a secondary in vitro
assay. This may imply that the repertoire of ovine γδ T cells, in contrast to other species, is naturally skewed towards the recognition of components within a preparation of Mycobacterial antigen, or components that structurally resemble these antigens. A proliferative response was seen by γδ T cells in a secondary response to the soluble protein antigen such as ovalbumin.

The limited proliferation observed in vitro by ovine γδ T cells to these exogenously added antigens may reflect an inherent poor ability of the cells to respond to antigen, or alternatively the incorrect choice of antigen presenting cells in these assays. It is established that a threshold level of approximately 50-300 antigenic peptide/MHC complexes are required for αβ T cell activation (Williams and Beyers 1992, Jorgensen et al 1992), but it remains to be established if γδ T cells require similar amounts of peptide/MHC complexes to induce their activation. If γδ T cells require more peptide/MHC complexes than their αβ counterparts, then with respect to this issue antigen presenting cells known to express elevated levels of the classical MHC molecules may have been more appropriate in the assays. Potential candidates are dendritic cells as it is established that these cells in comparison to other antigen presenting cells express from 10-100 fold more MHC molecules (Bhardwaj et al 1992). In addition, these cells express molecules that have the potential to act as presentational elements which are not found on all antigen presenting cells. Such examples are the class I associated CD1 molecules (Bujdoso et al 1989a).

It was observed that proliferation of ovine γδ T cells within a population of PBMC occurs during the first 5 days of culture in both a primary and secondary in vitro response with Mycobacterium antigens and in a secondary response to ovalbumin and PPD. In view of this an investigation of the optimum culture period of ovine γδ T cells was established. This demonstrated that maximum proliferation of γδ T cells was observed in a 3 day assay relative to a 5 day proliferation assay. With respect to the above data, and that obtained in sections 4.2, which clearly demonstrates that subsequent to Con A activation in vitro ovine γδ T cells express CD25 prior of the αβ T cell subset, it may be concluded that γδ T cells are activated prior to αβ T cells and responded to Mycobacterium tuberculosis and ovalbumin prior of the αβ lineage in the
assays established here. The implication is that this is a general feature of γδ T cells as activation of these cells is presumably via antigen and presentational element triggering the TCR molecule they express. In addition, the observation that supplementation of the cultures with exogenous IL-2 5 days after antigen activation of PBMC resulted in a preferential expansion of αβ T cells may be explained again in accordance with the data reported in section 4.2. This showed that 5 days after Con A activation 50% of the γδ population had lost CD25 expression, whilst the majority of the αβ lineage expressed CD25, therefore the number of cells within the former population that could respond to IL-2 was greatly reduced, resulting in the preferential expansion of the αβ T cell lineage.

The results from the study of potential presentational elements of ovine γδ T cells implies the presence of both MHC class I and II restricted ovine γδ T cells. It is well established that the majority of peptides derived from intracellular or endogenous antigens are presented to T cells by MHC class I molecules (Townsend and Bodmer 1989, Morrison et al 1988b) and peptides derived from exogenous antigens are presented to T cells by MHC class II molecules (Unanue and Allen 1987). Despite this, reports have described the presentation of exogenous antigen to murine T cells in association with MHC class I molecules on the surface of appropriate antigen presenting cells (Rock et al 1992, Rock et al 1990, Carbone and Bevan 1989, Staerz et al 1987). These antigen presenting cells were shown to be MHC class II-positive, implying that exogenous antigen was not expressed in association with MHC class I due to inability to express MHC class II molecules.

Possible explanations for the presentation of exogenous antigen with MHC class I molecules in this thesis and in the reports listed above are that (i) peptides present in the antigen preparation did not require processing for efficient binding to MHC molecules and bind empty MHC class I molecules on the surface of the antigen presenting cells within the cultures, alternatively (ii) exogenous antigen is internalised by the antigen presenting cell and enters the endosomal compartments where it is processed into antigenic peptides. If some peptides are generated with a specialised sequence at one terminus, such as a signal sequence, this may enable the peptide to translocate into the endoplasmic reticulum and associate with MHC class I molecules during their
biosynthesis. Data in favour of the latter hypothesis is from recent investigations on the TAP transporter genes, which encode proteins located within the endoplasmic reticulum (Monaco et al 1990, Trowsdale et al 1990, Spies et al 1990). The TAP proteins are responsible for the transport of cytosolic peptides generated by the proteasome into the endoplasmic reticulum where they associate with MHC class I molecules (Kleijneer 1992, Powis et al 1992). Cell lines deficient in these TAP genes are unable to transport the majority of peptides into the endoplasmic reticulum but some peptides are able to enter the endoplasmic reticulum (Powis et al 1991, Attaya et al 1992), associate with MHC class I molecules and form stable complexes at the surface of the antigen presenting cell. It has been shown that these peptides translocate into the endoplasmic reticulum via a signal sequence located at the amino terminus of the peptide (Townsend 1992, Wei and Creswell 1992). An analogous situation may be operating within the in vitro assay described here, with the subsequent presentation of exogenous antigen in association with MHC class I.

Whilst MHC class I and II molecules do not appear to serve generally as presentational elements for human and murine γδ T cells, some clones expressing this TCR molecule which are reactive with allogeneic MHC class I and class II products have been reported (Bluestone et al 1988, Matis et al 1989). These clones were obtained from nude mice after strong selection by allogeneic stimulation, and exhibit a broad cross-reactivity for the products of different alleles of class I and class II MHC genes. These clones may have been derived from rare cells that are specific for non-classical MHC proteins but were selected by in vivo and/or repeated in vitro stimulation on the basis of their cross reactivity with classical MHC proteins. Based on a similar hypothesis, the response observed in this investigation of ovine γδ T cells to antigen in association with classical MHC molecules may reflect the response of a subset of ovine γδ T cells whose receptors crossreact with conventional MHC class I and II molecules but in fact have a higher affinity for an autologous MHC class I-like molecule. The data obtained here may support this hypothesis as the weak in vitro proliferative responses observed may have been a consequence of ligand recognition via crossreactivity with the presentational elements, hence not inducing maximum proliferation.
The inability of the anti-γδ TCR monoclonal antibody, 86D, to inhibit the proliferation of ovine γδ T cells to antigen in vitro may be the consequence of two possibilities, either (i) monoclonal antibody 86D binds a region of the TCR distinct from the region that is involved in peptide binding enabling both the peptide and the monoclonal antibody to bind the TCR in a co-ordinate manner with no subsequent effect on T cell proliferation, or alternatively (ii) that ovine γδ T cells were stimulated by superantigens which bind outside the TCR peptide binding groove. The latter is an unlikely explanation in view of the fact that to date, ovalbumin has not been regarded as a superantigen. However it has been suggested that a population of human γδ T cells, those that express Vγ9 Vδ2, are expanded by either a superantigen (Pfeffer et al 1992) or powerful antigens (De Libero et al 1991) within Mycobacteria antigens. This is based on the observations that (i) this subset represents a minor population of γδ T cells within the thymus, (ii) the diversity of the nucleotide sequence at the V-D-J junction of the Vδ2 chain implies that this population is polyclonal, and (iii) all Vγ9 Vδ2 γδ T cells display lytic activity to Daudi Burkitt's lymphoma cells which presumably express the superantigen.

Collectively this investigation has shown that ovine γδ T cells respond in vitro to both Mycobacterium tuberculosis and ovalbumin in association with classical presentational elements. These observations together with the expanded V gene repertoire and prominence of γδ T cells within the ovine immune system argue that this T cell lineage has a more elaborate functional role within this species in comparison to other species and may represent a lineage perhaps analogous to the αβ T cell lineage.
Lymphocytes migrate between lymphoid tissues and other sites via the blood and lymphatic vessels. There are two main physiological functions of lymphocyte migration: firstly, it enhances the presentation of antigen to the appropriate set of antigen-specific lymphocytes, and secondly, it disseminates the immune response throughout the body. Lymph nodes are important components of the secondary lymphoid tissues. Greater than 90% of lymph node lymphocytes are derived by migration of cells from the blood across high endothelial venules, while the remaining 10% comprise lymphocytes that migrated either from epithelial sites of lymphoid tissue viaafferent lymph (Hall and Morris 1964). Lymph nodes comprise relatively discrete T and B cell dependent areas with their relevant accessory cells, essential for antigen processing and presentation in the case of T cells. These include follicular dendritic cells (FDCs) for presentation to B cells (Van Rooijen 1990) and interdigitating dendritic cells which present antigen to T cells (Steinman and Nussenzweig 1980). The former are involved predominantly in immune complex localisation and antigen presentation within germinal centres whereas the latter present antigen within paracortical T cell areas of lymph nodes.

The traffic of accessory cells to lymph nodes represents an important mechanism of antigen transport. Studies on different lymphoid dendritic cells in the sheep have clearly shown they are capable of transporting antigen in an immunogenic form from a site of intradermal injection of antigen (Bajdoso et al 1989a). Dendritic cells have high avidity Fc receptors for IgM and IgG which serves to localise antigen following intradermal challenge (Harkiss et al 1990). By utilising antigen-antibody complexes dendritic cells in vivo may efficiently concentrate and process small amounts of antigen. Further features of dendritic cells include their high level of MHC class II expression (Bhardwaj et al 1992) and alteration in the level of expression of presentational elements such as MHC class I and II and CD1 as a consequence of the induction of the immune responses (Hopkins et al 1989).
INTRODUCTION.

Lymphocytes migrate between lymphoid tissues and other sites via the blood and lymphatic vessels. There are two main physiological functions of lymphocyte migration: firstly, it enhances the presentation of antigen to the appropriate sets of antigen specific lymphocytes; and secondly, it disseminates the immune response throughout the body. Lymph nodes are important components of the secondary lymphoid tissues. Greater than 90% of lymph node lymphocytes are derived by migration of cells from the blood across high endothelial venules, whilst the remaining 10% comprise lymphocytes that migrated either from epithelial sites or lymphoid tissue via afferent lymph (Hall and Morris 1965). The architecture of lymph nodes is such that they comprise relatively discrete T and B cell dependent areas with their relevant accessory cells, essential for antigen processing and presentation in the case of T cells. These include follicular dendritic cells (FDCs) for presentation to B cells (Van Rooijen 1990) and interdigitating dendritic cells which present antigen to T cells (Steinman and Nussenzweig 1980). The former are involved predominantly in immune complex localisation and antigen presentation within germinal centres whereas the latter present antigen within paracortical T cell areas of lymph nodes.

The traffic of accessory cells to lymph nodes represents an important mechanism of antigen transport. Studies on afferent lymph dendritic cells in the sheep have clearly shown they are capable of transporting antigen in an immunogenic form from a site of intradermal injection of antigen (Bujdoso et al 1989a). Dendritic cells have high avidity Fc receptors for IgM and IgG which serves to localise antigen following intradermal challenge (Harkiss et al 1990). By utilising antigen-antibody complexes dendritic cells in vivo may efficiently concentrate and process small amounts of antigen. Further features of dendritic cells include their high level of MHC class II expression (Bhardwaj et al 1992) and alteration in the level of expression of presentational elements such as MHC class I and II and CD1 as a consequence of the induction of the immune responses (Hopkins et al 1989).
The analysis of \( \gamma \delta \) T cell localisation within various species has established the rarity of these cells in lymph nodes but suggests their localisation at epithelial sites. This compartmentalisation is particularly pronounced in murine epithelia where the majority of T cells express a \( \gamma \delta \) TCR (Stingl et al 1987a, Stingl et al 1987b, Koning et al 1987). It was proposed that \( \gamma \delta \) T cells mediate immunological surveillance of epithelia where they recognise and respond in the appropriate manner to either invading pathogens, autologous transformed cells or autologous stress proteins expressed by stressed cells (Janeway et al 1988, Tonegawa et al 1989). This hypothesis was proposed in view of the observations that epithelial \( \gamma \delta \) T cells exhibit highly restricted V gene usage, preferential pairing of TCR chains, and lack of diversity at the junctions creating populations of cells with virtually identical TCR (Allison and Havran 1991).

A salient feature of investigating the immune system of the sheep is that the lymphatic vessels of these relatively large experimental animals are readily cannulated and enable an investigation of \( \gamma \delta \) T cell phenotype and function within these compartments. It may be hypothesised that \( \gamma \delta \) T cells recognise their ligand, of either endogenous or exogenous origin, at epithelial sites on the surface of the appropriate antigen presenting cell, such as dendritic cells (Bujdoso et al 1990). If this is the case then afferent lymph may be characterised by the presence of activated \( \gamma \delta \) T cells expressing IL-2 receptors. As an alternative hypothesis, ovine \( \gamma \delta \) T cells may only participate within an immune response subsequent to \textit{in vivo} antigen challenge. If so then \( \gamma \delta \) T cells would localise within lymph nodes and appear activated in efferent lymph. The cannulation of lymphatic vessels draining either epithelial sites (afferent lymph) or lymph nodes (afferent lymph) will allow an investigation of these hypotheses.

An issue associated with the functional studies on afferent lymph cells is that single afferent lymphatics are small and difficult to cannulate, having a low output of cells and lymph fluid. To overcome this, peripheral lymph nodes in sheep can be ablated and subsequently cannulate the pseudo-afferent lymphatic vessel that arises as a consequence of afferent lymphatic vessels re-anastamosing with the former efferent duct (Hopkins et al 1985). The pseudo-afferent lymphatic vessel is relatively easy to cannulate and routinely provides a greater output of cells than a cannulated true afferent...
lymphatic vessel.

The majority of efferent lymphocytes comprise those that entered the node from the blood as well as cells produced as a result of clonal expansion within the lymph node. The latter population are responsible for the establishment of immunological memory and dissemination of the immune response to other lymphoid organs (Hall 1967). It remains to be established if lymphocytes derived from afferent lymph leave lymph nodes via efferent lymphatics. The cannulation of afferent and efferent lymphatic vessels, primarily to analyse the γδ T cell populations in these compartments, allows investigations into this issue.

This investigation has established the percentages of γδ T cells within afferent and efferent lymph and peripheral blood to determine if ovine γδ T cells preferentially localise within certain lymphoid compartments. CD25 expression by γδ T cells within each of these compartments was investigated to assess their activational status. In addition, the output and phenotypic changes of ovine γδ T cells within afferent and efferent lymph subsequent to a secondary in vivo antigen challenge were investigated. Finally, the intranodal distribution and number of γδ T cells within lymph nodes undergoing immune responses was analysed to determine whether γδ T cells have an immunological function within fixed lymphoid tissue.
RESULTS.

7.1. Differences In The Percentage of $\gamma\delta$ T Cells Between Various Lymphoid Compartments.

Lymphocytes continually recirculate between the bloodstream, tissues, lymph nodes and lymph, re-entering the bloodstream via the thoracic duct. This provides potential for selective migration of lymphocytes between these compartments. The percentage of $\gamma\delta$ T cells in PBMC, afferent and efferent lymphatic vessels of 18 month old animals was investigated by single colour immunofluorescence to determine if ovine $\gamma\delta$ T cells preferentially localise within any of these compartments. The results are shown in Table 1.

The data shows that the highest level of $\gamma\delta$ T cells was recorded in afferent lymph where 13.6% of mononuclear cells expressed a $\gamma\delta$ TCR. Lower levels of $\gamma\delta$ T cells were seen in peripheral blood and efferent lymph where approximately 7-8% of cells stained with the monoclonal antibody 86D. The similarities in the percentages of $\gamma\delta$ T cells within efferent lymph and peripheral blood may be explained by the observation that 85-90% of efferent lymph lymphocytes are derived from the blood supply to the lymph nodes (Hall and Morris 1965).

Afferent and efferent lymph are characterised by a higher percentage of CD4-positive cells compared to peripheral blood, whereas the percentage of CD8-positive cells is approximately equal within each lymphoid compartment. In addition, afferent lymph was unique in comparison to the other lymphoid compartments in that it not only had the highest level of $\gamma\delta$ T cells but also contained a population of dendritic cells which are potent antigen presenting cells for T cells. The presence of dendritic cells was determined by analysis of the forward and side scatter dot profiles of afferent lymph cells on a FACS analyser. Figure 1 shows that dendritic cells are characterised on dot plots by increased forward and side light scatter relative to lymphocytes. Collectively, the data suggests that ovine T cell subsets are not randomly distributed between the vascular and lymphatic compartments (Mackay et al 1988b, Bujdoso 1989a).
TABLE 1. Lymphocyte Composition Of Various Lymphoid Compartments (n=6).

<table>
<thead>
<tr>
<th></th>
<th>CD4</th>
<th>CD8</th>
<th>γδ</th>
<th>T19</th>
<th>slg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td><strong>23.3</strong> (4.0)</td>
<td><strong>13.4</strong> (6.1)</td>
<td><strong>6.9</strong> (2.3)</td>
<td><strong>6.3</strong> (2.5)</td>
<td><strong>63.7</strong> (7.9)</td>
</tr>
<tr>
<td>Efferent Lymph</td>
<td><strong>51.3</strong> (9.4)</td>
<td><strong>12.8</strong> (4.4)</td>
<td><strong>7.9</strong> (3.9)</td>
<td><strong>6.9</strong> (3.2)</td>
<td><strong>27.4</strong> (12.4)</td>
</tr>
<tr>
<td>Afferent Lymph</td>
<td><strong>48.8</strong> (2.2)</td>
<td><strong>11.9</strong> (3.3)</td>
<td><strong>13.6</strong> (2.5)</td>
<td><strong>11.2</strong> (2.2)</td>
<td><strong>23.3</strong> (4.3)</td>
</tr>
</tbody>
</table>
Figure 1.
Afferent lymphocytes were collected from a cannulated afferent lymphatic vessel of an 18 month old Finnish Landrace animal and analysed by flow cytometry for their forward and side light scatter profiles.
7.2. γδ T Cells Within Afferent Lymph Express CD25.

It is suspected that γδ T cells encounter their ligand at epithelial sites (Janeway et al. 1988, Tonogawa et al. 1989). The ligand for γδ T cells may be derived from either an invading pathogen or endogenous derived material at epithelial sites. In this case, that γδ T cells within afferent lymph may express an activated phenotype. The investigation of this hypothesis (Naucler et al. 1992). Two colour immunofluorescence analysis was utilized to study CD25 expression by γδ T cells in different lymphoid compartments.

The results are shown in Figure 2.

The data clearly shows that in afferent lymphatic vessels approximately 50% of γδ T cells were activated, as defined by the high percentage of the CD25 expression by afferent lymph, indicating the presence of this population of γδ T cells as low from the lymph nodes or peripheral blood expressing CD25. This was not true for the remaining γδ T cells as approximately 40% of this T cell population in peripheral lymphoid blood expressed CD25, while the 10% of γδ T cells in all of these compartments do not express CD25.

7.3. Changes In The T Cell Composition Of Lymph Compartments Following In Vivo Antigen Challenge.

The data reported above shows distinct differences between afferent and efferent lymph in two respects, in that both the percentage of afferent lymph γδ T cells and the fraction of these cells that are activated are reduced in afferent lymph. These observations were made on lymph draining superficial (afferent lymph) or lymph node (efferent lymph) draining sites undergoing the secondary in vivo antigen load, of antigen stimulation. To observe the effect of immunosuppressive challenge to these sites, animals primed to antigen were subsequently given a secondary in vivo challenge into the drainage area of a conformed afferent or efferent lymphatic vessel. Changes in afferent and efferent lymph were monitored for a number of parameters.

FIGURE 1. Scatter profiles Of Ovine Afferent Lymph Cells.
7.2. γδ T Cells Within Afferent Lymph Express CD25.

It is suggested that γδ T cells encounter their ligand at epithelial sites (Janeway et al 1988, Tonegawa et al 1989). The ligand for γδ T cells may be derived from either an invading pathogen or endogenous derived material at epithelial sites. If this is the case then γδ T cells within afferent lymph may express an activated phenotype. The availability of monoclonal antibodies specific for CD25 has enabled an investigation of this hypothesis (Naessens et al 1992). Two colour immunofluorescence analysis was utilised to study CD25 expression by γδ T cells in different lymphoid compartments. The results are shown in Figure 2.

The data clearly shows that in afferent lymphatic vessels approximately 50% of γδ T cells were activated, as defined by their expression of CD25. This high percentage of the CD25 expression by afferent lymph γδ T cells is a unique feature of this population of γδ T cells as less than 10% of γδ T cells within either efferent lymph or peripheral blood expressed CD25. This was not the case for the CD4-positive cells as approximately 40% of this T cell subset in afferent and efferent lymph and peripheral blood expressed CD25. Less than 10% of the CD8-positive lymphocytes within all of these compartments expressed CD25.

7.3. Changes In The T Cell Composition Of Lymph Compartments Following In Vivo Antigen Challenge.

The data reported above shows distinct differences between afferent and efferent lymph in two respects, in that both the percentage of afferent lymph γδ T cells and the fraction of these cells that are activated was higher than observed in efferent lymph. These observations were made on lymph draining epithelial (afferent lymph) or lymph node tissue (efferent lymph) in the absence of any intentional antigen challenge to these sites. These observations therefore, represent the output and activation state of γδ T cells draining sites undergoing the normal, or background level, of antigen stimulation. To observe the effect of intentional antigenic challenge to these sites, animals primed to antigen were subsequently given a secondary in vivo challenge into the drainage area of a cannulated afferent or efferent lymphatic vessel. Changes in afferent and efferent
Afferent and efferent cells and PBMC were prepared from 18 month old Finnish Landrace animals and analysed by two colour immunofluorescence for co-expression of CD25 with either γδ TCR, CD4 and CD8. Cells were stained with IL-A111 (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when IL-A111 and 86D were used cells were treated with IL-A111 alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Finally cells were reacted with biotinylated 86D followed by phycoerytherin-streptavidin and analysed by flow cytometry. The immunofluorescence profiles are shown.
FIGURE 2. CD25 Expression By Lymphocytes Obtained From Afferent and Efferent Lymph and PBMC.

(i) Afferent Lymph  (ii) Efferent Lymph  (iii) PBMC

γδ

CD4

CD8

CD25 (green fluorescence)
lymph in terms of (i) the ability of lymphocytes obtained from lymph to respond in vitro to exogenous IL-2, (ii) γδ T cells output, and finally (iii) the fraction of γδ T cells expressing CD25 were then investigated.

The statistical analysis of the significance of the output of both γδ T cells and the number of CD25-positive γδ T cells was based on a test where a range was calculated from the control values of each animal at the 95% confidence interval. Statistical significance was taken as any value outside of this range. A limitation of this test of significance is that single time points are compared to the control values hence no standard error is available on such values. However, the data from all experimental animals were analysed in this way and within an experimental group the same trends of statistical significance were shown.

The FCS and SSC of afferent and efferent lymph differ. Efferent lymphocytes have a lower FSC value compared to afferent lymphocytes as shown in Figure 3(i) and Figure 3(ii). As efferent lymphocytes become activated they acquire a high FSC and SSC value and are regarded as activated or blast cells [Figure 3(iii)]. This allows the analysis of both resting (low FSC and SSC) and activated (high FSC and SSC) cells in this compartment. In afferent lymph, the lymphocytes have a broader FSC and SSC profiles which suggests activated cells are continually present. In addition, the lymphocyte profile overlap with the dendritic profile, hence it is not possible to differentiate resting and activated cells in afferent lymph and as a consequence the two populations were considered as one. Some dendritic cells would be included in this analysis of afferent lymphocytes but as dendritic cells comprise only 1-5% of afferent lymph, the presence of some of these cells would be minimal.

7.3.1. Proliferative Response Of Afferent Lymph CD25-Positive Lymphocytes To IL-2.

Three animals were analysed for this investigation. Two animals were challenged with *Mycobacterium tuberculosis* whilst the remaining animal was challenged with ovalbumin. A representative set of data for one *Mycobacterium tuberculosis* challenged
Figure 3.
Afferent and efferent cells were prepared from 18 month old Finnish Landrace animals and analysed by flow cytometry for their forward and side light scatter profiles. Live gates were established for the analysis of various lymphocyte populations: (i) total lymphocytes within afferent lymph; (ii) total lymphocytes within efferent lymph; and (iii) activated lymphocytes within efferent lymph.

(i) Afferent Lymph Cells

(ii) Resting Efferent Lymph Cells

(iii) Activated Efferent Lymph Cells

To investigate how challenge with antigen affected an immune response in the common eider, birds were challenged with antigen in the presence of exogenous IL-2 as described in section 2.6.3. The proliferative response of these lymphocytes in response to IL-2 was measured, and the results were expressed as the percentage of cells which constituted a form of the IL-2 receptor-expressing activated (Walder and Smith 1987). The results indicate that IL-2 enhances the proliferation of T cells (data not shown).

2.3.2. Changes in Afferent Lymph of T Cell Output Following a Secondary in Vivo Antigen Challenge.

The number of lymphocytes obtained per hour from afferent lymphatic vessels following antigen challenge was determined. The results are shown in Figure 3. The data suggest that there was a significant decrease in the output of cells above control levels, which was significant across all time points (p < 0.05). On day 3, the output of cells was significantly increased above control levels, which was not observed on days 5, 6, and 7 following a secondary in vivo antigen challenge. These changes were consistent with those reported for T cells, the T cell subset consisting of afferent lymphatic vessels.
animal is shown as the other two antigen challenged animals showed trends similar to those reported here.

To investigate if a secondary in vivo challenge with antigen effected an immune response in the cannulated animal, afferent lymphocytes collected before and after the antigen challenge were cultured in vitro in the presence of exogenous IL-2 as described in section 2.6.6. The data in Figure 4 shows the proliferative response of these lymphocytes in the presence of 100 pM IL-2. This concentration of IL-2 binds to high affinity IL-2 receptors comprising α and β chains which constitute the form of the IL-2 receptor expressed by T cells after antigen specific activation (Wang and Smith 1987, Bujdoso et al 1987b).

24 and 48 hours after antigen challenge the proliferative response of unfractionated lymphocytes to IL-2 was equivalent to that observed prior of the challenge. In contrast, between 3 and 6 days post in vivo challenge an increase in proliferation to IL-2 was observed, but this decreased to control values on day 7. A noticeable feature of the data obtained between 3 and 6 days, is that background proliferation was increased relative to the other time points. Analysis of the stimulation indices of the proliferative responses showed the majority of these were similar or below, but never higher than those obtained prior to secondary in vivo antigen challenge (data not shown).

7.3.2. Changes In Afferent Lymph γδ T Cell Output Following a Secondary In Vivo Antigen Challenge.
The number of lymphocytes obtained per hour from afferent lymphatic vessels following antigen challenge was determined. The results are shown in Figure 5. Twenty four hours after antigen challenge there was a significant decrease in the output of cells (p < 0.05). On days 2 and 3 cell output increased above control levels, which was significant on day 2 (p < 0.05), but between 3 and 4 days decreased below control values and remained at this level for days 5, 6 and 7 following a secondary in vivo antigen challenge. To determine if these changes were a consequence of differences in either the number of γδ T cells or αβ T cells, the T cell subset composition of afferent
Figure 4.

Afferent lymphocytes were collected from a cannulated afferent lymphatic vessel of an 18 month old Finnish Landrace animal following a secondary *in vivo* challenge with *Mycobacterium tuberculosis* around the drainage area of the cannulated vessel. $10^5$ cells/well were cultured with 100 pM of exogenously added IL-2 in flat-bottom plates. The assays were incubated for 3 days and proliferation was measured by the uptake of $^3$H-thymidine over the last 7 hours of culture.
FIGURE 4. *In Vitro* Proliferative Response Of Afferent Lymphocytes To IL-2 Following Antigen *In Vivo* Challenge.

![Graph showing proliferation response over days after antigen challenge.](image)

**KEY:**
- Background
- 100pM IL-2
Afferent lymphocytes were collected from a cannulated afferent lymphatic vessel of an 18 month old Finnish Landrace animal following a secondary *in vivo* challenge with *Mycobacterium tuberculosis* around the drainage area of the cannulated vessel. Cells were counted and the cell output per hour was calculated. Statistical analysis was carried out as described in section 7.3.
FIGURE 5. Cell Output From An Afferent Lymphatic Vessel Following *In Vivo* Antigen Challenge.

* $p < 0.05$ relative to controls.
Figure 6.
Afferent lymphocytes were collected from a cannulated afferent lymphatic vessel of an 18 month old Finnish Landrace animal following a secondary *in vivo* challenge with *Mycobacterium tuberculosis* around the drainage area of the cannulated vessel. Cells were stained with either monoclonal antibodies 86D, ST4 or ST8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the output per hour of \(\gamma\delta\) TCR-positive, CD4-positive and CD8-positive T cells was calculated. Statistical analysis was carried out as described in section 7.3.
FIGURE 6. T Cell Subset Output From An Afferent Lymphatic Vessel Following In Vivo Antigen Challenge.

KEY:

- □ - γδ T Cells
- □ - CD4-Positive T Cells
- □ - CD8-Positive T Cells

* p < 0.05 relative to controls.
lymph was determined. Figure 6 shows that twenty four hours post \textit{in vivo} antigen challenge a significant decrease in the output of afferent lymph $\gamma\delta$ T cells was observed ($p < 0.05$) but on days 2 and 3 post antigen challenge $\gamma\delta$ T cell output had increased to control values. Between days 4 and 7 the output of $\gamma\delta$ T cells was significantly lower than control values ($p < 0.05$) but similar to that obtained 24 hours after challenge with antigen. The data implies that afferent lymph $\gamma\delta$ T cell output was reduced subsequent to antigen challenge. Similarly, a decrease in the output of afferent lymph $\alpha\beta$ T cells was observed 24 hours following antigen challenge, but in contrast to the $\gamma\delta$ subset an increase was observed in their output on days 2 and 3. Between days 4 and 7 the output of CD4-positive T cells was lower than control values but CD8-positive T cells returned to the levels observed prior to challenge.

7.3.3. The Output of CD25-Positive $\gamma\delta$ T Cells Decreases Following \textit{In Vivo} Antigen Challenge.

An analysis of CD25 expression by $\gamma\delta$ T cells draining a site of \textit{in-vivo} antigen challenge was carried out to determine if an increased number of these cells were activated. The results are shown in Figure 7.

A significantly lower number of CD25-positive $\gamma\delta$ T cells was observed at all time points investigated compared to pre-challenge values ($p < 0.05$). The percentage of CD4-positive and CD8-positive T cells expressing CD25 followed a similar trend to that shown by the $\gamma\delta$ T cells, with the exception of the CD8-positive population where an increase in the number of CD25-positive T cells was observed at day 3 post challenge. Collectively, it appears that over the time period investigated the number of CD25-positive T cells within afferent lymph decreases following antigen challenge.

7.3.4. Proliferative Response Of CD25-Positive Efferent Lymphocytes To IL-2.

The aim of this section of the investigation was to determine if ovine $\gamma\delta$ T cells participate in the immune response within a secondary \textit{in vivo} challenged peripheral
Afferent lymphocytes were collected from a cannulated afferent lymphatic vessel of an 18 month old Finnish Landrace animal following a secondary *in vivo* challenge with *Mycobacterium tuberculosis* around the drainage area of the cannulated vessel. Cells were analysed by two colour immunofluorescence for co-expression of CD25 with either $\gamma\delta$ TCR, CD4 or CD8. Cells were stained with IL-A111 (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were subsequently treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when IL-A111 and 86D were used cells were treated with IL-A111 alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the output per hour of $\gamma\delta$ TCR-positive, CD4-positive and CD8-positive T cells co-expressing CD25 was calculated. Statistical analysis was carried out as described in section 7.3.
FIGURE 7. Output Of CD25-Positive T Cells From An Afferent Lymphatic Vessel Following In Vivo Antigen Challenge.

![Graph showing output of CD25-Positive T Cells.](image)

**KEY:**
- γδ T Cells
- CD4-Positive T Cells
- CD8-Positive T Cells

* *p < 0.05 relative to controls.*
lymph node. This was assessed as previously described in section 7.3. Four animals were analysed in the investigation, two animals were challenged with *Mycobacterium tuberculosis* and the other two with ovalbumin. A representative set of data for one of the *Mycobacterium tuberculosis* challenged animals and one of the ovalbumin challenged animals is shown. Similar trends were observed with the other two animals from each antigen challenged group.

The response of efferent cells to exogenously added IL-2 was established to determine that *in vivo* antigen-challenge did in fact effect an immune response within the node. The assays were established as described in section 2.6.6. The results are shown in Figure 8 and illustrate the proliferative response of the lymphocytes in the presence of 100 pM of IL-2 for the reasons stated in section 7.3.1.

The response of unfractionated efferent lymph cells to IL-2 twenty four hours after challenge was comparable to control proliferation. Between days 2 and 6 post antigen challenge, proliferation of efferent lymph lymphocytes was increased in the presence of IL-2 (no data is available for day 4) above control values. On day 7 the proliferative response was similar to the controls. It appears that *in vivo* antigen challenge increased the ability of the lymphocytes to proliferate to IL-2 *in vitro*.

7.3.5. Secondary *In Vivo* Antigen Challenge Increased the Number of γδ T Cells In Efferent Lymph.  
Figure 9 shows the output of total and activated cells in efferent lymph. 24 hours after an *in vivo* secondary antigen challenge the output of total lymphocytes was similar to the controls, but increased on day 2, and subsequently decreased over the next 6 days. A significant increase of cell output, relative to control values, was observed between days 2-5 (*p* < 0.05). A similar trend in the output of activated lymphocytes was observed, again with a significant increase in the output between days 2-5 (*p* < 0.05). In the ovalbumin challenged animals the maximum number of activated lymphocytes was observed on day 3, whilst in *Mycobacterium tuberculosis* challenged animals this was observed on day 4.
Efferent lymphocytes were collected from the cannulated efferent lymphatic vessels of 18 month old Finnish Landrace animals following secondary *in vivo* challenge with either *Mycobacterium tuberculosis* or ovalbumin around the drainage area of the lymph node. $10^5$ cells/well were cultured with 100 pM of exogenously added IL-2 in flat-bottom plates. The assays were incubated for 3 days and proliferation was measured by the uptake of $^3$H-thymidine over the last 7 hours of culture.

**Figure 8.**
FIGURE 8. In Vivo Proliferative Response of Different Lymphocytes to IL-2 Following In Vivo Antigen Challenge.
Figure 9.
Efferent lymphocytes were collected from the cannulated efferent lymphatic vessels of 18 month old Finnish Landrace animals following secondary in vivo challenge with either *Mycobacterium tuberculosis* or ovalbumin around the drainage area of the lymph node. Cells were counted and the cell output per hour from the lymph node of both total and activated lymphocytes were calculated. Statistical analysis was carried out as described in section 7.3.
FIGURE 9. Total and Activated Efferent Cell Output From In Vivo Antigen Challenge Lymph Nodes.

Days After Antigen Challenge

- Mycobacterium tuberculosis
- Ovalbumin

KEY:

- * p < 0.05 relative to controls.

<table>
<thead>
<tr>
<th>Days After Antigen Challenge</th>
<th>8</th>
<th>7</th>
<th>6</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2</th>
<th>1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Activated Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Total Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
If γδ T cells are activated and clonally expanded within the lymph node as a consequence of antigen recognition then an increase in the output of γδ T cells from an antigen challenged lymph node may be seen. To assess this, the output within the total and activated populations of efferent lymph of the various T cells subsets was determined. The results of this investigation are shown in Figure 10 and 11.

Figure 10 shows that after antigen challenge the number of γδ T cells within the total population of efferent lymphocytes decreased, but on day 2 a small but significant increase in their number was apparent (p < 0.05). The output of CD4-positive T cells within efferent lymph 24 hours after in vivo antigen challenge increased slightly whilst the output of CD8-positive T cells either remained similar or decreased slightly compared to control levels. 2 days after challenge there was a large increase in the output of αβ T cells by the lymph node which was due mainly to the CD4-positive population but decreased gradually over the next 5-6 days when their output was similar to controls.

The output of γδ T cells within the activated efferent lymphocyte population following in vivo antigen challenge of the node is shown in Figure 11. The maximum number of activated γδ T cells was observed on day 2, and was significantly different from control values (p < 0.05). Thereafter the numbers of γδ T cells decreased to pre-challenge levels. A similar trend was shown by the CD8-positive population. In contrast, the time point when maximum output of activated CD4-positive T cells occurred was dependent on the antigenic challenge of the animal, day 2 in ovalbumin challenged animals whilst day 3 in Mycobacterium tuberculosis challenged animals. The number of CD4-positive blasts did not return to control values over the time investigated.

The changes observed within the total population of efferent lymphocytes are reflected in the ratio of αβ / γδ T cells shown in Table 2. An increase was observed in the ratio following antigen challenge but decreased 7-8 days later. Analysis of the ratio of CD4 / CD8 cells demonstrated an analogous trend over the same time suggesting that the changes in both ratios was primarily a consequence of an increased output of the CD4-positive population.
Figure 10.
Efferent lymphocytes were collected from the cannulated efferent lymphatic vessels of 18 month old Finnish Landrace animals following secondary in vivo challenge with either *Mycobacterium tuberculosis* or ovalbumin around the drainage area of the lymph node. Cells were stained with either monoclonal antibodies 86D, ST4 or ST8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the output per hour from the lymph node of γδ TCR-positive, CD4-positive and CD8-positive T cells within the total population was calculated. Statistical analysis was carried out as described in section 7.3.
FIGURE 10. T Cell Subset Output From In Vivo Antigen-Challenged Lymph Nodes.

**KEY:**
- ■ CD4-Positive T Cells
- □ CD8-Positive T Cells
- △ γδ T Cells

Days After Antigen Challenge

Number of Cells \(\times 10^6\) Hour\(^{-1}\)

Mycobacterium tuberculosis

Ovalbumin

\(p < 0.05\) relative to controls.
Figure 11.
Efferent lymphocytes were collected from the cannulated efferent lymphatic vessels of 18 month old Finnish Landrace animals following secondary *in vivo* challenge with either *Mycobacterium tuberculosis* or ovalbumin around the drainage area of the lymph node. Cells were stained with either monoclonal antibodies 86D, ST4 or ST8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry and the output per hour from the lymph node of γδ TCR-positive, CD4-positive and CD8-positive T cells within the activated population was calculated. Statistical analysis was carried out as described in section 7.3.
**FIGURE 11. Activated T Cell Subset Output From In Vivo Antigen-Challenged Lymph Nodes.**

**KEY:**

* $p > 0.05$ relative to controls.

- CD8 positive T cells
- CD4 positive T cells
- $\gamma$ T cells

**X-axis:** Days After Antigen Challenge

**Y-axis:** Number of Cells x 10^6 Hour^-1
Efferent lymphocytes were collected from cannulated efferent lymphatic vessels of 18 month old Finnish Landrace animals challenged *in vivo* with either *Mycobacterium tuberculosis* or ovalbumin around the drainage area of the lymph node. Cells were stained with either monoclonal antibodies 86D,ST4 or ST8 followed by anti-mouse immunoglobulin-FITC and analysed by flow cytometry.

**Table 2.**

Efferent lymphocytes were collected from cannulated efferent lymphatic vessels of 18 month old Finnish Landrace animals challenged *in vivo* with either *Mycobacterium tuberculosis* or ovalbumin around the drainage area of the lymph node. Cells were stained with either monoclonal antibodies 86D,ST4 or ST8 followed by anti-mouse immunoglobulin-FITC and analysed by flow cytometry.
TABLE 2. Percentage Of T Cells In Total Efferent Lymph Leaving An In Vivo Antigen Stimulated Lymph Node.

**Mycobacterium tuberculosis Challenge**

<table>
<thead>
<tr>
<th>T cell subset</th>
<th>Days after antigen challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>CD4</td>
<td>48.2</td>
</tr>
<tr>
<td>CD8</td>
<td>9.0</td>
</tr>
<tr>
<td>γδ</td>
<td>15.4</td>
</tr>
<tr>
<td>CD4 : CD8</td>
<td>5.4</td>
</tr>
<tr>
<td>αβ : γδ</td>
<td>3.7</td>
</tr>
</tbody>
</table>

**Ovalbumin Challenge**

<table>
<thead>
<tr>
<th>T cell subset</th>
<th>Days after antigen challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>CD4</td>
<td>44.9</td>
</tr>
<tr>
<td>CD8</td>
<td>14.4</td>
</tr>
<tr>
<td>γδ</td>
<td>5.4</td>
</tr>
<tr>
<td>CD4 : CD8</td>
<td>3.1</td>
</tr>
<tr>
<td>αβ : γδ</td>
<td>10.9</td>
</tr>
</tbody>
</table>
7.3.6. A Larger Fraction of Efferent Lymph $\gamma\delta$ T Cells Express CD25 Following *In-Vivo* Secondary Antigen Challenge.

The data reported above implies that following secondary *in vivo* antigen challenge an increased output of $\gamma\delta$ T cells can be observed within both total and activated lymphocytes of efferent lymph. The percentage of $\gamma\delta$ T cells expressing CD25 within these two populations was assessed by two colour immunofluorescence analysis. The results are shown in Figure 12 and 13.

Twenty four hours subsequent to an *in vivo* secondary challenge the number of CD25-positive $\gamma\delta$ T cells within the total lymphocyte population (Figure 12) was similar to pre-challenge levels. After 2 days, a significant increase in the number of CD25-positive $\gamma\delta$ T cells was observed ($p < 0.05$), thereafter, returning to control levels. The number of CD4-positive and CD8-positive T cells expressing CD25 at time points 1 and 2 days showed the same trends as that seen with $\gamma\delta$ T cells. Similarly, the number of CD25-positive $\gamma\delta$ T cells within the activated population showed an analogous trend with respect to time as that observed for $\gamma\delta$ T cells within the total population, in that a significant increase in the number of CD25 expressing $\gamma\delta$ T cells was observed 2 days after antigen challenge of the lymph node ($p < 0.05$) (Figure 13). In contrast to the total population, this number stayed either constant or increased slightly 3 days after challenge. Between days 4 and 7 the number of CD25 expressing $\gamma\delta$ T cells decreased to control levels. The expression of CD25 by $\alpha\beta$ T cells within the activated population followed an analogous trend to that obtained with the activated $\gamma\delta$ T cell population.

7.4. Changes in The Percentage of Ovine $\gamma\delta$ T Cells in Antigen Challenged Lymph Nodes.

Data reported in previous section 3.6. of this thesis implies a greater reliance on $\gamma\delta$ T cells within young sheep. A significant proportion of their lymphocytes comprise cells expressing a $\gamma\delta$ TCR and the number of these cells is increased upon exposure to pathogens. In addition, section 6.3. established that this T cell lineage has the ability to respond to antigens *in vitro*. In view of these observations it might be expected that
Efferent lymphocytes were collected from the cannulated efferent lymphatic vessels of 18 month old Finnish Landrace animals following secondary in vivo challenge with either *Mycobacterium tuberculosis* or ovalbumin around the drainage area of the lymph node. Cells were analysed by two colour immunofluorescence for co-expression of CD25 with either γδ TCR, CD4 or CD8. Cells were stained with IL-A111 (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were subsequently treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when IL-A111 and 86D were used cells were treated with IL-A111 alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the output per hour from the lymph node of γδ TCR-positive, CD4-positive and CD8-positive T cells co-expressing CD25 within the total population was calculated. Statistical analysis was carried out as described in section 7.3.
**Figure 12.** CD25-Positive T Cell Subset Output From In Vivo Antigen Challenged Lymph Nodes.

![Graph](image)

**Key:**
- CD8-Positive T Cells
- CD4-Positive T Cells
- γδ T Cells

<table>
<thead>
<tr>
<th>Days After Antigen Challenge</th>
<th>Number of Cells x 10^6 Hour⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>20</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>20</td>
</tr>
</tbody>
</table>

*p < 0.05 relative to controls.*
Efferent lymphocytes were collected from the cannulated efferent lymphatic vessels of 18 month old Finnish Landrace animals following secondary *in vivo* challenge with either *Mycobacterium tuberculosis* or ovalbumin around the drainage area of the lymph node. Cells were analysed by two colour immunofluorescence for co-expression of CD25 with either γδ TCR, CD4 or CD8. Cells were stained with IL-A111 (IgG1) in association with either 86D (IgG1), SBU-T4 (IgG2a) or SBU-T8 (IgG2a) which were all biotinylated. When monoclonal antibodies of different isotypes were used cells were subsequently treated with anti-mouse IgG1-FITC and phycoerytherin-streptavidin. Alternatively, when IL-A111 and 86D were used cells were treated with IL-A111 alone followed by anti-mouse IgG1-FITC and then 10% normal mouse sera. Cells were finally reacted with biotinylated 86D followed by phycoerytherin-streptavidin. Cells were analysed by flow cytometry and the output per hour from the lymph node of γδ TCR-positive, CD4-positive and CD8-positive T cells co-expressing CD25 within the activated population was calculated. Statistical analysis was carried out as described in section 7.3.
FIGURE 13. Activated CD25-Positive T Cell Subset Output From In Vivo Antigen Challenged Lymph Nodes.

KEY:

Days After Antigen Challenge

Number of Cells x 10^6 Hour^-1

Mycobacterium tuberculosis

Ovalbumin

p > 0.05 relative to controls.

CD8-Positive T Cells

CD4-Positive T Cells

Other T Cells

KEY:

p < 0.05 relative to controls.

CD4-Positive T Cells

CD8-Positive T Cells

Other T Cells

FIGURE 13. Activated CD25-Positive T Cell Subset Output From In Vivo Antigen Challenged Lymph Nodes.
during an immune response these cells localise to fixed lymphoid tissue where cellular interactions are known to occur.

This hypothesis was investigated by the challenge of animals with antigen around the drainage area of both the prefemoral or prescapular lymph nodes on day 0, which was repeated 28 days later. 3 animals were used in the investigation and were primed with either *Mycobacterium tuberculosis*, ovalbumin or PBS on the above time points. The left prefemoral and prescapular lymph nodes were excised from each animal on day 7, whilst the right were excised on day 35. Single cell suspensions of lymphocytes were prepared from each node as described in section 2.4.3. The aims of the investigation were (i) to determine the intranodal distribution of γδ T cells in antigen challenged nodes, and (ii) to determine the percentage of γδ T cells within a single cell suspension obtained from antigen challenged nodes.

7.4.1. Distribution of Ovine γδ T cells in Lymph Nodes.

Analysis of the intranodal distribution of γδ T cells was made to determine if these cells localise to a particular cellular compartment during an immune response. This may provide some implication on the role this T cell lineage impart within fixed lymphoid tissue during challenge of the host with antigen. Sections of each lymph node were prepared for immunohistology as described in section 2.9.1. The results of the investigation are shown in Figures 14 and 15.

Immunohistochemical analysis of antigen challenged and control nodes showed no significant differences in the localisation of γδ T cells between the nodes. In addition, there was no obvious increase in the number of γδ T cells within challenged nodes as determined by conventional microscopy. A noticeable feature of the localisation of γδ T cells was that they were absent from areas of the lymph node predominated by αβ T cells (Figure 14). Generally γδ T cells were absent from the lymphoid follicles and paracortex, which are the prime locations of CD4-positive and CD8-positive cells, respectively. Figure 15 shows that γδ T cells were positioned in linear arrays, 1 to 2 cells deep, parallel to the marginal sinus and trabeculae, scattered in the basal cortical
Figure 14. Finnish Landrace sheep (n=3) were injected around the drainage area of popliteal and prefemoral lymph nodes with 1mg of either heat-killed *Mycobacterium tuberculosis* or ovalbumin in 1ml of PBS. Control animals were injected with 1ml of PBS. Lymph nodes were excised after 7 days and cryosections were prepared and stained either with monoclonal antibodies 86D, SBU-T4 or SBU-T8 followed by anti-mouse anti-IgG biotinylated monoclonal antibody and finally horse radish peroxidase conjugated to streptavidin. Positive cells were visualised by conventional microscopy.
FIGURE 14. The Localisation of γδ T Cells in Lymph Nodes.

(a) γδ TCR-Positive Cells

(b) CD4-Positive Cells

(c) CD8-Positive Cells
Figure 15.
Finnish Landrace sheep (n=3) were injected around the drainage area of popliteal and prefemoral lymph nodes with 1mg of either heat-killed *Mycobacterium tuberculosis* or ovalbumin in 1ml of PBS. Control animals were injected with 1ml of PBS. Lymph nodes were excised after 7 days and cryosections were prepared and stained with monoclonal antibody 86D followed by anti-mouse immunoglobulin-FITC. Positive cells were visualised by fluorescent microscopy.
FIGURE 15. The localization of γδ T Cells in Lymph Nodes.
areas and in the peripheral margins of the medullary cords but actually within the node parenchyma tissue. γδ T cells in the cortex and medullary cords were separated from the marginal sinus, the trabecular sinus and the medullary sinus by a layer of γδ-negative cells and did not form the boundary of the parenchymal tissue. Occasionally γδ T cells were found randomly positioned within cortical lymphoid follicles, an area associated with B cell differentiation. Small numbers of γδ T cells were present within the interfollicular areas.

An observation which distinguished antigen challenged nodes from control nodes was that γδ T cells were absent in areas around the follicles adjacent to the marginal sinus. This is likely to be a consequence of enlargement of lymphoid follicles during an immune response, with the result that cells directly above the follicle were pushed against the marginal sinus, making their visibility by immunohistochemical analysis difficult.

7.4.2. Proliferative Response of Lymph Node Lymphocytes To Antigen.
To show that antigen priming of lymph nodes had taken place, single cell suspensions were prepared from lymph node tissue and cultured in vitro in the presence of the immunising antigen. The results are shown in Figure 16.

Animals primed with either Mycobacterium tuberculosis or ovalbumin showed an antigen specific response only to the priming antigen. Control animals were unable to elicit an in vitro proliferative response to either antigen.

7.4.3. γδ T Cell Composition of Lymph Nodes.
A comparison of the percentage of γδ T cells within the cell suspensions was made by single colour immunofluorescence analysis. The average percentage of γδ and αβ T cells and the ratio of αβ / γδ T cells was calculated for the two nodes excised from each animal at the time points investigated. Similar trends were shown when lymph nodes were excised after either a primary (day 7) or secondary challenge (day 35),
Figure 16.
Finnish Landrace sheep (n=3) were injected around the drainage area of popliteal and prefemoral lymph nodes with 1mg of either heat-killed *Mycobacterium tuberculosis* or ovalbumin in 1ml of PBS. Control animals were injected with 1ml of PBS. Lymph nodes were excised after 7 days and single cell suspensions of lymphocytes prepared. Viable cells were isolated by density centrifugation and $10^5$ cells/well were cultured with various concentrations of either *Mycobacterium tuberculosis* or Ovalbumin in flat-bottom plates. The assays were incubated for 3 days and proliferation was measured by the uptake of $^3$H-thymidine over the last 7 hours of culture.

Mycobacterium tuberculosis.

KEY:

- Mycobacterium tuberculosis Challenged Animal
- Ovalbumin Challenged Animal
- Control Animal

Concentration of Antigen µg ml⁻¹
consequently data for only the former is included in this thesis and is shown in Table 3. Included in brackets are the range of percentages and range of ratios obtained.

In the *Mycobacterium tuberculosis* challenged animal an increased percentage of $\gamma \delta$ T cells was apparent, whilst a similar percentage was seen with cells prepared from the ovalbumin challenged animal and the control animal. The data shows that lymph nodes isolated from an animal primed with *Mycobacterium tuberculosis* had a reduced ratio of $\alpha \beta / \gamma \delta$ T cells relative to the control nodes. The average ratio was 3.8 within a range of 3.4 to 4.2, whilst the control nodes yielded a ratio of 15.2, within a range of 13.0 to 17.3. The average ratio of $\alpha \beta / \gamma \delta$ T cells obtained from the ovalbumin challenged animal was 6.5 within a range of 5.5 to 7.5. The data may imply a preferential expansion of $\gamma \delta$ T cells, relative to $\alpha \beta$ T cells, in antigen challenged nodes, in particular when the nodes were primed and challenged with *Mycobacterium tuberculosis*.

The above investigation compares changes in $\gamma \delta$ T cells of antigen challenged nodes between animals. A study was established to show that the above data was a consequence of antigen challenge to the lymph nodes, rather than variation within animals. Left lymph nodes (both prefemoral and prescapular nodes) were excised from a group of six animals on day 0 and subsequently two animals were selected at random and immunised with either *Mycobacterium tuberculosis* or ovalbumin. Control animals were treated with PBS. The right lymph nodes were removed 7 days later. Lymph node tissue was processed and analysed as above. Table 4 shows the average ratio of $\alpha \beta / \gamma \delta$ T cells of lymph nodes excised prior and 7 days subsequent of an *in vivo* antigen challenge. The data is tabulated in a format where animals were grouped according to the *in vivo* antigenic challenge they received on day 0. Included in brackets are the range of the ratios obtained.

Subsequent to *in vivo* antigen challenge the ratio of $\alpha \beta / \gamma \delta$ T cells decreased in the *Mycobacterium tuberculosis* challenged animals, whilst both ovalbumin challenged animals and control animals showed no decrease between the time points investigated.
Table 3.
Finnish Landrace sheep (n=3) were injected around the drainage area of popliteal and prefemoral lymph nodes with 1mg of either heat-killed Mycobacterium tuberculosis or ovalbumin in 1ml of PBS. Control animals were injected with 1ml of PBS. Lymph nodes were excised after 7 days and single cell suspensions of lymphocytes prepared. Viable cells were harvested by density centrifugation and stained with either monoclonal antibodies 86D, 19.19, ST4, ST8 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry. Included in brackets are the range of percentages and ratios obtained.
TABLE 3. Percentage Of Lymphocytes In Antigen Stimulated Lymph Nodes (n=2).

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>CD4</th>
<th>CD8</th>
<th>γδ</th>
<th>T19</th>
<th>sIg</th>
<th>αβ / γδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.8</td>
<td>20.9</td>
<td>3.7</td>
<td>3.0</td>
<td>33.7</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>(31.5-38.0)</td>
<td>(19.2-22.6)</td>
<td>(3.5-3.9)</td>
<td>(2.8-3.1)</td>
<td>(31.1-36.3)</td>
<td>(13.0-17.3)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>12.7</td>
<td>14.7</td>
<td>12.7</td>
<td>12.8</td>
<td>40.0</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>(27.0-40.3)</td>
<td>(13.4-15.9)</td>
<td>(11.9-13.4)</td>
<td>(12.3-13.3)</td>
<td>(33.6-36.3)</td>
<td>(3.4-4.2)</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>31.3</td>
<td>10.1</td>
<td>6.5</td>
<td>6.0</td>
<td>55.5</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>(30.8-31.8)</td>
<td>(9.9-10.3)</td>
<td>(5.4-7.6)</td>
<td>(4.9-7.1)</td>
<td>(54.9-56.0)</td>
<td>(5.5-6.5)</td>
</tr>
</tbody>
</table>
Table 4.
The left hand side prefemoral and prescapular lymph nodes were excised from Finnish Landrace sheep (n=6). Single cell suspensions of lymphocytes prepared and viable cells were harvested by density centrifugation. 1mg of either heat-killed *Mycobacterium tuberculosis* or ovalbumin in 1ml of PBS was injected around the drainage area of the right hand side popliteal and prefemoral lymph nodes. Control animals were injected with 1ml of PBS. Seven days later lymph nodes were excised and single cell suspensions of lymphocytes prepared. Viable cells were harvested by density centrifugation. All cells were stained with either monoclonal antibodies 86D, 19.19, ST4, ST8 or VPM8 followed by anti-mouse immunoglobulin-FITC. Cells were analysed by flow cytometry. The ratio of αβ / γδ T cells are shown. Included in brackets are the range of ratios observed.
TABLE 4. Ratio Of αβ / γδ T Cells In Lymph Nodes After In Vivo Antigen Challenge (n=2).

<table>
<thead>
<tr>
<th>Antigen used For Challenging</th>
<th>Ratio αβ / γδ T cells in Unchallenged nodes</th>
<th>Ratio αβ / γδ T cells in Challenged nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>7.6 (5.5-8.7)</td>
<td>4.5 (3.6-5.6)</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>7.5 (7.1-8.1)</td>
<td>7.8 (6.2-9.8)</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>10.0 (8.3-11.2)</td>
<td>10.2 (5.7-15.5)</td>
</tr>
</tbody>
</table>
DISCUSSION.

This Chapter has focussed on establishing the role of ovine γδ T cells during in vivo antigen challenge with heat-killed *Mycobacterium tuberculosis* and ovalbumin. A comparison of the percentage of γδ T cells within PBMC, afferent and efferent lymph provides potential to obtain information on the recirculation patterns of these cells to determine if they appear to localise within one lymphoid compartment relative to others. It has previously been established that afferent and efferent lymph compartments have a greater percentage of CD4-positive cells compared to peripheral blood (Mackay et al 1988b, Bujdoso 1989a). This supports the concept of selective T cell migration across subcutaneous endothelium and lymph node endothelial venules, resulting in the non-random distribution of T cell subsets between blood and the lymphatic system. In addition this study was designed to investigate if increased numbers of γδ T cells expressing CD25 could be detected in lymphatic vessels, either afferent or efferent, following antigen challenge of the area drained by that particular lymphatic vessel. An increase in the number of activated γδ T cells would indicate that these cells had recognised their ligand as a consequence of antigen challenge. Information on the percentages of γδ T cells within lymphoid compartments in association with the activational status of these cells may bear reflection on both the ligand they recognise and its localisation. Finally, the intranodal distribution and the percentage representation of γδ T cells within antigen challenged lymph nodes was analysed.

Following the challenge of animals with antigen around the drainage area of the cannulated vessel the cellular output of afferent lymph was variable. An initial decrease in the output was observed, followed by a transient increase, thereafter the number of cells in afferent lymph decreased with the duration of the cannulation. This implies that immediately after challenge lymphocytes were retained within epithelial tissue and the small increase in cell output subsequent to this maybe a consequence of these cells leaving epithelial sites rather than the expansion of lymphocytes in the skin. In contrast, there was no initial decrease in cell output of efferent lymph following antigen challenge, levels remained comparable to controls. Over the next few days elevated numbers of cells were apparent within efferent lymph. These data are not in agreement with others,
which demonstrate that secondary challenge of lymph nodes results in a marked reduction in the number of cells leaving the node via efferent lymph, a phenomenon known as cell shutdown (Hall and Morris 1965). Following this initial period the increase in cell output reported here is likely to comprise lymphocytes that are recruited to the node and those generated within the node in response to antigen (Hay and Hobbs 1977).

The investigation of CD25 expression by γδ T cells isolated from various lymphoid compartments demonstrated that prior of antigenic challenge approximately 50% of afferent lymph ovine γδ T cells are activated. In vivo antigen challenge with the antigens tested was unable to increase either the output of γδ T cells or the number of CD25-positive γδ T cells within the afferent lymphatic vessels. The inability to see an increase in the number of activated γδ T cells leaving an epithelial site challenged with either Mycobacterium tuberculosis or ovalbumin may merely reflect the low frequency of γδ T cell clones which recognise these antigens whose activation cannot be detected above the immune response already occurring in afferent lymph. The fact that such a high percentage of γδ T cells are already activated suggests that the ligand(s) these cells recognise are present in at least skin epithelium prior to an experimental antigen challenge. This observation supports the hypothesis that some γδ T cells may recognise self ligands such as stress proteins.

Following in vivo antigen challenge there was no significant change in the number and phenotype of the CD4-positive T cell subset within afferent lymph, whilst a small increase was observed in both the number of CD8-positive T cells and those CD8-positive T cells co-expressing CD25. This suggests that in vivo antigen challenge initiates predominantly a CD8 response within afferent lymph.

In contrast to the data reported above for afferent lymph this study has shown that following a secondary in vivo antigen challenge of lymph nodes, a small increase in the number of γδ T cell was apparent in lymph draining the node. These γδ T cells were present in both the small resting lymphocyte and lymphoblast populations. Most importantly there was an increase in the number of γδ T cells expressing CD25 within
both populations of γδ T cells. The increase in the number of CD25-positive γδ T cells leaving an antigen stimulated lymph node implies these cells have become activated as a consequence of antigen challenge to the node. As the majority of efferent lymph cells are derived from peripheral blood, it is unlikely these activated γδ T cells have originated from afferent lymph. Rather, these γδ T cells may be derived from peripheral blood after having undergone antigen-specific clonal expansion in the antigen-stimulated lymph node. If this is the case then the majority of the activated γδ T cells entering a lymph node from afferent lymph appear not to leave via the efferent lymph vessel.

Whilst small changes were observed within the γδ T cell subset within efferent lymph upon in vivo antigen challenge of the node, a significant increase was observed in the number of the αβ T cell subset within the small lymphocyte and blasting lymphocyte populations. In a similar manner to the γδ T cell subset, the αβ T cell subset showed an increase in CD25 expression following antigen challenge. It is noteworthy that the increased output of the αβ T cell subset was predominantly an increase in the output of CD4-positive cells.

The appearance of activated lymphocytes of all three T cell subsets within efferent lymph was correlated with an increased in vitro proliferative response of these cells to IL-2. Despite an increase in the appearance of activated cells it is not known whether they are antigen specific. An investigation of the antigen specificity of these activated cells would be required to establish this.

This study has demonstrated that in vivo antigenic challenge of lymph nodes appears to increase the percentage of γδ T cells, and the ratio of αβ / γδ T cells within the lymph node tissue. Profound differences were observed when the node was challenged with Mycobacterium tuberculosis but the changes observed with ovalbumin were less dramatic. These changes appear to be antigen specific as an in vitro proliferative response of these cells to the priming antigen was shown although no indication of whether γδ or αβ T cells are the responding population was established. This increase in the percentage of γδ T cells and changes in the ratio may be a consequence of three possibilities: (i) γδ T cells are clonally expanded in the lymph node upon recognition of
their specific ligand, (ii) γδ T cells are selectively retained within the lymph node after in vivo antigen challenge, or (iii) there is a selective decrease in the number of αβ T cells within the lymph node following in vivo antigen challenge.

Immunocytochemistry of lymph node histology demonstrated that ovine γδ T cells localise predominantly within the peripheral areas of the node parenchyma, arranged in linear arrays parallel to the marginal sinus, trabeculae and medullary cords, areas known to be associated with the reticuloendothelial system. The absence of γδ T cells from the areas of the node classically associated with T cell ligand recognition and activation raises the question of whether this lineage encounter their ligand(s) on the surface of antigen presenting cells within lymph nodes. If not, an alternative explanation is that this T cell lineage mediate other functions within lymph nodes, such as helper functions by interacting with other lymphocytes, assisting their activation or clonal expansion. The absence of γδ T cells from lymphoid follicles implies that these cells, if their aim is to provide help, mediate this function in a distinct manner to that of the CD4-positive population which provide help during the differentiation of B cells into plasma cells within the follicles. In contrast human γδ T cells localise within lymph nodes in a manner analogous to that of the αβ lineage (Groh et al 1989), with the exception that γδ T cells represent a smaller fraction of the T cell population relative to αβ T cells. This implies that ovine and human γδ T cells either recognise a different array of antigens within the lymph node or mediate functions distinct to the species.

Previously the localisation of ovine γδ T cells has been interpreted to suggest that these cells play no significant role within the lymph node and are merely trafficking through the node (Mackay et al 1989). It was implied that the majority of these cells are derived from afferent lymph, based on their poor ability to migrate from the blood into lymph nodes (Mackay et al 1988b), transiting quickly into areas associated with lymphocyte migration, and finally into the medulla. It is noteworthy that this statement is based on the authors observation that, in contrast to this study, ovine γδ T cells were found within the marginal and trabecular sinus rather than within the node parenchyma.
An explanation for the distinct distribution of $\gamma\delta$ T cells within lymph nodes is that these cells are not merely trafficking through the node but are interacting with cells at these sites. Likely candidates are macrophages of the reticuloendothelial system. In addition this study has shown an increase in the number of activated $\gamma\delta$ T cells in transit from an antigen challenged lymph node, implying that this lineage have encountered their ligand, presumably, on the surface of an antigen presenting cell within the lymph node. This provides further evidence that this lineage of T cells have a defined function within the lymph node rather than a transitional one through the node and back into the vascular system.

The presence of activated $\gamma\delta$ T cells in afferent lymph in the absence of an intentional in vivo antigenic challenge may suggest that the ligand these cells recognise are continually present in the epithelium which the afferent lymphatics drains. Whether the ligand, presumably comprising peptide and presentational elements on the surface of antigen presenting cells present in epithelium, that $\gamma\delta$ T cells recognise is an exogenous derived component such as that from a bacterium, or is an endogenous derived component such as stress proteins is as yet unclear. A likely possible in vivo model of $\gamma\delta$ T cell activation within the ovine species is that $\gamma\delta$ T cells localise at epithelial sites by migration from peripheral blood and recognise their immunogen on the surface of dendritic cells (Bujdoso et al 1990). These two subsets of cells then migrate to the draining lymph node for activation and or proliferation for subsequent effector function development.
CHAPTER 8

Final Discussion.

The discussion was an investigation of various T cells. The main objective of the research was to investigate the prevalence and properties of different T cells. This objective was achieved through isolation of various T cells from various sources and examination of their characteristics. A second objective was to investigate a possible role for these cells in the activation of other cells. A third objective was to investigate the role of various T cells in the activation of other cells. As a result, the investigators found that certain T cells were able to activate other cells, which in turn activated still other cells, creating a chain reaction.

One of the key findings was the role of T cells within the immune system. It was observed that T cells play a crucial role in the immune response, particularly in the activation of other immune system cells. This was supported by the findings of other studies, which had also indicated the importance of T cells in the immune response.

For more information, the reader is referred to the studies of other researchers. However, the role of T cells in the immune system is now discussed.
8.1 Objective Of This Thesis.

This thesis was an investigation of ovine $\gamma\delta$ T cells. The initial objective of this thesis was to investigate the prevalence and phenotype of ovine $\gamma\delta$ T cells. These studies were intended to establish a base of information on the expression of various cell surface molecules by ovine $\gamma\delta$ T cells, isolated from animals of different ages and antigenic burden. A second objective was to establish a purification technique for the isolation of $\gamma\delta$ T cells from ovine peripheral blood for the assessment of antigen recognition and analysis of the presentational elements used by these cells. A third objective of this thesis was to investigate the \textit{in vitro} activation of ovine $\gamma\delta$ T cells as they lack the expression of the co-receptor molecules CD4 and CD8 which are intimately associated with T cell activation pathways. Information from these first three objectives, namely phenotype, activation and antigen responsiveness, of $\gamma\delta$ T cells were then applied to a fourth objective which was to study these cells \textit{in vivo} by the technique of lymphatic cannulation. This discussion has focused primarily on two major aspects of the thesis, namely the \textit{in vitro} and \textit{in vivo} activation of ovine $\gamma\delta$ T cells.

8.2. $\gamma\delta$ T Cells And Their Role Within The Immune System.

It is collectively hypothesised that $\gamma\delta$ T cells recognise their ligand as a complex of either class I\textsubscript{b} or CD1 molecules in association with peptides at epithelial sites (Janeway et al 1988, Tonegawa et al 1989). Such peptides may be derived from intracellular bacteria, which invade epithelial sites of the host, via a pathway similar to that of the MHC class I antigen processing and presentation (Hedrick 1992). Upon ligand recognition $\gamma\delta$ T cells may then initiate a cytotoxic response that eliminates the transformed or infected target cells. However, some $\gamma\delta$ T cells recognise peptides associated with classical MHC class I or class II molecules but evidence that this is the case is limited (Matis and Bluestone 1991, Bluestone et al 1988, Matis et al 1989, Matis et al 1987). The evidence for such hypotheses is now discussed.
The localisation of γδ T cells near epithelial cells, during their thymic ontogeny may imply that by interacting with epithelial cells within the thymus, or with their extracellular matrix, the repertoire of the developing γδ T cell population is selected for the recognition of features that distinguish healthy epithelial cells from infected or transformed counterparts. In the sheep species it has been shown that during TCR rearrangement γδ T cells predominate in the medulla of the thymus (Hein and Mackay 1991), rather than within the cortex which is the prime location of αβ T cells. In particular, γδ T cells are seen in high density around, and occasionally within, the Hassall’s bodies (Mackay et al 1989, McClure et al 1989). Hassall’s bodies are generated by the differentiation of medullary epithelial cells as judged by morphological studies and the co-expression of specific cytokeratin polypeptides on Hassall’s bodies and epidermis (Goldstein and Mackay 1969, Von Gaudecker 1986). All cytokeratins that are expressed in a tissue-specific fashion elsewhere in the body are produced by cells of the thymic reticulum (Heid et al 1988). Other features of γδ T cell ontogeny, such as the timing of their TCR gene rearrangement further suggest this T cell lineage comprise a repertoire of receptors that recognise particular sets of cells and presentational elements to those of αβ T cells. γδ T cells rearrange their TCR prior to the development of some stromal elements implicated of significance in the development of αβ T cell (Hayes 1984).

In addition the absence of the cell surface expression of CD4 and CD8 on γδ T cells further suggests that γδ T cells do not recognise MHC molecules in the same manner as do αβ T cells. Several pieces of data have shown that the presentational elements for some γδ T cell clones comprise non-polymorphic molecules such as the class Ib (Wang et al 1991, Ito et al 1989, Vidovic et al 1989) or CD1 (Porcelli et al 1989) molecules. The former are known to be expressed by various tissues in a manner similar to MHC class I molecules, but in particular are enriched at various tissues populated by γδ T cells (Eghtesady et al 1992), whilst the latter are expressed primarily on the surface of cells known to be antigen presenting cells (Porcelli et al 1991).

Recent data has reported that a class Ib molecule H-2M3, (Wang et al 1991) and CD1b (Porcelli et al 1992) present immunogens to T cells in the form of processed peptides.
located within a putative peptide binding groove of these molecules. The presentation of peptides in association with class I b molecules (Pamer et al 1992) and CD1 molecules (Porcelli et al 1992) was made to αβ TCR expressing T cell lines. In view of this data it is likely that the γδ T cell line reported by Porcelli et al (1989), which lysed Molt-4 cells did so by recognition of specific peptide associated with CD1c. It is suggested that the class I b molecule H-2M3 preferentially binds peptides derived from prokaryotic organisms (Shawar et al 1990, Fisher Lindahl et al 1991) based on the observations that H-2M3 present peptides containing a formylated N-terminal methionine.

Some of the data reported in this thesis supports the above prototypic hypothesis for γδ T cell function and some of the data suggests γδ T cell functions may differ between species. These aspects of the thesis are now discussed further.

8.3. Similarities And Differences Of γδ T Cells In Various Species.

Ruminants are characterised by the localisation of some γδ T cells at various epithelial tissues, such as the skin, gut, oesophagus, tongue and vaginal epithelium (Hein and Mackay 1991). In chickens, γδ T cells are found in the intestinal epithelium but not the skin (Bucy et al 1988), whilst human γδ T cells are sparse at all epithelial sites (Groh et al 1989). In contrast, murine γδ T cells show a marked tropism for the epithelial microenvironment (Allison and Havran 1991). Ruminant skin γδ T cells which localise primarily in the dermal layers and adjacent to epithelium lining the hair follicles and their associated glands but murine γδ T cells localise in the epidermal layers of the skin (Bergstresser et al 1985). In addition, murine γδ T cells located at such sites, with the exception of intestinal γδ T cells (Guy-Grand et al 1991), are further characterised by the use of the same TCR V gene segments yielding γδ T cells with limited diversity. To date, it remains to be established if γδ T cells located at epithelial sites in other species express γδ TCR with limited diversity. A feature characteristic of all species so far investigated is that γδ T cells represent only a minor population within the secondary lymphoid tissue (Vroom et al 1991), with the exception of the avian species where up to 30% of splenic T cells express a γδ TCR (Sowder et al 1988).
The circulating pool of \(\gamma\delta\) T cells differs between species and may indicate varied levels of reliance on the \(\gamma\delta\) T cell subset in the immune system of different species. This thesis has shown that \(\gamma\delta\) T cells may constitute a major subpopulation of peripheral T cells of sheep and extends the preliminary observations by others which suggested that ruminants in general have higher levels of these cells compared to other species (Hein and Mackay 1991). In humans and mice \(\gamma\delta\) T cells constitute only 1-5% of the peripheral blood lymphocyte pool (Groh et al 1989). Data reported in this thesis has shown the predominance of \(\gamma\delta\) T cells in neonatal lambs. This is in contrast to what is reported in young individuals of other non-ruminant species (Parker et al 1990).

Neonatal ruminants, in comparison to newborn humans and mice, show precocious activity, resulting in far greater exposure to foreign antigens implying that an increased level of \(\gamma\delta\) T cells evolved in response to physiological differences. Evidence that increasing pathogenic burdens results in an increased percentage of \(\gamma\delta\) T cells within ovine peripheral blood, is also reported in this thesis where an investigation was carried out of the level of \(\gamma\delta\) T cells within young lambs exposed to various antigenic burdens.

Analysis of the V gene usage of ovine \(\gamma\delta\) T cells established that, in contrast to their human and murine counterparts, these cells of the ovine species express a greater repertoire of antigen receptors which is contributed to by the increased diversity in both variable and constant region gene segments (Hein and Dudler 1993). Variable region diversity results mainly from the use of a large family of duplicated V\(\delta\) genes that have retained two distinct hypervariable segments comparable to the complementarity determining regions present in other antigen V genes. The repertoire of rearranged V\(\gamma\) and V\(\delta\) gene segments expressed in peripheral \(\gamma\delta\) T cells in the sheep varies at different stages of development and differs markedly between fetuses and adult animals. This implies that sheep V\(\gamma\) chains have been intensely selected during evolution, probably at sites involved in ligand recognition (Hein and Dudler 1993). In contrast, human peripheral blood \(\gamma\delta\) T cells comprise two populations, the major population (70-90% of total \(\gamma\delta\) T cells) utilise the V\(\gamma9\) gene segment, whilst the minor population (10-30% of total \(\gamma\delta\) T cells) utilise the V\(\delta1\) gene segment (Sturm et al 1989).
A common feature of the majority of γδ T cells in all species so far examined is that they do not express the co-receptor molecules CD4 and CD8. The expression of CD4 and CD8 molecules by αβ T cells is now known to couple TCR triggering to T cell activation via protein tyrosine kinases (PTK) associated with the cytoplasmic domain of these co-receptor molecules (Janeway 1992). This inability by the majority of γδ T cells to express the cell surface glycoproteins CD4 and CD8 may imply that the activation of these cells is regulated by different mechanisms. Data obtained in this thesis suggests that this is the case.

8.4. In Vitro Activation Of Ovine γδ T Cells.

An in vitro comparison of the rate of activation of γδ and αβ T cells has shown that ovine γδ T cells were activated by Con A prior to αβ T cells as assessed by expression of IL-2 receptor α chain, CD25. As yet, no similar comparison has been reported for the activation rate of γδ and αβ T cells in other species, hence it remains to be established if these activation characteristics apply to γδ T cells within different species or whether they are unique to ovine γδ T cells. In addition, an investigation of the expression of the nuclear antigens, termed Ki67 (Gerdes et al 1991), which provide a marker of proliferating cells, showed distinct differences between the two T cell lineages. Whilst both lineages expressed Ki67 antigens 48 hours after Con A activation, the immunofluorescence intensity of Ki67 expression by γδ T cells was lower than that of their αβ counterparts.

If this difference in the rate of activation between γδ and αβ T cells is peculiar to ovine γδ T cells it may be because of the unique molecule, namely T19, shown by this thesis to be expressed by the majority of γδ but not αβ T cells within the sheep. This molecule may serve as an activation molecule on γδ T cells as does the CD4 and CD8 on αβ T cells. To date no homologue of this molecule has been found on the surface of γδ T cells from other species although the genes encoding T19 have been discovered in other species (Metzelaar et al 1992). The recent cloning of the T19 molecule has established that its protein structure is characteristic of a type I integral membrane protein with an extracellular domain consisting of 11 scavenger receptor cysteine-rich-repeats.
This family of type I integral membrane proteins include the prototypic macrophage scavenger type I receptors (Freeman et al 1990), CD5 (Burgess et al 1992) and CD6 (Arufo et al 1991). These macrophage scavenger type I receptors bind a wide variety of polyanions, including chemically modified proteins and lipids and certain polynucleotides (Freeman et al 1990). CD5 physically associates with the TCR/CD3 complex of T cells (Osman et al 1992) and represents a substrate of p56\textsuperscript{lek}, implying a direct role for this molecule during T cell activation.

Based on the findings of the immunobiochemistry of CD5 and the homology between CD5 and the T19 molecule it is tempting to speculate that the latter may have a key role in the signal transduction pathway and subsequently the activation of ovine γδ T cells. Phosphorylation of the T19 protein by a PTK, such as p56\textsuperscript{lek}, may induce a conformational change(s) within T19 that results in an enhanced pathway of signal transduction across the cell membrane of γδ T cells. Alternatively, phosphorylation of the T19 molecule may activate other PTK which subsequently phosphorylate other cellular proteins responsible for transmitting signals to the nucleus. If future investigations demonstrate both a direct role of the T19 molecule in T cell activation and no apparent difference in the rate of CD25 expression by γδ T cells of other species, these findings may imply that γδ T cells within ruminants have evolved in such a manner that enables these lymphocytes to become activated prior to αβ T cells.

It has been shown that human γδ T cells constitutively express the β-chain of the IL-2 receptor (Aparicio et al 1989). This component of the IL-2 receptor is implied to be the signal transducing element, which upon binding its ligand initiates the synthesis of CD25 (Tsudo et al 1987), yielding an IL-2 receptor of high affinity. As yet, no monoclonal antibody is available to the ovine β-chain of the IL-2 receptor to verify if a similar situation occurs on ovine γδ T cells. However, if an analogous situation does occur it may imply that CD25 synthesis and expression by γδ T cells occurs prior to that of αβ T cells as a consequence of the signal transduction pathway via the β-chain of the IL-2 receptor inducing CD25 expression at an increased rate relative to the TCR / CD3 complex on αβ T cells.
An alternative explanation for the activation rate of γδ and αβ T cells is offered by the observation that γδ T cells differ in the δ-chain of the CD3 proteins compared to αβ T cells (Brenner et al. 1986). If the differences between the CD3δ chain of γδ and αβ T cells results in a more efficient transduction of signals across the cell membrane in γδ T cells compared to αβ T cells it may reduce the time required for signals to be transmitted to the nucleus of the former cells inducing CD25 expression at a more rapid rate.

Evidence in favour of the hypothesis that changes within the CD3 complex initiates differences in T cell signal transduction is from the work of Mizoguchi et al. (1992), where they report that T cells from tumour bearing mice had impaired immune responses as a consequence of a decreased expression of CD3γ chain. Based on the high degree of homology between the CD3 γ and δ chains (Gold et al. 1987), it is feasible to suggest that changes in either the structure or the level of surface expression of the δ chain by γδ and αβ T cells may bear similar consequences on T cell signalling.

An investigation of the above hypothesis that differences in the CD3 complex may induce different rates of activation of γδ and αβ T cells requires monoclonal antibodies generated to components of CD3. One aim of this thesis was to generate monoclonal antibodies to ovine CD3 (see appendix). Unfortunately due to the inability of mice immunised with affinity purified CD3ε to generate an immune response to this protein and shortage of time to complete the expression of ovine CD3γ chain was expressed in the yeast Ty system, no monoclonal antibodies were generated. However, in the event of obtaining such reagents, they would have enabled a direct comparison of the rate of activation of γδ and αβ T cells via crosslinking of their CD3 molecules and immunoprecipitation of the CD3 components enabling a comparison of these proteins derived from either γδ or αβ T cells. For such an investigation, protocols established within this thesis for the isolation of distinct populations of T cells could be utilised for the isolation of γδ and αβ T cell populations.

The rapid appearance of CD25 on the surface of γδ T cells is similar to that described for memory T cells (Taga et al. 1991). The data of this thesis has established that the majority of ovine γδ T cells do not express CD45RA the isoform of CD45 which has been used to describe naive T cells (Thomas 1989). This feature was characteristic of
the majority of ovine γδ T cells investigated, irrespective of animal age or their antigenic challenge. In contrast, the majority of αβ T cells in neonatal animals expressed CD45RA, but in older animals less T cells expressed this molecule. In the absence of a monoclonal antibody specific for the CD45RO isoform of CD45, suggested in other species to describe T cells of a memory phenotype (Horgan et al 1992, Vitetta et al 1991), it cannot be shown conclusively that ovine γδ T cells express CD45RO in the absence of CD45RA expression. However, based on two observations made in humans that: (i) the majority of fetal and adult γδ T cells express the CD45RO phenotype (Braakman et al 1991); and (ii) the majority of cells devoid in expression of CD45RA express CD45RO (Sanders et al 1988), it may be assumed an analogous situation occurs in the ovine species implying that ovine γδ T cells are of a memory phenotype and do express CD45RO or the equivalent ovine homologue. If this is the case it is possible that CD45 isoforms bind different ligands and thereby activate variable rates of CD45 phosphatase activity, which subsequently induce differences in the rate of activation between cells expressing these variant isoforms.

It has been shown that the transfer of CD4-positive T cells of either CD45RA or CD45RO phenotype, into athymic nude rats, generates cells of the opposite CD45 phenotype (Sparshott et al 1991, Bell and Sparshott 1990). These observations raise doubt about the validity of the theory that CD45 isoform expression delineates T cells of either naive or memory phenotype. Rather CD45 may reflect a physiological phenotype of recently activated T cells and that interconversion of isoforms is neither unidirectional nor irreversible (Rothstein et al 1991). If this is correct it may imply that γδ T cells, within the ovine species at least, are a population of lymphocytes which continually encounter their ligand within the immune system. For this implication to be valid, ovine γδ T cells at an early stage of ontogeny must express the CD45RA phenotype and only acquire the CD45RO phenotype upon ligand recognition. In the absence of an investigation of CD45 isoform expression by ovine γδ T cells within the thymus it can not be concluded that ovine γδ T cells express the CD45RA phenotype at an early stage of development. However evidence suggesting that ovine γδ T cells do convert their CD45 isoform expression is from data within this thesis, where it was shown that a small population of ovine γδ T cells express CD45RA but subsequently lose this
molecule after Con A activation. This might be analogous to the situation in the human species where human peripheral blood γδ T cells that express a TCR utilising the Vγ9 Vδ2 gene segments all express CD45RO, whilst those cells that express a TCR utilising the Vδ1 gene segments express the CD45RA phenotype, but express CD45RO after PHA activation (Braakman et al 1991). This suggests that human peripheral blood γδ T cells which express the Vδ1 gene segment have not encountered their ligand and hence do not express CD45RO, implying the acquisition of this phenotype only occurs upon γδ T cell activation.

8.5. In Vivo Activation Of Ovine γδ T Cells.

Whilst this thesis reports novel information on the difference in the in vitro activation of ovine γδ and αβ T cells it also reports that the in vivo aspect of γδ T cell activation which showed that the majority of γδ T cells draining epithelial sites are activated, as assessed by CD25 expression. The presence of activated γδ T cells was seen in afferent lymph in the absence of any intentional antigen challenge of the animal in the drainage area of the cannulation. It should be remembered that a very small percentage of blood borne and efferent lymph γδ T cells expressed CD25. Two hypothesis can be proposed to explain the higher level of CD25-positive γδ T cells in afferent lymph. Firstly, that there is an enrichment of activated γδ T cells and other T cells from blood through epithelial tissue to afferent lymph. This is somewhat supported by the observation that a significant number of CD4-positive and CD8-positive T cells are activated in afferent lymph. Secondly, the presence of activated γδ T cells in afferent lymph may suggest that the ligand these cells recognise are continually present in the epithelium which the afferent lymphatics drains. Whether the ligand, presumably comprising peptide and presentational elements on the surface of antigen presenting cells present in epithelium, that γδ T cells recognise is an exogenous derived component such as that from a bacterium, or is an endogenous derived component such as stress proteins is as yet unclear.

If γδ T cells merely recognise an endogenously presented cellular component such as a stress protein derived peptide then the population of activated γδ T cells in afferent lymph compartment...
lymph would presumably have a limited diversity of \( \gamma \) and \( \delta \) chain V gene products. A similar argument has then to be applied to the repertoire of activated \( \alpha \beta \) T cells in afferent lymph and the question that can be asked is that do all activated CD4-positive cells represent those activated by a limited set of endogenous antigens? It is more likely that the activation of these cells, both \( \gamma \delta \) and \( \alpha \beta \) in afferent lymph represents the normal background immune response by T cells to endogenous and exogenously derived material. This could be tested by an anchored PCR analysis of V region usage in \( \gamma \delta \) and \( \alpha \beta \) T cells from this lymph compartment.

A significant feature associated with the presence of activated \( \gamma \delta \) T cells within ovine afferent lymph is that this lymphoid compartment also contains dendritic cells, known to be potent antigen presenting cells that express both MHC class I and II molecules at higher levels than other antigen presenting cells (Steinman 1991) and express non-polymorphic molecules such as CD1 molecules (Bujdoso et al 1990, Bujdoso et al 1989a, Hopkins et al 1989). As yet, no evidence reports that dendritic cells express class Ib molecules, but based on the recent observations of Eghtesady et al (1992), that some class Ib molecules are expressed in a manner analogous to MHC class I molecules it seems highly likely that dendritic cells express class Ib molecules.

A likely possible in vivo model of \( \gamma \delta \) T cell activation within the ovine species is that \( \gamma \delta \) T cells localise at epithelial sites by migration from peripheral blood and recognise their immunogen on the surface of dendritic cells (Bujdoso et al 1990), in association with either class Ib or CD1 molecules or classical MHC molecules as presentational elements located at these sites. These two subsets of cells then migrate to the draining lymph node for activation and or proliferation for subsequent effector function development, such as cytotoxicity. Data in this thesis has shown that ovine \( \gamma \delta \) T cells do not express perforin, a protein associated with target cell lysis or contain cytoplasmic electron dense granules characteristic of cytotoxic cells in other species (Koizumi et al 1991). However these observations do not eliminate the possibility that ovine \( \gamma \delta \) T cells mediate cytotoxic functions as other components not located in cytoplasmic electron dense granules, such as TNF (Cairns et al 1992), may enable the cytotoxicity of these cells.
It has been previously reported that ovine γδ T cells do not recognise their ligand on the surface of dendritic cells (Mackay et al 1988a). It is noteworthy that the authors drew this conclusion from the observation that γδ T cells do not appear to associate via cell-cell contact with dendritic cells when cytocentrifuged onto glass slides. In view of the experimental protocol utilised in the investigation it is quite clear that a more sophisticated experimental design is required to fully assess the physiological functional implication of antigen presentation by dendritic cells to γδ T cells. The sheep provides unlimited potential for such studies, as not only are dendritic cells readily available from cannulated afferent lymphatic vessels, but in young animals at least, peripheral blood is characterised by its high level of γδ T cells providing an excellent source of these cells for such investigations.

As previously discussed, in contrast to afferent lymph γδ T cells, less than 10% of ovine efferent lymph γδ T cells are activated. However, an in vivo challenge with antigen around the drainage area of the lymph node, induced a small increase in the number of both γδ T cells with the lymph node tissue and the number of activated γδ T cells exiting the node. In respect, this may imply that the function of γδ T cells does not only include immune surveillance of epithelial sites and the recognition of a limited set of antigenic peptides but also mediate immune responses within lymphoid tissues to distinct protein antigens as do αβ T cells. Within lymph nodes γδ T cells localise in the proximity of the reticuloendothelial system, implying they may recognise their ligand on the surface of these cells. The absence of γδ T cells from areas classically associated with T cells indicates that γδ T cells do not provide B cell help within lymphoid follicles as do CD4-positive T cells. An immunological function of γδ T cells rather than one merely of epithelial surveillance is further implied in humans where no tropism of γδ T cells to epithelial sites is apparent. However, various disease states in humans elicit an increase in the percentage of γδ T cells within the periphery or the disease lesion. This suggests indirectly that γδ T cells are activated and are expanded at various sites, of which lymphoid tissue is inclusive.
8.6. Ligand Recognition By Ovine γδ T cells.

Whilst afferent lymph dendritic cells may be an important antigen presenting cell for γδ T cells in epithelial tissue, in vitro assays reported in this thesis showed that γδ T cells isolated from ovine peripheral blood responded to both Mycobacterium tuberculosis and ovalbumin antigens presented by autologous peripheral blood mononuclear cells. It is well established that cells within the peripheral blood mononuclear population with the ability to present antigenic peptides express classical MHC molecules (Unanue 1984, Lanzavecchia 1990) and hence may imply that ovine γδ T cells respond to antigenic peptides in association with classical MHC molecules. Further evidence to support this is from the in vitro assays carried out during this thesis, where anti-MHC class I and anti-MHC class II monoclonal antibodies were tested for their ability to inhibit in vitro proliferative responses to Mycobacterium tuberculosis and ovalbumin antigens. The data implies that some γδ T cells in the sheep recognise their antigen in association with both MHC class I and class II molecules.

The observation that neither monoclonal antibody to MHC class I or class II molecules when tested alone had the ability to inhibit all of the proliferative response seen by γδ T cells implies that some γδ T cells recognised their antigen in association with other presentational elements. These alternatives could be tested by establishing in vitro proliferation assays in the presence of both anti-MHC class I and anti-MHC class II monoclonal antibodies. Complete inhibition of proliferation in such assays would suggest that the majority of ovine γδ T cells recognise their ligand as a complex of immunogen in association with either MHC class I or class II molecules. In the absence of complete inhibition the data may imply that whilst some γδ T cells recognise their immunogen in association with classical MHC molecules, others may recognise their immunogen in association with other known presentational elements such as non-polymorphic molecules. Potential candidates are the class Ib or CD1 molecules, or as yet unidentified presentational elements. However, in the absence of characterised monoclonal antibodies specific for either ovine CD1 or class Ib molecules (ovine homologues of class Ib molecules remain unreported) this remains an exciting and eagerly awaited area of research to be investigated.
8.7. Ovine γδ T cells, Alpha or Omega?

The frequency and physiological distribution of γδ T cells in different animals define a quantitative spectrum ranging between species characterised by low levels of γδ T cells, such as rodents and humans and species with high levels of γδ T cells, such as ruminants. Although the reasons for these differences are not clear, the habitats of various species may exert specific evolutionary pressures. Ruminants are hosts for a large number of pathogenic viruses, bacteria, protozoa and metazoan parasites that assault mucosal surfaces or the skin, several of these organisms infect and then replicate in epithelial cells during the early pathogenesis of disease. To date, the majority of research into the ontogeny, repertoire, phenotype and function of γδ T cells is based on the analysis of species characterised by low levels of γδ T cells. Within these species the γδ population appears to be a highly skewed sample with respect to their repertoire and percentage representation within the lymphoid system. The analysis of γδ T cells within species characterised by high levels of these cells offers a greater understanding of the immunobiology of γδ T cells within the immune system.

The unique features observed in this thesis with respect to ovine γδ T cell immunobiology, relative to γδ T cells of other species, has lead to speculation that γδ T cells serve different roles within different species. This may imply that the immune system of the ovine species and other ruminants has evolved in such a way that immunity provided by the γδ T cell lineage is of a higher order with respect to that of γδ T cells of other non-ruminant species. Furthermore, if the in vitro activation characteristics of ovine γδ T cells described in thesis can be applied to an in vivo system, this further establishes that ovine γδ T cells represent a highly adapted lineage of T cells and may provide their host with a rapid response to invading pathogens. Collectively, in the ovine species at least, γδ T cells appear to be a lineage with a degree of complexity with regards to their immunobiology analogous to that of αβ T cells.

It is clear that ovine γδ T cells are activated at epithelial sites and may provide epithelial surveillance, but the view of this thesis is that this is not the only role of ovine γδ T cells. From data within this thesis it is implied that γδ T cells respond to antigens which they encounter on the surface of antigen presenting cells within secondary lymphoid
tissue, such as lymph nodes. If this is the case then it further emphasises that ovine $\gamma\delta$ T cells resemble the $\alpha\beta$ T cell lineage.

If ovine $\gamma\delta$ T cells represent a lineage of T cells of similar functional complexity as the $\alpha\beta$ T cell lineage then the obvious question to ask is why is this lineage not as prevalent in older animals? The studies of Hein et al (1990a) have established that the development of $\gamma\delta$ T cells is dependent on the presence of the thymus. Hence the decrease of these cell with age may be a consequence of the fact that the ovine immune system no longer has the ability to generate these cells.

Collectively, these aspects raise important questions about the increased relevance of $\gamma\delta$ T cells in species characterised by high levels of these cells, such as the sheep. Furthermore, the ability to cannulate the lymphatic vessels of the sheep is a salient feature associated with this experimental animal and emphasises an advantage of immunological research in species other than the human and mouse. In view of these points the sheep is a prime resource for research into $\gamma\delta$ T cell immunobiology with ample potential for further investigation into this elusive, but unique subset of T cells, which will remain a challenge until their enigma is resolved.
APPENDIX

Generation Of Reagents To Ovine CD3 Complex.
The TCR associates non-covalently with five polypeptide chains involved in signal transduction. These proteins are referred to collectively as the CD3 complex and are termed the γ, δ, ε, ζ and η chains (Clevers et al 1988). The CD3 complex couples antigen recognition to intracellular signal transduction pathways. The genes encoding CD3γ, δ and ε are structurally homologous and are predicted to have arisen from an ancestral precursor by gene duplication (Gold et al 1987). The analysis of the primary structure of the sheep CD3γ, δ and ε chains (Hein and Tonnacliffe 1990, Hein and Tonnacliffe 1993) has shown that these components of the TCR are significantly conserved across species. These proteins show a gradient of increased conservation towards their transmembrane and cytosolic regions. However the extracellular domain of the CD3ε chain is less conserved than the homologous region of the CD3δ and CD3γ chains (Hein and Tonnacliffe 1993). Two motifs of 8 and 32 amino acids are conserved within the C terminus of the human, murine and ovine CD3γ, δ and ε proteins, implying a pivotal role by these regions of the proteins in regulating the manner in which they associate with other components of the TCR / CD3 complex.

The availability of a monoclonal antibody specific for the ovine γδ TCR (Mackay et al 1989) has enabled a detailed characterisation of lymphocytes expressing this receptor for antigen. As yet there is no equivalent reagent to define αβ T cells in the sheep. αβ T cells are assumed to be the summation of the percentage of CD4-positive and CD-8 positive cells, as these co-receptor molecules are not expressed by the majority of ovine γδ T cells (this thesis). Alternatively, the evaluation of the number of αβ T cells is offered by subtracting the number of γδ TCR-positive cells from the percentage of CD3-positive T cells. Whilst no monoclonal antibody has been generated to any component of the ovine CD3 complex, at least one monoclonal antibody HMT3.1, specific for human CD3ε chain does cross react with ovine T cells. Unfortunately, this reagent recognises an internally expressed epitope of the CD3ε chain, hence limiting its in vitro immunological applications.
The availability of monoclonal reagents specific for the ovine \(\alpha\beta\) TCR and CD3 complex would enhance knowledge of T cell immunobiology within this species. These reagents would enable a comprehensive investigation of ovine T cells, at both the total T cell population and T cell subset level. In particular, monoclonal antibodies specific for the ovine \(\alpha\beta\) TCR and the \(\gamma\delta\) TCR could be used to investigate the selective activation of ovine T cells without the need to purify the two subsets. Conversely, these monoclonal antibodies could be used to positively select or deplete either population by MACS.

Monoclonal antibodies to the ovine CD3 complex would allow immunoprecipitation of this complex from T cells to determine if ovine \(\alpha\beta\) T cells differ in their CD3 complex relative to \(\gamma\delta\) T cells, as suggested of human T cells by Brenner et al (1986). Alternatively, immunoprecipitation studies may establish that proteins such as the T19 molecule are associated with either the CD3 or TCR on the surface of ovine \(\gamma\delta\) T cells.

An attempt was made to generate monoclonal antibodies to the sheep \(\alpha\beta\) TCR / CD3 complex by two strategies. Firstly, through the immunisation of mice with immunoprecipitated sheep TCR / CD3 proteins. The monoclonal antibody HMT3.1 was shown in Chapter 3 (Figure 8) to react with an internal epitope of sheep CD3 expressed by permeabilised T cells. As the permeabilisation of T cells did not involve denaturation of cellular proteins it was assumed that HMT3.1 reacted with the native conformation of the sheep CD3 molecule. If this was the case, it was possible that an affinity column of HMT3.1 monoclonal antibody could be used to purify sheep CD3 proteins, and under mild denaturing conditions, presumably in association with TCR proteins. This material could be used for the immunisation of mice for the generation of monoclonal antibodies to CD3 and \(\alpha\beta\) TCR structures through appropriate screening of the resultant fusion products on sheep T cell subsets. The second strategy was an approach using molecular biological techniques to isolate cDNA encoding an ovine CD3\(\gamma\) protein and derive the encoded protein through the use of a suitable expression system. The ovine CD3\(\gamma\) chain has been cloned and sequenced (Hein and Tonnacliffe 1990). From the published DNA sequence suitable primers for a polymerase chain reaction (PCR) were selected for the amplification of cDNA encoding this protein from T cell mRNA for expression via the yeast Ty system. The progress of this monoclonal antibody work is discussed in this Appendix.
Whilst both strategies for the generation of reagents were pursued during this thesis the immunopurification of CD3 / TCR complexes was completed but due to shortage of time the protocol utilising the Ty system was not completed by myself. My input into the project concerned the generation of the ovine CD3γ chain by PCR, the sequence analysis of the PCR product and its ligation into the pOGS40 vector. The transformation of yeast with the plasmid and insert and the expression and subsequent purification of the CD3γ chain protein was carried out by Dr Raymond Bujdoso and Dr Douglas Roy. The data obtained from this area of the work is reported briefly in section A.2.3. and merely provides an overview of the project as it stands to date.
RESULTS.

A.1. HMT3.1 Reacts With a 22 KDa Protein Expressed by Ovine T Cells.

HMT3.1 is a hamster monoclonal antibody specific for a cytoplasmic epitope of the human polypeptide of CD3e (Kubo, unpublished). The immunofluorescence studies described in section 3.3.1.4. (Figure 8) of this thesis established that this reagent reacted with all T cells, presumably with the equivalent protein expressed by sheep T cells. This was based on the observation that the percentage of permeabilised ovine lymphocytes immunofluorescently labelled by HMT3.1 was approximately equal to the percentage of cells that reacted with a combination of monoclonal antibodies specific for either the γδ TCR and CD2 or the γδ TCR, CD4 and CD8. Further evidence that HMT3.1 reacted with a CD3 protein of sheep T cells was shown by the molecular weight of the protein detected by the monoclonal antibody shown by SDS-PAGE and Western blot analysis of an ovine T cell lysate. Figure 1 shows that HMT3.1 reacted with a protein of approximately 22KDa within a lysate derived from sheep T cells. The control for this analysis was the reactivity of HMT3.1 with lysates prepared from the human T cell line Jurkat and the human B cell line Raji. Figure 1 demonstrates that the monoclonal antibody clearly reacts with a protein of approximately 22KDa present in the human T cell lysate but absent from a human B cell lysate. Proteins of molecular weights 28-80KDa in the human T cell lysate, and 75-80KDa in lysates of ovine lymphocytes and human B cells were due to non-specific binding as these proteins were also detected by normal hamster immunoglobulin.

The above data strongly suggests that monoclonal antibody HMT3.1 reacts with ovine T cells and recognises an epitope of a 22KDa protein. Based on a recent publication on the homology between ovine and human CD3e (Hein and Tunacliffe 1993), it is assumed that monoclonal antibody HMT3.1 crossreacts with the ovine homologue of the human CD3e chain protein.
An ovine lymphocyte lysate was prepared from peripheral blood mononuclear cells of an 18 month old Finnish Landrace sheep. Control lysates were prepared from the transformed human T and B cell lines, Jurkat and Raji, respectively. The presence of CD3γ chain was analysed by SDS-PAGE and Western blot analysis using the hamster monoclonal antibody HMT3.1. Background staining was established with normal hamster sera.
FIGURE 1. Identification Of Ovine CD3ε Chain By Western Blot Analysis.

KEY:

(i) Jurkat Cell Lysate
(ii) Raji Cell Lysate
(iii) Ovine T Cell Lysate

As HMT3.1 appears to react with the native CD3 complex of sheep T cells, and probably via the CD3ε protein it is possible that sufficient sheep CD3 antigen could be purified on an affinity column comprising this monoclonal antibody. Accordingly, attempts were made to generate sufficient HMT3.1 immunoglobulin to form such a column. Because of the inability of the HMT3.1 hybridoma to produce ascites fluid in mice, 12 litres of HMT3.1 hybridoma culture supernatant were generated and the secreted immunoglobulin was immunopurified on an anti-mouse immunoglobulin affinity column which was known to bind hamster immunoglobulin.

An anti-CD3 affinity column was generated by linking immunopurified HMT3.1 immunoglobulin to cyanogen bromide activated Sepharose 4B. A sample of ovine spleen lysate was prepared to provide a source of CD3 protein, and this was loaded onto the column. Material bound to the column was eluted and collected in 2ml fractions. The presence of ovine CD3ε chain within the eluted fractions was determined by SDS-PAGE and Western blot analysis as shown in Figure 2. From this data it appears that the ovine CD3ε chain detected by HMT3.1 is collected in fractions number 3-11. The most intense staining was seen in fractions 6 and 7 implying that an increased amount of ovine CD3ε chain was present within these fractions relative to other eluted fractions. In addition, Western blot analysis showed an array of protein bands with molecular weights in the range of 25-65KDa in fractions number 3-11. The absence of these bands within the positive (efferent lymph lysate) and negative controls (column eluted material blotted with normal hamster sera) suggests that these bands represent complexes of ovine CD3ε chain in the form of dimers and trimers. These complexes may have formed due to the high pH and salt concentrations required to elute the column. Alternatively, the higher molecular weight bands may represent the ε chain in association with other proteins of the TCR / CD3 complex. This is possible because the conditions used to prepare the spleen lysate were expected to retain complexes of CD3 and TCR components including the γ, δ, α and β chains.
Figure 2.
Ovine CD3γ chain protein was immunopurified from an ovine spleen lysate via a HMT3.1 affinity column as described in Materials and Methods. Material bound to the column was eluted and collected in 2ml fractions. Each fraction was analysed by SDS-PAGE and Western blot analysis for the presence of ovine CD3γ chain protein using the hamster monoclonal antibody HMT3.1. A positive control was established using an ovine lymphocyte lysate prepared from peripheral blood mononuclear cells of an 18 month old Finnish Landrace sheep. Background staining was established with normal hamster sera.
FIGURE 2. The Presence Of Ovine CD3ε Chain Within Fractions Eluted From A HMT3.1 Affinity Column.

HMT3.1 Supernatant

Normal Hamster Sera

KEY:

(i) Ovine T Cell Lysate

1 - 12 - Fractions Eluted From Affinity Column
The protein concentrations of fractions 3-11, inclusive, was determined by spectrophotometry (data not shown), thereafter they were pooled and treated as one sample. The immunopurified ovine CD3ε chain was acetone precipitated and used for the immunisation of mice as described in Materials and Methods.

A.1.3. Immunisation of Mice with Ovine CD3 Antigen.
Mice were immunised with the CD3ε protein for monoclonal antibody production. After the completion of the immunisation protocol and prior to establishing a fusion to generate an antibody secreting hybridoma cell line, a sample of sera was obtained from the mice and tested by immunofluorescence analysis for its reactivity to ovine lymphocyte surface antigens.

The antisera obtained from mice immunised with ovine CD3 did not display any reactivity to antigens expressed on the surface of ovine lymphocytes. The ability of the antisera to react with antigens expressed internally was not investigated as the sole purpose of the project was to establish reagents that could be utilised for the immunofluorescence analysis of antigens expressed at cell surface, hence avoiding the permeabilisation of cells. In addition this antisera also failed to react with proteins presented within a sheep T cell lysate as judged by Western blot analysis.

A.2. Using the Polymerase Chain Reaction (PCR) and the Ty Yeast System.
During the course of the above mentioned work an alternative strategy was employed to generate monoclonal antibodies to the CD3 complex. This involved a molecular biological approach and was designed to generate recombinant ovine CD3γ chain for the immunisation of mice. From the recently published cDNA sequence of the ovine CD3γ chain, shown in Figure 3, specific nucleotide primers were selected for use in a polymerase chain reaction (PCR) which would generate a significant proportion of the ovine CD3γ coding region. 5’ and 3’ primers were chosen for their optimum guanosine and cytosine base content, lack of hairpin structures and suitable annealing temperature.
Figure 3.
The nucleotide sequence of the ovine CD3γ chain protein is shown. The underlined and double-underlined areas represent the regions where the 5' and 3' PCR primers bind, respectively.
FIGURE 3. Nucleotide Sequence of Ovine CD3γ Chain.

1  GTAAAAGTGG ATGACAATCA AGAAGATGGT TCTGTAATTG TGATTTTGTG
51  CACCGGATGAA AAAAGATCA CATGGCTTAA AGATATGAAA GAAATAAGTT
101  CTGGAGACAC AAATAAAACCT ACTTTGGGATC TTGGAAGTAG TACCAAAGAC
151  CCTCGAGGAA TATATGAATG TAAAGGATCA AGTAACGAAT CAAAATCACT
201  CCAAAATATAT TATAGAATGT GTCAGAACTG CATTGAACCTG AACCTAGGCA
251  CTGTTGGCTGG CTTTATCTTC ACTGAAATCG TCAGCATTTC CCTCCTTGCT
301  GTTGGGGTCT ACTTCATTGC TGGACAGGAA GGAGTTGCCC AGTCAAGAGC
351  TTCAGACAAG CAGACGCTTG TGAACAATGA CCAGCTCTAC CAGCCCCCTTA
401  AGGAACGGGGA AGATAGCCAA TACAGCCACC TGAGGAACGGG GTGAACCCAA
451  GACTCAGACT AGATTTGGCT TTTCTGGAAA AATGGAATCA TATGGGATTG
501  CTGGACATTC TTGTGCAAGG TCTTCATGTT GACATATGTT TTTATTCCTC
551  TGGGTGGCTA CCTAGGAGTG AAGCAATGGT GGATGGGTATG GTAAATTATAT
601  CTATATCGAT TTAAGAAACT GCCAAAGTGT TTGGCAACCT GGGTTGCTCA
651  TTTTACATTC CCAGCAGCCA TAAAGGTTTG GGGTTCTTCT TTTTTCTCCT
701  CCTCACAAAAA GTTCTTTACT ATCTTTTTTA GTATAGCCAT TCTAGGGGAT
751  GTAAACAGGT ATCTTTATTAT GCCTTTAAAT TGCATTCTCTC TATGACTAAC
801  GATGTTGAAC ATCTTTTCAT GTGCTTTATG GTTCTTCTCA TCATATCTTT
851  AGTGAAGTGT GTTACCAAA CTTTTTAAAT TCGTTATATT GTTTTTCTTC
901  TTATATCATTGA TTGGAACATC TTTTAAAAA TTGGTTATAT TTTTTATGAT
951  AGCAGATATA TGAATTGCCA ATATATCCTAC CAGTCTGTGA TTTGTCTTTT
1001  CATTTTTCTTA ATGGTGCTCTT TTGAAATGCA AAGGTATTTA ATTTTGCTGA
1051  AATCTGTCTT ACTCTTTTTTTT TTCCCTTTATG GATTTGTGC CGTGTGTGATC
1101  GACTAAGAAA TCCCTGCAAA ACCCAAGATC ACAAGATGTT TTTTATAGTT
1151  TTATGCTCTAA CGATAAATGT TATTTGGAAC ATTAAGACTA TTTGTGAACAA
1201  ATACAAATTAA ATGCAATTGC AGCACCATAA AAAAAAA
Two sets of primers were chosen. One set which would allow the generation of cDNA encoding virtually the full length CD3γ chain (440 base pairs) and a second set encoding a significant proportion of the external domain of the protein (189 base pairs). The primers had additional sequences at either the 5' and 3' ends encoding BamHI restriction sites to enable the cloning of the PCR product into the unique BamH1 site of the vector pOGS 40. The 5' primer was also designed to contain the sequence encoding a Factor Xa enzyme cleavage site to allow the cleavage of the CD3γ chain peptide from the P1 fusion protein after the purification of Ty particles. This vector containing the CD3γ chain insert was subsequently ligated into the yeast retrotransposon Ty vector for expression of the CD3γ chain as a fusion protein with the fusion partner, P1. The predicted PCR products are shown in Figure 4.

A.2.1. Generation of Ovine CD3γ Chain cDNA by PCR.

The optimum concentration of magnesium chloride for the PCR, which is dependent on the individual primers and substrate used, was determined by titration. The titration of magnesium chloride for the PCR involving primers enabling full length CD3γ chain cDNA to be generated is shown in Figure 5. The ethidium bromide stained agarose gel shows the predicted PCR product of approximately 440 base pairs. Bands were visible with concentrations of magnesium chloride in the range of 3-5mM, but a band of maximum intensity was obtained at a concentration of 5mM magnesium chloride, which was used in subsequent PCR reactions. The concentration of magnesium chloride for the set of primers which resulted in a cDNA-PCR product encoding the external domain of the CD3γ chain was 2 mM (data not shown). The PCR product encoding the ovine CD3γ chain was determined by DNA sequencing.

A.2.2. Transformation of JM83 Bacteria With Ovine CD3γ chain cDNA.

The ovine CD3γ chain PCR products were modified to generate BamH1 5' and 3' ends by firstly "filling in" the ends of the PCR products with Klenow DNA polymerase enzyme followed by BamH1 digestion. The products were then independently ligated to BamH1-cut pOGS40 vector. Ligated plasmids were rescued by transformation of E.
**Figure 4.**  
A schematic representation of the predicted PCR products encoding CD3γ chain proteins using the two different sets of PCR primers.
FIGURE 4. Schematic Representation Of The PCR Products Encoding Ovine CD3γ Chain.

PCR product-1, 440 Base Pairs.

PCR product-2, 189 Base Pairs.
Figure 5.

A titration of the concentration of magnesium chloride for the PCR primers enabling full length CD3γ chain to be generated.
FIGURE 5. Generation Of Ovine CD3γ Chain cDNA By PCR.

Concentration of MgCl₂ mM

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coli strain JM83 and selection on LB plus ampicillin agar plates. Selected bacterial colonies were subsequently grown in liquid culture overnight and analysed by mini-prep plasmid DNA preparation and BamH1 digestion. The presence of inserts of the correct size were identified by agarose gel electrophoresis and ethidium bromide staining. The correct orientation of the inserts was confirmed by DNA sequencing across the pOGR40-CD3γ chain cDNA junction. Two colonies were selected for further investigation, pCD3γ FL-1 (full length insert) and pCD3γ TC-1 (truncated insert). Both colonies were grown in large scale liquid cultures and CD3γ FL-1 and CD3γ TC-1 plasmids isolated then purified by cesium chloride centrifugation for transformation of yeast spheroplast.

A.2.3. Expression Of Ovine CD3γ Chain cDNA In Yeast.

The protease deficient Saccharomyces cerevisiae strain BJ2168 was simultaneously transformed to leucine and uracil independence with plasmids either pCD3γ FL-1 or pCD3γ TC-1 and pUG41S. Plasmid pUG41S over expresses GAL4 protein under the control of a galactose inducible promoter (Lue et al 1987). Co-transformation of yeast with this vector and a PAL-promoter containing VLP plasmid (Kingsman et al 1990) results in increased expression levels since a major constraint on the level of protein expression in galactose inducible systems is the low level of GAL4 gene expression (Johnston and Hopper 1982), especially if the cell contains multiple copies of the expression plasmid (Baker et al 1987). Regulated over production of GAL4 increases protein expression levels and retains the inducibility of the system (Schultz et al 1987).

A number of yeast transformants containing either pCD3γ FL-1 or pCD3γ TC-1 grown in the presence of pUG41S were identified and when grown in the presence of the galactose were found to express a Ty fusion protein as shown in Figure 6. Two were selected for further analysis, T.CD3γ FL-1 and T.CD3γ TC-1.

When analysed by coomassie stained SDS-PAGE protein extracts of T.CD3γ FL-1 grown in the presence of galactose show a band of 67KDa which was not present in uninduced yeast. A similar analysis of protein extract of galactose-induced T.CD3γ TC-
Figure 6.

Yeast transformants containing cDNA encoding either full length or truncated CD3γ chain were grown in either the absence or presence of galactose. Yeast protein extracts were analysed by SDS-PAGE for the presence of the CD3γ chain fusion proteins.
FIGURE 6. Identification Of Ovine CD3γ Chain Fusion Proteins.

KEY:

(i) Fusion Protein pCD3γFL-1
(ii) Fusion Protein pCD3γTC-1
1 showed a prominent band of 60KDa not present in extracts of non-induced yeast. The molecular weight of wild type P1 in SDS-PAGE is 50KDa. The protein bands of 67KDa and 60KDa present in induced extract of T.CD3γ FL-1 and T.CD3γ TC-1 are the predicted sizes for the fusion proteins of Ty and the expressed CD3γ chain cDNA inserts.

Monoclonal antibody 5V6, specific for the TCR β chain TCR β, has identified the CD3 complex of the T cell lineage which has been used to enrich the specific T cell population. The TCR complex was found in extracts of non-induced yeast. The molecular weight of wild type PI in SDS-PAGE is 50KDa. The protein bands of 67KDa and 60KDa present in induced extract of T.CD3γ FL-1 and T.CD3γ TC-1 are the predicted sizes for the fusion proteins of Ty and the expressed CD3γ chain cDNA inserts.
DISCUSSION.

A panel of monoclonal antibodies specific for various ovine cell surface antigens have enable a detailed investigation of lymphocyte phenotype and function within this species. Monoclonal antibody 86D, specific for the ovine γδ TCR has enabled the characterisation of the γδ T cell lineage within the sheep, but the absence of a reagent specific for the ovine αβ TCR has imposed limitations on the analysis of this T cell lineage. Reagents to the αβ TCR and CD3 complex are required to enable a comprehensive investigation of the total T lymphocyte population within the ovine immune system. These reagents would enable the characterisation of their phenotype, function and the molecular association of receptor and co-receptor molecules within the cell membrane. The aim of the work described in this appendix was to generate reagents to the αβ TCR and CD3 protein complex to enable further characterisation of ovine T cells.

Reagents are available to the αβ TCR of the human and murine species but do not cross react with the ovine αβ TCR. Consequently, and in the absence of the nucleotide sequence of either the ovine α or β TCR chains at the initiation of this research project, strategies were established to generate monoclonal antibodies to the ovine CD3 complex. This was made possible by the availability of both a monoclonal antibody specific for the ε chain of human CD3 that cross reacts with a homologue expressed by ovine T cells, and secondly by the availability of the deduced nucleotide sequence of the CD3γ chain (Hein and Tunnacliffe 1990). The production of monoclonal antibodies in this investigation was based on two strategies: (i) the affinity purification of ovine TCR / CD3 complex utilising monoclonal antibody HMT3.1; (ii) to isolate cDNA encoding ovine CD3γ chain and derive the encoded protein through the use of a suitable expression system.

Both strategies have enabled the isolation of ovine CD3 proteins. Immunopurification of the TCR / CD3 complex yielded approximately 150μg of protein per isolation, whilst the TY-yeast system produced approximately 250μg of the CD3γ fusion protein per litre of yeast cultures. To date, mice have been immunised with the immunopurified protein...
whilst the CD3γ protein prepared by expression in the yeast Ty-system is yet to be used for the same purpose.

For reasons unknown mice immunised with immunopurified TCR / CD3 protein(s) did not generate an immune response to this immunogen. These results tend themselves to two possibilities. Firstly, that mice generated anti-ovine CD3ε chain specific monoclonal antibodies which only reacted with an internally expressed epitope present in the native form of the protein and not present following SDS-PAGE. Secondly, that the mice were not primed to the injected antigen. This would explain the apparent absence of antibodies specific for the proposed ovine CD3ε chain and may be a consequence of insufficient antigen used for the immunisation. Alternatively, the CD3 proteins may be sufficiently conserved between species to prevent priming of the mice with ovine CD3.

Evidence in favour of the latter is supported by a comparison of the nucleotide sequence of the CD3γ, δ and ε proteins in the human, murine and ovine species (Clevers et al 1988, Hein and Tunnacliffe 1990, Hein and Tunnacliffe 1993). These studies showed that components of the CD3 complex are highly conserved between species (Hein and Tunnacliffe 1990). In addition the genes encoding CD3γ, δ and ε are structurally homologous and are predicted to have arisen from an ancestral precursor by gene duplication (Gold et al 1987). The immunisation of mice with the CD3γ chain obtained from the yeast Ty expression system will hopefully provide further evidence as to whether the CD3 components are too conserved between species to enable the generation of monoclonal antibodies. If this is the case then it may explain the limited availability of reagents specific for the CD3 complex in other species.

Data in Chapter 4 of this thesis reported differences in the activation rates of γδ and αβ T cells. The availability of reagents to the ovine αβ TCR and CD3 complex would provide potential to enhance these preliminary investigations of the unique activation characteristics of the two T cell lineages. The recent publication of the nucleotide sequence of both ovine α (Hein et al 1991) and β (Grossberger et al 1992) TCR chains together with the yeast Ty system used in this appendix to generate large amounts of pure expression protein, provides excellent potential to generate anti-ovine αβ TCR
monoclonal antibodies by this strategy. The recent cloning of the genes encoding the T19 molecule (Wijngaard et al 1992) has established considerable homology between this molecule and CD5, a component known to be associated with T cell activation (Osman et al 1992). The availability of reagents to ovine CD3 and TCR will enable an investigation into this challenging area of ovine γδ T cell immunobiology and will assist in elucidating the role of the cell surface glycoprotein, T19. Immunoprecipitation studies may establish an association of this molecule with either the TCR or CD3 complex, indicative of a role for this molecule in the activation of the γδ T cell lineage.
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