THE ROLE OF DIFFERENTIATION ANTIGENS IN T CELL ONTOGENY AND IMMUNE FUNCTION

by

SARAH ELIZABETH BELL
ICRF Tumour Immunology Unit
Department of Biology
UNIVERSITY COLLEGE LONDON

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Abstract

Using the technique of murine foetal thymus organ culture as an *in vitro* model of thymic ontogeny, the role of the differentiation antigens CD4 and CD8, during development of the T cell repertoire has been examined. The developing subpopulations have been phenotyped in this culture system, and the *in vitro* recolonisation potential of purified progenitor populations from mouse foetal liver investigated. Selective cell depletion by density gradient centrifugation, and anti-heat stable antigen (HSA) antibody plus complement treatment, or cell sorting, has enabled a progenitor population to be identified within the HSA-negative subset of foetal liver.

Antisense oligodeoxynucleotides corresponding to the 5' region of the genes encoding CD4, or CD8 (α and β chains) have been used to specifically inhibit cell surface expression of these molecules in foetal thymus organ culture. The cellular uptake and stability of these oligonucleotides was assessed by 5' end labelling using [γ-^{32}P]-ATP, and analysis following various times of incubation with the labelled oligonucleotide. By incubation of foetal thymus lobes with a biotinylated oligonucleotide, and staining tissue sections with a streptavidin reagent, the oligonucletides were demonstrated to penetrate an intact thymus explant. The addition of CD4 antisense oligonucleotides appears to cause a specific decrease in cell surface expression of this molecule and the appearance of a population of $CD4^{-}CD8^{+}$ cells.

The functional importance of the domains of CD3, CD4, and CD8 has been investigated by constructing chimaeric molecules comprising the extracellular, transmembrane, and cytoplasmic domains of CD4, or CD8, and the γ chain of CD3. Hybrid constructs comprising the extracellular domain of CD8 and the transmembrane and cytoplasmic domains of CD3γ were successfully transfected into a T cell hybridoma. Although these molecules can be expressed, they appear to be retained within the cell. This is in agreement with recent studies which have identified a retention signal in the transmembrane domain of components of the TCR/CD3 complex, which targets individual components, particularly CD3γ, for ER degradation.

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Abbreviations:

APS : ammonium persulphate

ATP : adenosine tri-phosphate

BSA : bovine serum albumin

DAB : diaminobenzidine

dH_2O : distilled water

DMSO : dimethyl sulphoxide

DTT : dithiothreitol

EDTA : ethylene diamine tetra-acetic acid (tri- sodium salt)

FACS : fluorescence activated cell sorter

FCS : foetal calf serum

FTOC : foetal thymus organ culture

HEPES : N-2 hydroxyethylpiperazine - N'-2-ethanesulphonic acid

MEM-H : minimal essential medium plus HEPES

MOPS : 3-[N-morpholino] propanesulphonic acid

NaOH : sodium hydroxide

O.D. : optical density

PBS : phosphate buffered saline

PEG : polyethylene glycol

psi : pounds per square inch

ρ : density

2 ME : 2 mercaptoethanol

SDS-PAGE : sodium dodecyl sulphate polyacrylamide gel electrophoresis

TE : Tris / EDTA buffer

v:v : volume:volume

CHAPTER ONE

General introduction

1.1 The role of accessory molecules in immune function

The expression of cell surface receptors capable of distinguishing between self and non-self is a key feature of the cells of the immune system. The process of antigen recognition involves the subsequent translation of antigen binding into signals which control cellular responses. This provides a mechanism for regulating the maturation of T cells via thymic selection, and of initiating the immune response in mature cells. The T cell antigen receptor complex performs a central role in these processes.

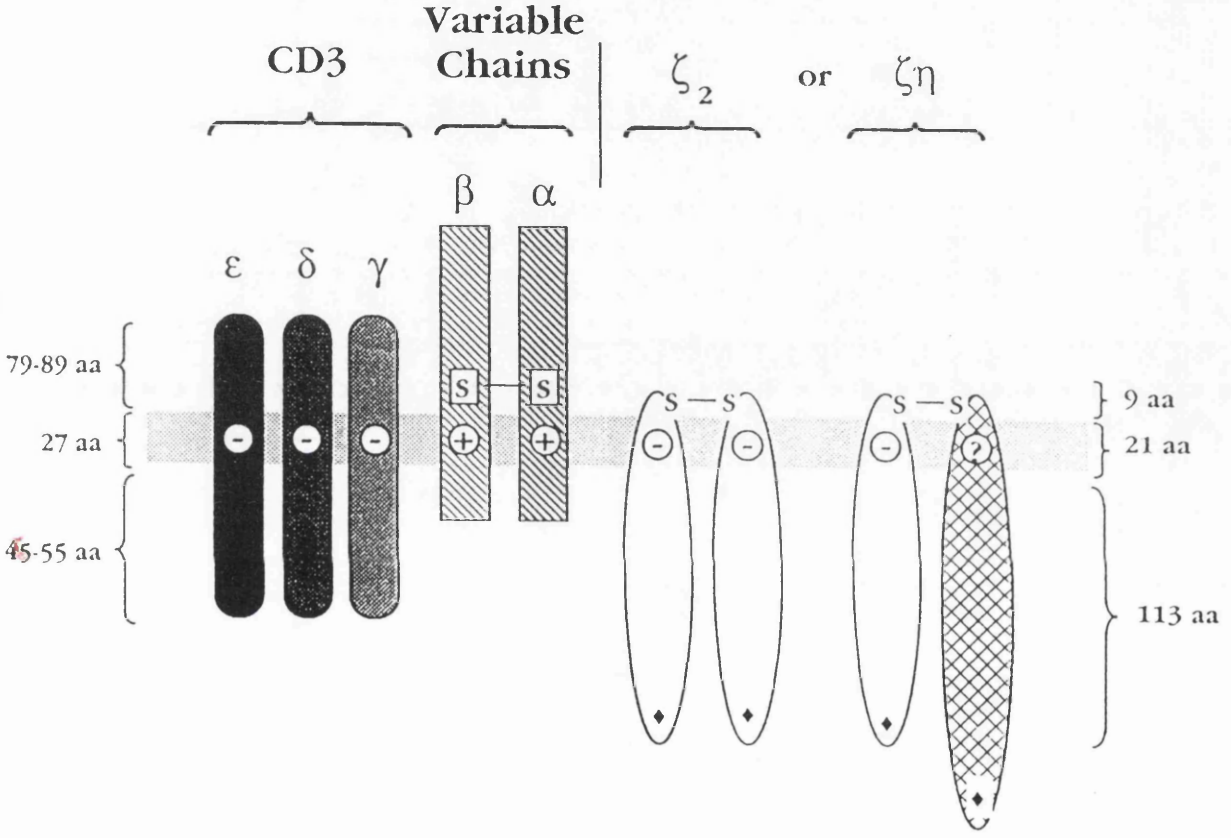
1.1.1 The T cell antigen receptor

The T cell antigen receptor is a complex composed of at least six or seven membrane-spanning polypeptides (reviewed by Ashwell & Klausner, 1990). The complex generally comprises a disulphide linked heterodimeric T cell receptor (α/β, or γ/δ), non-covalently associated with at least five invariant chains, γ, δ, ε, η, and ζ, which comprise the CD3 complex (Figure 1.1). The γ, δ, and ε CD3 chains are encoded by three homologous, clustered genes, on chromosome 11 in the mouse. The ζ family is genetically distinct from CD3, and comprises three proteins encoded by two genes, ζ, its alternatively spliced form η, and the γ chain of multisubunit Fcε receptors (Klausner & Samelson, 1991). The TCR/CD3 complex is assembled intracellularly, and expression of all seven chains is required for efficient surface expression of the T cell receptor/CD3 complex (reviewed by Ashwell & Klausner, 1990)

The T cell receptor (TCR) chains are highly polymorphic in their N-terminal domain, and are responsible for antigen recognition. Although the functions of the individual CD3 subunits are not known, it is assumed they participate in signal transduction. Evidence suggests that the cytoplasmic domain of the ζ chain is essential for antigen-stimulated signalling (Frank *et al.*, 1990), since it is phosphorylated on tyrosine following T cell stimulation. More recent evidence for the function of ζ has been obtained from the construction of a chimaeric molecule comprising the extracellular and transmembrane domain of CD8 and the cytoplasmic domain of the ζ chain. Transfection of this molecule into a T cell line enabled activation of the cells by

Figure 1.1 *Schematic diagram of the cell surface T cell receptor complex*

Reproduced from Ashwell & Klausner, 1990.



⊖, ⊕ Charged amino acid(s) in the transmembrane region (⊘ indicates unknown)

♦ Homology to a consensus nucleotide-binding sequence

antibody-mediated cross-linking of this chimaeric molecule (Irving & Weiss, 1991), supporting an important role for the TCR ζ chain in coupling the receptor to biochemical signalling pathways.

Many studies have been directed at investigating the early biochemical events which occur in response to TCR engagement. In addition to specific antigen presented by the appropriate MHC molecule, receptor-specific antibodies and mitogenic lectins have been used as stimuli to elicit TCR-mediated activation. Interaction of the TCR complex with antigen/MHC or ligation by monoclonal antibodies against the TCR or CD3, has been shown to induce the activation of at least two signal transduction pathways, involving phosphoinositol turnover and a rapid change in the intracellular concentration of calcium. At least two kinase pathways appear to be activated and several proteins phosphorylated in response to receptor engagement, including phosphorylation of CD3γ on serine, and early tyrosine phosphorylation of several intracellular substrates, particularly the TCR ζ chain. One of the kinase pathways involved is attributable to activation of protein kinase C, since it is possible to mimic the phosphorylation of CD3 by treatment of cells with phorbol ester or diacylglycerol (reviewed by Klausner & Samelson, 1991). Phosphorylation of the ζ chain, however, indicates an alternative tyrosine kinase pathway is also involved (Mustelin *et al.*, 1990). The components of the CD3 complex have subsequently been found to be substrates for phosphorylation by the *src*-family protein tyrosine kinase p56^{lck} (Barber *et al.*, 1989), particularly TCR ζ (Carrera *et al.*, 1988). These changes in protein phosphorylation are thought to be an initial step in the chain of signal transduction events. The significance of protein tyrosine kinase activation and phosphorylation in TCR signal transduction and initiation of later events, such as lymphokine secretion, however, still remains uncertain.

1.1.2 The accessory molecules CD4 & CD8

The accessory molecules CD4 and CD8 are members of the immunoglobulin gene superfamily and are present on mutually exclusive subsets of T cells. Murine CD4 is a 55kD monomeric integral membrane glycoprotein that binds to non-polymorphic

regions of major histocompatibility complex (MHC) class II antigens on antigen presenting cells (Doyle & Strominger 1987). CD8 exists as a disulphide-linked heterodimer comprising two chains, the CD8α (previously termed Ly 2) 38kD, and CD8β chain (Ly 3), 28kD, and acts as a receptor for MHC class I antigens on antigen presenting cells (Norment *et al.* 1988). The CD8α mRNA has been shown to give rise to two alternatively spliced forms, one encoding the CD8α full length chain, and the other a truncated or "tailless" form, CD8α' (reviewed by Parnes, 1989). Both CD4 and CD8 are thought to stabilise interactions between T cells and antigen presenting or target cells (reviewed by Parnes, 1989), and specific monoclonal antibodies to each of these molecules have been shown to inhibit antigen induced T cell activation (reviewed by Geppert *et al.*, 1990).

Although the affinity of CD4 and CD8 for the MHC class II or class I molecules, respectively (Doyle & Strominger, 1987; Norment *et al.*, 1988) may suggest the potential to promote adhesive interactions, the intimate involvement of CD4 and CD8 in TCR/MHC ligand interactions has complicated attempts to determine their relative contribution in signalling and adhesion. It is uncertain as to whether CD4 and CD8 bind to MHC molecules adjacent to those bound by the TCR/CD3 complex, or whether CD4, or CD8 and the TCR/CD3 complex need to bind the same MHC molecule simultaneously. However, the "co-receptor" model proposes that the assembly of a macromolecular complex involving the physical association of CD3 with CD4 or CD8, enables recognition by TCR/CD3 complex of nominal antigen bound to polymorphic MHC determinants, and CD4 and CD8 to bind to non-polymorphic determinants on the same MHC molecule (Janeway, 1988, 1989). Consistent with this model, comodulation of CD4 with the TCR/CD3 complex can be observed with certain anti-T cell receptor antibodies (Saizawa *et al.*, 1987; Anderson *et al.*, 1988; Rivas *et al.*, 1988; Kupfer *et al.*, 1987; Rojo *et al.*, 1989).

Further evidence supporting this co-receptor model has been provided from studies which have mapped the site of interaction between CD8 and class I MHC to the α_3 domain. These data suggest that in order to activate T cells, CD8 and the TCR must

interact with the same MHC molecule (Potter *et al.*, 1989; Salter *et al.*, 1990). Since the sites of interaction between CD4 and class II MHC have not been mapped, it is still unclear whether CD4 interacts with class II MHC molecules in a manner homologous to the CD8/class I interaction.

1.1.3 Signal transduction

Although transfection experiments have confirmed the influence of these accessory molecules on TCR interaction (Dembic *et al.*, 1987; Gabert *et al.*, 1987; Sleckman *et al.*, 1987), accumulating evidence suggests that CD4 and CD8 may be additionally involved in signal transduction pathways. Upon antibody binding, both CD4 and CD8 can generate an increase in intracellular calcium levels (Ledbetter *et al.*, 1987; Wacholtz *et al.*, 1989), although crosslinking appears to transduce an incomplete activation signal, since it does not induce proliferation or IL-2 production. Crosslinking CD4 or CD8 with CD3, or with MHC class I, however, augments the functional activation observed by crosslinking either molecule individually, generating an increase in intracellular calcium levels, and the production of IL-2 (Boyce *et al.*, 1988; Eichmann *et al.*, 1987; Emmrich *et al.*, 1986, 1988). The immobilisation of monoclonal antibodies to CD3 and CD4 or CD8 to plastic; coupling to sepharose beads; or the use of heteroconjugate antibodies, all result in enhanced T cell activation, in comparison to that induced by the use of individual monoclonal antibodies or homoconjugates alone (reviewed by Rudd *et al.*, 1989).

The demonstration that both CD4 and CD8 are associated with the *src*-family T cell specific protein tyrosine kinase $p56^{lck}$ (Veillette *et al.*, 1988; Barber *et al.*, 1989), provides further evidence to suggest a role for CD4 and CD8 in the activation of mature T cells. Antibody-mediated CD4 or CD8 cross-linking results in an increase in tyrosine phosphorylation and a rapid increase in $p56^{lck}$ tyrosine specific protein kinase activity (Veillette *et al.*, 1988). The interaction between CD4 or CD8 and $p56^{lck}$ is mediated by a cysteine motif in the cytoplasmic domain of CD4 and CD8α (Shaw *et al.*, 1990; Turner *et al.*, 1990). Mutational analysis of this motif has enabled the functional significance of this association in antigen-specific T cell activation to be examined

(Glaichenhaus *et al.*, 1991). This interaction therefore provides direct evidence for the involvement of CD4-p56^{lck} complexes in T cell activation, and supports a role for the CD4 molecule in signal transduction.

In the studies reported by Glaichenhaus *et al.*, only minimal T cell activation was observed in cells expressing CD4 molecules which could not bind p56^{lck}, as a result of mutating the cysteine motif (Glaichenhaus *et al.*, 1991). Although these studies may indicate only a minor adhesive role for CD4 in T cell activation, the assay system used does not directly address this question, nor the influence of the affinity of the TCR interaction. In contrast, previous experiments using a CD8-dependent T cell hybridoma have suggested that the alloreactive response to a class I MHC ligand is only partially dependent on the cytoplasmic domain of CD8α (Zamoyska *et al.*, 1989; Letourneur *et al.*, 1990). Whilst a partial response was seen with a truncated, "tailless" form of CD8α (CD8α'), perhaps suggesting some adhesive function for CD8 in this system, the inability of p56^{lck} to associate with a truncated CD8 molecule correlated with the observation that the response to antigen was impaired (Zamoyska *et al.*, 1989).

The association of CD4 and CD8 with p56^{lck} therefore suggests a potential mechanism for the costimulatory effect observed upon crosslinking these accessory molecules. The properties of CD4-p56^{lck} appear to differ from those of CD8-p56^{lck}, however, since treatment of cells with activators of protein kinase C cause the dissociation of p56^{lck} from CD4, together with CD4 internalisation, whereas this was not observed for the CD8-p56^{lck} complexes (Hurley *et al.*, 1989). These findings raise the possibility that the interaction of p56^{lck} with CD4 and CD8 may differ.

The association of the *src*-family protein kinase p56^{lck} with CD4 and CD8 has prompted the search for other associations between *src*-family members and cell surface receptors. Recent studies have identified another protein tyrosine kinase, p59^{fyn} physically linked to the TCR/CD3 complex (Rudd, 1990; Samelson *et al.*, 1990). This kinase has been functionally implicated in T cell activation, since transgenic mice overexpressing *fyn* appear to be highly responsive to antigenic stimulation (Perlmutter *et al.*, unpublished).

1.1.4 The role of adhesion molecules

The capacity of the T cell to be activated following recognition of antigen by the TCR/CD3 may be regulated by a number of adhesion molecules, including CD2, CD28, CD45, ICAM-1, and LFA-1 (Springer, 1990). These non-polymorphic molecules appear to increase the avidity with which a T cell interacts with a target cell, and may also participate in signal transduction. In particular, LFA-1 and CD2 are known to perform an adhesion function and to participate in the initial antigen-independent formation of conjugates between T cells and other cells (Spits *et al.*, 1986; Springer *et al.*, 1987). Crosslinking CD2 with certain pairs of anti-CD2 monoclonal antibodies has also been shown to provide a potent stimulus for T cell proliferation, thus implicating a role for CD2 in T cell activation (reviewed by Geppert *et al.*, 1990).

The CD28 molecule is also capable of providing co-stimulatory signals. CD28 is a 44 kD homodimer present on most $CD4^+$ T cells, and a subset of $CD8^+$ T cells. Antibodies to CD28 can be used to stimulate T cell proliferation in the presence of PMA, and have been shown to be co-mitogenic with anti-CD3 antibodies (Ledbetter *et al.*, 1985). T cells can be directly activated by the CD28 pathway (Ledbetter *et al.*, 1990), although this activation pathway appears to be distinct from that triggered by anti-CD3 monoclonal antibodies, and is independent of CD3 expression (June *et al.*, 1987; Weiss *et al.*, 1986). Engagement of CD28 by its ligand may therefore result in co-stimulation, and hence optimal activation of the cell after antigen/MHC recognition by the TCR. Furthermore, anti-CD28 monoclonal antibodies have been demonstrated to be comitogenic for thymocytes following crosslinking of CD3 with either CD4 or CD8 (Turka *et al.*, 1990). This mitogenic activity and the presence of the natural ligand for CD28 (BB-1/B7; Linsley *et al.*, 1990) on thymic tissue suggests that the CD28 pathway may be involved in thymic selection (Turka *et al.*, 1991).

Another potentially important regulatory molecule in T cell activation is the leukocyte common antigen (CD45). CD45 identifies a family of glycoproteins expressed at different levels on the surface of different types of haematopoietic cells (Ralph *et al.*, 1987), and the complex includes proteins of molecular weight 180 kD,

190 kD, 205 kD, and 220 kD, produced by alternate exon splicing of a single gene (Thomas & Lefrancois, 1988). Although the biological function of CD45 on haematopoietic cells is not known, two regions of the cytoplasmic domain have been shown to exhibit homology with a soluble placental protein phosphotyrosine phosphatase (Charbonneau *et al.*, 1988). This suggests that CD45 may be involved in the regulation of protein phosphorylation and signal transduction.

Evidence to support the role of CD45 in signal transduction has been provided by studies using T cells which have lost CD45, in which an increase in phosphorylation of the tyrosine kinase $p56^{lck}$, was observed (Ostergaard *et al.*, 1989). A tyrosine residue in the carboxy terminal domain of $p56^{lck}$, tyrosine 505, has been shown to be important in the regulation of kinase activity, since phosphorylation of this tyrosine residue inhibits protein kinase activity, whereas dephosphorylation activates this kinase. Hence the increase in tyrosine kinase activity observed by incubating CD45 with $p56^{lck}$ is probably due to dephosphorylation of tyrosine 505 (Mustelin *et al.*, 1989; Ostergaard *et al.*, 1989). Mutation or deletion of this tyrosine residue has been shown to confer oncogenic potential to $p56^{lck}$ (Amrein & Sefton, 1988; Marth *et al.*, 1988), suggesting a potential role of this kinase in the regulation of cell growth.

Antibody-mediated coclustering of CD45 with CD4 or CD8 has provided further evidence to implicate $p56^{lck}$ as a substrate for CD45 *in vivo*. Crosslinking CD45 with CD4 appears to regulate the dephosphorylation of $p56^{lck}$ on tyrosine and therefore its *in vitro* kinase activity (Ledbetter *et al.*, 1988). Similar results have been obtained from CD45 crosslinking with CD8 on cytotoxic T cell lines (Ostergaard & Trowbridge, 1990). Crosslinking CD45 and CD4 also increases intracellular levels of calcium, suggesting that interactions between CD45 and CD4 may cause an upregulation of the cell signalling machinery (Ledbetter *et al.*, 1988). In contrast, crosslinking CD45 with CD3, or CD2, causes a reduction in the levels of intracellular calcium (Ledbetter *et al.*, 1988). This may result in the dephosphorylation of the CD3 ζ chain, and therefore limit cell signalling. Whilst a ligand for CD45 has still not been

identified, the evidence implicates an important role for this molecule in signal transduction.

1.1.5 Induction of T cell responsiveness or tolerance?

Although the biochemical events that transduce the signals delivered to the T cell are not fully understood, engagement of these T cell surface molecules is known to induce or augment increases in the levels of intracellular calcium, and the generation of second messengers. Triggering via the TCR/CD3 complex appears to depend on the number of molecules engaged, the nature of crosslinking, and duration of engagement. Previous studies have found that T cell activation by soluble anti-CD3 antibodies requires costimulation with accessory cell signals, in order to generate an optimal response (Davis *et al.*, 1986; Williams *et al.*, 1985). Furthermore, CD3 signalling in the absence of the appropriate costimulation appears to inhibit responsiveness to subsequent stimulation.

If the ligand involved is the antigen-MHC complex presented by an antigen-presenting cell, additional nonspecific interactions appear necessary to induce activation (reviewed by Geppert *et al.*, 1990). In the absence of accessory cell signals, antigen recognition may fail to activate the T cell and induce unresponsivenes to subsequent antigen exposure. Hence these accessory signalling events appear to be crucial in determining whether a state of antigen responsiveness or tolerance is induced.

1.2 T cell ontogeny

Events in intrathymic development and post-thymic stages of T cell differentiation have been extensively studied, however relatively little is known regarding the nature of the bone marrow prothymocyte which homes to the thymus to initiate and maintain thymic lymphoid populations. Although the characterisation of progenitors on the basis of expression of phenotypic markers has successfully identified multipotent progenitors in the bone marrow and foetal liver (Spangrude *et al.*, 1989; Ikuta *et al.*, 1990), the timing of commitment of these progenitor cells and their lineage relationships are still unknown. The identification and isolation of

haematopoietic stem cells, however, will enable the requirements for growth and differentiation of these precursors to be determined. There is now accumulating evidence from such studies to suggest that precursor cells of both the myeloid and lymphoid lineages express receptors for several interleukins (Palacios & Pelkonen, 1988) and a candidate early haematopoietic factor has been recently identified (reviewed by Witte, 1990).

1.2.1 The thymus

The thymus is the major site of T cell lymphopoiesis and continually colonised by haemopoietic cells from fetal liver or bone marrow. The first wave of immigrant cells from fetal liver colonise the thymus at day 10-11 of gestation (Moore & Owen, 1967; Owen & Ritter, 1969) - the fetal liver being the major haemopoietic organ during embryonic development, a role subsequently fulfilled by the bone marrow after birth (Metcalf & Moore, 1971). Whilst the early differentiation stages of prethymic maturation remain to be elucidated, it appears there is some heterogeneity amongst the cells which seed the thymus, and thymic colonisation by several immigrant precursor populations appears to occur at different times during development (Jotereau *et al.*, 1987; Penit & Vasseur, 1989). The frequency of these progenitors, their variation with age, and organ distribution, are the subject of continuing investigation.

The immigration of these haematopoietic precursors into the thymus is thought to be under the influence of thymus derived chemoattractants, which have been shown to include β_2 microglobulin (β_2m) (Dargemont *et al.*, 1989; Deugnier *et al.*, 1989; Dunon *et al.*, 1990). Although the precise site of thymic entry is still controversial, prothymocytes are thought to localise to the thymic corticomedullary junction or subcapsule (Hirokawa *et al.*, 1989; Penit & Vasseur, 1988).

There is still debate as to whether the thymus microenvironment is an absolute requirement for all T cell precursors to develop into functional T cells, and evidence from congenitally athymic mice suggests that an extrathymic pathway of T lymphoid differentiation may exist (Hünig, 1983). Although the presence of T cell function in nude mice may be the result of an inefficient mechanism of thymus maturation,

followed by peripheral expansion, the presence of T cell specificites which are usually deleted in euthymic mice may argue in favour of extrathymic differentiation (Rocha, 1990; Rocha & von Boehmer, 1991).

1.2.2 Lineage diversification

The immigrant haematopoietic cells differentiate within the thymus to produce mature T lymphocytes. The developmental stages in thymic ontogeny have been characterised by expression of the T cell receptor and CD4 and CD8 co-receptors (reviewed by von Boehmer 1988; Boyd & Hugo, 1991), the earliest precursors lacking surface expression of the TCR, CD4 and CD8. Shortly after entry into the thymus, the immigrant precursor population starts to rearrange TCR gene segments, to generate a large number of clonally diverse cells (Strominger, 1989), first γδ and later β then α. A major question that still remains unresolved, however, is when and where the diversification of the αβ and γδ TCR lineages occurs. Evidence suggests that pro-T cell clones can be induced to differentiate into αβ or γδ TCR-expressing cells *in vitro* (Palacios *et al.*, 1989), although αβ cells do not appear to arise from γδ cells which have failed to undergo successful rearrangements, since cells rearranging TCR_{α} have not previously rearranged TCR_{δ} (Winoto & Baltimore, 1989). The latter data imply that there may be two lineages of cells which are committed to express either γδ TCR or αβ TCR, however, the γ TCR genes appear to be rearranged in both lineages (Winoto & Baltimore, 1989).

In ontogeny, the appearance of γδ TCR^+ cells precedes that of the αβ TCR^+ cells (Havran & Allison, 1988; and reviewed by Fowlkes & Pardoll, 1989), γδ TCR^+ cells first being detected by day 15 gestation in the mouse, whereas αβ TCR^+ cells do not appear until day 17. If the two lineages arise from a common precursor, this may suggest that the embryonic thymus preferentially induces γδ TCR expression, perhaps as a result of differences in the timing of appearance of stromal elements required to support either γδ TCR^+ or αβ TCR^+ cells. The appearance of γδ TCR^+ cells in congenitally athymic (nude) mice, may imply that these cells have different thymic requirements to those of the αβTCR lineage. The epithelial localisation of these γδ

TCR+ cells in peripheral tissues (dermal, and intestinal) in normal mice may reflect certain similarities between the thymus and these sites. Although the thymus may not represent the sole site for development of γδ cells (Takagaki *et al.*, 1989, Carding *et al.*, 1990, Parker *et al.*, 1990), there is evdence that γδ TCR cells undergo positive (Itohara & Tonegawa, 1990; Lafaille *et al.*, 1990) and negative selection in the thymus during maturation (Dent *et al.*, 1990; Bonneville *et al.*, 1990).

Induction of αβ TCR rearrangement appears to be limited to the thymic microenvironment, however, and TCR β chain rearrangement is initiated in the immature thymocyte population by day 16. During thymic ontogeny, rearrangement of the β TCR genes precedes rearrangement of the α TCR genes (Raulet *et al.*, 1985; Snodgrass *et al.*, 1985a, 1985b; Samelson *et al.*, 1985; Williams *et al.*, 1986). Productive rearrangement of β TCR genes appears to suppress further β rearrangement and to initiate TCR_{α} rearrangement. It is unclear, however, whether expression of α TCR genes may influence further rearrangement of Vα gene segments (Kisielow *et al.*, 1988).

Expression of a productively rearranged TCR β chain in transgenic mice prevents productive and non-productive rearrangement of endogenous Vβ gene segments, a phenomenon referred to as allelic exclusion (Blüthman *et al.*, 1988; Fenton *et al.*, 1988; Uematsu *et al.*, 1988). The mechanism of allelic exclusion is unknown, although it appears to require a productively rearranged β TCR gene but not the entireVβ region (Krimpenfort *et al.*, 1989). It is also uncertain as to how the receptor chains on the surface of immature thymocytes instruct the cell that a productive rearrangement has been made. The productive rearrangement of β TCR genes seems to be an important stage in T cell development, however, since the introduction of a rearranged β TCR gene into mice with severe combined immunodeficiency induces expression of CD4 and CD8, and transcription of the α TCR locus (Kishi *et al.*, 1991).

The ordered rearrangement of TCR genes during T cell ontogeny parallels the rearrangement of immunoglobulin (Ig) genes seen in B lymphocyte development, where rearrangement of the μ heavy chain in pre-B cells precedes the rearrangement of

the light chain (κ or λ). In addition to its role in the induction of light chain rearrangement, the presence of the membrane form of the μ chain is required for allelic exclusion, indicating that surface expression of the μ chain is an important event in B cell development (Manz *et al.*, 1988; Reth *et al.*, 1987). Thus expression of the μ chain and TCR β chain appears to fulfil a parallel role duirng B and T cell ontogeny.

1.2.3 Differentiation and repertoire selection in the thymus

The $CD4^-CD8^-$ population in the thymus can be subdivided into immature and more mature cells by expression of the heat stable antigen (HSA). Whilst the precise function of this marker is unknown, it has been used as a marker of haematopoietic differentiation in both the T and B lineages, and is identified by a number of antibodies which all appear to recognise the same antigenic determinant (Alterman *et al.*, 1990). The majority of $CD4^-CD8^-$ cells in the thymus are immature and express HSA, however, a minor subpopulation are HSA^- and express the αβ TCR, in association with the CD3 complex (Wilson *et al.*, 1988). In neonatal animals, some $CD4^-CD8^-$ HSA^- cells are also detected, a significant proportion of which express αβ TCR, however the immediate precursors of this population and their functional significance are unknown.

The developing thymocytes undergo a programmed sequence of differentiation events, resulting in the generation of mature T cells. Expression of CD4 and CD8 is developmentally regulated, with cell surface expression of TCR following the acquisition of CD4 and CD8. Cell transfer studies by Guidos *et al.* suggest that the $CD3^-CD4^-CD8^-$ precursors may pass through an intermediate stage expressing low levels of CD8 on the cell surface prior to expressing both CD4 and CD8 by day 16 of gestation in the mouse (Guidos *et al.*, 1989a). However, this may not be the sole pathway that maturing thymocytes follow in the development of double positive cells.

1.2.4 The role of the TCR in repertoire selection

The TCR specificity of individual T cells is determined during intrathymic development. The TCR β chain is first expressed in the cytoplasm by day 14 gestation, the product of successful rearrangement of the TCR variable (V), diversity (D) and joining (J) gene segments. Subsequent products of V-J rearrangements of the TCR α

locus combine with the products of TCR_β rearrangement, and the αβ TCR is first expressed on cells in the immature $CD4^+CD8^+$ population by day 17 of gestation (reviewed by Fowlkes and Pardoll, 1989). These double-positive cells comprise a diverse population each cell expressing a unique antigen receptor. The heterogeneity of the αβ T cell receptor expressed therefore generates the potential to interact with a large number of MHC molecules. This diverse repertoire is then subject to selection events in the thymus which shape the final mature T cell repertoire.

Significant cell death occurs during intrathymic development, and only a small proportion of the $CD4^+CD8^+$ (double-positive) intermediates expressing low levels of TCR on the cell surface survive to differentiate further into mature single positive, $CD4^+CD8^-$ or $CD4^-CD8^+$ cells bearing high levels of surface TCR (Fowlkes & Pardoll, 1989; Petrie *et al.*, 1990). These "mature" single-positive cells then exit to seed the periphery. Cell trafficking studies suggest that between 95 and 99% of the double positive population die within the thymus (Scollay & Shortman, 1984). A proportion of the cell death is attributable to unsuccessful rearrangement of the TCR, however, the remainder is due to selection processes.

It is unclear as to the mechanism by which an immature thymocyte expressing both CD4 and CD8 lose expression of one or the other of these molecules. Following expression of αβ TCR on $CD4^+8^+$ thymocytes, subsequent development is determined by the specifity of the αβ TCR. It has been proposed that the class of the MHC molecule bound by the receptor may determine the specificity of the cell interaction with MHC class I or class II, driving the selected cell to assume a $CD4^-8^+$ or $CD4^+8^-$ phenotype, respectively (referred to as the "instructive model", discussed by Robey *et al.*, 1991). Alternatively the appearance of cells may occur by a stochastic process, whereby CD4 or CD8 expression is randomly switched off (Ramsdell & Fowlkes, 1990).

Whichever mechanism is involved, it appears that if the receptor on immature thymocytes does not bind to any ligand in the thymus the cells may die. However, it has been proposed that if the receptor binds with a certain affinity to the appropriate

MHC ligands on the thymic epithelium, the thymocytes will be rescued from programmed cell death, or apoptosis, by a process of positive selection (reviewed by Blackman *et al.*, 1990). A high affinity interaction of receptor with intrathymic ligands may induce cell death of immature thymocytes with reactivity to self-antigen plus MHC, by a process referred to as negative selection, or may induce functional inactivation of these cells (reviewed by Ramsdell & Fowlkes, 1990).

1.2.5 Positive selection

Antigen recognition by T cells is known to be restricted by components of the MHC (Bevan & Fink, 1978; Zinkernagel *et al.*, 1978), and during ontogeny the developing thymocytes are "educated" in the recognition of self-MHC. Experiments using radiation chimaeras first suggested that the bone marrow cells used to reconstitute the irradiated host could respond to antigen presented by host MHC molecules. Subsequent experiments confirmed that this self-restriction was determined by the radioresistant thymic epithelium (reviewed by Blackman *et al.*, 1990). The term positive selection was given to this process of thymic "education", in which developing thymocytes capable of interacting with self-MHC on the thymic epithelium receive a signal to potentiate their subsequent differentiation to maturity.

The requirement for a direct interaction between the TCR on the developing thymocytes and the MHC on the thymic epithelium was demonstrated by injecting monoclonal antibodies against the TCR or MHC into pregnant mice and subsequent neonates, or by the addition of antibodies to foetal thymus organ cultures. The development of mature T cells with the corresponding TCR or MHC specificity was disrupted by this antibody treatment, as assessed by expression of the appropriate TCR or MHC, or by functional analysis (Kruisbeek *et al.*, 1985; McDuffie *et al.*, 1986; Born *et al.*, 1987; Marusic-Galesic *et al.*, 1988, 1989; Marrack *et al.*, 1988; Zuñiga-Pflücker *et al.*, 1989a). Similarly, the involvement of CD8 in positive selection has been demonstrated by *in vivo* treatment with anti-CD8 antibodies during development (Zuñiga-Pflücker *et al.*, 1990a).

Evidence for positive selection has accumulated following the advent of transgenic mouse technology. The introduction of functionally rearranged α and β chains for a T cell receptor with a particular antigen and MHC specificity, into the mouse germline, has enabled the expression of a single TCR with defined specificity on almost all thymocytes and peripheral T cells (Kaye *et al.*, 1989; Kisielow *et al.*, 1988; Sha *et al.*, 1988; Berg *et al.*, 1989). In these mice, immature thymocytes expressing the transgenic receptor develop irrespective of the MHC haplotype of the animal. Mature thymocytes and peripheral T cells, however, only accumulate in mice expressing the same MHC haplotype, as that of the mouse from which the transgenic TCR was derived. Furthermore, the proportion of CD4 or CD8 cells in the repertoire of these mice is strongly biased towards the accessory molecule expressed by the original T cell clone from which the TCR was isolated. Hence the TCR, MHC, CD4 or CD8 are all implicated as being involved in the selection process.

In normal non-transgenic mice, monoclonal antibodies directed against specific TCR V_β gene products have also been used to determine the influence of MHC haplotype on positive selection of T cells (MacDonald *et al.*, 1988). The use of monoclonal antibodies directed against specific V_β chains has allowed the fate of a population of developing T cells of known specificity to be examined. For example, V_β17a-expressing cells were found to recognise the class II MHC molecule in combination with a B cell-derived product (Marrack & Kappler, 1988), independently of other receptor components (Blackman *et al.*, 1988). By following the fate of V_β17a-expressing cells using V_β-specific antibodies, the influence of MHC haplotype on positive selection in normal mice has been determined, indicating that $V_\beta17a^+$, $CD4^+$ T cells are preferentially selected in mice of the H-2^q MHC haplotype, since there are more peripheral $V_\beta17a^+$, $CD4^+$ T cells in H-2^q than in H-2^b mice. Similar studies showed that expression of $V_\beta17a^+$, $CD4^+$ T cells in F1→ parent radiation chimaeras after reconstitution was determined by the MHC haplotype of the irradiated host, rather than that of the donor bone marrow cells (Blackman *et al.*, 1988). Such studies confirmed the earlier observations documenting the importance of the thymic cortical

epithelium in mediating positive selection, and provided evidence for the additional influence of non-MHC genes (Fry *et al.*, 1989a).

The interaction of the TCR, the MHC and the accessory molecules on the developing thymocyte and the thymic cortical epithelium are thus responsible for rescuing immature double-positive cells from cell death in order to generate a peripheral repertoire which is biased to recognise foreign antigen bound to self-MHC.

1.2.6 Negative selection

It is generally accepted that an essential requirement in the maintenance of the immune system is tolerance to self components. The random generation of TCR specificities gives rise to a diverse population of cells each bearing a unique antigen receptor, and will undoubtably include cells bearing autoreactive receptors. Evidence suggests that there may be a number of mechanisms to maintain self tolerance, and that these may operate at different developmental stages. Three general mechanisms by which tolerance is maintained have been proposed: clonal deletion, clonal anergy, and suppression of self-reactive T cell clones.

1.2.7 Tolerance mediated by clonal deletion

The mechanism of clonal deletion as a means of eliminating autoreactive T cell clones was first demonstrated using monoclonal antibodies to TCR chains with a specificity for self-antigen. Most T cells bearing a receptor containing the β chain of the $V_\beta 17$ family were found to be reactive to the class II MHC molecule I-E (Kappler *et al.*, 1987a, 1987b). Using $V_\beta 17$-specific antibodies, it was discovered that $V_\beta 17a$-expressing T cells were virtually absent in the periphery of mouse strains expressing I-E. This was the first demonstration that clonal deletion was a principal mechanism in achieving self-tolerance. Further evidence for clonal deletion was provided by analysing the involvement of other TCR V_β regions in the recognition of self-antigens.

Superantigens can be assigned to two general categories, those which are expressed endogenously (self-superantigens) and those which are exogenously derived (e.g. Staphylococcal enterotoxins; reviewed by Blackman *et al.*, 1990). In mice expressing the appropriate superantigen/MHC combination, Mls-1^a (minor lymphocyte

stimulating antigen-1) plus most class II MHC alleles, T cells which bear $V_\beta 8.1$, $V_\beta 6$, and $V_\beta 9$ are eliminated (Happ *et al.*, 1989, Kanagawa *et al.*, 1988; Kappler *et al.*, 1988; MacDonald *et al.*, 1988). Superantigens have also been used to induce clonal deletion experimentally, both *in vivo*, and *in vitro*. The injection of staphylococcal enterotoxin B into mice from birth (White *et al.*, 1989), or the addition of toxins to foetal thymus organ cultures (Finkel *et al.*, 1989; Yagi *et al.*, 1990; Jenkinson *et al.*, 1989), has been shown to cause the deletion of mature thymocytes expressing antigen-reactive V_β chains. Hence clonal deletion has been demonstrated in all V_β-superantigen systems examined. In contrast to the deletion of $V_\beta 17a^+$ T cells mediated by class II I-E molecules, a number of non-MHC encoded molecules, closely linked to the endogenous pro-virus Mtv-9, have been shown to mediate deletion of $V_\beta 5^+$ and $V_\beta 11^+$ T cells, by I-E (Bill *et al.*, 1989; Woodland *et al.*, 1990).

Recent evidence indicates that Mls and related genes specifying self-superantigens are encoded by retroviruses (Acha-Orbea *et al.*, 1991; Choi *et al.*, 1991; Dyson *et al.*, 1991; Frankel *et al.*, 1991; Marrack *et al.*, 1991; Woodland *et al.*, 1991). These reports have described a V_β selective element which is maternally transmitted and encoded by a mouse mammary tumour virus (MMTV). A B cell tumour which contains a member of a different retroviral family, a defective murine leukaemia virus (MuLV), also expresses a V_β selective element (Janeway, 1991). These data implicate a retroviral protein in the T cell response to V_β selective elements. An obvious question is why retroviruses encode V_β selective elements and why they are present in the mouse genome. It is possible that T cell activation by retroviral V_β selective elements may render them susceptible to retroviral infection. The presence of these viral sequences in the mouse genome may therefore suggest a strategy by which T cells capable of responding to these viral elements are eliminated. The biological significance of Mls antigens and other V_β selective elements therefore remains to be determined.

There is accumulating evidence to suggest that clonal deletion operates on immature thymocytes at the $CD4^+CD8^+$ stage of development. The elimination of $V_\beta 17a$-bearing T cells in $I\text{-}E^+$ mice deletes both the $CD4^+$ and the $CD8^+$ thymocytes,

although in the periphery it is only the $CD4^+$, $V_\beta 17a^+$ population which is I-E reactive (Kappler *et al.*, 1987a, 1987b; Burgert *et al.*, 1989). This suggests that the $CD8^+$, $V_\beta 17a^+$ cells are deleted at the double-positive stage, i.e. when the cells express both CD4 and CD8. Furthermore, deletion of $CD8^+$, $V_\beta 17a^+$ cells is prevented in I-E^+ mice which have been treated with antibodies to I-E or CD4 (Fowlkes *et al.*, 1988; MacDonald *et al.*, 1988). These data imply that the double-positive thymocytes are a target for clonal deletion, and that the participation of accessory molecules is involved. However, not all immature thymocytes appear to be susceptible to clonal elimination (Blackman *et al.*, 1990).

Mice expressing a transgenic αβ TCR and the appropriate specific antigen and MHC haplotype have also been used as a model to investigate clonal deletion (Kisielow *et al.*, 1988; Teh *et al.*, 1988, 1989). In contrast to the data from normal mice, the data obtained from these transgenic mice suggested that the majority of double-positive thymocytes and mature single-positive thymocytes were susceptible to deletion. This is probably a result of the transgenic receptor being expressed much earlier and at a higher density than in normal development, due to the introduction of TCR α and β genes which are already rearranged. In studies using transgenic mice containing a rearranged β chain only, receptor expression is restricted to the timing seen in normal development by rearrangement of the endogenous α chain, and consequently in these animals, the pattern of deletion follows that seen in normal mice (Berg *et al.*, 1989).

The differences between transgenic and normal mice have been further addressed using mice with a transgenic αβ TCR of dual specificity. The transgenic αβ TCR in these animals is specific for lymphocytic choriomeningitis virus (LCMV), plus D^b, in addition to the Mls-1^a reactivity conferred by the use of $V_\beta 8.1$ in the transgenic receptor (Pircher *et al.*, 1989a, 1989b). In Mls-1^a mice which express LCMV/D^b, approximately 50% of the immature thymocytes, and all of the mature thymocytes are deleted. In mice which express Mls-1^a but not LCMV, only the mature thymocytes are deleted (Pircher *et al.*, 1989a, 1989b). Thus the deletion of thymocytes at different maturational stages appears to be dependent on the expression of the different self

antigens (LCMV plus D^b, or Mls-1^a). Pircher *et al.* propose that the timing of deletion is determined by the affinity of the TCR for antigen, the deletion of the earlier immature double-positive thymocytes a consequence of a high affinity interaction between the TCR and LCMV+ D^b, whereas an interaction between a lower affinity antigen (Mls-1^a), causes deletion at a later stage of development. An alternative explanation may be that the timing of Mls-1^a expression may be delayed, or restricted to certain cells within the thymus, therefore influencing the timing of deletion of Mls-1^a-reactive cells.

Although the thymic cortical epithelium is responsible for positive selection, the identity of the cells mediating deletion is not known. However, the use of radiation bone marrow chimaeras or thymus grafting systems, has demonstrated that bone marrow-derived and thymic medullary epithelial cells participate in negative selection (Marrack *et al.*, 1988; Blackman *et al.*, 1990).

1.2.8 The paradox of selection

The models of selection based on self-recognition, thus present a paradox, in that the same receptor appears to fulfil contradictory roles. Two hypotheses have been proposed to account for this paradox, based on the affinity of the receptor, or based on a difference between the MHC molecules expressed on thymic cortical epithelium and those expressed elsewhere in the animal (referred to as the "altered ligand" hypothesis; Marrack & Kappler, 1987).

The "affinity" hypothesis postulates that thymocytes expressing both high and low-affinity receptors may be positively selected by interaction with self-MHC, whereas only those cells expressing high-affinity receptors are deleted (Sprent & Webb, 1987). Clones which have a low affinity for self will therefore survive deletion, and will constitute the peripheral T cell repertoire for foreign antigen plus self-MHC.

The elucidation of the structure of a class I MHC molecule led to the suggestion that in the absence of foreign peptide, the peptide binding site in the molecule may be occupied by self-peptides (Bjorkman *et al.*, 1987a, 1987b; Brown *et al.*, 1988). Theoretically, the binding of different self peptides could generate a large number of self peptide/self-MHC molecules. Various forms of the "altered ligand" hypothesis

have been proposed, however, the basic tenet demands that the MHC expressed by the thymic cortical epithelial cells is able to present a set of peptides that is unique to the thymic environment. With the assumption that these cells are incapable of inducing clonal elimination, this may explain how both positive and negative selection of thymocytes can occur, resulting in the production of mature cells capable of antigen/self MHC recognition.

There is some experimental evidence to support the hypothesis that the MHC expressed on thymic cortical epithelial cells is unique to the thymus. Firstly, an antibody has been raised against I-A, that recognises a unique determinant, in the presence of I-E. Although this antibody recognises I-A in peripheral tissues, it does not appear to be able to bind to I-A on thymus cortical epithelial cells (Murphy *et al.*, 1989). This may suggest that the MHC is different in the thymus, or that the determinant recognised is simply hidden in this environment. Furthermore, a number of T cell hybridomas have been isolated which will respond to antigen plus self MHC expressed by the thymus cortical epithelium, but not by other cells (Marrack *et al.*, 1989), which may suggest that these cells recognise a particular thymic determinant.

Both the affinity and altered ligand hypotheses require that the developing thymocytes are able to discriminate between the receptor interactions which induce positive selection, and those inducing clonal elimination. The affinity hypothesis overcomes this difficulty by proposing that weak interactions are positively selective, whereas higher affinity interactions are lethal to the cell. The second hypothesis requires that the thymocytes receive a different signal from engagement of the T cell receptor with the thymus cortical epithelial cells, than from engagement by other cell types. This may be accountable by different accessory signals, perhaps lymphokines, produced by the thymus cortical epithelial cells (Lorenz & Allen, 1989), or possibly the developmental status of the thymocyte, at the time it interacts with the cortical epithelial cells.

There is some evidence to support the latter proposal, suggesting that immature thymocytes may exist in a number of differentiative stages, with regard to their

sensitivity to clonal elimination. Clonal deletion may result from receptor engagement leading to increased levels of intracellular calcium, which may trigger thymocyte death (Smith *et al.*, 1989a). In agreement with this observation, Finkel *et al.* have shown that immature thymocytes exist in two stages, one in which receptor engagement by antigen does not induce a rise in intracellular calcium levels, and the cells survive; and another in which receptor engagement may cause an increase in intracellular calcium levels, and cell death (Finkel *et al.*, 1989). Treatment with anti-CD3 antibodies, however, increases intracellular calcium levels, and causes cell death in both populations (Finkel *et al.*, 1989; Smith *et al.*, 1989a). Perhaps the TCR and CD3 are not effectively coupled in the immature population which survives receptor engagement, or the necessary accessory signals for inducing a rise in intracellular calcium levels are absent at this stage (discussed by Finkel *et al.*, 1991). Presumably, these two populations must exist for a reason, and perhaps the population which survives receptor interaction may be the target of positive selection.

1.2.9 Peripheral tolerance

Although clonal deletion exists as a mechanism for maintaining tolerance to self-antigens expressed in the thymus, it does not explain how tolerance to peripheral self antigens is sustained. Clonal deletion may act on mature cells in the periphery, though experimental evidence would argue against this proposal. Deletion of Mls-1^{a}-reactive T cells expressing $V_{\beta}6$ and $V_{\beta}8.1$ is not observed following the injection of Mls-1^{a+} splenocytes into adult mice (Qin *et al.*, 1989), which argues against elimination of mature T cells. Furthermore, neonatal thymectomy inhibits clonal deletion of self-reactive $V_{\beta}11^{+}$ in I-E^{+} mice (Smith *et al.*, 1989b), and the failure to demonstrate clonal deletion of $V_{\beta}3^{+}$ and $V_{\beta}11^{+}$ cells in congenitally athymic mice expressing the corresponding self antigens, (Hodes *et al.*, 1989; Fry *et al.*, 1989b), suggests that clonal deletion is restricted to a thymic stage in T cell development.

Another possibility of maintaining tolerance to antigens not expressed in the thymus may be that circulating antigen-presenting cells can acquire self-antigens from the periphery and present these antigens to the developing thymocytes. Exogenous

antigen can be presented by thymic dendritic cells (Kyewski *et al.*, 1984), and if all peripheral antigen could be efficiently expressed by this mechanism, clonal deletion in the thymus would be sufficient for tolerance. This may be sufficient for class II presentation of exogenous antigens, however, class I presentation appears to be restricted to molecules synthesised endogenously (Moore *et al.*, 1988).

Class II MHC is only expressed on certain cell types, and it is known that class II-restricted T cells perform a critical role in the induction of peripheral B and T cell responses. It has therefore been suggested that self-reactive T cells will not be activated by the presentation of self molecules in the absence of class II expression, nor in the absence of the appropriate costimulatory signals. This proposal implies that if tolerance needs only to be established to class II reactive T cells, then presentation of class-II restricted antigens in the thymus would be sufficient, and peripheral mechanisms of tolerance are unnecessary.

1.2.10 Tolerance mediated by clonal anergy

In addition to deletional mechanisms of tolerance, functional inactivation (anergy) or suppression have been proposed as alternative regulatory processes. Anergy can be induced experimentally by manipulating the expression of self-antigens, for example, the injection of Mls-1^{a+} splenocytes into adult Mls-1^{b} mice does not cause deletion of Mls-1^{a}-reactive T cells, although this treatment renders the cells non-responsive to challenge (Ramensee *et al.*, 1989). An identical effect is mediated by the expression of I-E on peripheral cells in transgenic mice, on I-E reactive $V_{\beta}11^{+}$ and $V_{\beta}17a^{+}$ T cells (Ramsdell & Fowlkes, 1990).

Other experimental data suggest that binding of monoclonal antibodies to the TCR, or coreceptors such as CD4, in the absence of costimulatory signals from the antigen-presenting cell may also cause clonal inactivation rather than responsiveness (reviewed by Geppert *et al.*, 1990). The lack of appropriate costimulatory signals may also be involved in anergy induced experimentally by I-E expression in transgenic mice (Ramsdell & Fowlkes, 1990). In these mice, the T cells are rendered anergic even to

subsequent stimulation by "professional" antigen presenting cells (Markmann *et al.*, 1988).

In conclusion, the receptor repertoire of mature T cells appears to be limited by two contradictory processes which occur in the thymus, positive selection and clonal elimination. Although these two processes may be explained by several hypotheses, the existence of these two processes has yet to be directly proven. Tolerance to self is essential for the immune system, and clonal elimination in the thymus and anergy in the periphery presumably maintain this status. It is not fully understood why certain self antigens induce tolerance by different mechanisms, although potentially immune cells can clearly escape these regulatory processes to give rise to autoimmune disease.

1.2.11 Aims of this thesis

This thesis has two main aims. Firstly to identify lymphoid precursors in the murine foetal liver, and secondly to examine the role of CD4 and CD8 during thymic ontogeny, and to further investigate their role in immune function.

The identification of thymic precursors has facilitated the study of the early differentiation stages of prethymic maturation, and enabled investigation of the factors influencing normal T cell development. The different sites of haemopoiesis during embryonic and adult life suggest potential variation in the individual precursor populations. As an alternative to previous studies using the bone marrow or thymus, the foetal liver was exploited as a source of haematopoietic progenitors to examine the phenotype of thymic precursors in the developing embryo. The identification of such progenitors will enable the factors that regulate the differentiation of haematopoietic cells to be further defined.

Accumulating evidence has confirmed the role of the differentiation antigens, CD4 and CD8 in immune function, although their participation in the generation of functionally mature T cells in the thymus still remains unresolved. Although these molecules appear to interact with the TCR on mature T cells in antigen recognition, it is

likely that they function differently in signal transduction during the selection process. The contribution of CD4 and CD8 in thymic selection, and the extent of their interaction with the TCR on immature thymocytes, therefore remains to be elucidated. Whilst the acquisition of CD4 and CD8 during thymic ontogeny can be analysed *in vivo*, the development of *in vitro* model systems, such as foetal thymus organ culture, has facilitated experimental manipulation of the mechanisms involved in ontogeny. The combination of antisense oligonucleotides as a novel method of regulating molecular expression (in order to exclude coregulatory or signalling effects), with the *in vitro* system of thymus organ culture has been used to address the role of CD4 and CD8 in thymic development. The use of recombinant DNA technology and the availability of *in vitro* functional assay systems has also provided a means to dissect out individual T cell surface molecules to further investigate the role of these components in antigen recognition.

CHAPTER TWO

Materials and methods

2.1 Tissue Culture

For basic tissue culture of hybridomas RPMI 1640 medium (specifically developed for lymphocyte culture) containing 2% bicarbonate was used. With the exception of IMDM, all media - RPMI, MEM-H, and methionine-free, cysteine-free medium (used in metabolic labelling) was supplied by ICRF Cell Production Unit, Clare Hall, South Mimms, Hertfordshire, U.K. All foetal calf serum (mycoplasma tested, Gibco, U.K.) added to tissue culture media was incubated at 56°C for 30 minutes to heat-inactivate complement proteins. All cells were maintained at 37°C in an humidified tissue culture incubator with a constant 5% CO_2 atmosphere. Standard tissue culture grade plasticware was supplied by Falcon (U.K.) unless otherwise stated. Media supplements were as stated for individual cell types; glutamine (0.24M) was obtained from ICRF Cell Production Unit; 2-Mercaptoethanol (14.4M, BDH, U.K.) was used in media at a final concentration of $5x10^{-5}$ M, diluted in PBS to give a stock of $5x10^{-2}$ M, filter sterilised and stored at -20°C.

2.1.1 Reagents

General reagents were purchased from Sigma Chemical Company Ltd.(U.K.) unless otherwise specified.

G418 sulphate

G418 sulphate (Geneticin, Gibco BRL, U.K.) is toxic to eukaryotic cells and cells transformed with a plasmid carrying neomycin resistance were selected upon their resistance to G418. G418 sulphate was reconstituted in H_2O and sterilised by 0.2μ filtration and stored at -20°C.

Iscove's Modified Dulbecco's Medium (IMDM)

To 1 pack of IMDM powder with 25mM HEPES and L-glutamine (Gibco, U.K.), 900ml dH_2O were added and stirred for 1 hour at room temperature. 3.025g $NaHCO_3$, 10ml of Pencillin/Streptomycin solution (10 000 IU Penicillin/ml, 10 000 μg/ml Streptomycin, Gibco, U.K.), 1ml of a $5x10^{-2}$ M stock of 2-Mercaptoethanol

(BDH, U.K.), and 0.5 ml human transferrin (10mg/ml stock in PBS, Sigma) were added. The medium was stirred for a further hour, then the volume adjusted to one litre with dH_2O and filter sterilised into 200ml aliquots.

Phosphate Buffered Saline (Dulbecco's 'A')

NaCl	8.0g/l
KCl	0.25g/l
Na_2HPO_4	1.43g/l
KH_2PO_4	0.25g/l

The pH was adjusted to 7.2

Trypan Blue

All viable cell counts were performed by trypan blue exclusion. A stock of 0.2% w/v trypan blue was prepared in PBS / 3mM sodium azide. Four parts of 0.2% trypan blue were mixed with one part 4.5% saline to yield a stock of 0.16% trypan blue/0.9% sodium chloride for counting cells. Cells were mixed with trypan blue at a ratio of 1:1 (v/v) for counting.

Trypsin/Versene for Trypsinising cells

Trypsin : 0.25% in Tris saline pH adjusted to 7.7 with 1M HCl, filter sterilised and stored at -20°C.

	For 1 litre:
Trypsin	2.5g
NaCl	8g
KCl : 19% solution w/v in dH_2O	2ml
Na_2HPO_4	0.1g

dextrose	1g
Trizma base	3g
phenol red (1% w/v in dH_2O)	1.5ml
penicillin / streptomycin	10^5 U/0.1g

Versene : in PBS pH 7.2, autoclaved and stored at 4°C.

	for 1 litre
EDTA	0.2g
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
phenol red (1% w/v in dH_2O)	1.5ml

1 volume of trypsin was added to 4 volumes of versene prior to use.

2.1.2 Cell culture

DC 27.10 hybridoma cells

DC 27.10 cells (Gabert *et al.*, 1987) were maintained in RPMI supplemented with 8% FCS, 2-Mercaptoethanol ($5x10^{-5}$ M), glutamine (4mM), HEPES (10mM), and Gpt. selection (see below). All hybridoma cells were passaged by diluting approximately 1 in 10 into fresh medium every 3 days.

Gpt. selection	stock	working concentration
xanthine	25mg/ml (100x)	250μg/ml
hypoxanthine	3mg/ml (200x)	15μg/ml
mycophenolic acid (MPA)	2mg/ml (1000x)	2μg/ml

The above stocks were made up in H_2O, heated to 65°C and 10M NaOH added to aid dissolution, before sterilisation by 0.2μ filtration.

3T3 cells

Swiss 3T3 cells were supplied by ICRF Cell Production Unit, Clare Hall. Cells were maintained in RPMI supplemented with 5% FCS, and glutamine (4mM), and cultured almost to confluency before being divided and reseeded. The cells required trypsinisation for removal from plastic: the cells were washed twice in serum-free medium (MEM-H), 4ml of trypsin/versene added (to a 75cm^2 flask), incubated for 10 minutes at room temperature, with circulation of the medium. The cells were recovered by gentle tapping of the base of the flask and washed twice in MEM-H plus 5% FCS, by centrifugation at 200 xg, at room temperature. Cells were reseeded at approximately a 1 in 10 dilution with fresh medium.

Cryopreservation of cells

Cells were pelleted in the cold, resuspended in 1ml of ice-cold freezing mix (90% fetal calf serum, 10% DMSO, 0.2μ filtered to sterilise, and stored at -20°C) and aliquotted into ice-cold freezing vials. The cells were frozen at -70°C overnight, and transferred to liquid nitrogen for long term storage. The cells were recovered by thawing the vial rapidly, and the contents of the vial were added to a universal containing warm medium (37°C). The cells were washed immediately and the pellet resuspended in the appropriate culture medium.

TABLE 1 ***Antibodies used***

Name	*Class*	*Reference*	*Source*
145-2C11 anti-CD3ε	Hamster IgG	Leo *et al.* (1987)	kind gift from J. Bluestone
GK 1.5 anti-CD4 PE conjugate	Rat IgG2b	Dialynas *et al.* (1983)	Becton Dickinson
YTA 3.1 anti-CD4	Rat IgG2b	Qin *et al.* (1987)	kind gift from H. Waldmann
YTS 169.4 anti-CD8α	Rat IgG2b	Cobbold *et al.* (1984a)	kind gift from H. Waldmann
YTS 156.7.7 anti-CD8β	Rat IgG2b	Qin *et al.* (1989)	kind gift from H. Waldmann
53-6.7 anti-mouse CD8 FITC conjugate	Rat IgG2a	Ledbetter & Herzenberg (1979)	Becton Dickinson
M1-93.4 HL2 anti-CD45	Rat IgG2a	Springer *et al.* (1978)	ATCC (TIB 122)
YBM 5.10.4 anti-HSA	Rat IgM	Watt *et al.* (1987)	kind gift from H. Waldmann
M1/70.15.1 anti-macrophage marker, Mac 1/CD11b	Rat IgG2b	Springer *et al.* (1979)	Serotec, U.K.
IM7.8.1 anti-Pgp1	Rat IgG2b	Trowbridge *et al.* (1982)	kind gift from I. N. Crispe

7D4 anti-TAC (IL2R p55)	Rat IgM	Malek *et al.* (1983)	ATCC (CRL 1698)
Désiré-1 anti-TCR K^b KBC20 clonotype	Mouse IgG2b	Hua *et al.* (1985)	kind gift from A.-M. Schmitt-Verhulst
YTS 154.7 anti-Thy1 (all alleles)	Rat IgG2b	Cobbold *et al.* (1984b)	kind gift from H. Waldmann
T11D7e2 anti-Thy1.1	Mouse IgM	Lake *et al.* (1979)	ATCC (TIB103)
HO 13.4 anti-Thy1.2	Mouse IgM	Marshak-Rothstein *et al.* (1979)	ATCC (TIB 99)
YTH 89.1 anti-human glycophorin		Cobbold *et al.* (1984a)	kind gift from H. Waldmann

Fluorescein-conjugated anti-mouse immunoglobulin was purchased from Nordic Immunological Laboratories Ltd., U.K.

Second step reagents

For rat antibodies: fluorescein-conjugated affinipure goat anti-rat IgG (H+L) (Jackson Immunoresearch Labs., U.S.A.), was used.

For biotinylated antibodies: phycoprobe phycoerythrin-conjugated avidin (Biogenesis, U.K.) was used. For three colour staining, streptavidin-PE/Texas red "Tandem" reagent (Southern Biotechnology Associates Inc., U.S.A.) was used.

2.2 Antibody Preparation

2.2.1 Ammonium sulphate precipitation of immunoglobulin from culture supernatant

Cells were removed from culture supernatant by centrifugation or filtration. Solid ammonium sulphate powder was added to the supernatant to achieve 45% saturation, and stirred overnight at 4°C. The precipitate was recovered by centrifugation at 10,000 xg for 30 minutes in the cold. The precipitate was resuspended in a minimum volume of dH_2O and dialysed against 200 volumes of 1x PBS for at least 36 hours, changing the buffer at regular intervals. The first two changes were performed at room temperature, then the antibody dialysed overnight at 4°C. The dialysed material was then stored at -20°C. The protein concentration was estimated from spectrophotometric determination by measuring the O.D. at 280nm. The $O.D._{280}$ value was adjusted for the dilution factor and divided by 1.4 to give an estimate of the concentration of immunoglobulin in mg/ml.

2.2.2 Preparation of ascites

Nude mice (nu/nu) were injected intra-peritoneally with pristane, 0.5ml per mouse, and left for 4 days. The hybridoma cells were cultured to a density of 0.5-1.0 x 10^6 cells/ml maximum, and the cells injected intra-peritoneally in PBS (2 x 10^7 cells/mouse). The mice were observed between 7-14 days after injection, and ascites fluid removed from the peritoneal cavity. Ascites fluid was collected following injection with the hybridoma cells T11D7e2 (anti-Thy1.1) and directly coupled to biotin or fluorescein, as described.

2.2.3 Biotinylation and FITC coupling of antibodies

The antibody was diluted to 1mg/ml with bicarbonate buffer (17.3g sodium bicarbonate, 8.6g sodium carbonate, per litre pH ~9.3). The antibody was then dialysed against bicarbonate buffer for 2 hours at room temperature. For biotinylation 15µl biotin were added per mg of protein (Biotin succinamide ester, Pierce Ltd., U.K.;

10mg/ml stock diluted in DMSO). For FITC coupling 20μg FITC were added per mg protein (fluorescein isothiocyanate, Pierce Ltd., U.K.; 1mg/ml FITC isomer stock in DMSO). Each antibody was then rotated for 2 hours at room temperature or for 4 hours at 4°C, then dialysed extensively against PBS in the cold. A final concentration of 1% BSA, 0.05% sodium azide was added to biotinylated antibodies before storage at 20°C. FITC-coupled antibodies were stored without further addition at -20°C.

2.2.4 Coupling antibodies to CNBr-activated sepharose

The antibodies were dialysed in coupling buffer (0.1M $NaHCO_3$ pH 9.3, 0.5M NaCl) for approximately 1 hour, using 5-10mg of protein per ml of gel. The required quantity of CNBr-activated sepharose (1g per 3.5 ml final gel volume) was allowed to swell for 15 minutes in 1mM HCl, using 200ml in total per gram of gel by resuspending the gel in several aliquots of 1mM HCl and aspirating the liquid on a sintered glass filter between additions. The gel was washed with coupling buffer immediately before adding the protein using 5ml per gram of gel. The protein was mixed with the gel using a gel:buffer ratio of 1:2, and rotated at room temperature for 2 hours or overnight at 4°C. The $O.D._{280}$ of the supernatant was measured to determine the amount of antibody bound to the gel. The gel was allowed to settle and the supernatant removed, the blocking buffer (0.2M glycine pH 8.0, 0.5M NaCl) added, and the gel rotated for 2 hours at room temperature or overnight at 4°C in 25ml in a universal. Excess adsorbed protein was removed by washing the gel onto a sintered glass filter with 200ml coupling buffer, followed by 200ml acetate buffer (0.1M sodium acetate, 0.1M acetic acid, plus sodium chloride to 0.5M, pH 4), and 200ml coupling buffer. The gel was finally washed in PBS and resuspended in PBS containing 0.05% azide.

2.2.5 Protein A sepharose (PAS)

0.5g PAS (PAS CL-4B; Pharmacia, U.K.) were placed into a centrifuge tube and made up to 5ml with lysis buffer. The gel was left to swell approximately 10

minutes, aliquotted into microfuge tubes and washed several times with lysis buffer (centrifuged for 1 minute at 2,200 xg) before use.

2.2.6 Coupling of rabbit anti-rat Ig to sepharose

To 75ml of purified rabbit anti-rat Ig, 1.5 ml PAS were added and rotated for a minimum of 1 hour at 4°C. The rabbit anti-rat sepharose was washed 3x in lysis buffer before use and stored at 4°C.

2.3 Immunofluorescent staining of cell suspensions for FACS analysis

2.3.1 Direct immunofluorescent staining using conjugated antibodies

Cells were resuspended in 100µl of a saturating concentration of antibody (100µg/ml), diluted in 0.2µ filtered PBS/0.5% BSA/0.05% sodium azide. Cells plus antibody were kept on ice for 40 minutes then subjected to a large volume wash using filtered PBS and centrifuged at 250 xg at 4°C for 7 minutes. For propidium iodide staining, propidium iodide (0.5µg/ml) was included in the final wash. The supernatant was aspirated and the cells resuspended in a final volume of 200-400µl of PBS/BSA/azide. Dead cells and debris were excluded from the analysis using low-angle light scatter and propidium iodide staining. Analysis was performed on a Becton Dickinson FACScan analyser fitted with a 488nm argon laser, and using FACScan Research software Version A.

2.3.2 Indirect immunofluorescent staining

Cells were incubated with the first layer antibody as for direct staining, then subjected to a large volume wash as described and the cells resuspended in 100µl of the second layer antibody (diluted to the appropriate concentration determined by titration, in PBS/BSA/azide). Cells were incubated for 30 minutes on ice, then washed as before. Cells were finally resuspended in PBS/BSA/azide as for direct staining. Stained samples were then analysed as above.

2.3.3 Internal staining of cells in suspension

The cells were washed twice in cold RPMI and resuspended in 1ml of ice-cold 50% ethanol/RPMI, immediately mixed by repeated vortexing for 1 minute, then placed on ice for 5 minutes before washing three times with ice-cold RPMI, and stained as described for indirect/direct immunofluorescent staining.

2.4 Immunohistochemical analysis

2.4.1 Gelatin coating of slides ("subbing") for cryostat sections

The gelatin (0.01%) was dissolved in water at approximately 65°C. After the solution had cooled chrome alum (0.01%) was added, mixed, and the solution stored at 4°C. Slides to be "subbed" were rinsed in detergent, then thoroughly in water, and dipped into the subbing solution, then allowed to dry several hours. The slides were stored dry in a dust-free container at room temperature.

2.4.2 Immunohistochemical staining of tissue sections

The frozen sections were stored at -20°C until required, then brought to room temperature before staining. The sections were fixed in acetone for 30 minutes at room temperature, then washed by immersion for 10 minutes in Tris-buffered saline (150mM NaCl, 5mM Tris, 4.4mM HCl). The sections were incubated in a 1:20 dilution of normal mouse serum (if using rat anti-mouse antibodies) for 20 minutes, or in Tris-buffered saline alone if using mouse anti-mouse antibodies. The sections were washed with Tris-buffered saline, then the first layer antibody added to the preparation and incubated for 1 hour at room temperature in a humid box. The first layer antibody was rinsed from the slides by immersion in Tris-buffered saline for 15 minutes. The second layer peroxidase conjugate was pre-incubated with normal mouse serum, if using a rat-anti-mouse reagent, and then applied to the sections and incubated for 30 minutes at room temperature as before. Excess antibody was removed by washing in Tris-buffered saline as before, for 15 minutes. The substrate for the peroxidase conjugate, diaminobenzidine (DAB) was prepared by dissolving 6mg DAB tetrahydrochloride in

10ml of 0.05M Tris.Cl buffer pH 7.6. A 3% solution of hydrogen peroxide in dH_2O was freshly prepared and 0.1ml added to 10ml of DAB. If a precipitate appeared, the mixture was filtered through Whatman No. 1 paper, before application to the specimen and incubation for 10 minutes. The reaction was stopped by washing in Tris-buffered saline then in running tap water for 2 or 3 minutes. The sections were counterstained with haematoxylin, the slides dipped into alcohol insoluble Harris haematoxylin for 30 seconds, and washed in running tap water for 2-3 minutes. The slides were then dipped into 0.5% glacial acetic acid/99.5% ethanol for 10 seconds, washed with running water for 3 minutes, then dehydrated through 70% ethanol once, twice through 100% ethanol, and finally dipped into 1.1.1.trichloroethane tetrachloroethylene (CNP, Pentone, U.K.) before permanent mounting in DPX (BDH, U.K.).

2.5 Foetal Thymus Organ Culture

2.5.1 Recovery of embryos and dissection of thymic lobes

CBA/J and AKR mice were time-mated and supplied by ICRF Cell Production Unit, Clare Hall, Hertfordshire, U.K. Embryos were obtained from timed matings by caging young adult males and females during the dark period of a controlled light/dark regime and checking for a vaginal plug at the end of the dark period. The day of detection of a vaginal plug was termed day 0 and individual thymus lobes were dissected from day 14 embryos.

The uterus was removed from the abdomen of a pregnant mouse and transferred to a sterile 90mm petri dish (bacteriological grade). The uterus was then dissected under sterile conditions in a flow cabinet, and the individual conceptuses removed. These were transferred to a dish containing sterile PBS or medium (MEM-H) to wash off as much contaminating maternal blood as possible. After transfer of each conceptus to a fresh dish of medium the embryos were liberated using watchmakers forceps (straight number 4 or 5, Ideal-Tek, Switzerland) to tear open the membranes. Before proceeding, a few minutes were allowed for partial exsanguination via umbilical

vessels. Dissection of thymus lobes from individual embryos was performed in a fresh dish containing sufficient medium to cover them, using a low power dissecting microscope. The embryos were decapitated, held ventral side uppermost using one pair of forceps, whilst the tip of the second pair of forceps was used to produce an incision along the midline of the thorax. The forceps used to hold the body in position were then used to open the thorax to display its contents. The "thoracic tree" (heart, lungs and trachea) was removed by grasping just above the heart and stripping from the thorax in a cranial direction. Isolated thoracic trees were collected into medium containing 5% FCS. Examination of these isolated rudiments revealed the thymus lobes, one located each side of the tracea just above the heart. Individual lobes were then isolated leaving behind as much of the surrounding mesenchyme as possible. Excess mesenchyme was removed by gentle teasing with watchmakers forceps. The isolated lobes were placed into IMDM supplemented with 5% FCS until all lobes had been collected.

2.5.2 Basic organ culture procedure

The foetal thymus lobes were cultured in 60mm sterile petri dishes (60 x 15mm, Nunclon Delta, Inter Med, Denmark) supported by a sterile polycarbonate nucleopore filter (0.8μm pore size, 13mm diameter, Nucleopore, Costar, U.K.), the latter sterilised by autoclaving at 120°C (15 psi) for 20 minutes in a glass petri dish. Four filters were placed into each dish, and a maximum of up to 8 lobes were placed onto each filter. Lobes were transferred from the collecting dish onto the filters using watchmakers forceps. The petri dishes containing the lobes were placed inside a clear plastic bioassay dish (24cm x 24cm, Nunc, Denmark), and cultured at 37°C in an humidified incubator with 5% CO_2 atmosphere.

After the required period of culture, from 1 to 21 days, the lobes were harvested and a cell suspension produced from the lobes by gentle disruption in ice-cold MEM-H plus 5% FCS using a mini-homogeniser (Uniform, 0.1 ml, Jencons, U.K.). The cells released from the lobes were washed in MEM-H and resuspended in a small volume of medium, usually 200-500μl, and viable cell counts performed by trypan blue exclusion.

Cells isolated from lobes cultured for varying time periods were stained using monoclonal antibodies against various cell surface markers and analysed as described previously.

2.6 Recolonisation of alymphoid lobes

Thymus lobes were depleted of their lymphoid elements using 2 deoxyguanosine (dGuo: 9mM stock prepared in PBS, Sigma, U.K.; Jenkinson *et al.*, 1982). Organ cultures were set up essentially as described, except they were cultured for the first 5 days in the presence of dGuo (1.35mM). After this time the lobes were recovered and washed in a large volume of dGuo-free medium for at least 2 hours in a 37°C incubator to remove residual dGuo. Recolonisation was performed by association of donor precursor cells (AKR, Thy1.1) with alymphoid lobes (CBA, Thy1.2) in hanging drop culture. Cell suspensions were prepared from donor tissue - either mouse foetal liver or foetal thymus. Day 12 or 14 gestational foetal livers were collected and gently expressed through sterile monofilament nylon mesh (125μm mesh size, sterilised by soaking in 70% ethanol and allowed to dry in a sterile cabinet), then passed through a 25 gauge needle, washed by centrifugation, and resuspended in medium (MEM-H) before viable cell counts were performed. Day 14 foetal thymocytes were prepared by teasing apart fetal thymus lobes using watchmakers forceps and a fine cataract knife (B 418, John Weiss & Son Ltd., London, U.K.), or a combination of gentle expression through monofilament nylon mesh, and teasing with forceps. The cells were washed, and resuspended for counting as previously described.

The cells used to recolonise the alymphoid lobes were resuspended in IMDM plus 10% FCS to the required final cell concentrations. Into each well of a Terasaki microwell plate (60 x 10μl Nunclon Delta, Inter Med, Denmark) 10-20μl of the donor cell suspension were pipetted. Control wells contained medium alone. Alymphoid thymus lobes were transferred, one lobe per well, to the microwell plate.

The plate was then carefully inverted and placed inside a clear plastic box with a flush fitting lid (Stewart Plastics, U.K.), on a platform consisting of a 96 well plate, and approximately 4 ml of sterile water were added to the box to retain an humidified atmosphere. Lobes were cultured in a hanging drop for 48 hours, then washed in fresh medium to remove any adherent cells, before being replaced on nucleopore filters in 60mm petri dishes as described, and subcultured for a further 6 to 12 days to allow the precursor cells to expand and differentiate within the thymus explant. Recolonised lobes were harvested and a single cell suspension prepared as previously described for non-manipulated lobes. The recovered cells were stained using a panel of monoclonal antibodies to characterise their cell surface phenotype, and analysed on a Becton Dickinson FACScan Analyser as described.

2.7 Foetal liver depletion

2.7.1 Antibody plus complement lysis

The liver cells were counted and diluted anti-HSA antibody (YBM 5.10.4) added to a final concentration of 100µg/ml, 1ml per $5x10^7$ cells and incubated on ice for 45 minutes. The cells were then washed three times in MEM-H and resuspended in a 1:10 dilution of rabbit complement (Low-Tox rabbit complement, Cedarlane Labs., Ontario, Canada) using 1ml per $5x10^7$ cells and incubated at 37°C for 30 minutes. The cells were washed as before and resuspended in MEM-H. Cell viability was established by trypan blue exclusion, dead cells being removed by applying the cells to a ficoll gradient. The cells were resuspended in 5ml of medium (> $2x10^7$ cells/ml) and underlayered with 2ml of ficoll (Lympholyte M, Cedarlane Labs., Ontario, Canada) at room temperature. The ficoll gradient was centrifuged at 500 xg for 20 minutes at room temperature. The live cells banded at the interface and the dead cells and erythrocytes pelleted at the bottom of the tube. The cells at the interface were carefully recovered into a large volume of medium and washed by centrifugation at 400 xg for 10 minutes to pellet the lymphocytes, then washed

twice more at 220 xg. The cells were recounted and resuspended in IMDM plus 10% FCS at the required cell concentration for recolonisation.

2.7.2 Discontinuous BSA Gradient Separation (Raidt, 1980)

BSA stock solutions were prepared using 35% Bovine Serum Albumin (BSA), sterile (sterile buffered albumin, Sigma Ltd., U.K.), stored 4°C. In order to prepare four gradients of the albumin concentrations shown, the following were mixed aseptically:

BSA Concentration	Volume of 35% stock	MEM-H (serum-free)
35%	4.0ml	0ml
29%	4.0ml	0.82ml
26%	3.2ml	1.11ml
23%	3.0ml	1.50ml
10%	1.5ml	3.75ml

The swinging buckets and caps were sterilised by rinsing with 70% ethanol and dried in a sterile hood. The cellulose nitrate centrifuge tubes were sterilised by filling with 70% ethanol for 10 minutes, then washing well with sterile MEM-H.

The dilutions of 35% albumin stocks were prepared using sterile serum-free medium (MEM-H) as the diluent. A suspension of the cells to be separated was prepared and washed twice with MEM-H. To one part packed cells, 9 parts of 35% BSA were added. The cells were gently resuspended and 1ml of this cell suspension placed in a 5ml centrifuge tube and carefully overlayered with an equal volume (1ml) of each of the BSA solutions, starting with the most concentrated (29%) first, and finishing with the 10% albumin solution. The centrifuge tubes were loaded into the buckets and the caps secured, then the tubes centrifuged at 20,000 xg for 30 minutes at 4°C. Each of the discrete bands was harvested with a sterile Pasteur pipette into a tube

containing 20ml of MEM-H. The cells were pelleted by centrifugation at 200 xg for 10 minutes, then washed a further 2-3 times before use.

2.7.3 Enrichment using magnetic bead separation

The MACS (magnetic cell separation system, Miltenyi Biotec, Germany) was used to further enrich for $HSA^{-/+}$ cells, essentially using the sterile protocol recommended by the manufacturers. The appropriate column size was chosen for the number of cells loaded (B1), and the autoclaved column placed in the separator in a sterile hood. The column was filled with 70% ethanol, then the ethanol replaced with PBS/1% BSA. A 22G (0.7 x 30) needle was attached to the column to achieve a flow rate of approximately 1.5ml/minute. The foetal liver cells were separated by BSA density gradient fractionation, and fractions A and B then stained in a final volume of 200µl for 10 minutes on ice with a biotinylated anti-HSA monoclonal antibody (YBM 5.10.4, diluted in PBS to 50µg/ml, and sterilised by 0.2µ filtration). The cells were subjected to a large volume wash with sterile PBS (120 xg, 4°C, 5 minutes), then resuspended in sterile FITC-conjugated streptavidin (Biogenesis, U.K.). The cells were incubated for a further 10 minutes then washed again before incubation with the superparamagnetic MACS microbeads (5µl per 10^8 cells, diluted 1:50 in PBS, sterilised by 0.2µ filtration, and added to a final concentration of 1:100). A small aliquot was removed and reserved on ice prior to addition of the beads (as the non-separated control). The cells plus beads were incubated on ice for 5 minutes, then 800µl cold PBS/1% BSA added. The cells labelled with the beads were applied to the column (pre-cooled by washing with 2-3 volumes ice-cold PBS/1% BSA), and were allowed to pass through the column under gravity. The column was washed with 3-5 column volumes of cold PBS/1% BSA, and the eluent collected as the "non-magnetic" fraction. This fraction was enriched for HSA^- cells. The needle attached to the column was replaced with one of a larger diameter, 20G (0.9 x 40), to achieve a flow rate of approximately 6ml/minute. The column was washed with a further 5 column volumes of PBS/BSA, to remove any weakly bound cells, non-specifically retained, and the

washes collected. The column was removed from the separator and the needle removed. The bound cells (the HSA^+ enriched fraction) were washed out of the column and collected as the "magnetic" fraction, using a further 3-5 column volumes of cold PBS/BSA. The cells in each fraction were collected by centrifugation at 200 xg and washed before viable cell counts were performed. An aliquot of each of the 4 fractions was then analysed by flow cytometry to assess the degree of enrichment, in comparison to the unseparated sample.

The enriched fractions were then used as a recolonising population to seed alymphoid thymus lobes. The lobes were harvested after 10 days in culture and analysed as previously described.

2.7.4 Enrichment by sterile flow cytometric sorting

Flow cytometric sorting was used in order to obtain pure HSA^- and HSA^+ liver cell populations. Foetal liver cells were separated by density gradient fractionation and fractions A and B labelled with an anti-HSA antibody as previously described. The cells were finally resuspended in PBS/5% FCS and kept on ice whilst sorted. Sterile cell sorting was kindly performed by ICRF staff at the ICRF department in Lincoln's Inn Fields, using a Becton Dickinson FACStar Plus machine. Live gates were set on forward and side scatter, and on the negative and positive populations as shown in Chapter 4). The HSA^- cells comprised approximately 10% of each population. The cells recovered were washed and counted, and the HSA^- population of both fractions resuspended in IMDM plus 10% FCS at a final concentration of 10^6 cells per ml (seeded at 10^4 cells per lobe), and the HSA^+ population, at a final concentration of 10^7 cells per ml (seeded at 10^5 cells per lobe), before being cultured with alymphoid lobe in the recolonisation assay, as described. Cells were harvested from the lobes after 10 days in culture, and analysed as previously described.

2.8 Antisense oligonucleotides

2.8.1 Oligonucleotide synthesis

Oligonucleotides were synthesised by the ICRF Oligonucleotide Synthesis service at Clare Hall, by standard phosphoramidate chemistry on the Applied Biosystems DNA Synthesiser Model 380, using modified phosphorothioate linkages in the internucleosidic phosphonate bond. The original oligonucleotide preparations of completely modified analogues were synthesised using hydrogen phosphonate chemistry, in which the phosphorothioate modification involved sulphurisation using carbon disulphide, and was initially performed at the end of the synthesis, to convert the trivalent sulphur to a pentavalent form. Subsequent oligonucleotides syntheses of analogues containing a single linkage at each end of the molecule were performed using phosphoramidate chemistry to improve the efficiency of synthesis. These oligonucleotides included the sulphurisation reagent on the column used for synthesis.

Antisense (AS) oligonucleotides were synthesised complementary to the leader sequence and extending 23 nucleotides downstream fron the initiation of translation start codon of the genes encoding murine CD4, CD8α and CD8β. Control oligonucleotides comprising a random sequence of the same proportion of bases as the equivalent antisense sequence were also synthesised. For the CD8 control, a single control oligonucleotide was synthesised, comprising an average of the base composition of both the CD8α and CD8β antisense oligonucleotides.

Four types of oligonucleotide were synthesised, comprising normal bases, or oligonucleotides containing phosphorothioate linkages throughout the molecule (a), a single modified linkage at the 5' and 3' termini (b), or a single linkage at the 3' end of the molecule (c). Only the product oligomer contains a 5' terminal protecting group, the dimethoxytrityl moiety, since all other species in the crude reaction mixture will have been acetylated at the 5' position during capping. This subsequently enabled the oligonucleotides to be purified by trityl-on chromatography. Reverse phase high-performance liquid chromatography (HPLC) was used to separate the tritylated from

non-tritylated species, the former, being more hydrophobic, was retained longer on the column. HPLC was performed using a linear gradient of increasing acetonitrile concentration, 20-50% over 30 minutes in 0.1M triethylammonium acetate. The elution of the the dimethoxytritylated oligonucleotide generally occurs at an acetonitrile concentration of 35-40%. The dimethoxytrityl fraction was collected, detritylated, and supplied fully deprotected as an ethanol precipitate. The concentration of the oligonucleotide supplied was determined by measuring the absorbance at 260nm.

The ethanol precipitate was recovered by microcentrifugation (10,000 xg) at 4°C for 15 minutes, washed twice with 70% ethanol, and allowed to dry in a sterile cabinet. Initially the DNA pellet was resuspended in TE buffer, however, subsequent batches were resuspended in dH_2O, to give a 1mM stock. Later batches were resuspended in IMDM to give a 5mM stock, and all stocks were stored at -20°C.

2.8.2 Sequences of oligonucleotides used (5'-3'):

		Mol. wt.
CD4 AS	CTAAGAGAGATGGCTCGGCACAT	7574
CD4 C	CAGTGCTAAGGACATGAGACCTG	7574
CD8α AS	AAGCGGGTCAACGGTGAGGCCAT	7615
CD8β AS	ACCAGCCAGAGCCATGGCTGCAT	7495
CD8 C	GCTGGTACCGAAGGCAATCCGAC	7535

2.9 Stability and uptake of the oligonucleotides

2.9.1 Radiolabelling of oligonucleotides

Oligonucleotides were 5' end labelled using T4 polynucleotide kinase and γ-^{32}P[ATP] (ICN Radiochemicals, specific activity 7000Ci/mmol) (Wickstrom *et al.*,

1988). One microgram of oligonucleotide was incubated with 250μCi γ-^{32}P[ATP], in 1x kinase buffer and with 5 units of T4 polynucleotide kinase, at 37°C for one hour. The reaction was heated at 68°C for 10 minutes to denature the enzyme. To determine the amount of incorporated radioactivity, 1μl was removed, diluted 1 in 10, and 1μl spotted onto each of two DE 81 (2.4cm diameter) Whatman filter paper discs. One of the filters was untreated, whilst the other filter was washed five times with 0.5M Na_2HPO_4, each wash for 5 minutes, then two one minute washes with dH_2O, and finally washed twice for one minute with 95% ethanol, to remove unincorporated radioactivity. The filters were then dried for 2 minutes in an 80°C oven and placed into a scintillation vial with scintillation fluid (Aquasol, NEN Research products, U.S.A.) and the associated radioactivity determined in a beta counter. If the percentage incorporation of γ-^{32}P was greater than 50%, the reaction was stopped. The labelled oligonucleotide was purified and unincorporated radioactivity removed using a Biospin 6 column (size exclusion ~5bp, Biorad, U.K.) following the manufacturers instructions. After purification, 1μl of the labelled oligonucleotide was removed and the incorporated radioactivity determined by counting in a beta-counter.

2.9.2 Determination of uptake and stability of oligonucleotides

A suspension of young adult thymocytes in IMDM plus 10% heat-inactivated FCS was dispensed into 1ml aliquots (2 x 10^6 cells/ml) into each well of a 24 well flat-bottomed plate. 5μl of labelled oligonucleotide were added to each aliquot of 2 x 10^6 thymocytes (equivalent to 10^6 cpm for the oligonucleotides (a) and (b); and 10^7 cpm for (c)) and the cells plus oligonucleotide incubated for various periods of time at 37°C in 5% CO_2, from 4 to 48, or 52 hours. After the indicated periods of incubation, the cells were harvested and pelleted by microcentrifugation (2,200 xg) for 2 minutes in a benchtop microfuge. The supernatant was removed and transferred to a fresh screw-capped 1.5ml tube and placed on ice. The cell pellet was washed twice with 0.5ml Tris-buffered saline (150mM NaCl, 10mM Tris.HCl, pH 7.4) and the washes saved and stored in a 1.5ml microfuge tube on ice. The cell pellet was then subjected to an acid

wash (100µl of 20mM HCl, 150mM NaCl, pH 1.7; Watts *et al.*, 1987) for 2 minutes on ice, to remove any oligonucleotide bound to the cell membrane. 400µl of Tris-buffered saline were added and the cells centrifuged for 3 minutes at 2,200 xg. The supernatant was transferred to a fresh 1.5ml tube and stored on ice. The cell pellet was then lysed using Tris-buffered saline containing 1% SDS, and extracted once using phenol/chloroform (1:1, v:v). The organic phase was then re-extracted using an equal volume of Tris-buffered saline and the aqueous phases pooled.

Associated radioactivity in aliquots of the supernatant, washes, acid wash and cell lysate was determined by liquid scintillation counting in a beta counter. Aliquots of equivalent volumes of supernatant and cell lysate were dried in a rotary vacuum desiccator and resuspended in 20µl of loading buffer (80% deionised formamide, 0.01% bromophenol blue, 0.01% xylene cyanole FF, 1x TBE). The samples were then boiled for 5 minutes and cooled on ice, before being loaded and electrophoresed on a 20% denaturing polyacrylamide gel (pre-run for 15 minutes). An aliquot of a 5' end-labelled 22bp length oligonucleotide was included as a size marker, and the gel run at 200V, with 1x TBE running buffer. Following electrophoresis, the positions of the dye fronts on the gel were measured and the gel was soaked in 10% acetic acid, 3% glycerol for 30 minutes to remove the urea. The gel was washed for a further 30 minutes in dH_2O containing 3% glycerol, then dried using a Biorad gel drier, model 583, for 2 hours at 80°C, then autoradiographed against pre-flashed film (Fuji, X-100, Japan) with intensifying screens at -70°C. The distance the dye fronts had migrated gave an estimate of size; the position reached by the bromophenol blue dye front corresponding to approximately 8bp, and that reached by the xylene cyanole FF dye front, corresponding to approximately 28 bp (Becker *et al.*, 1989).

2.9.3 Denaturing polyacrylamide gel electrophoresis (PAGE)

PAGE gels (20%) were used to analyse aliquots of the labelled oligonucleotides. Gels were prepared to a final concentration of 20% acrylamide (Sequagel, U.K.), 1x TBE, and 7M urea. The acrylamide, TBE and urea in a final

volume of 10 ml were mixed by stirring under vacuum to dissolve the urea, then 60µl 10% ammonium persulphate and 3µl of TEMED added, before pouring.

2.10 Determination of oligonucleotide effects on the specific inhibition of protein synthesis in thymocytes

2.10.1 Metabolic labelling

Young adult thymocytes were dispensed at 2 x 10^7 cells per well into a 24 well flat-bottomed plate and incubated at 37°C for 2 hours in IMDM plus 10% heat-inactivated FCS with oligonucleotide, or medium alone, and preincubated for 2 hours before culture in methionine-free, cysteine-free medium. Each cell sample was washed twice in serum-free medium (MEM-H), then resuspended at a concentration of 2 x 10^7 cells/ml in methionine-free and cysteine-free medium containing 3% (v/v) dialysed FCS (dialysed against PBS with 2 to 3 changes at room temperature followed by dialysis overnight at 4°C to remove amino acids), 1% glutamine, 5 x 10^{-5} M 2-Mercaptoethanol, and 1% HEPES. The oligonucleotide was replaced and the cells incubated for 2 hours. To each sample 0.5 mCi of ^{35}S labelled-methionine/cysteine were added (Tran ^{35}S-label, specific activity 1000Ci/mmole, ICN Radiochemicals, U.K.), and the cells incubated for a further 2 hours at 37°C. The cells were harvested and washed twice with PBS. The cell pellet was resuspended in lysis buffer at 2 x 10^7 cells/ml buffer (1% NP-40, 10mM Tris pH 7.5, 1mM EDTA pH 8.0, 0.15M NaCl, 10mg/ml BSA) containing protease inhibitors (1µM leupeptine, 200 µM PMSF, and 5mM iodoacetamide) and placed on ice for 15 minutes. The lysate was centrifuged (10,000 xg) for 5 minutes at 4°C and the supernatant transferred to a fresh tube. TCA precipitation was performed using 10µl of supernatant to give an estimate of total protein. An equal volume of cold 25% TCA was added to the labelled supernatant and placed on ice for 45 minutes. The precipitate was then vacuum filtered onto a Millipore filter (0.2µ) and washed twice with cold 12.5% TCA. The filter was air dried in a fume cupboard, then placed inside a scintillation vial with scintillation fluid and the associated

radioactivity determined in a β counter. The remainder of the lysate was either stored at -70°C until required or precleared immediately.

The lysate was precleared to remove labelled irrelevant proteins. 50μl of rat Ig sepharose, 25μl normal rabbit serum, 50μl protein A sepharose, and 50μl mouse Ig sepharose were added and the lysate rotated for at least 2 hours at 4°C. The lysate was centrifuged (2,200 xg) for 3 minutes and the supernatant transferred to a fresh tube. 100μl PAS and 100μl rabbit anti-rat sepharose were added to the supernatant and the tube rotated for at least 2 hours at 4°C. The contents were centrifuged again as above and the supernatant transferred to a fresh tube. The two preclearing steps were repeated twice more. The supernatants were stored at -70°C or the precipitation continued.

2.10.2 Specific immunoprecipitation

The thawed supernatants were passed through a 0.2μm filter and each sample divided into four fractions. The specific antibodies anti-CD8α (YTS 169.4), anti-CD8β (YTS 156), or anti-CD4 (YTA 3.1), all coupled to sepharose were then added to the appropriate fraction, antibodies were washed three times with lysis buffer immediately before use and 40μl added to the labelled supernatant. The antibody plus supernatant were rotated for an hour at 4°C, then 50μl of PAS were added and rotation continued for a further hour at 4°C. A non-specific antibody (YTS 89.1, recognising human glycophorin) was used to control for non-specific precipitation, using the same procedure as for the specific antibody in this step. After the appropriate incubations the tubes were centrifuged and the pellets retained and washed as follows.

Each pellet was washed sequentially in 1ml high salt lysis buffer (lysis buffer containing 0.5M NaCl and 200μM PMSF, 5μM leupeptin, 5mM iodoacetamide); 1ml high salt lysis buffer underlayered with 100-200μl of 30% sucrose (w/v in high salt lysis buffer); 1ml low salt lysis buffer/0.1% SDS containing PMSF; then 1ml of TET buffer (10mM Tris pH 7.6, 5mM EDTA, 0.1% Triton X-100). The pellet was collected by microcentrifugation (2200 xg) and the first supernatant retained. The supernatants from the subsequent washes were discarded. The samples were divided into two at this

last washing stage. Both washed pellets were stored at -70°C if required, or half of each sample resuspended in 20-25µl of sample buffer (2% SDS, 10% glycerol, 0.0625M Tris pH 6.8, 5% 2ME, 0.01% bromophenol blue).

Protease inhibitors

	final conc.	stock 1000x	solvent	storage
phenylmethylsulphonyl -fluoride (PMSF)	200µM	0.2M	isopropanol	RT
iodoacetamide	5mM	0.5M	DMSO	-20°C
leupeptin	5µM	5mM	dH_2O	-20°C

2.11 SDS/Polyacrylamide gel electrophoresis

(SDS/PAGE: Laemmli, 1970)

Metabolically labelled, and immunoprecipitated material was analysed using SDS/PAGE. PAGE gels were prepared as follows.

2.11.1 SDS/polyacrylamide gel preparation

A gel of the desired percentage was prepared using the components described below:

Lower gel	7.5%	10%	12.5%	15%
Lower gel buffer [a]	7.5ml	7.5ml	7.5ml	7.5ml
30% acrylamide	7.5ml	10ml	12.5ml	15ml
dH_2O	15ml	12.5ml	10ml	7.5ml

[a] Lower gel buffer: 1.5M Tris base, 0.4% SDS, pH adjusted to 8.8 with HCl

The gel buffer and acrylamide and dH_2O were mixed and degassed for 10-15 minutes, before adding 100µl of 10% ammonium persulphate (APS) and 10µl TEMED.

The gel was poured, then overlayered with 2-butanol saturated with dH_2O and allowed to polymerise for 45 minutes. The butanol was washed off with dH_2O, and drained well before pouring the stacking gel.

Stacking gel	4.5%
Upper gel buffer [b]	2.5ml
30% acrylamide	1.5ml
dH_2O	6ml

[b] Upper gel buffer: 0.5M Tris base, 0.4% SDS, pH adjusted to 6.8 with HCl

[c] SDS/PAGE running buffer (5X): 144g glycine, 5g SDS, 30g Tris base, per litre (pH 8.3)

The acylamide and gel buffer were mixed gently, before the addition of 60µl of 10% APS, and 10µl of TEMED. The gel was then poured onto the lower gel and the comb placed in position. The stacking gel was allowed to polymerise for 20-30 minutes, before the comb was removed and the wells rinsed with 1x running buffer[c] to remove any non-polymerised acrylamide.

The samples were boiled for 2-3 minutes, briefly centrifuged, and cooled on ice before loading on an SDS/PAGE gel. ^{14}C-labelled "Rainbow" protein molecular weight markers (Amersham International plc., U. K.) were used as size markers, diluted with sample buffer, and boiled 2-3 minutes before loading on a 12.5% acrylamide gel. Electrophoresis was performed in 1x SDS/PAGE running buffer at 25mA through the stacking gel, and subsequently at 20mA until completion.

Following electrophoresis, the gel was fixed in 30% methanol/10% acetic acid for 30 minutes. The gel was washed for 30 minutes in dH_2O containing 3% glycerol, before "enhancement" with 1M sodium salicylate/3% glycerol, for a further 30 minutes. The gel was dried using a Biorad gel drier, model 583, for 2 hours at 80°C, then autoradiographed against pre-flashed film (Fuji, X-100, Japan) with intensifying

screens at -70°C. Scanning densitometry of autoradiographs was performed using a Chromoscan 3 (Joyce Loebel, Germany).

2.12 Analysis of uptake of oligonucleotides into thymus lobes

Oligonucleotides were synthesised as described, with the attachment of a biotin molecule at the 5' end, via an amino linkage, in order to verify oligo uptake by day 14 foetal thymus lobes in organ culture. Day 14 thymus lobes were cultured in a hanging drop in an inverted microwell plate, in a volume of 10μl of IMDM plus 10% FCS, and biotinylated oligonucleotide added to a final concentration of 50μM. The lobes were incubated with oligonucleotide for 24 hours then snap frozen in Tissue-Tek O.C.T. compound (Miles Scientific, U.S.A.) in liquid nitrogen, cryostat-sectioned (10μm thickness), air dried, and stored at -20°C until required. Immunohistochemical staining was performed as described previously (section 2.4) using a streptavidin-peroxidase conjugate (Dako Ltd., U.K.) to visualise the biotin. The sections were observed under phase conditions using a Leitz microscope and photographed.

2.13 Analysis of the inhibitory effects of antisense oligo-nucleotides in foetal thymus organ culture

Foetal thymus lobes from day 14 gestation embryos were cultured using the thymus organ culture technique on polycarbonate filters as described earlier, or using a modification of the technique, placing a single lobe in 10μl of medium into each well of a microwell plate. The oligonucleotides were added to the medium to a final concentration as shown, or with the same volume of the buffer used to resuspend the oligonucleotides in medium alone, then incubated in hanging drop culture as described previously, for a period of between one to four days. Oligonucleotide plus nutrient supplement was added daily to each well, and following culture the lobes were harvested and gently disrupted in a mini-homogeniser as previously described, to produce a single cell suspension. After washing, the cells were resuspended in a final volume of 500μl MEM-H plus 5% FCS and viable cell counts were established by

trypan blue exclusion. Cells were then stained for immunofluorescent analysis as previously described.

Nutrient supplement added to lobes in hanging drop culture

essential amino acids 50x	15ml
non-essential amino acids 100x	7.5ml
glutamine 20mM stock	7.5 ml
10% dextrose (D+ Glucose)	15ml
2M NaOH	2.5ml

These were made up to 100ml with IMDM, filter sterilised and stored at -20°C. 1.5ml heat-inactivated FCS and 0.6ml 7.5% sodium bicarbonate in dH_2O were added to 3ml of the supplement, then filter sterilised before use.

2.14 Molecular Biology

2.14.1 Reagents

All buffer stocks used in molecular biology were made up in autoclaved, double-distilled water and using DNA grade reagents. All RNA buffer stocks were made using diethylpyrocarbonate-treated double-distilled, autoclaved water, and RNA grade reagents. Equipment was autoclaved where possible to destroy deoxyribonuclease activity, and autoclaved glassware or disposable plasticware was used for all solutions. All enzymes used were obtained from Pharmacia (U.K.).

Agarose Gel Dye 10x:

10% Ficoll (w/v)

25% Glycerol (w/v)

Tris EDTA buffer 10mM:1mM (T:E)

0.25% bromophenol blue

0.25% xylene cyanole

Agarose gel DNA markers:

DNA gel markers λ DNA-Hind III fragments (for estimation of DNA concentration) or 1kb DNA ladder (Gibco BRL, U.K.) were used, 1μg of marker was loaded onto the gel.

Agarose Gel Electrophoresis buffers

Tris Acetate (TAE) 50x

	per litre
Tris Base	242g
glacial acetic acid	57.1ml
0.5M EDTA (pH 8.0)	100ml

Used at 1x concentration

Agarose gels were usually prepared at 1% with 1x TAE buffer using electrophoresis grade Agarose NA (Pharmacia, U.K.). The DNA was visualised by staining with ethidium bromide (10mg/ml stock in dH_2O), at a final concentration of 0.5μg/ml, for 15 minutes, then destained in dH_2O for a further 15 minutes.

Antibiotics

Ampicillin: A 50mg/ml (1000x) stock of the sodium salt was prepared in dH_2O, filter sterilised and stored at - 20°C. Ampicillin was added to liquid media or plates to a final concentration of 50μg/ml. Plates were stored at 4°C for only 1-2 weeks before use.

Tetracycline: A 12.5mg/ml (1000x) stock of tetracycline hydrochloride was prepared in ethanol / dH_2O (50% v/v), sterilised by filtration and stored at -20°C in the dark. Tetracycline was added to autoclaved media cooled to approximately 40°C, to a final concentration of 12.5μg/ml. The plates were stored at 4°C in the dark.

Deionised Formamide

200ml formamide were added to 10g monobed resin "Amberlite" MB1, and stirred for 30 minutes in a fume cupboard at room temperature. The formamide was

filtered through Whatman No. 1 filter paper to remove the resin, and stored in the dark at 4°C.

Denhardt's Buffer 100x

2% ficoll (w/v)

2% polyvinylpyrrolidone (w/v)

2% BSA (w/v)

filter sterilised, and stored at -20°C.

Ethanol wash solution (Geneclean, BIO 101, Inc., U.S.A.)

50% Ethanol

0.1M sodium chloride

10mM Tris pH 7.5

Buffers for Hexamer Radiolabelling

TM	DTM	OL
250mM Tris.HCl pH 8.0	100µM dATP	1mM Tris.HCl pH 7.5
22mM magnesium chloride	100µM dGTP	1mM EDTA
50mM 2-Mercaptoethanol	100µM dTTP	90 O.D.units/ml hexamers
	dNTPs made up inTM	

LS (stored in 12µl aliquots at -20°C)

1M HEPES pH 6.6	25 parts
DTM	25 parts
OL	7 parts

IPTG (isopropyl-β-D-thio-galactopyranoside)

A 100mM stock (23.8mg/ml) was prepared just prior to use and kept on ice. Aliquots were stored at -20°C.

T4 polynucleotide kinase buffer (10x)

500mM Tris.HCl pH 7.6

100mM $MgCl_2$

50mM DTT

1mM spermidine

1mM EDTA pH 8.0

The above were mixed, filter sterilised and stored at -20°C. ATP was added to 10mM immediately before use (ATP was omitted when labelling with [γ-^{32}P] ATP).

Ligation buffer (10x)

0.5M Tris.HCl (pH 7.5)

50mM $MgCl_2$

50mM dithiothreitol

ATP was added fresh each time to final concentration of 1mM.

LB (Luria broth)

10g Tryptone

10g NaCl

5g Yeast Extract

For LB plates, agar was added at 15g/l, or 8g/l for top agar. Antibiotics were added when the media had cooled to approximately 40°C, before pouring the plates.

PEG/sodium chloride

20% polyethylene glycol 6000

2.5M sodium chloride

Phenol

To a 100g bottle of redistilled phenol (Gibco, BRL, U.K.) 50ml of 1M Tris (pH 7.6) were added and the phenol placed at 37°C until dissolved (1-3 hours). 5ml m-cresol, 0.2ml 2 ME, and 10mg 8-hydroxyquinoline, were added, shaken, and the

phenol left overnight at room temperature. The phenol was stored at room temperature and used within 1-2 months .

Restriction Enzyme Buffers (10x)

	NaCl	Tris.HCl pH 7.5	$MgCl_2$	dithiothreitol	BSA
Low	0	100mM	100mM	10mM	1mg/ml
Medium	0.5M	100mM	100mM	10mM	1mg/ml
High	1M	500mM	100mM	10mM	1mg/ml

stored at -20°C.

RNA lysis buffer

10mM Tris pH 8.0

0.14M sodium chloride

3mM magnesium chloride

RNase A

Stock : 10mg/ml RNase A in TE buffer (pH 7.4). Boiled for 15 minutes (to remove any DNase activity), allowed to cool, and stored at -20°C.

RNA Gel Running Buffer (10x MOPS)

0.4M MOPS (3-morpholineopropanesulphonic acid sodium salt) pH 7.0

0.1M sodium acetate

0.01M EDTA pH 8.0

pH adjusted to 7.0, then autoclaved.

RNA Gel

(1.2% Agarose / formaldehyde gel)

2.4g Agarose

20 ml 10 x MOPS

144 ml dH_2O

Microwaved to dissolve, allowed to cool, then (in fume hood) 36ml formaldehyde and 5µl of ethidium bromide (5mg/ml) added before pouring.

RNA loading buffer

30% Ficoll

1mM EDTA pH 8.0

0.25% bromophenol blue

0.25% xylene cyanole FF

stock autoclaved and kept for RNA work only.

RNA sample buffer

40µl 10 x MOPS

70µl 40% (w/v) formaldehyde

200µl deionised formamide

10% SDS (sodium dodecyl sulphate)

100g of electrophoresis grade SDS were dissolved in 900ml of H_2O, and heated to 68°C to assist dissolution. The pH was adjusted to 7.2 with HCl, and the volume was finally adjusted to 1 litre.

Sodium iodide solution (saturated with Na_2SO_3)

90.8g NaI

1.5g Na_2SO_3

in 100ml dH_2O

The above were mixed and filtered through Whatman No. 1 paper. 0.5g Na_2SO_3 in a dialysis bag was added to the NaI solution to saturate with Na_2SO_3. NaI was stored in the dark at 4°C.

SSC buffer 20x

3M sodium chloride

0.3M sodium citrate.$2H_2O$

The pH was adjusted to 7.0 with 1M HCl, and made up to 1 litre with dH_2O, then autoclaved.

STE Buffer

10mM Tris.HCl pH 7.5

0.1M sodium chloride

1mM EDTA

Transformation buffer solution 1

30mM potassium acetate

50mM manganese chloride

100mM potassium chloride

10mM calcium chloride

15% glycerol w/v

The pH was adjusted to 5.8 with 0.2M acetic acid, the buffer filter sterilised and stored at 4°C.

Transformation buffer solution 2

10mM MOPS pH 7.0

(a 100mM stock MOPS was used with the pH adjusted to 7.0 with KOH)

75mM calcium chloride

10mM potassium chloride

15% glycerol w/v

Filter sterilised and stored at 4°C

Tris-Borate Buffer (TBE)

5x stock	per litre:
Tris base	54g
boric acid	27.5g
0.5M EDTA (pH 8.0)	20ml
Working concentration:	0.089M Tris-borate

0.089M boric acid

2mM EDTA

Tris/EDTA Buffer (TE)

10mM Tris pH 7.4

1.0mM EDTA pH 8.0

For TE pH 8.0, 1M Tris pH 8.0 was used.

X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside)

A 2% stock was prepared in dimethylformamide. Aliquots were stored at -20°C.

2.15 Bacterial cell maintenance

Bacterial cells were maintained in LB medium with the addition of the appropriate antibiotic selection for maintenance of plasmid DNA. Cells were grown at 37°C with vigorous shaking.

2.15.1 Cryopreservation of bacteria

An 0.85ml aliquot of an exponential phase culture of bacteria was placed in a sterile freezing vial, 0.15ml of sterile glycerol were added and mixed well. The cells were frozen and stored at -70°C. The cells were recovered by streaking a loopful of the stock onto an appropriate media plate and isolating individual colonies.

2.15.2 Preparation of competent cells

A 40ml culture of bacterial cells was grown to exponential phase at 37°C with shaking (*E. coli* strain XL1-Blue, Stratagene, U.K., to $O.D._{600} \sim 0.3$). The cells were pelleted by centrifugation at 700 xg for 10 minutes. The supernatant was discarded and the bacterial pellet resuspended, adjusting to half the original culture volume i.e. 20ml, with Solution 1. The cells were placed on ice for 30 minutes, before being pelleted by centrifugation. The supernatant was discarded, the pellet resuspended and the volume adjusted to 4ml with Solution 2. The cells were placed on ice for at least 60 minutes,

the competence increasing with longer incubation times upto 2-3 hours. The cells were aliquotted on ice and then stored at -70°C until use.

2.15.3 Transformation of competent cells

Frozen stocks of competent cells were thawed on ice and used immediately. Eppendorf tubes containing the DNA in a volume not exceeding 20µl (1-25ng) were pre-chilled on ice and 200µl of the thawed competent cells added, swirling gently to mix. The cells plus DNA were incubated on ice for 40 minutes, then heat shocked by placing the tubes at 42°C for 90 seconds. The tubes were chilled by replacing on ice for 10 minutes. 800µl of LB medium were added to each tube, and the cells incubated at 37°C for 60 minutes with shaking to allow expression of antibiotic resistance. Aliquots of the transformation mixture were plated out onto pre-warmed LB agar plates containing the appropriate antibiotic selection. The plates were allowed to dry, then inverted and incubated at 37°C overnight. Individual colonies were grown in selective media for small scale plasmid preparation.

2.16 Preparation of plasmid DNA

2.16.1 Plasmid miniscreen

An overnight culture of bacteria was prepared in 5 ml of LB medium plus the appropriate antibiotic. The cells were poured into a microfuge tube (1.5ml) and the rest of the bacteria saved at 4°C. The bacteria were pelleted by microcentrifugation for 2 minutes (10,000 xg) the supernatant discarded, and the pellet resuspended in 100µl of buffer 1 (62.5mM glucose, 31mM Tris pH 8.0, 12.5mM EDTA, 2.5mg/ml lysozyme), and mixed by vortexing or pipetting. After incubation on ice for 10 minutes, 200µl of NaOH/SDS (0.2M sodium hydroxide, 1% SDS) were added, the contents mixed by inversion, and placed on ice for 5 minutes. 150µl of 3M sodium acetate (pH 4.8) were added, the mixture vortexed and placed on ice for a further 5 minutes. The lysate was microcentrifuged for 5 minutes (10,000 xg) at room temperature and 400µl of the supernatant collected into a fresh tube - avoiding the bacterial pellet. The lysate was

extracted with an equal volume of phenol and the aqueous phase recovered into a fresh tube, and extracted with an equal volume of chloroform and the aqueous phase retained. The DNA was precipitated by adding 1 ml of cold 100% ethanol, mixing, and leaving at room temperature for 5 minutes. The DNA was recovered by microcentrifugation for 10 minutes in the cold. The supernatant was removed and the pellet was washed with 70% ethanol at room temperature. The DNA pellet was dried for 5 minutes in a rotary vacuum desiccator, and finally resuspended in 100µl of TE buffer.

2.16.2 Large scale plasmid preparation

A 250ml overnight culture of bacteria was grown in selective medium, then pelleted by centrifugation at 4,000 xg at 4°C for 15 minutes. The culture pellet was resuspended in 6ml of freshly prepared 25mM Tris.HCl pH 7.5, 10mM EDTA pH 8.0, 15% sucrose, 2mg/ml lysozyme, and incubated on ice for 20 minutes. 12ml of 0.2M NaOH/1% SDS were added, the contents mixed carefully by inversion, and placed on ice for 10 minutes. 7.5ml of 3M sodium acetate (pH 4.6) were added, mixed by inversion and placed on ice for 20 minutes. The lysate was centrifuged at 27,000 xg at 4°C for 15 minutes and the supernatant transferred to a fresh tube, avoiding the precipitate. 50µl of RNase A (1mg/ml) were added and the supernatant incubated at 37°C for 20 minutes. The digest was extracted twice with an equal volume of phenol : chloroform (1:1, v/v) by centrifugation at 1,500 xg, the aqueous phase recovered, and 2 volumes of absolute ethanol added. The tube was then placed at -70°C for 30 minutes to precipitate the DNA. The DNA was pelleted by centrifugation at 2,000 xg at 4°C for 30 minutes, the supernatant discarded, and the pellet resuspended in 1.6ml of dH_2O. The DNA was transferred to a 15ml centrifuge tube and 0.32ml of 5M NaCl were added, followed by 2ml of 13% PEG, the contents mixed and placed on ice for 60 minutes. The DNA was pelleted at 13,000 xg at 4°C for 10 minutes and the supernatant discarded. The remains of the PEG were removed using a drawn out Pasteur pipette, and the pellet washed with 70% ethanol. The DNA pellet was dissolved in 400µl of

dH_2O or TE buffer, and extracted with phenol : chloroform (1:1, v/v), at least 5 times until no white protein precipitate was visible. The DNA was then reprecipitated with ethanol (plus 250mM NaCl) and the DNA finally resuspended in 200µl TE buffer (pH 7.4).

2.17 DNA manipulation

2.17.1 Spectrophotometric determination of DNA and RNA concentration

An estimate of the concentration and purity of DNA preparations was determined by measuring the O.D. value of a known dilution of the preparation at 260nm and 280nm in a spectrophotometer. The O.D. value at 260 nm gives an estimate of the concentration of nucleic acid in the sample. An O.D. $_{260}$ value of 1 is equivalent to approximately 50µg/ml double-stranded DNA; 40µg/ml single-stranded DNA or RNA; or 20µg/ml oligonucleotide. The O.D. $_{260}$/O.D. $_{280}$ value gives an estimate of purity of the nucleic acid. Pure DNA gives an O.D. $_{260}$/O.D. $_{280}$ value of 1.8; pure RNA gives an O.D. $_{260}$/O.D. $_{280}$ value of 2.0. Contamination with protein or phenol reduces this value.

2.17.2 Estimation of nucleic acid concentration by comparison to a known standard, λ/Hind III DNA

A known volume of the DNA was analysed by 1% agarose gel electrophoresis, by comparison to a standard amount (1µg) of lamba DNA digested with Hind III (Gibco BRL, U.K.), run as a parallel sample, and the gel stained with ethidium bromide. Comparison of the DNA preparation with a band of equivalent intensity from the lambda/Hind III standard gave an approximation of the concentration of the DNA loaded.

2.17.3 Restriction enzyme digestion

All restriction enzyme digests were performed at 37°C for 1-2 hours. BssH II (CP Laboratories, U.K.) digests were performed subsequent to other enzyme digestions, and incubated at 50°C. The enzyme was added (at approximately 1 unit per

μg DNA) to the DNA (0.2-1μg) in a final volume of less than 20μl. The DNA was resuspended in restriction enzyme buffer of the required salt concentration (no salt, medium 50mM salt, or high 0.1M salt) for the enzyme used, and diluted to the final reaction volume using dH_2O.

2.17.4 Ligations

Ligations were performed at 16°C for 12-16 hours, in 1x ligation buffer. Vector plus fragment DNA were combined at a ratio of 3:1, and 10 units of T4 DNA ligase (Pharmacia, U.K.) were added per μg vector DNA.

2.17.5 Geneclean Procedure for purification of DNA (Geneclean, BIO 101 Inc., U.S.A.)

The band required was excised from the agarose gel (~ 200μl slice) into an Eppendorf tube, 2-3 volumes of sodium iodide stock solution (saturated with sodium sulphite) added and the gel slice incubated for 5 minutes at 45-55°C with vortexing to dissolve the agarose. The glassmilk suspension was added ~ 5μl to solutions containing 5μg or less of DNA, mix and incubated on ice for 5 minutes. The glassmilk plus DNA was pelleted for 5 seconds at 10,000 xg in a microcentrifuge, and the supernatant discarded. The glassmilk was washed three times with 200μl of ice-cold ethanol wash solution, pipetting to mix, then microcentrifuged at 10,000 xg for 5 seconds and the supernatant discarded. The pellet was briefly respun to remove the last remaining traces of the ethanol wash solution. The DNA was eluted by resuspending the pellet in TE buffer or dH_2O in a volume of 10μl, and incubating the tube at 45-55°C for 2-3 minutes. The pellet was collected by microcentrifugation (10,000 xg) for 30 seconds and the supernatant containing the DNA carefully removed and transferred to a new tube. This was repeated once more, the supernatants pooled and the white pellet discarded.

2.18 Preparation of bacteriophage M13

2.18.1 M13 Transformation

The required fragment was ligated to the M13 vector overnight at 16°C, and a 5ml overnight culture of XL1-B in LB plus tetracycline prepared. 20ml of fresh LB plus tetracycline were inoculated with 0.2ml of the overnight culture of XL1-B, and incubated with shaking at 37°C for 1.5 to 2 hours to an O.D. $_{660}$ of 0.3. The top agar was melted and placed at 42°C to remain molten, and the required number of LB plates brought to 37°C.

The competent cells were thawed on ice and 5µl of ligation mix (1-25ng DNA) placed into chilled microfuge tubes. To each tube, 200µl of thawed competent cells were added and incubated on ice for 40 minutes. The cells were heat shocked at 42°C for 90 seconds, then the tubes returned to ice for 10 minutes. Whilst the cells were being heat-shocked, 40µl of 100mM IPTG, 40µl of 2% Xgal in dimethylformamide, and 200µl fresh exponential phase *E. coli* host cells (XL1-B) were prepared for each tube. 280µl of this mixture of IPTG, Xgal plus bacterial cells were added to each tube, plus 3ml of molten agar, and the contents of each tube plated onto a pre-warmed agar plate. The plates were allowed to solidify at room temperature before inversion and incubation at 37°C overnight.

2.18.2 Preparation of single stranded M13 template DNA (Sequenase Method)

A fresh exponential culture of host cells (XL1-B) was grown at 37°C with shaking, and diluted 1:100 in 1.5 ml of LB medium plus tetracycline (12.5µg/ml) and infected with a purified M13 plaque, transferring the plaque from a plate using a sterile Pasteur pipette. The cells plus plaque were incubated at 37°C with shaking for 5-8 hours. The contents were transferred to a microfuge tube and pelleted by microcentrifugation (10,000 xg) for 10 minutes at room temperature. The bacteriophage supernatant was removed into a fresh tube and 1/9 volume of 40% PEG 8000 and 5M

sodium acetate (pH 7.0) was added. The bacteriophage were allowed to precipitate for 15 minutes at 4°C, then microcentrifuged (10,000 xg) for 10 minutes in the cold, and the supernatant removed and discarded. All traces of the PEG were removed with a drawn-out Pasteur pipette. The bacteriophage pellet was resuspended in 0.2ml of TE buffer and extracted with an equal volume of phenol : chloroform (3:1, v/v), vortexed, and microcentrifuged (10,000 xg) for 3 minutes. (The phenol was equilibrated with 0.05 M Tris HCl (pH 7.5) immediately prior to use). The upper aqueous layer was recovered, and the phenol : chloroform extraction repeated, followed by a final extraction with 200µl of chloroform. To the aqueous phase 0.1 volume of 3M sodium acetate (pH 6.0), and 2.5 volumes of absolute ethanol were added, and the contents chilled to -70°C for 30 minutes. The DNA was recovered by centrifugation (10,000 xg) for 15 minutes in the cold, and washed with 90% ethanol. The pellet was allowed to air dry and was finally resuspended in 15-45 µl dH_2O.

2.19 Site-directed mutagenesis

Unique restriction enzyme sites were introduced by site-directed mutagenesis using a modification of the method of Nakamaye & Eckstein (Nakamaye & Eckstein 1986), using the oligonucleotide-directed *in vitro* mutagenesis system supplied by Amersham (Amersham International plc., U.K). The mutant oligonucleotide containing base mismatches was annealed to the single stranded template in M13mp18 and extended using Klenow polymerase in the presence of T4 DNA ligase to generate a mutant heteroduplex. During *in vitro* synthesis a thionucleotide was incorporated into the mutant strand, by replacing dCTP in the nucleotide mix with dCTPαS, generating one phosphorothioate and one non-phosphorothioate strand. Any remaining single-stranded template DNA was removed by filtration through a nitrocellulose filter in 0.5M NaCl (the single-stranded material binding to the filter). Certain enzymes are unable to cleave phosphorothioate DNA, such as Nci I. This enzyme was used to produce single-strand nicks in the non-mutant DNA. These nicks provided a site for exonuclease III to digest all or part of the non-mutant strand (exonuclease digestion from the free 3' ends

in a 3' to 5' direction ensures only the non-mutant strand is digested). The mutant strand was then used as a template to resynthesise the double-stranded closed circular molecule by repolymerisation with DNA polymerase I. The double-stranded mutant DNA was then transformed into competent XL1-B cells, and the resultant plaques used to infect bacteria. Double-stranded DNA was prepared from the infected bacteria and the introduction of the unique enzyme site verified by appropriate restriction enzyme digestion.

2.20 Transfection of eukaryotic cells

2.20.1 Protoplast Fusion (Oi *et al.*, 1983)

A 50ml culture of bacteria containing the plasmid of interest (pHβ Apr-1-neo plus the relevant insert) was grown at 37°C in LB plus antibiotic to an O.D. $_{550}$ of 0.35-0.60 (exponential phase). To this, 125μg/ml chloramphenicol (40mg/ml stock in 95% ethanol) were added, and the cells grown with shaking at 37°C for 12-16 hours. The bacterial cells were recovered by centrifugation of 25ml of the culture at 1,500 xg and resuspended in 1.25ml of chilled 20% sucrose in 0.05M Tris (pH 8.0), plus 0.25 ml lysozyme (5mg/ml in 0.25M Tris pH 8.0, prepared immediately prior to use), then incubated on ice for 5 minutes. 0.5ml of 0.25M EDTA (pH 8.0) were added and the cells incubated for a further 5 minutes on ice. After the addition of 0.5ml of 0.05M Tris (pH 8.0) the cells were incubated at 37°C for 10 minutes. 10ml of RPMI, 10% sucrose, 10mM magnesium chloride were added and the protoplasts held at room temperature for a further 10 minutes.

The eukaryotic cells (DC27.10 or 3T3) were harvested by centrifugation and resuspended in MEM-H media. Viable cell counts were performed and the cells diluted to a final concentration of between $5x10^6$ to $1x10^7$ cells per sample in 3ml MEM-H. 3ml of this cell supension were mixed with 5ml of protoplasts and then centrifuged at 1,000 xg at 25°C for 5 minutes. The cells plus protoplasts were resuspended in 1ml of warm RPMI, 45% PEG solution (pH 8.0; PEG 1500, Sigma, U.K.) over approximately 1 minute, then 9ml of warm (37°C) RPMI were added over

approximately 2 minutes, and the cells plus protoplasts centrifuged at room temperature at 300 xg for 5 minutes. The pellet was resuspended in 50 ml of DC27.10 or 3T3 medium containing 50μg/ml gentamycin. The cells were plated out in 1ml aliquots into a 24 well flat-bottomed plate, and incubated at 37°C for 48 hours in a 5% CO_2 atmosphere.

After 48 hours the supernatant was aspirated and replaced with 1ml medium plus G418 (the required concentration previously determined by titration). The cells were incubated at 37°C and started to die after 2 days under selection. The selection medium was replaced every 48 hours with a higher dose of G418 as necessary, until all the cells died. (Neomycin selection was performed at concentrations of G418 (Geneticin) upto 1mg/ml active concentration, determined by titration on the recipient cells). Most of the cells appeared dead by day 6 of selection. The cells were then incubated for a week to allow resistant clones to grow out in the selection medium. Resistant cells growing out in single wells were harvested and stained using monoclonal antibodies to detect expression of the molecule encoded by the transfected DNA, or expanded to prepare RNA for Northern blotting.

2.20.2 Electroporation

DNA for transfection was prepared by the large scale plasmid preparation as described (2.16.2). An estimate of the concentration and purity of the DNA preparation was determined as described previously.

The DNA (20-40μg) was linearised using an appropriate restriction enzyme with a single site in the vector (pHβ Apr-1-neo was restricted in the ampicillin resistance gene using Sca I), and the digest extracted once with phenol:chloroform (1:1, v/v). The DNA was ethanol precipitated with 500μg sonicated salmon sperm DNA as a carrier, washed with 70% ethanol and resuspended in 100μl of sterile TE buffer. The cells to be transfected were grown to an exponential phase, harvested, and washed in sterile PBS. Viable cell counts were determined by trypan blue exclusion and the cells adjusted to a concentration of 5 x 10^6 cells in 700μl of sterile PBS. The cells were transferred to a

sterile electroporation cuvette (BioRad, U.K.) with the DNA (or PBS alone as a control). The cells plus DNA were incubated on ice for 10 minutes, then electroporated using a BioRad Gene Pulser (BioRad, U.K.) at 25μfarads/550V and replaced on ice for a further 10 minutes. The cells were added to 50ml of DC27.10 medium, aliquotted, 1ml per well of a 24 well plate and cultured for 2 days at 37°C before selection medium was added. Selection was performed as described for transformation by protoplast fusion.

2.21 Preparation of total cellular RNA

2.21.1 RNA miniprep.

For each sample, $2x10^6$ cells were harvested by centrifugation at 4°C, 300 xg, for 7 minutes. The cell pellet was washed twice in RNA lysis buffer (4°C) and the pellet resuspended in 100μl of lysis buffer before being transferred to a 1.5ml microfuge tube. To lyse the cells, 150μl of lysis buffer containing 1% NP-40 (final concentration 0.5%) were added, mixed and the tube placed on ice for 2 minutes. The lysate was microcentrifuged (10,000 xg) at 4°C for 6 minutes and the supernatant transferred to a fresh tube. The supernatant was extracted twice with hot phenol (40°-50°C), centrifuging at 10,000 xg for 10 minutes at room temperature, then extracted with chloroform : isoamylalcohol mix (24:1, v/v). Sodium chloride and magnesium chloride were added to give a final concentration of 0.25M, and 0.01M, respectively, and 800μl of absolute ethanol were then added to precipitate the RNA. The RNA was placed at -70°C for 30 minutes, and the precipitate collected by microcentrifugation (10,000 xg) at 4°C for 30 minutes. The pellet was washed with 70% ethanol and stored at this stage, if required, under 70% ethanol, or dried briefly in a rotary vacuum desiccator. The pellet was finally resuspended in 4.5μl of RNAse-free dH_2O and 15.5μl of RNA sample buffer added. The sample was heated to 70°C for 5 minutes, cooled on ice, and 2μl of dye, and 0.5μl ethidium bromide(10mg/ml) added before electrophoresis.

2.22 Northern blotting

2.22.1 Electrophoresis of RNA

The gel tray and gel box were washed in SDS, then with DEPC-treated water. The 1.2% agarose/formaldehyde gel was electrophoresed in 1x MOPS at 50V (or overnight at 15V) with constant recirculation of the buffer.

The gel was photographed, then soaked for 1 hour in 20x SSC. The RNA was transferred by blotting overnight in 10x SSC onto nylon membrane (Hybond N, Amersham International plc., U.K.). The filter was then soaked for 30 minutes in 5x SSC and U.V. fixed for 3 minutes. The positions of the ribosomal RNA bands were marked in pencil on the blot.

2.22.2 Radiolabelling of DNA probe

The DNA probe (50ng) was prepared by restriction enzyme digestion to yield the appropriate fragment and the fragment gel purified and isolated by the Geneclean protocol (section 2.17). The DNA was radiolabelled using the hexamer radiolabelling technique (Maniatis *et al.*, 1982).

50ng of DNA were mixed with dH_2O as appropriate to a final volume of 8 µl. The DNA was placed in a boiling water bath for 5 minutes to denature the DNA, then placed on ice to prevent reannealing. The rest of the reagents (11.4µl LS, 5µl ^{32}P dCTP (50µCi), 0.5µl Klenow (2.5 units)) were added and incubated at room temperature for at least 3 hours. 200µl of TE buffer were added and the sample placed on ice. Unincorporated nucleotides were removed using a spun column (Maniatis *et al.*, 1982).

2.22.3 Northern blot hybridisation

The U.V. cross-linked filter was sealed into a plastic bag together with approximately 10-12ml of prehybridisation solution (50% deionised formamide (v/v), 5x Denhardts, 2% SDS (or 0.5%), 6x SSC. 50µg/ml sonicated salmon sperm DNA were denatured by heating in a boiling water bath for 5 minutes, chilled on ice, then

added to the prehybridisation solution. The filter was then pre-hybridised for at least 2 hours at 42°C.

The probe was labelled as described above, and the desired amount of probe (approximately $1x10^7$ cpm) was added to 50µg/ml sonicated salmon sperm DNA and boiled for 5 minutes. Poly dA and poly dC (2µg) were included before the probe was added to the prehybridisation fluid. The blot was incubated at 42°C overnight (for at least 12 hours) in a shaking water bath.

The hybridisation solution was discarded and the filter removed. The filter was subjected to two 15 minute washes, with 2xSSC, 0.05%SDS at room temperature. Stringent washes were performed using 0.1xSSC, 0.05% SDS buffer at 50°C with several changes of buffer over 1hour. The filter was monitored, and if the counts were down to background levels, the filter was wrapped in Saran wrap and exposed to film at -70°C; if not, the filter was washed further.

CHAPTER THREE

Foetal Thymus Organ Culture

3.1 Introduction

The thymus originates as an alymphoid epithelial rudiment which is continually colonised by haemopoietic cells. These haemopoietic cells originate in the liver during foetal life, and subsequently arise in the adult bone marrow. The first wave of lymphoid progenitor cells migrates from the foetal liver to colonise the murine thymus between days 10-11 of gestation (Moore & Owen, 1967). These immigrant cells express neither CD4 nor CD8, nor the T cell antigen receptor, and appear to act as the precursors for all the other thymocyte subsets (reviewed by Fowlkes & Pardoll, 1989). The thymus is the main site for T lymphocyte generation, providing the necessary microenvironment for the differentiation of immunologically naive precursors, inducing rearrangement and expression of T cell receptor genes, and positive and negative selection of the developing cells.

Thymocytes undergo a programmed sequence of differentiation events during embryonic development, resulting in the generation of mature antigen-specific T cells. Previous studies have used monoclonal antibodies against cell surface molecules to classify functionally distinct subpopulations of cells arising during thymic ontogeny *in vivo*. The earliest defined population, at day 14 of gestation in the mouse, contains cells which are CD4, CD8 and T cell receptor (TCR) negative. This triple-negative population of thymocytes is heterogeneous, and has been shown to contain a number of subsets by phenotypic and functional characterisation (reviewed by Fowlkes & Pardoll, 1989; and Nikolic-Zugic, 1991). Precursor cells within this $CD4^-CD8^-$ population give rise to $CD4^+CD8^+$ (double-positive) cells by day 16 of gestation. Although productive messenger RNA transcripts of T cell receptor chains can be detected in the cytoplasm of these double-positive cells, low levels of the TCR are not expressed on the cell surface until day 17-18 gestation. A substantial number of these double-positive cells are destined to die in the thymus, and it is believed that their fate is determined by positive and negative selection events to generate functionally mature T cells (reviewed by Fowlkes & Pardoll, 1989). The double-positive cells subsequently lose surface expression of either CD4 or CD8, by a mechanism as yet not understood, to produce

cells with a mature $CD4^+CD8^-$ or $CD4^-CD8^+$ (single-positive) TCR^+ phenotype by day 18-19 gestation. These cells then exit from the thymus to seed the periphery.

The ability to remove and maintain thymus lobes from 14 day mouse embryos in culture has enabled an *in vitro* model of thymic development to be established (Mandell & Kennedy, 1978; Jenkinson *et al.*, 1982). This experimental system of foetal thymus organ culture has subsequently provided a means of investigating the differentiation potential of precursors in the murine foetal thymus, *in vitro*. Recent studies using this system have addressed the effects of the addition of recombinant lymphokines, such as IL-1 (DeLuca & Mizel, 1986), IL-2 (Ceredig *et al.*, 1989; Plum *et al.*, 1990; Skinner *et al.*, 1987; Waanders and Boyd, 1990), and IL-7 (Watson *et al.*, 1989), and monoclonal antibodies, against the IL-2R (Jenkinson *et al.*, 1987; Plum & De Smedt, 1988), MHC class II (DeLuca, 1986), CD4/8 (Zuñiga-Pflücker *et al.*, 1989b), and the TCR (Born *et al.*, 1987; Owen *et al.*, 1988; Smith *et al.*, 1989a; Yachelini *et al.*, 1990) on the development of maturing thymocyte subpopulations in an attempt to understand the factors required for normal T cell development.

At the beginning of this study, relatively little work had been performed in the characterisation of the developing subpopulations of thymocytes in organ culture. The initial aim was to compare the phenotype of the cells developing in organ culture to those observed *in vivo*, and to monitor the kinetics of development. To address this, thymus lobes from day 14 embryos were maintained in organ culture for various periods of time up to 21 days then the cell surface phenotype of the thymocytes analysed by two colour flow cytometry using monoclonal antibodies against CD4, CD8, Thy1, heat stable antigen (HSA), CD3 and the low affinity IL-2R (p55).

3.2 Changes in cellularity and phenotype during organ culture

During organ culture the cell recovery increased from approximately 10^4 thymocytes per lobe at day 14 of gestation to 2×10^5 cells per lobe after only 4 days in culture. As shown in Figure 3.1, the cellularity increased to a maximum of 4×10^5 cells per lobe after a week, then declined to between $1\text{-}2 \times 10^5$ cells/lobe after 3 weeks of

culture. Following 3 weeks in culture the thymus lobes appeared to lose definition in their gross morphology, becoming flattened and "diffuse" with a less defined lobular appearance.

3.2.1 Changes in Thy1, CD4 and CD8 expression

Following a single day in culture the developing cells showed a similar phenotype to that of day 14 gestation thymocytes, expressing heat stable antigen (HSA) and low levels of Thy1 (Figure 3.2a), and the majority of cells lacking surface expression of CD4, CD8 (Figure 3.2b) and CD3, (Figure 3.4a). By day 4 *in vitro* the level of expression of Thy1 had increased to that seen in the adult thymus, whilst HSA expression remained unchanged (Figure 3.2a). During culture the thymocytes acquired CD4 and CD8, with the four subpopulations of cells defined by CD4 and CD8 expression as found in the adult thymus (namely $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$) appearing within 4 days in culture (Figure 3.2b).

The proportion of $CD4^+CD8^+$ (double positive) thymocytes increased to a maximum of approximately 70% between day 8 and day 10 of culture, although did not reach and maintain the levels seen in the adult thymus (~80% $CD4^+CD8^+$). In contrast to *in vivo* development, an increased proportion of $CD4^-CD8^-$ (double negative) cells was observed during organ culture (30-40% by 20 days; Figure 3.3a), and after 14 days in culture the percentage of CD8 single positive cells reached 20-30% (in contrast to 5-6% of CD8 single positives found in a normal CBA adult thymus). As seen in Figure 3.3(c), this corresponded to an increase from approximately 1 x 10^4 $CD4^-CD8^+$ cells per lobe after 4 days, to 3.5 x 10^4 cells/lobe at 14 days in culture, reaching a maximum of 4 x 10^4 cells/lobe by 3 weeks in culture. The number of CD4 single-positive cells increased from 4.5 x 10^4 cells per lobe at day 4 of culture to a maximum of 5-6 x 10^4 /lobe at day 14, decreasing thereafter to between 2-3 x 10^4 by 3 weeks in culture (Figure 3.3c).

3.2.2 IL-2R expression and acquisition of CD3

In addition to the appearance of CD4 and CD8 expressing cells, acquisition of CD3 was observed by day 4 in culture (Figure 3.4a), illustrating T cell maturation. The thymocytes, initially CD3 negative, began to express low levels of CD3 within 4 days in culture, the majority of the $CD3^{lo}$ cells being contained within the $CD4^+CD8^+$ population. Higher levels of CD3 expression were seen by day 6 *in vitro*, corresponding to the appearance of more mature CD4 or CD8 single positive cells, and the characteristic adult CD3 phenotype was attained by day 8.

The developing thymocytes showed transient expression of the low affinity IL-2Rα (p55), expression of this receptor preceding that of the T cell receptor in thymic development. Initially the majority of cells were IL-$2R\alpha^+$, however, there was a progressive decrease in expression during development, most cells becoming IL-2Rα negative by day 8 *in vitro*, as CD3 expression increased (Figure 3.4a & b). This pattern of expression corresponded to that observed *in vivo*, with few IL-2Rα positive cells detected in the adult thymus.

3.3 Discussion

The cell recovery from the lobes during organ culture increased rapidly as a result of proliferation of thymic precursors, although did not reach the value seen *in vivo*. The cellularity reached a maximum after 8 days *in vitro*, however, subsequently decreased with longer periods in culture. The overall decrease in cell recovery with time may be a result of the limited capacity for expansion of the precursors within a day 14 foetal thymus lobe. In addition, exhaustion of endogenous factors by the maturing cells which would normally exit from the thymus may arrest the subsequent maturation of other precursor cells.

Day 14 thymocytes are negative for CD4, CD8, and the T cell antigen receptor, and the data illustrate that the immature cells present at this stage of gestation have the capacity to proliferate and differentiate *in vitro* into mature thymocytes, generating all the subpopulations defined by CD4 and CD8 expression as seen in a normal adult

thymus. Acquisition of CD4, CD8 and CD3 in organ culture followed the developmentally ordered sequence that is seen *in vivo*. Following longer periods *in vitro* (> 10 days) there appeared to be an increase in the proportion of $CD4^-CD8^-$ and CD8 single-positive cells recovered from the thymus lobes. The overall decrease in cellularity of the lobes observed in longer term cultures correlated with a reduction in the number of $CD4^+CD8^+$ and CD4 single-positive cells. Since the actual number of $CD4^-CD8^-$ cells did not alter dramatically over 2-3 weeks in culture (Figure 3.3b), the reduction in total cellularity would account for the relative increase observed in the percentage of this population. However, this would not explain the observed increase in the proportion of CD8 single positive cells in longer term cultures, since this was accompanied by an increase in the actual number of $CD4^-8^+$ cells per lobe (Figure 3.3c). Hence it is important to relate differences in percentages to actual cell numbers wherever possible.

The increase in the number of CD8 single-positives in culture is in agreement with functional studies which have shown an increase in the frequency of alloreactive cytolytic T cell precursors during longer periods of organ culture (Ceredig *et al.*, 1982). The CD8 single-positive cells normally comprise only 5-6% of the total thymocytes, and perhaps under these conditions there is still further capacity for expansion in this subpopulation, in contrast to the CD4 single-positive subset. One possible explanation for the accumulation of this population may be the inability of the maturing cells to emigrate from the thymus explant. The greater proportion of $CD4^-8^+$ cells seen *in vitro* may also result from differential maintenance of the developing cells by the microenvironment in the foetal thymus explant, subtly altering the kinetics of generation of maturing cells in comparison to the *in vivo* situation.

The acquisition of CD3 by developing thymocytes and transient expression of the low affinity IL-2R in organ culture followed a similar pattern to that seen *in vivo*, expression of IL-2Rα preceding surface expression of the TCR/CD3 complex. Furthermore, the expression of IL-2Rα and CD3 appeared to be mutually exclusive, an observation recently confirmed by Guy *et al.* (Guy *et al.*, 1991). The expression of the

TCR during thymic development appears to be a critical requirement for positive and negative selection events resulting in the production of mature, self-tolerant T cells (Fowlkes & Pardoll, 1989), however, the significance of expression of IL-2R prior to that of the TCR, and the involvement of the IL-2/IL-2R pathway in thymic development is uncertain.

The functional significance of IL-2Rα expression on immature thymocytes is controversial, particularly since this receptor does not appear to be able to internalise IL-2, and is able to bind IL-2 with only low affinity (Lowenthal *et al.*, 1986; Ceredig *et al.*, 1985). To add further confusion, conflicting functional data have been published, several studies indicating a lack of proliferative response by murine foetal thymocytes to IL-2 alone (Raulet, 1985; Habu *et al.*, 1985; von Boehmer *et al.*, 1985; Guy *et al.*, 1991), whereas others report that foetal thymocytes exhibit IL-2-stimulated proliferation (Hardt *et al.*, 1985, 1986; Ceredig *et al.*, 1989; Toribio *et al.*, 1988, 1989; Watson *et al.*, 1989). Similarly, in organ culture, conflicting reports show that antibodies specific for the IL-2R either do (Jenkinson *et al.*, 1987; Tentori *et al.*, 1988), or do not influence thymic differentiation (Plum & De Smedt, 1988).

Although there is little evidence to suggest that significant quantities of IL-2 are produced in the thymus, recent data from Zuñiga-Pflücker *et al.* demonstrate by *in situ* hybridisation that expression of IL-2 and IL-2R messenger RNA appears to be developmentally regulated, and that foetal thymocytes show both high and low affinity IL-2R binding. In the absence of an antibody against murine IL-2Rβ (p75), however, the presence of both α and β chains on the surface of immature thymocytes cannot be confirmed. It has also been observed that IL-2 can stimulate proliferation *in vitro*, and that *in vitro* blocking of the IL-2R on thymus explants causes a reduction in cell yield (Zuñiga-Pflücker *et al.*, 1990b). This suggests that the IL-2R on immature thymocytes is indeed functional, and implicates IL-2 as a growth factor for early thymocytes.

It has been proposed that two waves of lymphoid precursors emigrate to the foetal thymus, the first between day 10-13 of gestation, and the second starting from day 18 onward (Jotereau *et al.*, 1987). The removal of thymus lobes at day 14 of

gestation would therefore allow development of only the first wave of precursors in organ culture, although this did not appear to result in the loss of any subpopulation in the lobes maintained *in vitro*. In addition, the differentiation of thymocytes in day 14 thymus organ cultures proceeds without any further influx of thymic precursors and in the absence of any exogenous factors, which may account for some of the heterogeneity observed between development *in vivo* and in organ culture.

Studies by Ceredig also addressing the question of the developmental potential of thymocytes in organ culture were published during the course of this work, and the data are comparable in both studies (Ceredig, 1988). The strain of mice used in this study were CBA/J, and those used by Ceredig were (C57BL/6 x BALB/c)F_1, therefore slight discrepancies observed in the cell yields and percentages of markers expressed are probably a result of strain variation. In conclusion, although there may be small differences in the developing subpopulations observed in longer term organ cultures, short term organ culture of foetal thymus lobes appears to provide an acceptable model system for studying early events in thymocyte development.

Figure 3.1 *Average cell recovery per lobe during organ culture.*

The average cell yield per lobe recovered after various times in organ culture is shown. The values shown at each time point are the results from a representative experiment, comprising a minimum of forty individual lobes analysed up to day 4 of culture, and twenty lobes thereafter. The cells recovered from the lobes were pooled and the average cell yield per lobe calculated. Repeat sets of experiments showed the same trend, although slight variations were observed in absolute cell numbers.

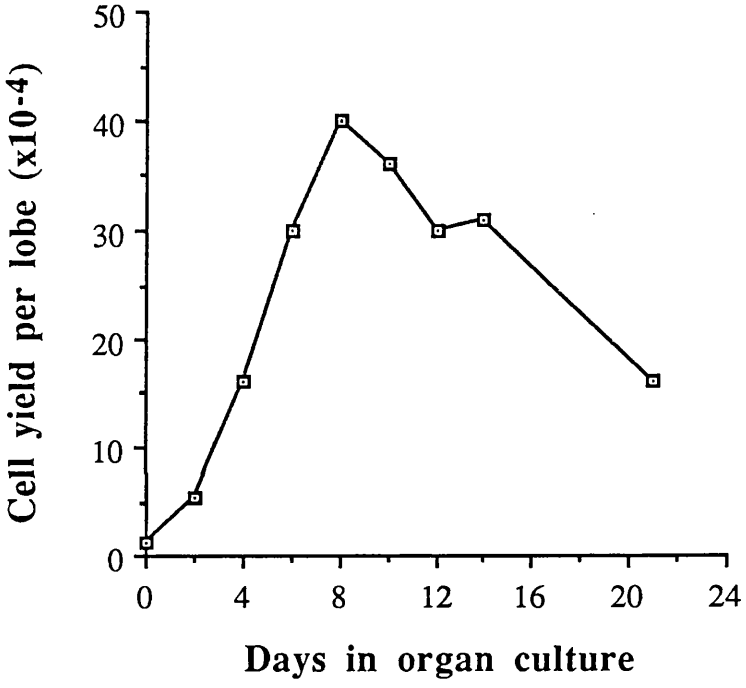

Cell yield per lobe (x10-4)
50
40
30
20
10
0
0
4
8
12
16
20
24
Days in organ culture

Figure 3.2 *Phenotype of the developing thymocytes in organ culture.*

The cell surface phenotype of thymocytes cultured from 1 to 8 days in organ culture is shown. A minumum of fifty lobes were harvested at day 1, and twenty lobes at 4, and 8 days of culture. The phenotype of young adult thymocytes (average age 6 weeks) is shown for comparison. (a) Changes in Thy1 and heat-stable antigen (HSA) expression. Cells were stained using a FITC-conjugated anti-Thy1 monoclonal antibody (YTS 154), biotinylated anti-HSA monoclonal antibody (YBM 5.10.4), plus PE-conjugated second step reagent. (b) Changes in CD4 and CD8 expression during culture. Cells were stained using a PE-conjugated anti-CD4 monoclonal antibody.(GK 1.5) and FITC-conjugated anti-CD8 monoclonal antibody (53.6.7). A minimum of 3000 events were collected per sample and live gates were set using forward and low angle light scatter, and propidium iodide exclusion. The markers in (a) were set on cells incubated with the second step reagent only, or in (b), on an unstained population. Fluorescence intensity is represented on a log scale and the percentage value in each quadrant is shown.

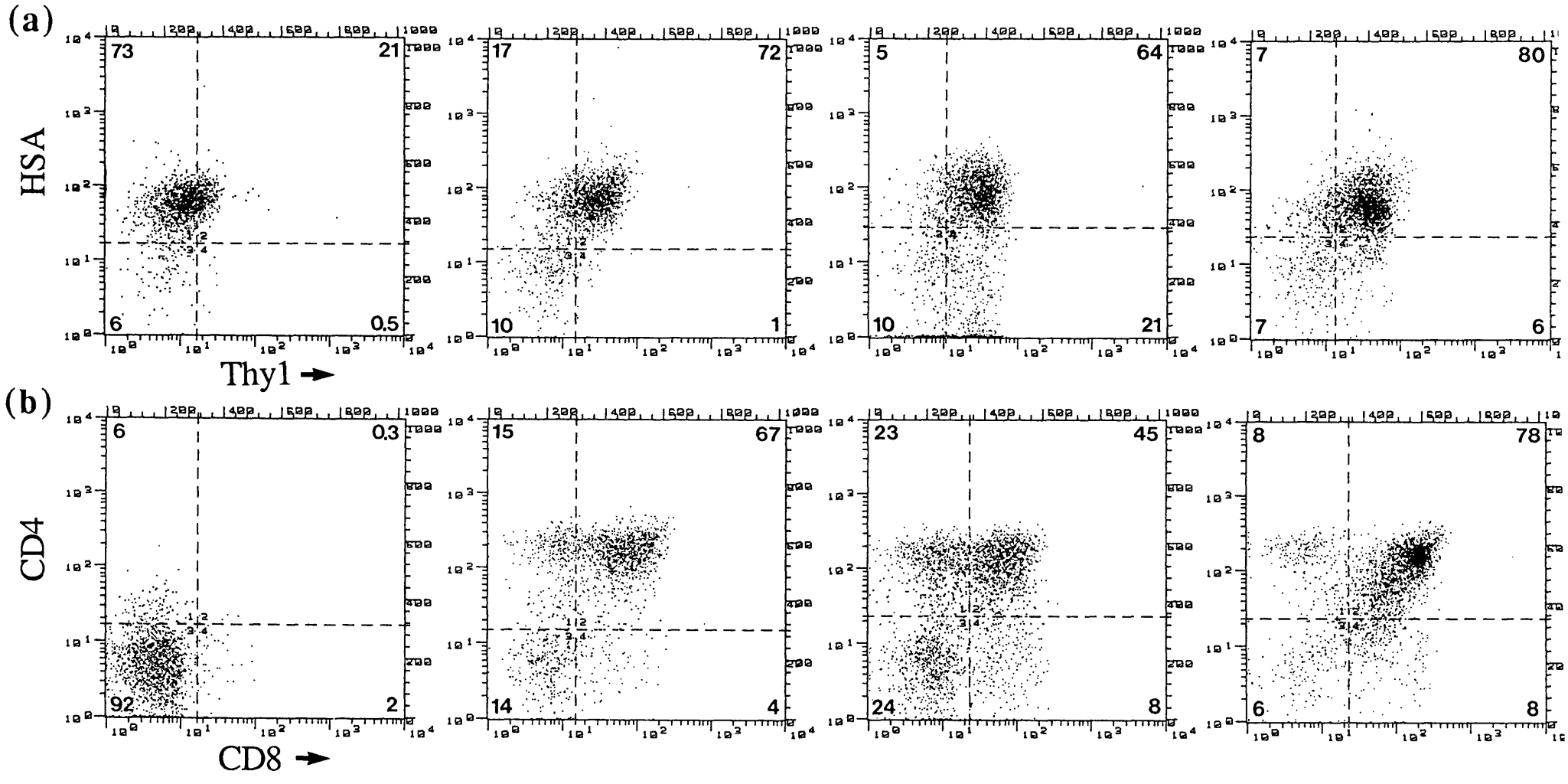
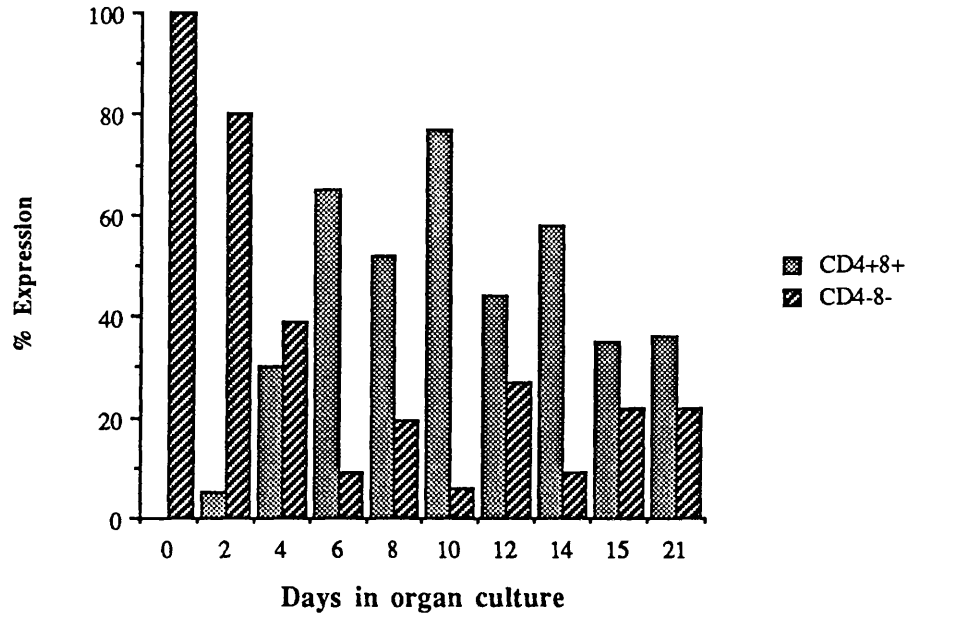
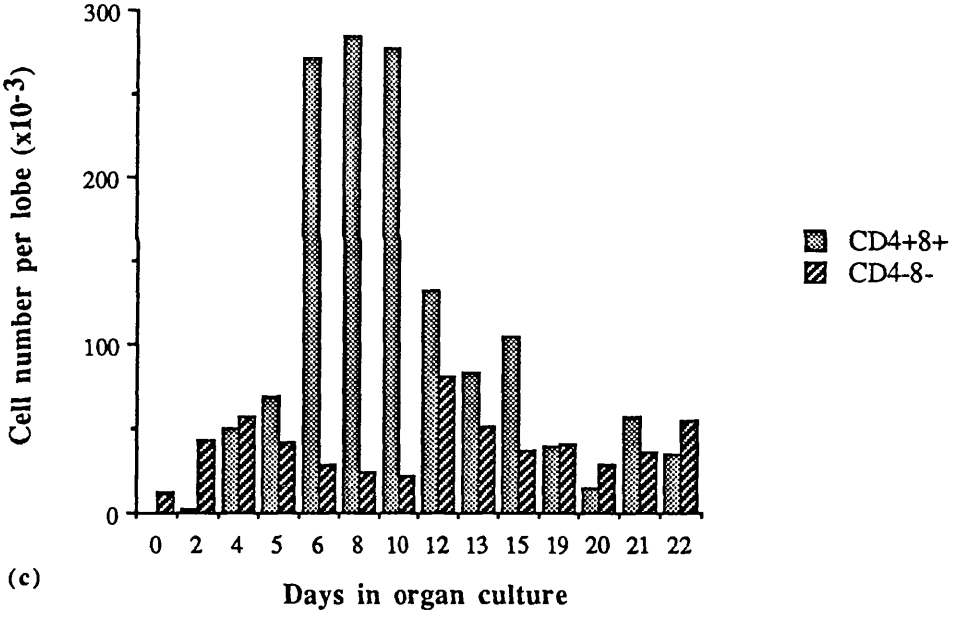

Day 1
Day 4
Day 8
Adult
(a)
HSA
Thy1 →
73
21
6
0.5
17
72
10
1
5
64
10
21
7
80
7
6
(b)
CD4
CD8 →
6
0.3
92
2
15
67
14
4
23
45
24
8
8
78
6
8

Figure 3.3 *Changes in CD4 and CD8 expression during organ culture.*

(a) Changes in the percentage of $CD4^-CD8^-$, and $CD4^+CD8^+$ cells during culture. The values are from a single representative set of experiments, using a minimum of forty lobes at time points up to 4 days, and twenty individual lobes thereafter. Cells were stained as in the legend to Figure 3.2. (b) Changes in absolute cell number per lobe of $CD4^-8^-$ or $CD4^+8^+$ cells during culture. (c) Changes in the absolute cell number per lobe of $CD4^+8^-$ or $CD4^-8^+$ single positive cells during culture. The values shown were calculated by multiplying the percentage of cells in each phenotype by the total number of cells harvested per lobe.


100
80
60
40
20
0
% Expression
0 2 4 6 8 10 12 14 15 21
Days in organ culture
CD4+8+
CD4-8-

(b)


300
200
100
0
Cell number per lobe (x10-3)
0 2 4 5 6 8 10 12 13 15 19 20 21 22
Days in organ culture
CD4+8+
CD4-8-

(c)

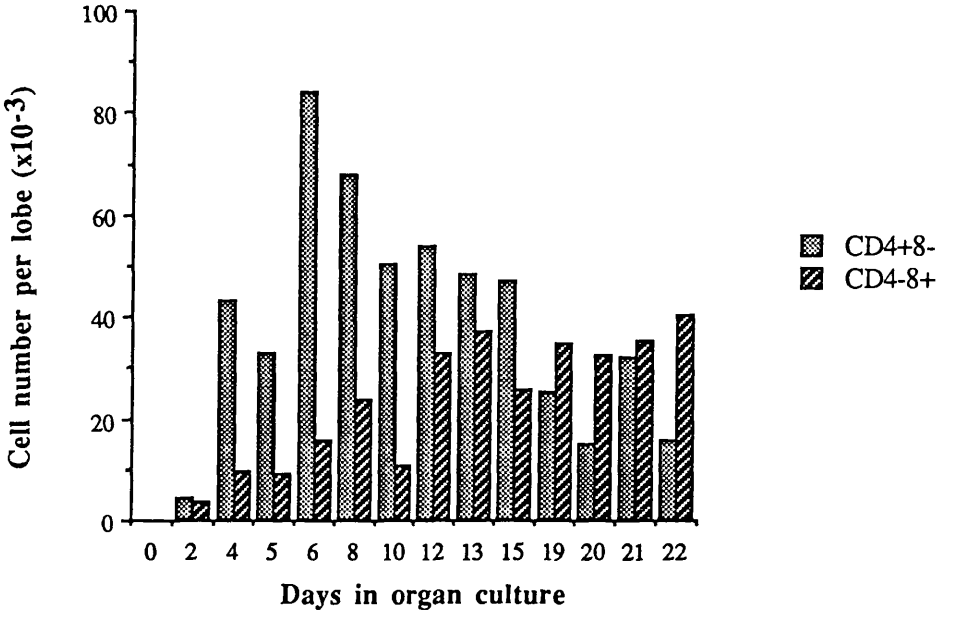
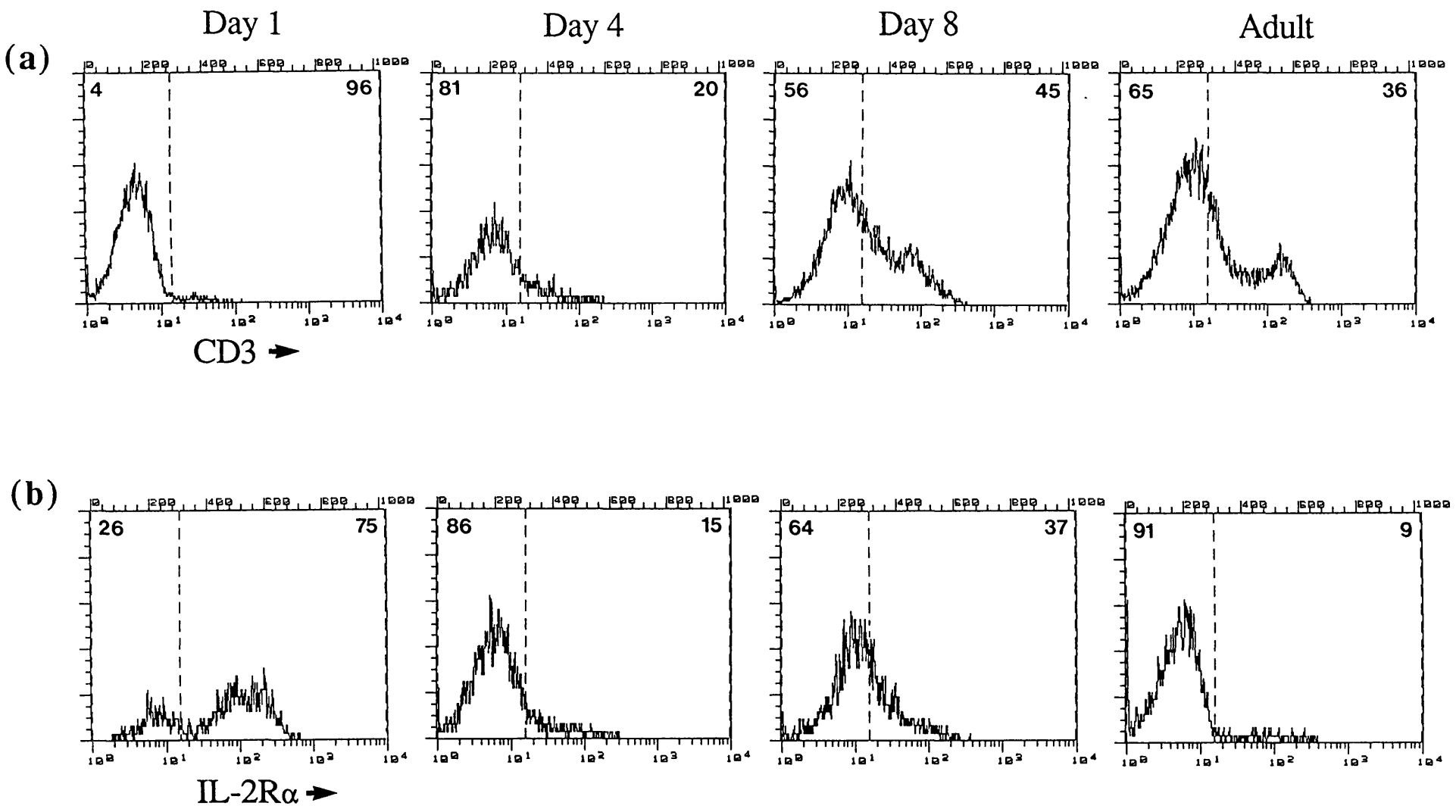

100
80
60
40
20
0
Cell number per lobe (x10-3)
0 2 4 5 6 8 10 12 13 15 19 20 21 22
Days in organ culture
CD4+8-
CD4-8+

Figure 3.4 *Changes in CD3 and IL-2Rα expression during organ culture.*

The cell surface phenotype of thymocytes cultured from 1 to 8 days in organ culture. (a) CD3 expression was analysed using a FITC-conjugated anti-CD3ε monoclonal antibody (2C11). Fluorescence intensity is represented on the abscissa (log scale) and cell number on the ordinate (linear scale). (b) Expression of the low affinity IL-2Rα (p55) from day 1 to day 8 of organ culture. Cells were stained with an anti-IL-2Rα monoclonal antibody (7D4) plus a goat anti-rat second step reagent. Cells were analysed as in the legend to Figure 3.2. The markers were set on cells stained with the second step reagent alone and the percentage values in each subpopulation are shown. All histograms are represented on the same scale.


Day 1
Day 4
Day 8
Adult
(a)
4
96
81
20
56
45
65
36
CD3
(b)
26
75
86
15
64
37
91
9
IL-2Rα

CHAPTER FOUR

Lymphoid precursor activity in murine foetal liver

4.1 Introduction

Haematopoietic stem cells have been generally defined by their capacity for self-renewal, and their ability to generate all types of haematopoietic cells. The search for haematopoietic stem cells was first initiated following the observation that animals which receive lethal doses of irradiation suffer bone marrow failure unless "rescued" by injection of unirradiated bone marrow cells (Ford *et al.*, 1956). It was subsequently shown that these animals are reconstituted in all myeloid and lymphoid cell types by bone marrow cells of donor origin (Micklem *et al.*, 1966). The first quantitative experiments using limiting numbers of bone marrow cells in the reconstitution of lethally irradiated mice identified clonal colonies of myeloid-erythroid cells in the spleen and in the bone marrow (Till & McCulloch, 1961), indicating the multipotentiality of the haematopoietic progenitors. The detection and enumeration of early myeloid-erythroid progenitors using this spleen colony forming assay (CFU-s) has therefore provided a method for measuring the frequency of a population of haematopoietic stem cells in suspension.

The identification of haematopoietic progenitor cells has relied largely on the establishment of functional assays for stem cells or their progeny. A number of *in vivo* selective adoptive transfer systems have been developed to assay clonogenic precursors of haematopoietic lineage cells. These include the intravenous (i.v.), and intra-thymic (i.t.) transfer of limiting numbers of cells to reconstitute lymphoid subpopulations in irradiated or non-irradiated recipient mice (Kadish & Basch, 1976, 1977; Goldschneider *et al.*, 1986; Guidos *et al.*, 1989b). The additional development of tisssue culture systems that can support and maintain committed progenitors *in vitro* (Whitlock & Witte, 1982; Jenkinson *et al.*, 1982), has enabled the requirements for normal haematolymphoid development to be addressed.

In order to understand the mechanisms of development of the haematopoietic system, an important aim has been to identify and isolate a pure population of self-renewing pluripotent stem cells. The characterisation of mouse haematopoietic stem cells has concentrated on candidate populations isolated from the bone marrow and the

thymus, although the cells obtained from these two sources appear to vary in lineage commitment.

Initial strategies to identify stem cell populations used the bone marrow as a source of multipotential progenitor cells (Visser *et al.*, 1984; Müller-Sieburg *et al.*, 1986; Spangrude *et al.*, 1988). Precursor populations were isolated by enrichment using a number of approaches, including density gradient sedimentation, sensitivity to anti-mitotic agents, the uptake of the mitochondrial selective dye, rhodamine 123, and by depletion and cell sorting using monoclonal antibodies against mature lymphoid and haematopoietic cell antigens (reviewed by Spangrude, 1989). The latter approach has enabled the elimination of cells which express antigens characteristic of a mature lineage (Spangrude *et al.*, 1988). Paradoxically, although the CD4 antigen is expressed on immature thymocytes and mature T cells, cell sorting of bone marrow cells expressing this marker has demonstrated that a $CD4^+$ population includes most of the multipotential haematopoietic stem cells (CFU-s), and myeloid progenitors (CFU-c) in the bone marrow (Fredrickson & Basch, 1989). To date, however, there are no specific markers for haematopoietic stem cells, although multipotent progenitors have also been identified within a population defined by Ly6A.2 (previously termed stem cell antigen-1 or Sca-1) expression in the bone marrow (Spangrude *et al.*, 1988, 1989). An additional marker, Sca-2, has been used to identify progenitor cells at a later stage of differentiation (Spangrude *et al.*, 1989). The use of such markers has enabled the identification of a stem cell population of $Thy1^{lo}$, mature lineage marker negative ($B220^-$, $Gr\text{-}1^-$, $Mac\text{-}1^-$, $CD4^-$ and $CD8^-$), $Sca\text{-}1^+$ expressing cells (representing <0.1% of total bone marrow) which have the capacity to rescue lethally irradiated mice, and subsequently restore all blood cell types in the survivors (Spangrude *et al.*, 1988).

In general, the most immature pluripotent progenitors have been identified in the bone marrow, with the capacity to generate all haematopoietic lineages, both myeloid and lymphoid, and only recent attention has been focussed on the foetal liver (Ikuta *et al.*, 1990; Jordan *et al.*, 1990). In contrast to haematopoietic progenitors isolated from the bone marrow, precursor cells identified in the day 14 foetal, and adult thymus

appear pre-committed to the lymphoid pathway, and limited in their capacity for self-renewal. It is also possible that progenitors in the embryo and adult exhibit different developmental potentials, and that the pluripotential capacity is restricted with age (Ikuta *et al.*, 1990). This may in part be due to different growth requirements, and different factors in the foetal versus adult microenvironment.

In order to characterise the various stages in haematopoietic development, attention has also concentrated on more committed progenitors, and their progeny in relation to the pluripotent haematopoietic stem cell. Although the time-course of appearance of different precursor populations has enabled them to be tentatively arranged in a developmental sequence, it is still uncertain as to when a particular progenitor population becomes lineage-restricted and what stimuli are essential for this to occur.

Lymphoid progenitors have generally not been fully characterised, however, in the absence of specific markers, certain antibodies have been useful in defining pro-T cell activity, such as those recognising Sca-1 (Spangrude *et al.*, 1988; van der Rijn *et al.*, 1989) and Joro (Palacios *et al.*, 1990). T cell precursor cells have been previously isolated from the $CD3^-CD4^-CD8^-$ population of thymocytes (reviewed by Fowlkes & Pardoll,1989), and more recently, prothymocytes expressing low levels of CD4 or CD8 have been identified in this subset (Wu *et al.*, 1991; reviewed by Scollay *et al.*, 1988). Many of the earliest prothymocytes, however, appear to have partially rearranged T cell antigen receptor genes (Pearse *et al.*, 1989), indicating restriction to the T lymphoid lineage.

Although the bone marrow has received most attention as a source of multipotential haematopoietic stem cells, the foetal liver is the major haematopoietic organ during embryonic life and is the principle site of B lymphopoiesis before initiation of B cell development in the bone marrow (Metcalf & Moore, 1971; Abrahamson *et al.*, 1977). The ability to reconstitute the lymphoid pathway in mice with severe combined immunodeficiency syndrome (Denis *et al.*, 1987; Bosma *et al.*, 1989), further demonstrates the lymphoid potential of this tissue.

Embryonic liver was therefore chosen as a suitable alternative to bone marrow as a source of lymphoid progenitor cells that would exclude the possibilty of contamination with mature cells. The initial aim was to identify and characterise a relatively pure lymphoid progenitor population, with the subsequent intention of manipulating these cells to gain more information about the factors influencing their development.

4.2 Identification of lymphoid precursor activity in murine foetal liver

In order to investigate the lymphoid precursor potential in murine foetal liver cells, the thymus organ culture system was chosen to provide a suitable microenvironment to allow the differentiation of T lineage progenitors. It has been previously demonstrated that treatment of embryonic day 14 thymus lobes with deoxyguanosine selectively depletes the lymphoid elements, whilst the stromal elements remain intact (Jenkinson *et al.*, 1982). By culturing an alymphoid thymus explant in direct contact with a suspension of precursor cells one can investigate the ability of the the precursors to migrate into and repopulate the thymus lobe to produce a 'chimaeric thymus' (Jenkinson *et al.*, 1982; Williams *et al.*, 1986), using different allelic markers to enable the donor and host cells to be distinguished.

Foetal liver from both day 12 and day 14 mouse embryos was used for this study for several reasons. Initially material was taken at day 12 of gestation since it was desirable to isolate early lymphoid precursors and this is the earliest stage that the liver can be easily distinguished and dissected from the other tissues in the developing embryo. One drawback, however, is the low cell recovery at this age (5×10^5 per liver in AKR mice). Since differentiated T, B and myeloid lineage cells are essentially still absent from the liver at day 14 gestation, and the cellularity has substantially increased by this stage to approximately 8×10^6 per liver, material was subsequently used from embryos at this gestational age.

4.2.1 Recolonisation with total foetal liver

Preliminary experiments were performed using the total cell population in day 12 or day 14 foetal liver, and progenitors giving rise to the T lymphoid lineage were identified on the basis of their ability to repopulate an alymphoid thymus lobe. Initially, foetal liver cells were titrated into alymphoid thymus explants and the cell recovery per lobe determined, as shown in Figure 4.1. Whole foetal liver from both day 12 and day 14 embryos contained cells with the capacity to repopulate a thymus explant. The minimum number of cells required to observe successful recolonisation (on the basis of an increase in overall size and cellularity compared to control levels) was 10^5 cells seeded per lobe. The minimum sensitivity of detection of cells recovered was approximately 2 x 10^3 cells, and it was arbitrarily decided that a recolonisation event would not be scored unless the cell recovery per lobe was at least twice this value. The maximum cellularity of control lobes (to which medium alone was added) was always below this value.

4.2.2 Anti-HSA enrichment of progenitors

Although the total foetal liver population from both day 12 or day 14 embryos was found to possess the capacity to recolonise deoxyguanosine-treated lobes and to give rise to cells expressing Thy1, CD4 and CD8, it was important to remove any irrelevant cells in order to purify the thymic progenitors in this tissue. The majority of haematopoietic cells in the embryonic liver and bone marrow express heat stable antigen (HSA), however it is believed that the early progenitors may lack this antigen, or only express it at low levels (Scollay *et al.*, 1984). Subsequent experiments therefore investigated the depletion of the foetal liver population by antibody plus complement lysis using a monoclonal antibody against HSA, as described in Chapter 2.

Anti-HSA antibody plus complement treatment of day 12 foetal liver resulted in depletion of between 80-95% of the starting population (the enrichment varied slightly between experiments), although a small percentage of HSA^+ cells invariably remained after depletion, suggesting some of the antibody had bound to, but not lysed the cells.

The recolonisation capacity of this enriched population in comparison to untreated foetal liver was determined in organ culture. Data from a representative experiment are given in Table 2, showing a comparison of the cell recovery from lobes recolonised with total and anti-HSA enriched foetal liver. Analysis of lobes recolonised with the anti-HSA depleted foetal liver over various periods of culture showed almost identical kinetics and phenotypic distribution of CD4 and CD8 as recolonisation with whole foetal liver (Figure 4.2).

Table 2 *Comparison of the cell recovery from thymus lobes repopulated with whole, or antibody-depleted foetal liver*

	Cell Yield per lobe (x 10^{-4})	
Day of recolonisation	**whole foetal liver**	**anti-HSA depleted liver**
8	0.7	1.9
10	2.6	3.8
14	15	15
17	13	50
Cell number seeded per lobe (x10^{-4})	10	1.5

Anti-HSA depletion therefore enriched for the progenitor population in day 12 foetal liver. This population yielded a similar frequency of recolonisation in comparison to untreated foetal liver, however, enrichment appeared to select for cells with a greater proliferative capacity, resulting in an increased expansion of cells within an alymphoid lobe.

The cells recovered from thymus lobes recolonised with the enriched population showed thymic maturation, as assessed by acquisition of Thy1, CD4 and CD8 (Figure 4.3). Different Thy1 allelic markers for donor AKR (Thy1.1) cells and recipient CBA (Thy1.2) lobes were used to demonstrate that the recolonising cells were of donor origin and not simply a result of expansion of pre-existing cells within the thymus explant. The same progenitor population in foetal liver failed to acquire Thy1, CD4 or CD8 if cultured in the absence of an alymphoid lobe, however, even in the presence of exogenous factors (con A supernatant, or WEHI-3 conditioned medium). The thymic microenvironment therefore appears to be a prerequisite for lymphoid development of these cells.

4.2.3 Density gradient separation of day 14 foetal liver cells

In order to further enrich for the progenitor cells detected using this assay system, five discontinuous albumin density gradients (30%, 29%, 26%, 23% and 10% albumin) were used to separate liver cells from day 14 embryos into four discrete subpopulations on the basis of the buoyant density of the component cells. These fractions were designated A, B, C, and D, and Figure 4.4 shows the location of each fraction and the percentage of the total cell number recovered in each fraction.

4.2.4 Characterisation of the density gradient fractions

Although no distinct subpopulations could be distinguished on the forward and side scatter profiles of each fraction, all four populations appeared be fairly heterogeneous on the basis of size and granularity (Figure 4.5). In addition, after placing the cells into tissue culture for several hours, both fractions A and B were seen to contain adherent as well as non-adherent cells, whereas C and D contained only non-adherent cells. The FSC profile for fraction B suggested that this fraction may contain higher proportion of large cells than in A, C and D. The formation of cell aggregates due to the presence of adherent cells in this fraction may account for this observation. The four gradient fractions were further characterised on the basis of their cell surface

phenotype, by monoclonal antibody staining and flow cytometric analysis, and on their repopulation ability *in vitro*.

Immunofluorescent analysis confirmed all four fractions to be negative for cell surface expression of CD4, CD8, CD3, Thy1, and membrane-bound immunoglobulin. All fractions contained cells which expressed HSA and phagocytic glycoprotein-1 (Pgp-1; Figure 4.6), although levels of expression varied between the gradient fractions. The cells in fractions C and D expressed high levels of HSA (the mean fluorescent intensity for the HSA^{hi} population was 127.5 and 137, respectively) whereas cells in the other two fractions showed less intense staining of this antigen (A, mean fluorescent intensity: 96.5; B: 92; Figure 4.6). Furthermore, the cells in fractions A and B could be subdivided into subpopulations of cells that were HSA-negative, those expressing low levels of HSA (HSA^{lo}), or those with relatively higher levels of expression (HSA^{hi}). Although the staining profiles were similar for both populations, fraction A appeared to contain a larger proportion of $HSA^{-/lo}$ cells (39%) than B (24%).

The staining profiles for fractions A and B also showed two distinct populations of Pgp-1 expressing cells, those negative or expressing low levels of Pgp-1 ($Pgp\text{-}1^{-/lo}$), and those expressing higher levels of this molecule ($Pgp\text{-}1^{hi}$). Fraction A contained a higher percentage of $Pgp\text{-}1^{-/lo}$ (90%) than $Pgp\text{-}1^{hi}$ cells (10%). However, in fraction B, a higher proportion of the cells (~30%), were contained within the $Pgp\text{-}1^{hi}$ subset. The cells contained in fractions C and D showed a unimodal distribution of Pgp-1 expression with fluorescence intensity intermediate to that of the two populations observed in fractions A and B (Figure 4.6). In addition to the differences in expression of HSA and Pgp-1, a small but discrete percentage of cells in fraction A (5%) and fraction B (3%) expressed CD45 - the common determinant of the leukocyte common antigen family, whilst the cells in fractions C and D were completely negative for this marker.

4.2.5 Recolonisation of alymphoid lobes using the albumin gradient fractions

All four gradient fractions were used as recolonising populations to seed alymphoid thymus lobes over a 10 day period. The lobes were assayed at various time points in organ culture and day 10 of recolonisation was chosen as an acceptable period of culture for subsequent experiments. After this period the recolonised lobes could be easily distinguished from the non-recolonised lobes on the basis of overall size and morphology. The cell recovery was also sufficient to allow immunofluorescent analysis to be performed on the repopulating cells, and the four subpopulations defined by CD4 and CD8 expression could be readily visualised by this stage.

As can be seen in Figure 4.7, only fractions A and B had the capacity to successfully recolonise a deoxyguanosine-treated lobe over this period, whereas fractions C and D gave very little cell recovery above control (non-recolonised) levels. Immunofluorescent analysis showed that cells recovered from lobes repopulated with both fraction A and B had acquired expression of Thy1.1 and HSA, and differentiated to exhibit the four CD4/CD8-defined subpopulations as found in a normal adult thymus (Figure 4.8). The cells recovered also expressed CD45, and Pgp-1 (Figure 4.8).

4.2.6 Anti-HSA depletion of the albumin gradient fractions

Subsequent experiments combined the two approaches used previously to enrich for the progenitor population in foetal liver. Day 14 material was first fractionated on a discontinuous albumin gradient, and the fractions enriched using anti-HSA plus complement treatment. This two-stage enrichment was only performed on the gradient fractions A and B, since C and D did not contain any precursor activity. This enrichment yielded a recolonising population with a higher precursor frequency than the non-depleted fractions, since alymphoid lobes seeded with the anti-HSA depleted samples gave higher cell recoveries than those seeded an equivalent number of progenitors from the untreated population. The results from a representative experiment are shown in Table 3.

Table 3 *Comparison of the recolonisation potential of untreated, or antibody depleted gradient fractions*

	Number of cells seeded ($x10^{-5}$)	Cell yield per lobe ($x10^{-4}$)
A untreated	5	4.14
B untreated	5	6.1
A anti-HSA depleted	0.3	11.4
B anti-HSA depleted	0.3	10.5

The cells recovered from the lobes repopulated with the anti-HSA depleted fractions also acquired expresssion of Thy1.1 and HSA (Figure 4.9), CD4, and CD8 (Figure 4.10), suggesting that the thymic precursor activity was contained within the HSA-negative (or HSA^{lo}) fraction.

4.2.7 Enrichment of albumin gradient fractions by MACS separation

The results of recolonisation studies using anti-HSA depleted day 14 foetal liver therefore indicated that precursor activity was contained within the HSA-negative fraction, although it was possible that the repopulation observed was due to the presence of a few contaminating HSA-positive cells in this fraction. As mentioned previously, it was difficult to obtain a completely HSA-negative population following depletion of the foetal liver cells using anti-HSA antibody plus complement, since not all of the cells which had bound antibody were lysed. In order to enable a cleaner enrichment to be achieved, an alternative separation approach was employed. After fractionation of the foetal liver on a discontinuous albumin gradient, the individual populations were enriched for HSA expression by magnetic bead separation, into HSA^+ and HSA^- populations (described in Chapter 2). The separations obtained were

still not complete and a number of positive cells were isolated with the depleted population (Figure 4.11). Alymphoid lobes recolonised with the population enriched for HSA^- cells yielded a greater cell recovery, however, than those recolonised with the HSA^+ enriched population, as seen with anti-HSA antibody plus complement treatment. In addition, the lobes recolonised with the HSA^- enriched fraction yielded cells expressing Thy1.1, CD4 and CD8, whereas the few cells recovered from the HSA^+ recolonised lobes were mainly $Thy1.1^-$, and therefore not of donor origin (Figure 4.12). The few $Thy1.1^-$ cells observed in the HSA^- enriched fraction are probably due to contaminating HSA^+ cells. The data were therefore consistent with the proposal that the precursor activity was contained within the HSA^- population.

4.2.8 Enrichment of gradient fractions by flow cytometric sorting

Enrichment of albumin gradient fractions of foetal liver using antibody and complement depletion, or by magnetic cell separation was therefore not completely effective as a method of obtaining a purified population of HSA-negative cells. Although these studies indicated that the progenitor activity was contained within the HSA-negative population of day 14 foetal liver cells, the possibility that the precursor activity observed was due to a small number of contaminating HSA-positive cells could still not be excluded. In order to formally exclude the possibility that the HSA-positive population contained any precursor activity, day 14 foetal liver cells were initially separated by density gradient fractionation, and subsequently enriched by sterile flow cytometric separation, into HSA-negative and HSA-positive fractions.

The foetal liver cells were fractionated into HSA^- and HSA^+ populations, as shown in Figure 4.13, the HSA^- cells representing approximately 3% of the foetal liver population in A, and 11% in B. The cells expressing low levels of HSA were excluded from the populations sorted. Analysis of the sorted populations showed that both HSA^- and HSA^+ cells expressed Pgp-1, and in both HSA-negative fractions (A and B), approximately 50% of the cells expressed CD45 (data not shown).

Both sorted populations were seeded into alymphoid lobes and the lobes harvested after 10 days (details of recolonisation are given in the legend to Figure 4.14). The lobes recolonised with the HSA^- population gave a higher efficiency of recolonisation, and a greater cell recovery than those recolonised with the HSA^+ population; 80-90% of the total number of lobes seeded with HSA^- cells were successfully recolonised, whereas less than 5% of the total number of lobes seeded with the HSA^+ population gave a cell recovery above control levels, the latter population thus containing very little progenitor activity. Furthermore, the cells recovered from lobes repopulated with the HSA^- cells were $Thy1.1^+$, i.e. of donor origin, and had acquired expression of HSA (Figure 4.14), CD4, CD8, and CD45 (Figure 4.15). The few cells recovered from the HSA^+ repopulated lobes lacked Thy1.1 expression. The thymic progenitor activity in foetal liver was therefore confirmed to be contained within the HSA-negative population.

4.3 Discussion

During foetal life haematopoiesis initially occurs in the yolk sac and subsequently in the liver after day 11 of gestation. The foetal liver remains the major haematopoietic organ until day 15 when the foetal spleen and bone marrow assume this role, the bone marrow becoming the major haematopoietic site after birth (Metcalf & Moore, 1971). The foetal liver is thus the source of immigrant precursors to the embryonic thymus, and hence a protocol was established in order to isolate thymic progenitors from this tissue. Using the thymus organ culture technique of Jenkinson *et al.* (Jenkinson *et al.*, 1982) it has been possible to characterise a population of progenitor cells which has the capacity to recolonise an alymphoid thymus lobe *in vitro* and develop along the T lymphoid lineage.

The heat stable antigen (HSA) was first identified by its resistance to thermal denaturation (Springer *et al.*, 1978), and was subsequently shown to be recognised by a series of monoclonal antibodies, including J11d (Bruce *et al.*, 1981), M1/69 (Springer *et al.*, 1978), B2A2 (Scollay *et al.*, 1984) and YBM 5.10.4 (Watt *et al.*,

1987), which appear to recognise the same antigenic determinant (Alterman *et al.*, 1990). Its precise function is unknown, although it has been suggested as a marker of haematopoietic differentiation, since it is expressed on most cells in the bone marrow, on eosinophils, neutrophils, primary B cells, erythrocytes, and on immature cortical thymocytes, however, it is not expressed by mature T cells nor memory B cells (Holmes & Morse, 1988). Evidence suggests that HSA may be a potential marker for thymic progenitors, since $CD4^-CD8^-$ thymocytes which express HSA have been demonstrated to have reconstitution potential on intrathymic transfer, whereas HSA-negative thymocytes do not (Crispe *et al.*, 1987). In addition, recent studies by Wu *et al.* have identified an early precursor in adult thymocytes which is also active in thymic reconstitution assays, and expresses HSA, Pgp-1 and low levels of CD4 (Wu *et al.*, 1991). Although the evidence suggests that the most immature cells in the thymus are $CD3^-CD4^-CD8^-$ and HSA^+, the status of this molecule on immigrant precursors from the foetal liver or bone marrow is uncertain. Scollay *et al.* have identified precursors in the bone marrow that are HSA^- (Scollay *et al.*, 1984), which may suggest that acquisition of HSA may occur just before or immediately after thymic entry. It was therefore of interest to investigate the role of this marker in development.

Since both the foetal liver and the bone marrow fulfil a haematopoietic role, it appears likely that both tissues may contain similar precursor activity. The identification of HSA-negative precursors in bone marrow therefore prompted the initial investigation of the significance of HSA expression on precursor cells in foetal liver. The first approach used was to simply deplete the HSA-expressing cells in foetal liver and assay the recolonisation capacity of this population. Depletion of the majority of the foetal liver cell population by antibody and complement lysis appeared to enrich for progenitor activity, assessed by *in vitro* recolonisation of alymphoid thymus lobe. In order to deplete more than 80% of the starting population, however, at least two rounds of complement lysis were required. To improve the efficiency of this approach, other strategies were investigated to remove irrelevant cells in this tissue before HSA

depletion. Fractionation using a discontinuous albumin gradient was found to enable efficient separation of the blast cells from the "resting" cell population.

The four fractions A, B, C, and D obtained by density gradient separation all expressed a similar phenotype, all negative for Thy1, CD4, CD8, CD3 and surface immunoglobulin, however, all expressed HSA and Pgp-1 although at different levels on the cell surface. The similarity between the phenotype of C and D may suggest that they comprise common cell types, although neither population contains progenitor activity in this assay system. Although fractions A and B both have the capacity to recolonise an alymphoid lobe *in vitro*, they appear to have different expansion capacities, perhaps indicating that they may contain precursor populations at different developmental stages. The pattern of HSA and Pgp-1 expression may also suggest that they are related populations, and in accordance with the proposal that the earliest precursors are HSA-negative, the differences observed in levels of expression may imply that fraction B contains precursors at a slightly later stage of development to those contained in A. The similarities between the two fractions are unlikely to be a result of cross-contamination during collection, however, since they form discrete bands on the gradient, and fractionate at different densities (A between 10% and 23% albumin gradients; and B between 23% and 26% albumin gradients), suggesting that the two populations contain distinct cell types.

Further enrichment of the gradient fractions using antibody plus complement depletion or magnetic cell separation also showed that the cells with recolonisation potential were contained within the HSA-depleted fraction. This was consistent with the proposal that precursor activity was contained within the HSA-negative population, although the evidence was not conclusive. Flow cytometric sorting of the gradient fractions on the basis of HSA expression subsequently confirmed that the early thymic progenitor activity was contained within the HSA^- population of both A and B, and little if any repopulation capacity in the HSA^+ fraction (Figure 4.14 & 4.15).

The early lymphoid precursor population identified in this study was therefore found to be Pgp-1 positive, HSA^-, $Thy1^-$, $CD3^-$, $CD4^-$ and $CD8^-$, and represented

approximately 1-3% of total foetal liver. Pgp-1 (CD44) is a polymorphic integral membrane glycoprotein, and has been postulated to be involved in matrix adhesion, lymphocyte activation, and lymphocyte homing (Trowbridge *et al.*, 1982). Pgp-1 has been extensively studied as a marker of T lymphocyte subsets and their precursors, and its acquisition by precursors destined for the thymus has been previously documented (Hyman *et al.*, 1986; Lesley *et al.*, 1985). The expression of Pgp-1 on lymphoid progenitors in the foetal liver is therefore consistent with these data. Furthermore, the addition of anti-Pgp-1 antibodies to long term bone marrow cultures has been reported to inhibit the production of lymphoid and myeloid cells (Miyake *et al.*, 1990). This finding implies that Pgp-1 may be critically important for cell interactions necessary for haemopoiesis in the bone marrow.

Analysis of the HSA-negative sorted population also showed that approximately 50% the population expressed CD45 (expressed by most cells of the haematopoietic and lymphoid lineage), and following recolonisation, all of the repopulating cells had acquired expression of this marker. This implies that the precursor population identified contains more than one cell type. It would therefore be useful to determine whether the precursor activity was contained in the $CD45^+$ or $CD45^-$ fraction, and whether this expression was correlated to developmental status or lineage restriction. Although the expression of the different isoforms of CD45 on the HSA^- progenitor cells was not determined, this should be the subject of further investigation in order to clarify the situation regarding isoform expression on early precursors. Evidence suggests that bone marrow stem cells are CD45-positive (Spangrude *et al.*, 1989), and that immature precursors in the thymus express high molecular weight isoforms of CD45 (Goff *et al.*, 1990), although there are no reports of the CD45 status of early precursors in the embryonic liver. The data presented here, however, imply that lymphoid progenitors in the foetal liver may express CD45.

In addition to haematopoietic progenitor cells, the foetal liver contains hepatic cells and their precursors, however, it is likely that the hepatic cells will be sensitive to the mechanical disruption employed to dissociate the tissue. Although hepatic precusor

cells and myeloid cells may survive this treatment and be present in the progenitor population isolated, the *in vitro* assay system of thymic organ culture is unlikely to provide the necessary microenvironment for their growth and maintenance. Furthermore, the cells which are able to repopulate the thymus must first migrate into the alymphoid explant, and subsequently expand and differentiate within the lobe, thus selecting for precursors which possess both the capacity to "home" and the ability to repopulate a thymic explant.

In vitro culture of the anti-HSA plus complement enriched progenitor population on a cloned thymic stromal cell monolayer (Larsson *et al.*, 1991) failed to allow development of T lymphocytes, as defined by the acquisition of Thy1, CD4 and CD8. However, the liver-derived progenitors were apparently able to mature along the monocyte/macrophage pathway, as assessed by Giemsa staining, with or without the addition of exogenous factors, such as con A supernatant, or WEHI-3 conditioned medium (data not shown). This may suggest that the foetal liver cells isolated by anti-HSA depletion were perhaps bipotential, and not only restricted to the lymphoid pathway. It therefore appears that the thymic microenvironment is a prerequisite for T lymphocyte development, providing the appropriate signals (e.g. cell surface accessory molecules, and matrix for presenting growth factors) to commit the cells to the T lineage pathway. The recolonisation assay in the thymus organ culture system thus appears to provide a useful approach to identifying thymic progenitors, and although it does not appear to support B cell development, in contrast to *in vivo* systems, it enables relatively simple isolation and identification of the donor progeny.

A crucial question remaining to be addressed from this study, however, is whether the foetal liver progenitors identified have undergone rearrangement of their T cell antigen receptor genes, which would suggest commitment of these cells to the T lymphocyte pathway, or whether these cells still retain the capacity to develop along the B lymphoid lineage. Furthermore, it is important to determine what differences exist between the precursor populations in the foetal liver and bone marrow. The *in vivo*

reconstitution capacity of this population needs to be addressed in order to resolve these questions.

Although most attention has been focussed on the bone marow and the thymus as a source of precursor cells, it is also important to compare the progenitor activity of both foetal and adult haematopoietic stem cells in order to be able to fully understand the mechanisms of differentiation and self-renewal. At the time this work was commenced, very little work had been published regarding progenitor activity in murine foetal liver. Subsequent studies, however, have identified haematopoietic stem cells in this tissue (Jordan *et al.*, 1990; Ikuta *et al.*, 1990), and isolated a haematopoietic stem cell in foetal liver with the same phenotype to that already documented in the bone marrow, i.e. $Thy1^{lo}$ Lin^- $Sca\text{-}1^+$ (Ikuta *et al.*, 1990). The HSA and Pgp-1 phenotype of these cells was not reported. The ability to isolate haematopoietic progenitors from this tissue suggests that foetal liver provides a valuable source of material for future studies addressing stem cell function to complement those currently involving bone marrow progenitors.

Figure 4.1 *Titration of foetal liver cells into alymphoid lobes.*

The number of precursor cells seeded per lobe was titrated and the subsequent cell recovery determined. The titration shows a representative experiment, using day 14 foetal liver cells and recolonisation over a period of 10 days. The minimum number of cells required to be seeded to give a successful recolonsiation event above control levels was determined to be 10^5 cells per lobe. The minimum sensitivity of detection of cells recovered was approximately 10^3 cells. Non-recolonised lobes always gave a maximum cellularity below this value.

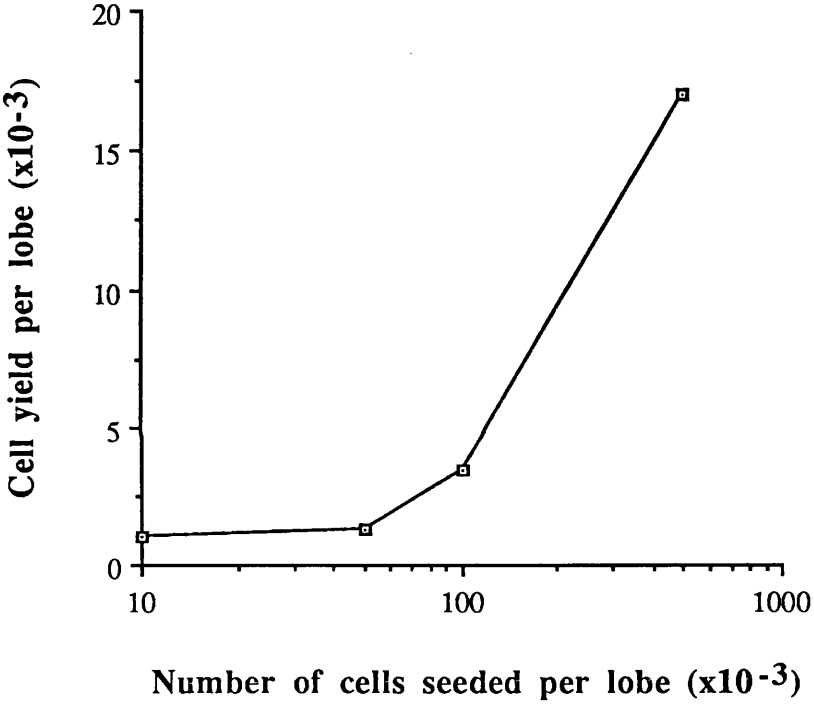

20
15
10
5
0
10
100
1000
Cell yield per lobe (x10-3)
Number of cells seeded per lobe (x10-3)

Figure 4.2 *Comparison of the kinetics and phenotypic distribution of lobes recolonised with whole vs. anti-HSA depleted day 12 foetal liver.*

Alymphoid lobes were recolonised with equivalent concentrations of precursor cells, either whole (untreated) day 12 foetal liver cells, or foetal liver cells following anti-HSA antibody plus complement depletion, and cultured for 8 to 17 days. At each time point shown the lobes were harvested and the cells recovered were stained for CD4 and CD8 expression. The cells were analysed by flow cytometry and the percentage of CD4 and CD8 expressing cells determined. The solid panels represent the phenotype of cells recovered from lobes repopulated with whole (untreated) foetal liver, the hatched panels represent the phenotype of cells recovered from lobes repopulated with anti-HSA depleted foetal liver.

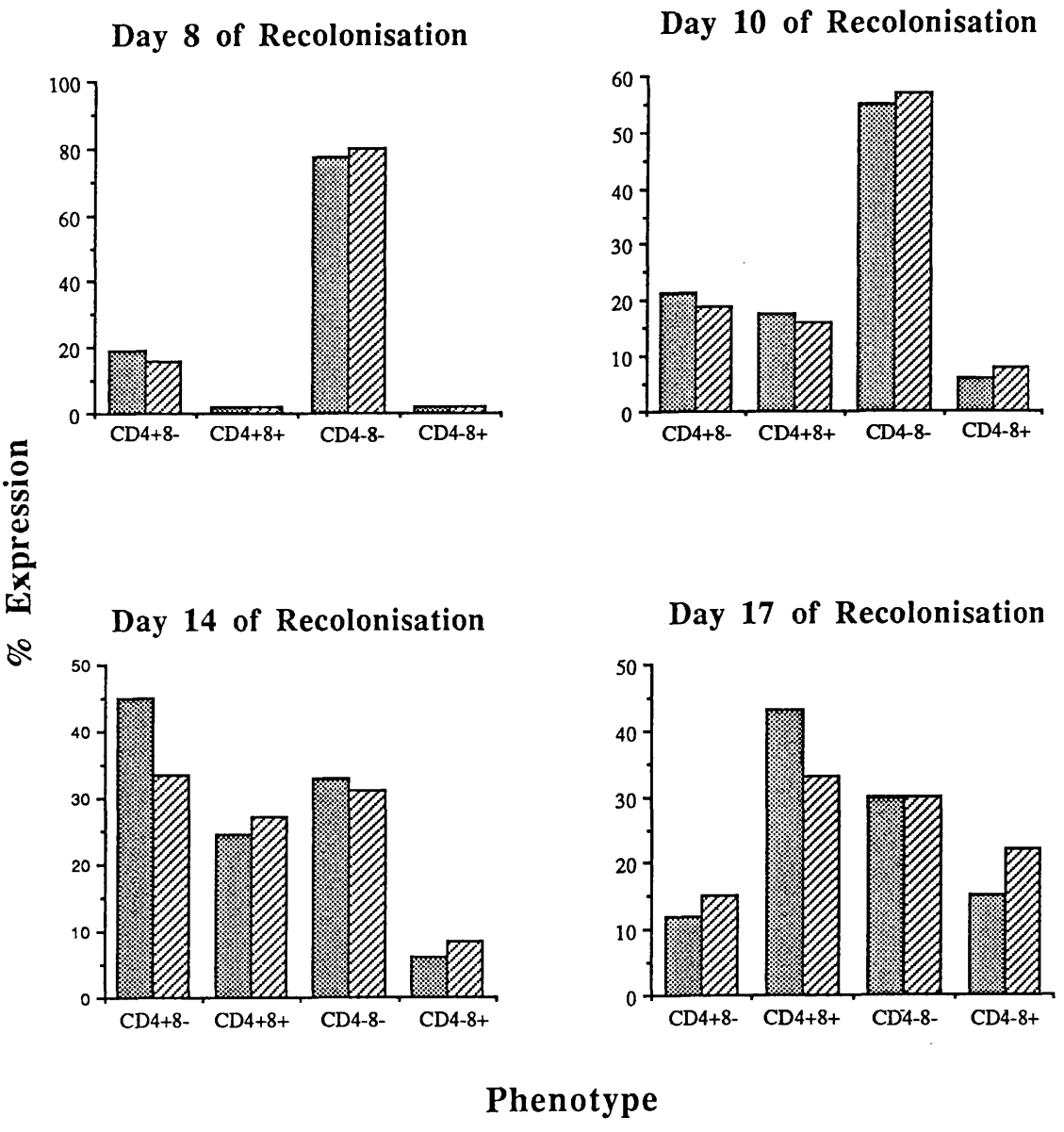
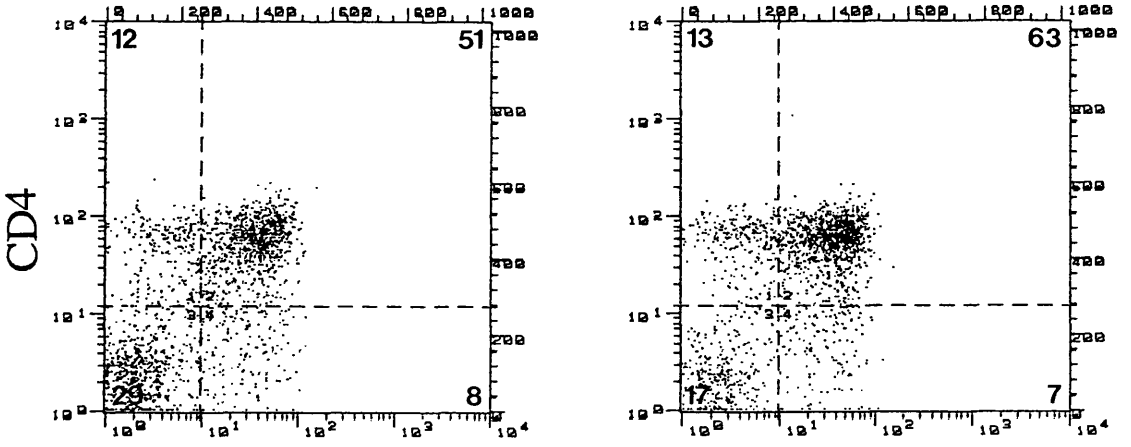

Day 8 of Recolonisation
Day 10 of Recolonisation
Day 14 of Recolonisation
Day 17 of Recolonisation
% Expression
Phenotype
CD4+8-
CD4+8+
CD4-8-
CD4-8+
whole foetal liver
HSA-depleted

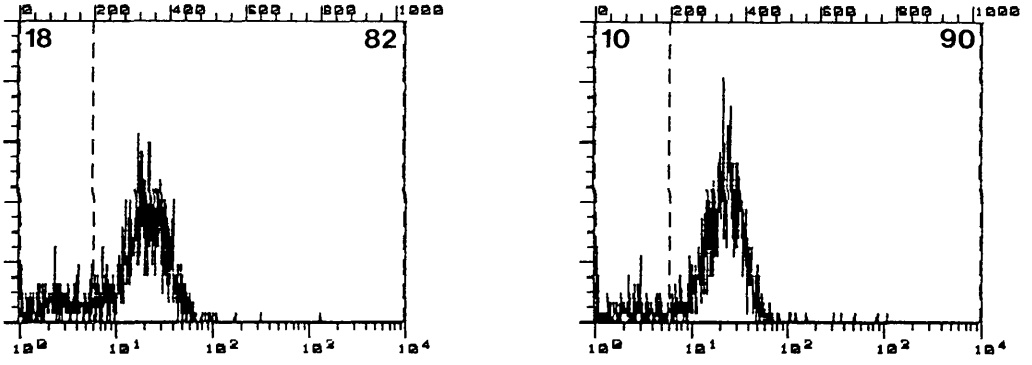
Figure 4.3 *Acquisition of Thy1, CD4 and CD8 by anti-HSA depleted foetal liver cells.*

The cells recovered from alymphoid lobes (CBA: Thy1.2) repopulated with anti-HSA depleted foetal liver cells (AKR: Thy1.1) were analysed for expression of Thy1.1, HSA, CD4 and CD8. The phenotype of cells recovered from alymphoid lobes repopulated with day 14 foetal thymocytes is shown for comparison. The cells recovered were stained using a FITC-conjugated anti-Thy1.1 monoclonal antibody (T11D7), and a biotinylated anti-HSA monoclonal antibody (YBM 5.10.4), plus phycoerythrin-conjugated streptavidin second stage reagent; or a FITC-conjugated anti-CD8 monoclonal antibody (53.6.7), and a PE-conjugated anti-CD4 monoclonal antibody (GK 1.5). Flow cytometry was performed as described previously, and a minimum of 3000 events were analysed for each sample. Fluorescence intensity is represented on a log scale.

HSA-depleted foetal liver

day 14 thymocytes





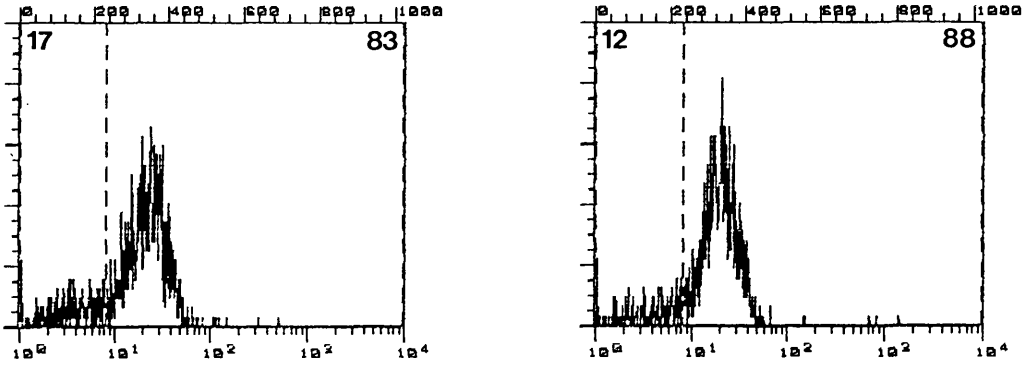


Figure 4.4 *Separation of 14 day foetal liver cells on the basis of their buoyant density, by discontinuous albumin gradient fractionation.*

A suspension of day 14 foetal liver cells was fractionated on a dicontinuous albumin gradient and each fraction was recovered from the interface between two density gradients, as shown. The fractions recovered were designated A, B, C, and D. The cell recovery in each fraction is expressed as a percentage of the total, and is the mean value from four separate experiments.

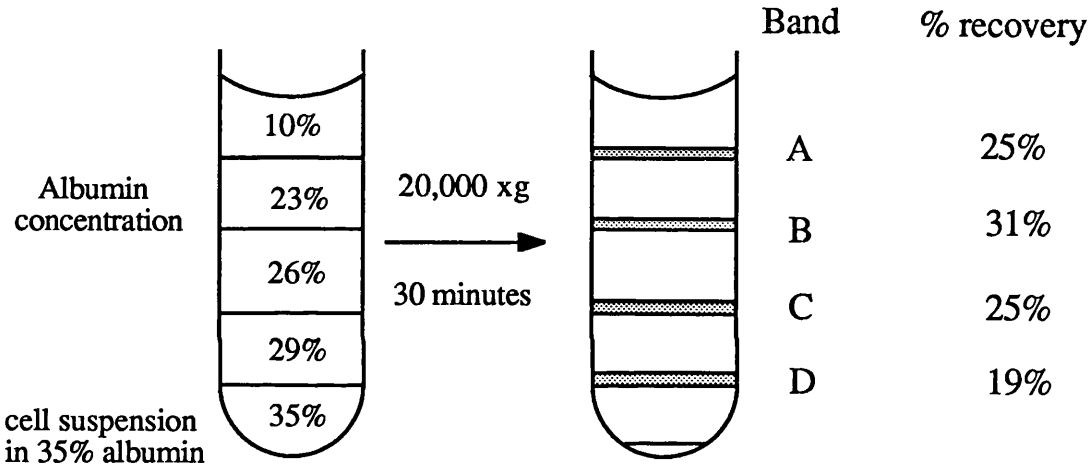

Band
% recovery
10%
23%
26%
29%
35%
Albumin concentration
cell suspension in 35% albumin
20,000 xg
30 minutes
A
25%
B
31%
C
25%
D
19%

Figure 4.5 *FSC and SSC profiles of the four fractions obtained by discontinuous albumin gradient separation of day 14 foetal liver.*

The forward and side scatter profiles of ungated samples of each of the four fractions obtained by discontinuous albumin gradient separation are shown. FSC and SSC are represented on the abscissa on a linear scale, and cell number is shown on the ordinate. The fractions appear fairly heterogeneous, although the modal value of each of the profiles varied (FSC: A 101; B 117; C 93; D 104; SSC: A 71; B 74; C 82; D 72). The profiles were analysed using Consort 30 software.

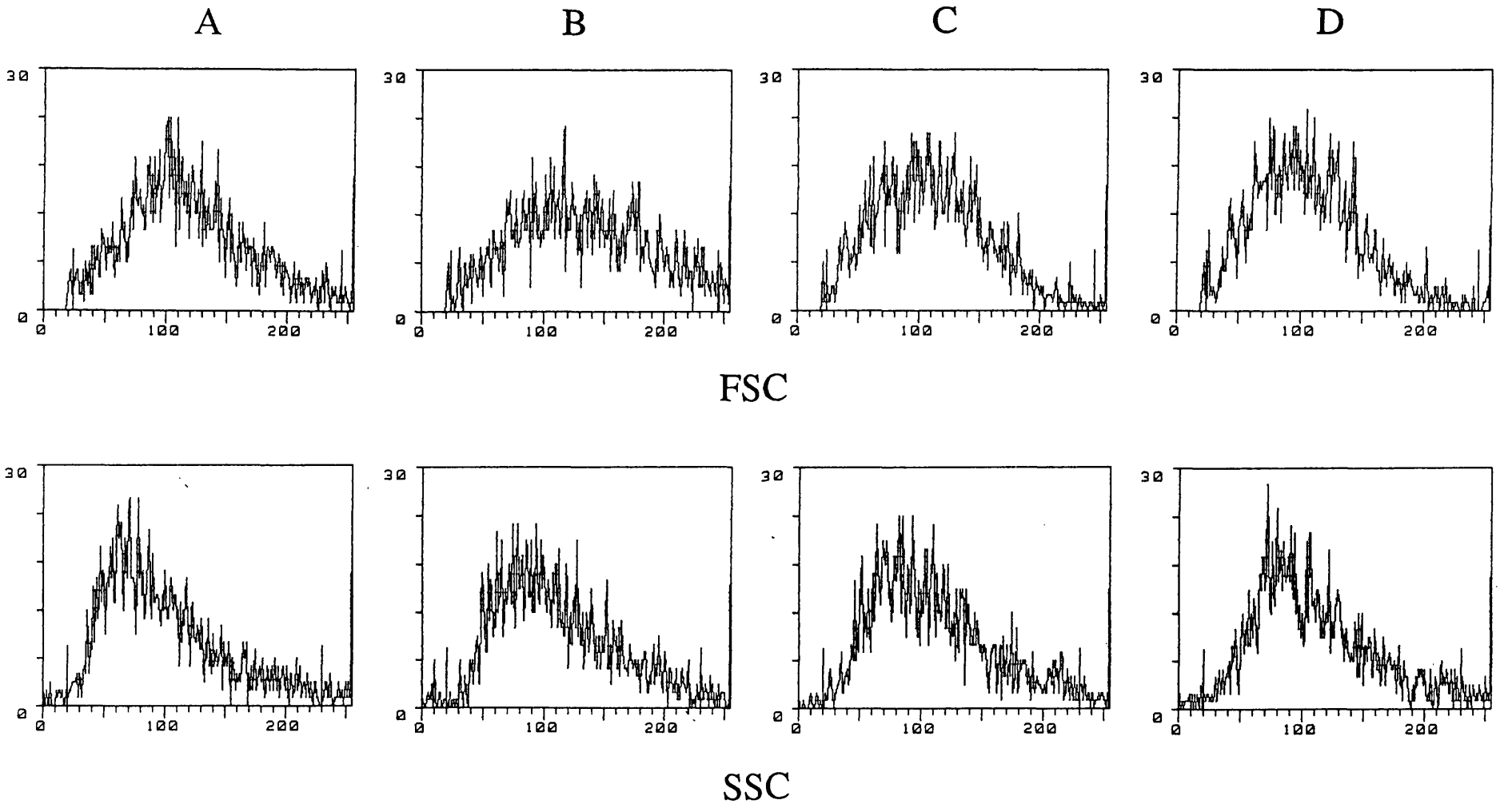

A
B
C
D
30
0
0
100
200
FSC
SSC

Figure 4.6 *Cell surface phenotype of the four fractions obtained by discontinuous albumin gradient separation of day 14 foetal liver.*

Flow cytometric analysis of HSA and Pgp-1 expression of each fraction. Cells in each fraction were stained using a FITC-conjugated anti-HSA monoclonal antibody (YBM 5.10.4), or a FITC-conjugated anti-Pgp-1 monoclonal antibody (IM7.8.1); the control shows staining with a FITC-conjugated anti-mouse Ig antibody. Fluorescence intensity is shown on the abscissa, and cell number on the ordinate. All histograms are represented on the same scale. Multiple histogram analysis was performed using FACScan research software.

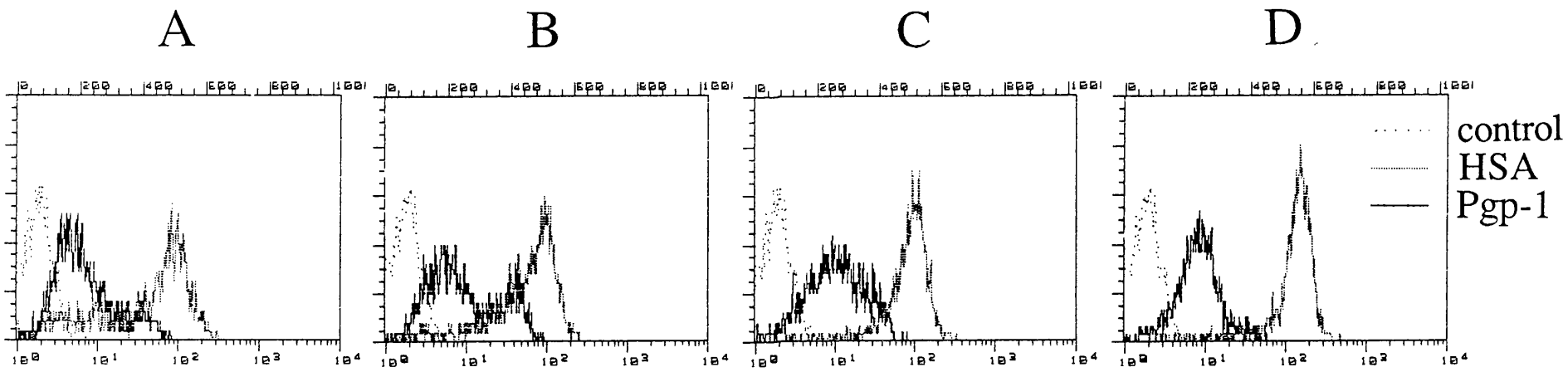

A
B
C
D
control
HSA
Pgp-1

Figure 4.7 *In vitro recolonisation potential of albumin gradient fractions.*

Cells from each albumin gradient fraction of day 14 foetal liver were used to recolonise alymphoid lobes, seeded at 2 x 10^5 cells per lobe, and cultured for 10 days. The lobes were harvested and the number of cells recovered per lobe in each experimental group calculated. The cell yields are presented as the mean value of data obtained from three separate experiments, and the standard deviations are shown.

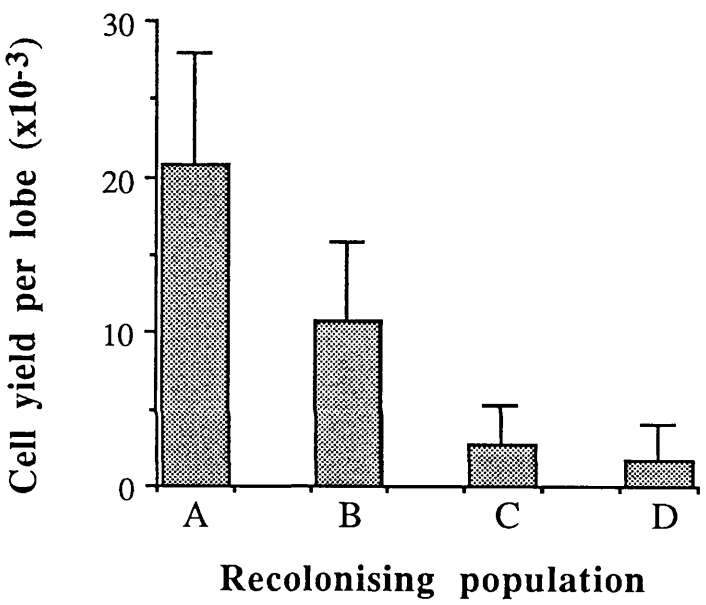
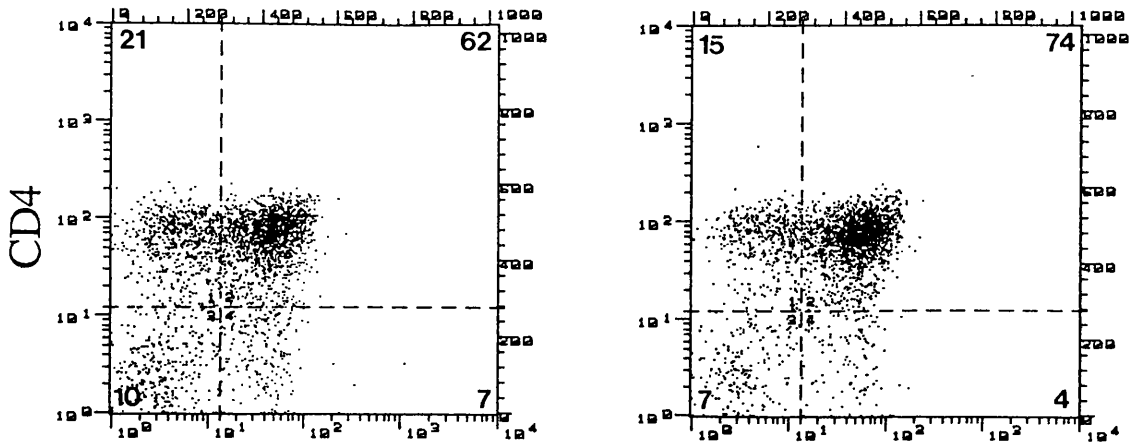
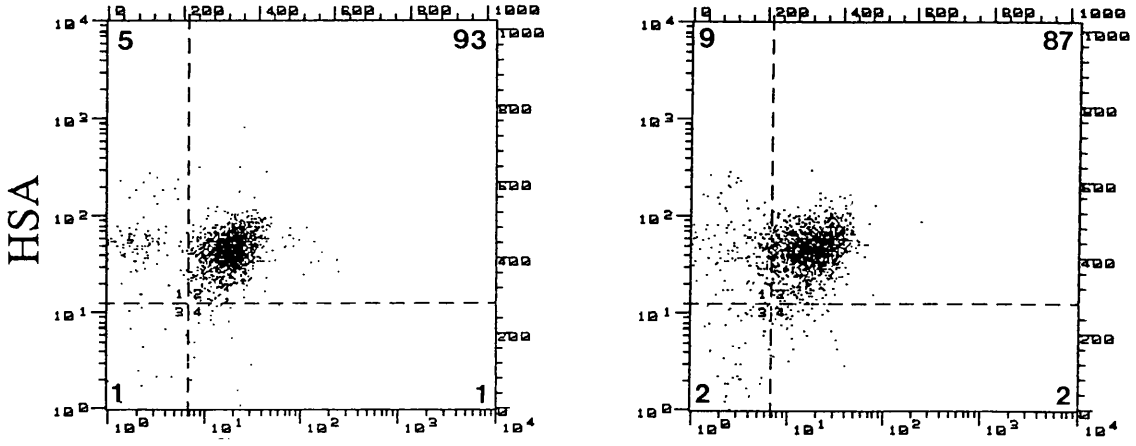
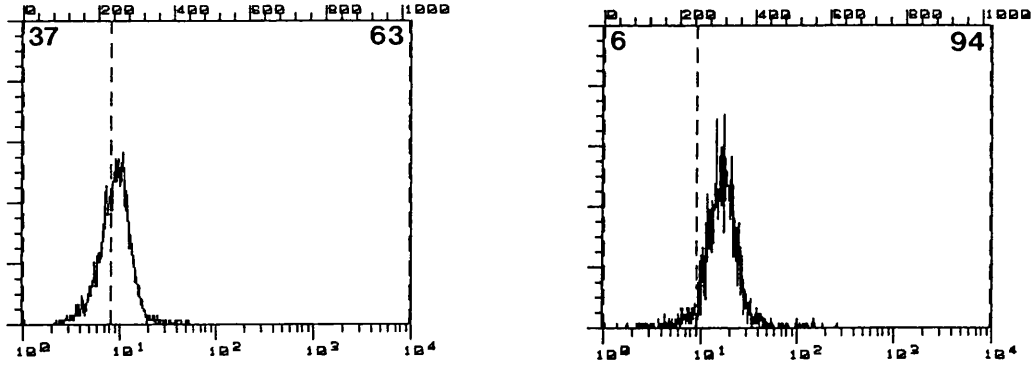

Cell yield per lobe (x10-3)
30
20
10
0
A
B
C
D
Recolonising population

Figure 4.8 *Phenotype of cells recovered from alymphoid lobes repopulated with albumin gradient fractions A and B.*

Cells from each albumin gradient fraction, A and B, of day 14 foetal liver were used to recolonise alymphoid lobes, seeded at 2 x 10^5 cells per lobe, and cultured for 10 days. Alymphoid CBA lobes (Thy1.2) were recolonised with foetal liver cells from AKR mice (Thy1.1). The cells recovered from lobes recolonised with fraction A, or fraction B were stained for expression of Thy1.1, HSA, CD4 and CD8 as described in the legend to Figure 4.3; or with a biotinylated anti-Pgp-1 antibody (IM7.8) plus PE-conjugated streptavidin, and FITC-conjugated anti-CD45 antibody (M1/93). Fluorescence intensity is represented on a log scale on the two-dimensional plots. The percentage expression in each quadrant is shown. All histograms are represented on the same scale, with fluorescence intensity shown on the abscissa, and cell number on the ordinate.


CD4
21
62
10
7
15
74
7
4
CD8


HSA
5
93
1
1
9
87
2
2
Thy1.1


37
63
6
94
Pgp-1

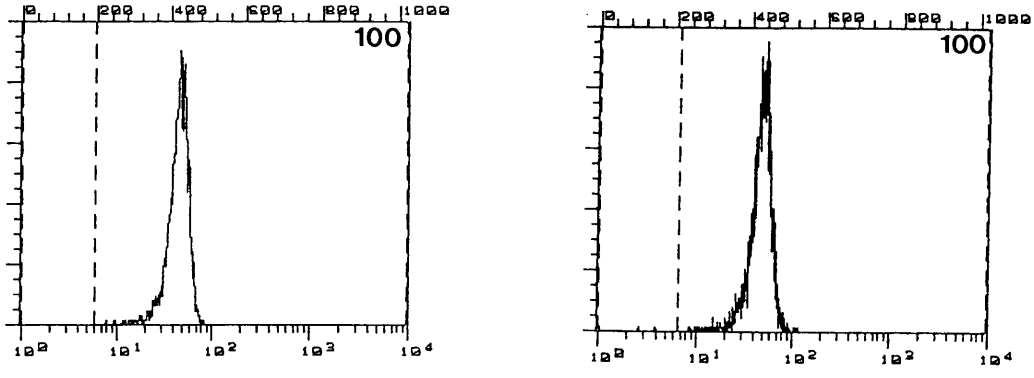
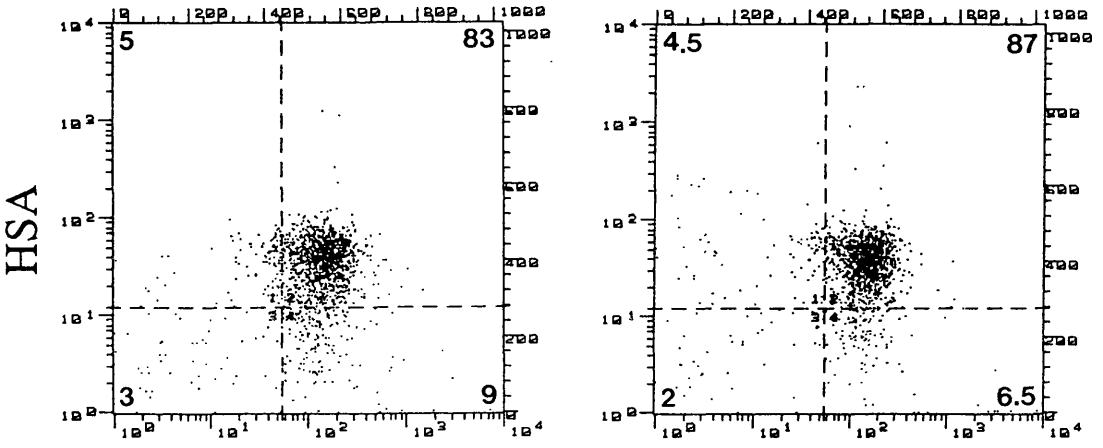

100
100
CD45

Figure 4.9 *Expression of Thy1.1/HSA on lobes repopulated with anti-HSA depleted albumin gradient fractions, A and B.*

Day 14 AKR foetal liver cells were first separated by discontinuous albumin gradient separation and fractions A and B subsequently depleted using anti-HSA antibody plus complement lysis. Both depleted populations contained 6% of the starting population. Untreated and anti-HSA depleted samples were resuspended in the same volume to give the same concentration of progenitors. The depleted populations were used to recolonise alymphoid CBA lobes over a period of 10 days. The cells recovered from recolonised lobes were analysed for expresssion of Thy1.1 and HSA as described in the legend to Figure 4.3.

A untreated

B untreated



A HSA-depleted

B HSA-depleted

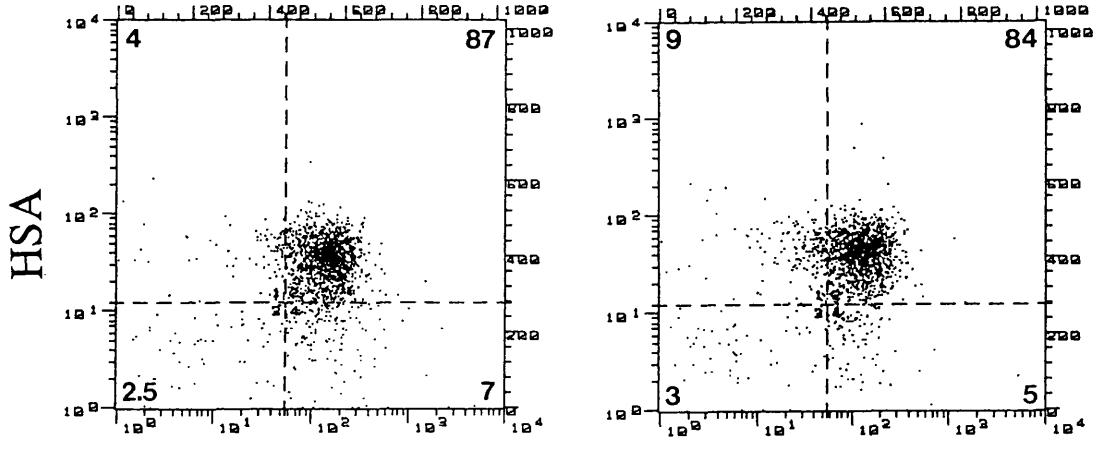
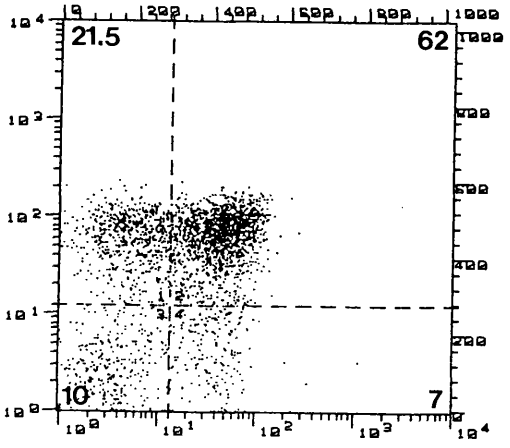


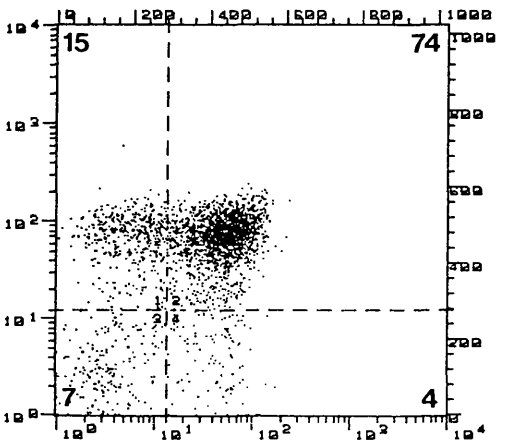
Figure 4.10 *Expression of CD4 and CD8 on lobes repopulated with anti-HSA depleted albumin gradient fractions, A and B.*

Anti-HSA depleted fractions A and B were used to recolonise alymphoid lobes as described in the legend to Figure 4.9. The cells recovered from recolonised lobes were analysed for expression of CD4 and CD8 as described in the legend to Figure 4.3.

A untreated



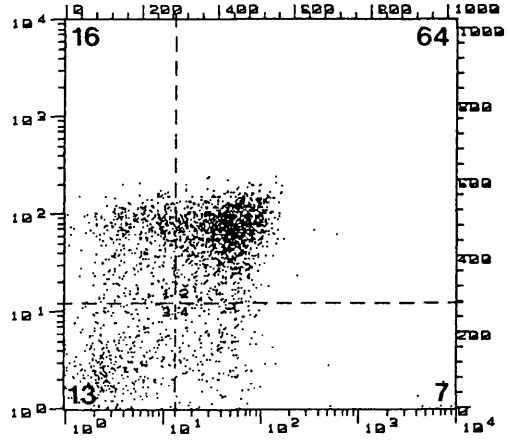
B untreated



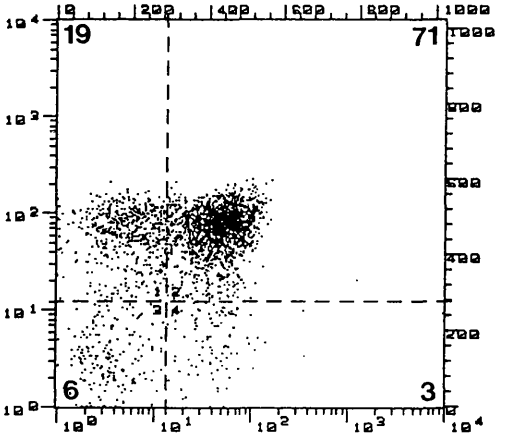
CD4

CD8

A HSA-depleted



B HSA-depleted



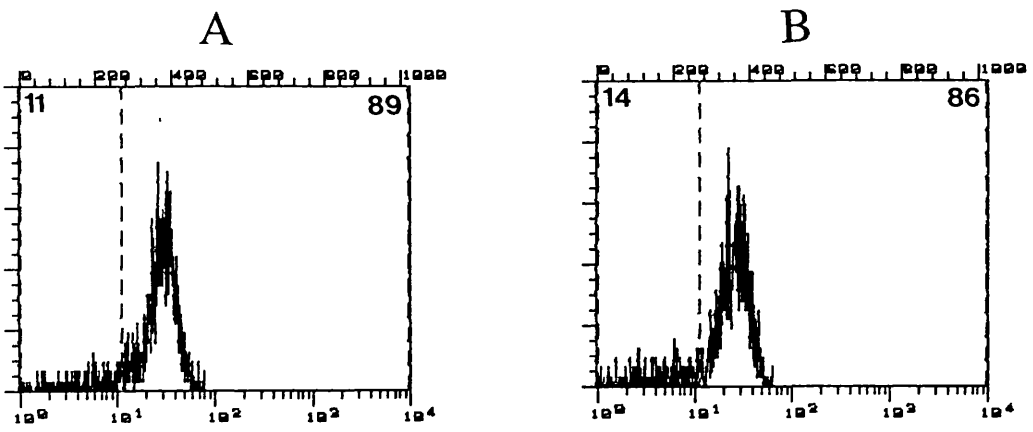
CD4

CD8

Figure 4.11 *MACS separation of albumin gradient fractions, A and B.*

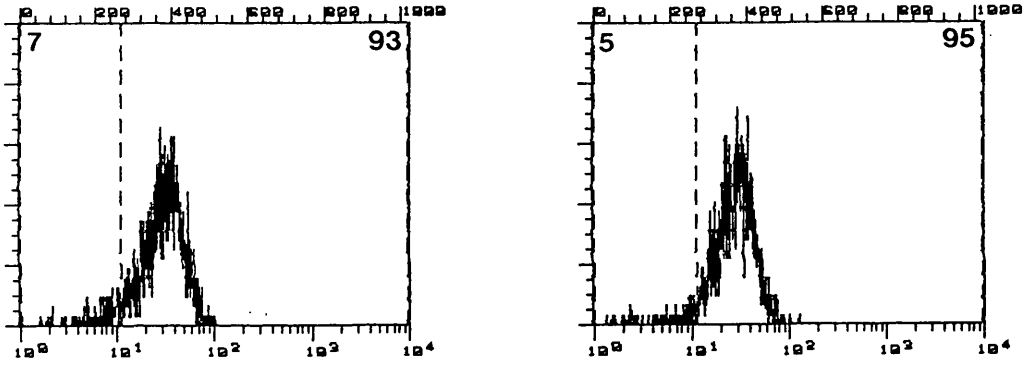
The profiles of the HSA-enriched populations of fractions A and B are shown: (a) unseparated; (b) HSA^+ enriched; and (c) HSA^- enriched fractions. Albumin gradient fractions A and B were further enriched on the basis of HSA expression using magnetic bead separation. Cells were incubated with a biotinylated anti-HSA antibody, FITC-conjugated streptavidin second stage reagent, and biotinylated magnetic beads. The labelled cells were then separated by passing through a strong magnetic field. The unseparated; HSA^+ enriched, magnetic fraction; and HSA^- enriched, non-magnetic fraction, were analysed by flow cytometry to determine the efficiency of separation. Fluorescence intensity is represented on the abscissa, and cell number on the ordinate. All histograms are shown on the same scale.

(a)



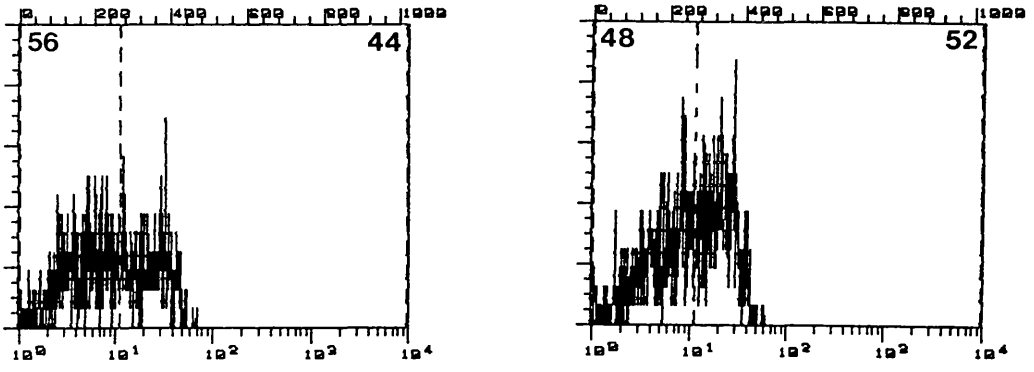
HSA

(b)



HSA

(c)



HSA

Figure 4.12 *Thy1.1/HSA expression of cells recovered from lobes recolonised with fractions enriched by MACS separation.*

Alymphoid thymus lobes were recolonised with gradient fractions A and B, enriched for HSA^+ or HSA^- cells, as described in the legend to Figure 4.11. Cells were stained for expression of Thy1.1 and HSA as described in the legend to Figure 4.3.

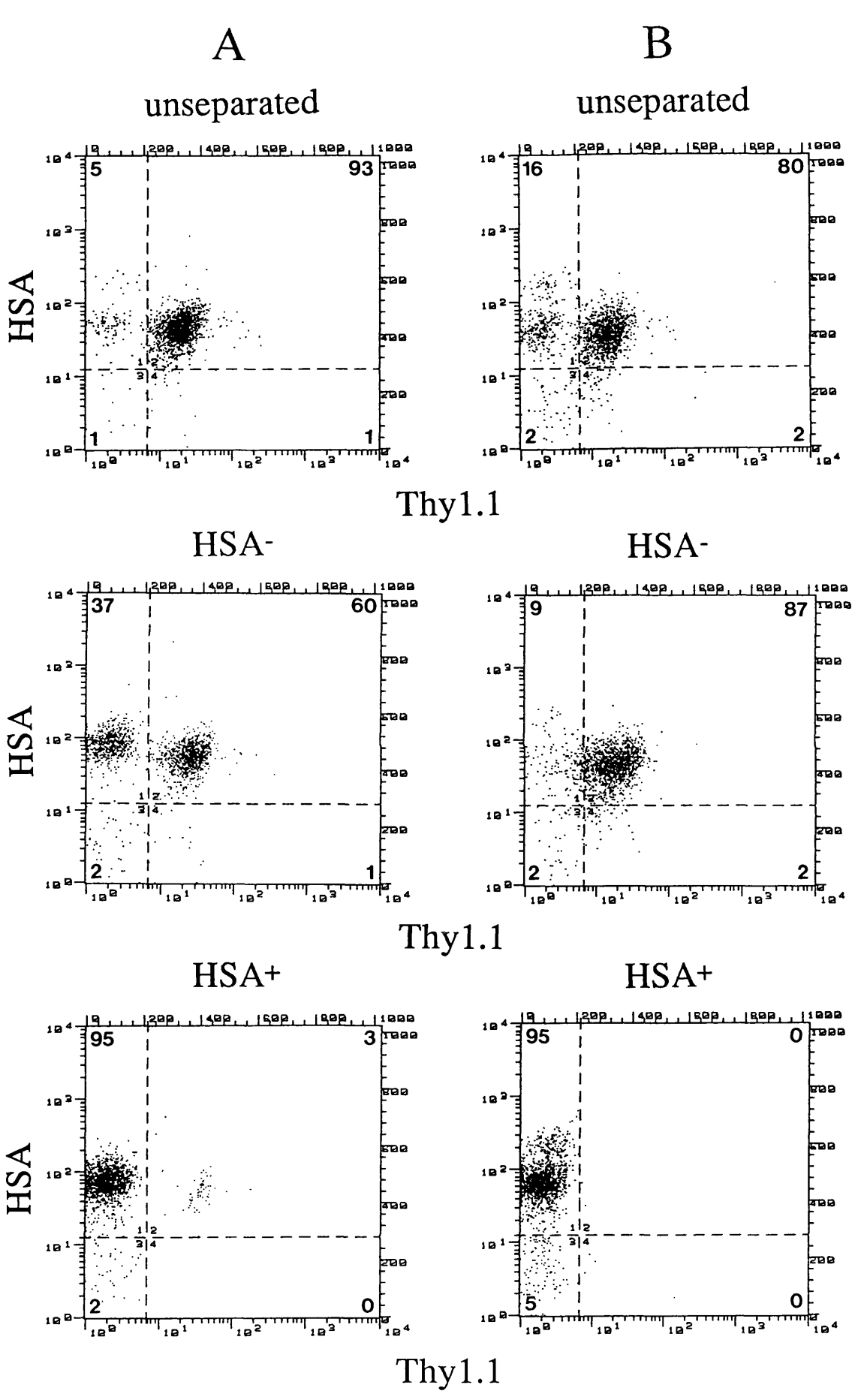

A
B
unseparated
unseparated
HSA
5
93
1
1
16
80
2
2
Thy1.1
HSA-
HSA-
37
60
2
1
9
87
2
2
Thy1.1
HSA+
HSA+
95
3
2
0
95
0
5
0
Thy1.1

Figure 4.13 *Flow cytometric separation of albumin gradient fractions A and B.*

Sterile flow cytometric separation of albumin gradient fractions A and B into HSA-negative and HSA-postive populations. Cells were stained using an anti-HSA antibody and sterile sorted on a FACStar plus. The cells were separated into HSA^- (left sorted, L) and HSA^+ (right sorted, R) expressing cells as shown. The HSA^- cells represented 3%, and the HSA^+ cells represented 91%, of the total fraction A population; the HSA^- cells represented 11%, and the HSA^+ cells represented 77%, of the total fraction B population.

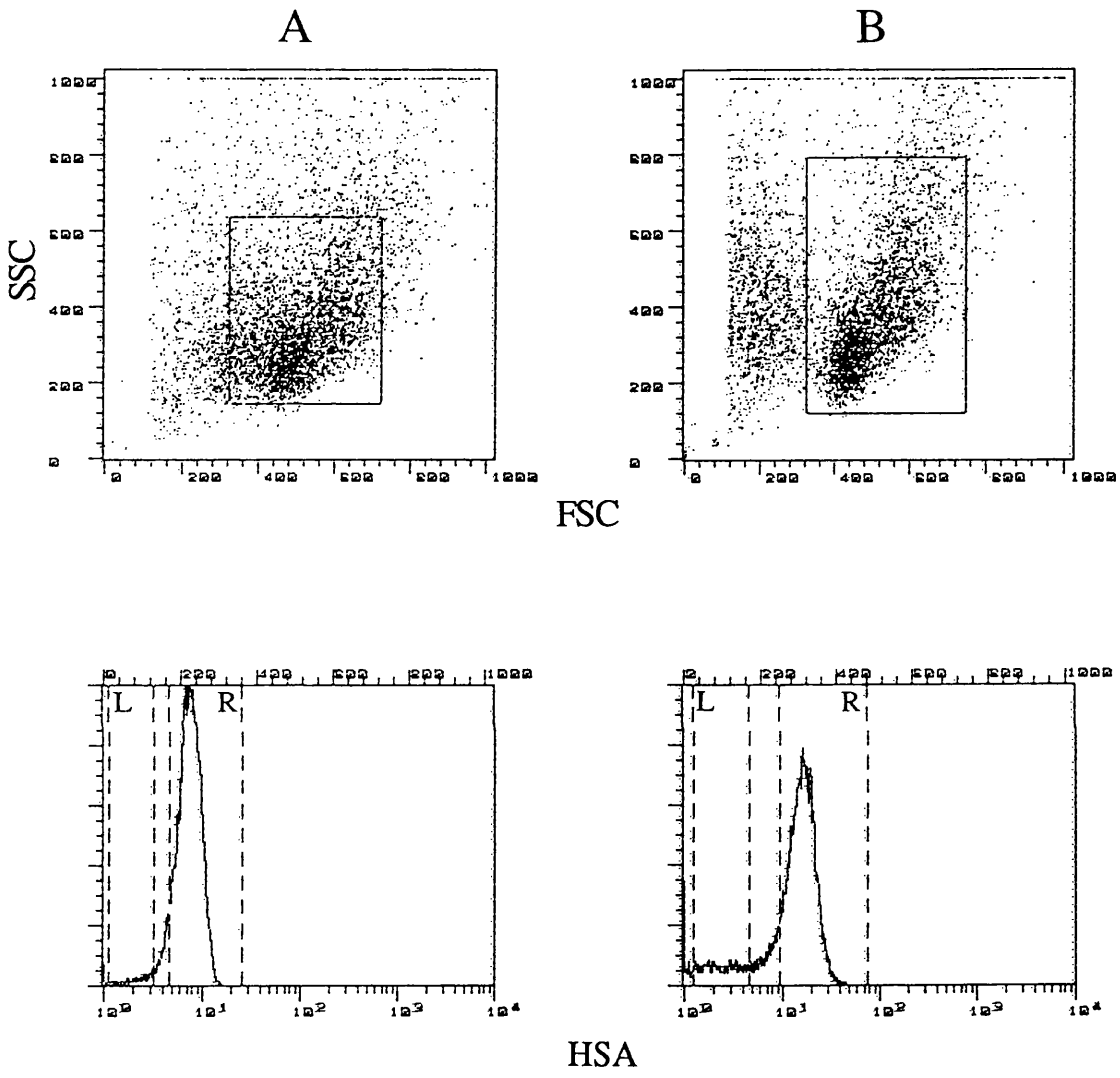
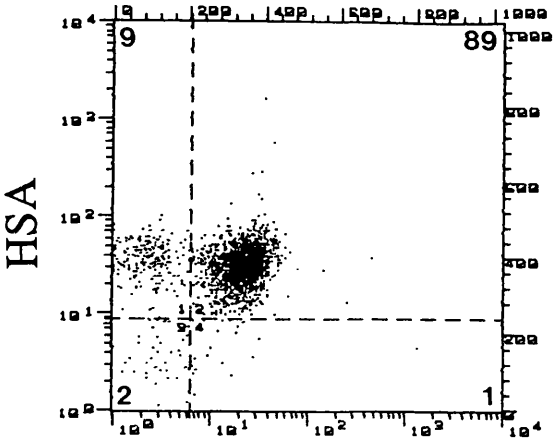

A
B
SSC
FSC
L
R
L
R
HSA

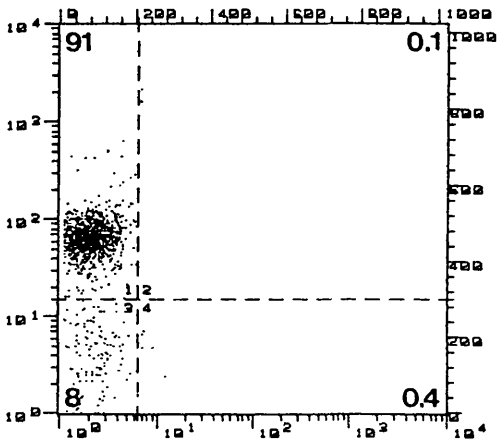
Figure 4.14 *Expression of Thy1.1/HSA on cells recovered from lobes recolonised with HSA-sorted day 14 foetal liver populations.*

Sterile flow cytometric separation was performed on the albumin gradient fractions A and B. These HSA-positive and HSA-negative populations were seeded into alymphoid thymus lobes in order to determine their recolonisation potential. Since the HSA-negative population represented only a small fraction of the total population of both A (3%) and B (11%), alymphoid lobes were seeded with only 10^4 HSA^- cells per lobe, or 10^5 HSA^+ cells per lobe. The cells recovered from each experimental group (lobes recolonised with A HSA^+, A HSA^-; or B HSA^+, B HSA^-, cells) were counted and stained with anti-Thy1.1 antibody and anti-HSA antibody, as in the legend to Figure 4.3. There were insufficient cells recovered from the B HSA^+ recolonised lobes to analyse Thy1.1/HSA expression.

A HSA-



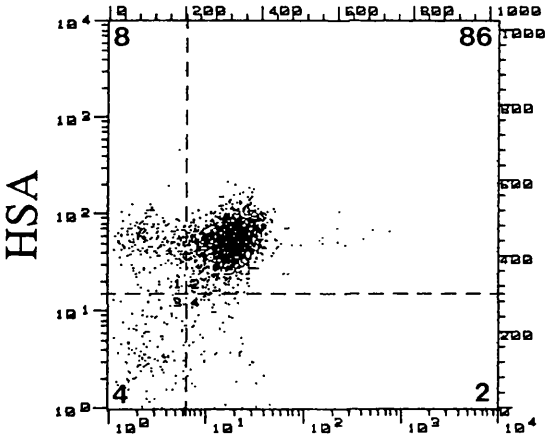
A HSA+



HSA

Thy1.1

B HSA-



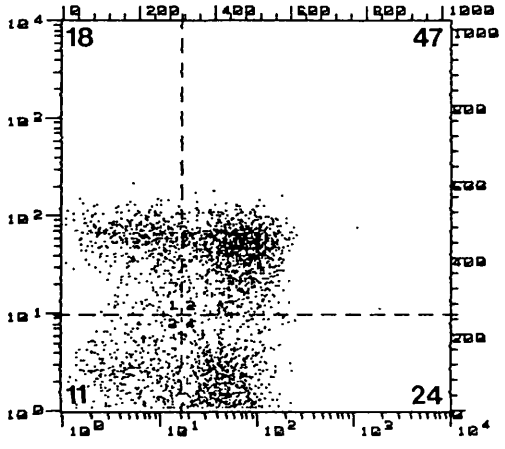
HSA

Thy1.1

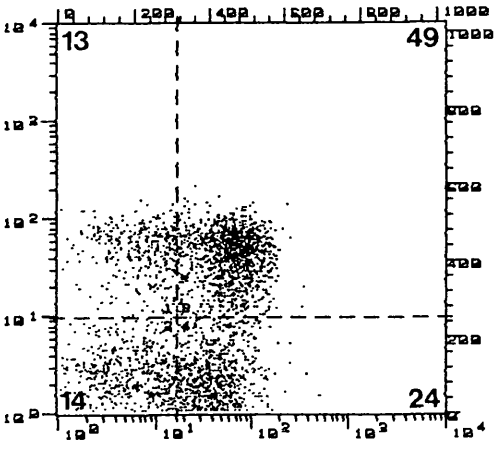
Figure 4.15 *Phenotype of cells recovered from lobes recolonised with HSA-sorted day 14 foetal liver populations.*

The phenotype of cells recovered following recolonisation with the HSA-sorted population from gradient A (as described in the legend to Figure 4.14) is shown. Cells from lobes recolonised with the A HSA^- fraction were stained with antibodies against CD4, CD8, CD45 and Pgp-1, as described in the legend to Figure 4.8. The number of cells recovered from the B HSA^- repopulated lobes was insufficient to allow analysis of CD45 and Pgp-1 expression by these cells.

A HSA-

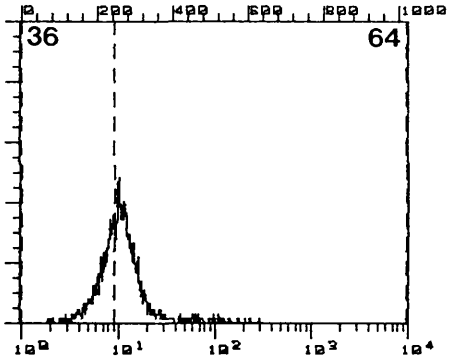


B HSA-

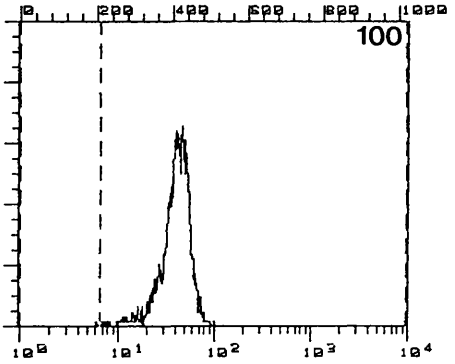


CD4

CD8



Pgp-1



CD45

CHAPTER FIVE

Inhibition of CD4 and CD8 expression in foetal thymus organ culture using antisense oligonucleotides

5.1 Introduction

In order to assess the function of a particular gene product it is informative to inhibit expression of the gene of interest. In prokaryotic systems naturally occurring mutant cell lines lacking a specific gene product often exist, however, in mammalian systems the difficulty of deriving such cell lines has initiated the search for other means of specifically regulating gene expression. The expression of certain bacterial genes has been shown to be regulated by naturally occurring complementary RNAs (Green *et al.*, 1986), in which the complementary RNA hybridises to the normal messenger RNA and thus blocks recognition and processing by the translational apparatus in the cell. No specific examples have been found where antisense RNA is used naturally as a control mechanism in eukaryotic cells.

Antisense inhibition of specific genes has been demonstrated by the artificial introduction of plasmids containing antisense RNA constructs into eukaryotic cells (Izant & Weintraub, 1984). The complementary sequence is introduced by transfection of the cells with a plasmid containing the gene of interest in the opposite orientation with respect to the promoter. This approach requires the efficient introduction of the plasmid DNA into the cells and the antisense RNA to be stably maintained at high levels, in order to reach saturating concentrations within the cell. It has often proved difficult, however, to attain the hundred-fold excess of antisense over endogenous sense message which is generally required to produce stable intracellular RNA/RNA duplexes.

An alternative approach using chemically synthesised oligonucleotides complementary to viral genes has been shown to be effective in preventing infection of cells by a number of viruses (Stephenson & Zamecnik, 1978; Zamecnik *et al.*, 1986). Previous studies have demonstrated that although transfection of DNA into lymphocytes is relatively inefficient, short synthetic oligonucleotides can readily penetrate lymphoid or myeloid cells (Gewirtz *et al.*, 1988; Harel-Bellan *et al.*, 1988a; Matsukura *et al.*, 1987). *In vitro* studies suggest that these antisense oligonucleotides act by specifically inhibiting translation of messenger RNA (Blake *et al.*, 1985a; Harel-

Bellan *et al.*, 1988b). The choice of sequence of the complementary oligonucleotide appears to be important, the results of other workers indicating that inhibition of translation is more readily achieved when the oligonucleotide sequence is complementary to the 5' region of the gene (Blake *et al.*, 1985a; Melton, 1985), where presumably the message is less folded to allow ribosomal interaction. Furthermore, the antisense oligonucleotide forms an intracellular DNA/RNA duplex, providing a substrate for ribonuclease H, which specifically degrades the messenger RNA in the duplex (Harel-Bellan *et al.*, 1988b; Walder & Walder, 1988), thus increasing the efficacy of this approach.

In addition to earlier studies demonstrating inhibition of viral gene expression, antisense oligonucleotides have also been shown to be effective in the specific inhibition of components of the lymphoid system, particularly in the inhibition of lymphokine gene expression (Harel-Bellan *et al.*, 1988b; Maier *et al.*, 1990), in the modulation of expression of T cell surface antigens (Zheng *et al.*, 1989; Flood *et al.*, 1990), and the regulation of perforin expression in cytolytic T lymphocytes (Acha-Orbea *et al.*, 1990).

The technique of antisense inhibition therefore appeared to provide a novel approach to investigate the role of molecules involved in thymic development. An obvious choice of targets for inhibition were the differentiation antigens CD4 and CD8. These molecules are acquired during thymocyte development and have been implicated as being intimately involved in antigen recognition and in the generation of functionally mature cells. Previous studies to investigate the role of these and other antigens during T lymphocyte development have focussed on the use of antibodies to inhibit expression of CD4, CD8, CD3 and components of the T cell receptor in foetal thymus organ culture (Born *et al.*, 1987; Smith *et al.*, 1989a; Yachelini *et al.*, 1990; Zuñiga-Pflücker *et al.*, 1989). The use of antibodies to modulate expression, however, can yield inconsistent results (Jenkinson *et al.*, 1987; Plum & De Smedt, 1988), and certain antibodies have the capacity to deliver a signal to cells upon binding to their ligand (Bank & Chess, 1985; Wassmer *et al.*, 1985; McCarthy *et al.*, 1988), which may

influence the developmental process. The technique of antisense oligonucleotide inhibition offered a means of investigating the role of CD4 and CD8 during thymocyte development, specifically inhibiting expression of these molecules at the messenger RNA level, to avoid any complications which may arise as a result of ligand binding.

5.2 Experimental design

Using foetal thymus organ culture as an *in vitro* model of thymic ontogeny, antisense oligonucleotides corresponding to the 5' region of the genes encoding CD4 or CD8 were employed to specifically inhibit the expression of these molecules during T cell development. CD8 exists normally as a disulphide-linked heterodimer, comprising two separate gene products, the CD8α and CD8β chains (Ledbetter *et al.*, 1981). Although CD8α is known to be involved in the T cell response to antigen, no function has yet been ascribed to CD8β (Letourneur *et al.*, 1990). Oligonucleotides were synthesised for each of these chains in order to investigate the individual contribution of both α and β. Antisense oligonucleotides were synthesised complementary to the leader sequence of the genes encoding CD4, CD8α or CD8β, and extending 23 nucleotides downstream from the ATG initiation of translation codon. This sequence was chosen as a suitable candidate for antisense inhibition on the basis of previous studies to inhibit expression of cell surface molecules (Blake *et al.*, 1985a; Zheng *et al.*, 1989).

In the use of oligonucleotides, it was important to consider the possible effect and toxicity of deoxynucleotide concentration within the cell (Penit & Papiernik, 1986; Jenkinson *et al.*, 1982; Kizaki *et al.*, 1988). Control oligonucleotides were therefore synthesised comprising a random sequence of the same proportion of bases as contained within the equivalent antisense sequence. For the CD8 control, however, a single control oligonucleotide was synthesised, since the α and β antisense sequences contained a similar proportion of individual bases. The CD8 control therefore comprised an average of the base composition of both the CD8α and CD8β antisense oligonucleotides. None of the control oligonucleotide sequences were found to be the reverse complement of any murine nucleotide sequence in the EMBL database, and all

the sequences were examined to exclude any motifs suggesting possible secondary structure formation.

In order to achieve maximal efficiency in the use of antisense oligonucleotides in thymic organ culture it was important to consider the cell permeability of such reagents and their relative stability *in vitro*. A number of oligonucleotide modifications have been developed to overcome the problems of degradation by nuclease activity. Oligonucleotides with modified phosphodiester bonds, such as phosphorothioate, methylphosphonate, or phosphoramidite derivatives can be routinely synthesised and are relatively resistant to nuclease attack (Agrawal *et al.*, 1988; Blake *et al.*, 1985b; Matsukura *et al.*, 1987; Sarin *et al.*, 1988). The cell permeability and hybridisation efficiency, however, has limited the use of certain of these reagents. Although methylphosphonate analogues show poor solubility due to their hydrophobic nature, and are unable to stimulate ribonuclease H activity, phosphorothioate analogues are readily soluble, comparatively resistant to nucleases and can direct ribonuclease H mediated degradation of RNA at the site of hybridisation (Matsukura *et al.*, 1987; Stein & Cohen, 1988). The literature therefore suggested that of the modifications available, phophorothioate analogues would be a suitable candidate for initial study.

The oligonucleotides in culture would be required to be maintained for sufficient periods to allow the existing target protein levels to decrease by natural turnover within the cell. During incubation the oligonucleotides would be susceptible not only to degradation by nucleases within the cell, but also by nucleases present in the serum contained in the culture medium. Unfortunately the presence of serum is necessary for the maintenance of thymocytes in organ culture, thus precluding the use of serum-free media. The nucleases in foetal calf serum have been shown to be predominately 3'-phosphodiesterases, with relatively little 5'-phophodiesterase or endonuclease activity (reviewed by Tidd, 1990). This suggested that in order to attain greater stability, it would be advantageous to synthesise reagents containing modified phosphodiester linkages at the 3' and 5' termini. Oligonucleotides were therefore synthesised using a variety of phosphorothioate modifications to determine their relative stability in culture.

These contained phosphorothioate linkages (a) throughout the molecule, (b) a single linkage at both the 5' and 3' ends of the molecule, and (c) at only the 3' terminus, as shown in Figure 5.1. Oligonucleotides comprising completely non-modified bases were also synthesised for comparison.

5.3 Uptake and Stability

In order to assess the the stability of the various oligonucleotide analogues in culture, the oligonucleotides were 5' end-labelled using γ-^{32}P-[ATP]. The oligonucleotides with a single phosphorothioate linkage at the 3' end, or a single linkage at the 5' and 3' end of the molecule appeared to label with a higher specific activity during the same period of labelling than the oligonucleotide containing phosphorothioate linkages throughout, perhaps as a result of steric effects due to the presence of a sulphur atom replacing an oxygen in the internucleosidic bond. The oligonucleotides were labelled to a sufficient specific activity, however, to allow analysis of their stability *in vitro*. The labelled oligonucleotides were incubated with a single cell suspension of thymocytes for various periods of time to determine the stability and efficiency of uptake of the DNA. Aliquots of the culture supernatant, the cell washes, and cell lysate were analysed in a beta-counter to determine the associated radioactivity, and aliquots of the cell lysate and culture supernatant were also analysed using denaturing PAGE to determine the stability of the various analogues. Adult thymocytes were chosen for this and subsequent metabolic labelling studies as an alternative to using foetal thymus lobes, since they provided a convenient source of cells of similar phenotype that were easier to manipulate than intact thymus explants.

Aliquots of the cell lysates were analysed to determine the associated radioactivity, and the amount of radiolabel incorporated by the cells was seen to reach a maximum within 4 hours. This level was subsequently maintained during the 52 hour period of incubation. The percentage uptake of labelled material was calculated by dividing the counts in the cell lysate by the combined counts in the supernatant, washes and lysate. There was very little difference in the efficiency of uptake observed for each

oligonucleotide. For all three oligonucleotides the percentage uptake was approximately 0.1%, indicating that the mechanism of uptake of oligonucleotide was relatively inefficient.

From the autoradiographs of the 20% denaturing gels shown in Figure 5.2, it can be seen that the oligonucleotide containing phosphorothioate linkages throughout the molecule (Figure 5.2a, upper panel) was the most stable in the culture supernatant, and could still be detected after a period of 52 hours. The other oligonucleotides were less stable, although the presence of a phosphorothioate linkage at both the 5' and 3' ends of the oligonucleotide enabled the molecule to remain intact in the supernatant for up to 48 hours (Figure 5.2b), whereas the oligonucleotide with a single linkage at the 3' end of the molecule could be detected in the supernatant for no longer than 24 hours (Figure 5.2c).

A similar pattern was apparent in the cell lysates (Figure 5.2, lower panel), with the oligonucleotide containing phosphorothioate linkages throughout the molecule stable for the entire 52 hour period of culture (Figure 5.2a). The oligonucleotide with a single modified linkage at both the 5' and 3' ends of the molecule could be detected at 32 hours of culture (Figure 5.2b), whereas the oligonucleotide with a single linkage at the 3' end of the molecule, remained present for no longer than 16 hours (Figure 5.2c). The low signal seen in the cell lysate in (a) is due to the relatively low specific activity of labelling of this oligonucleotide. The low efficiency of uptake of oligonucleotide by the cells was seen for all three analogues. The variation in intensity between the lanes is probably accountable by errors inherent in pipetting small volumes of radiolabelled material.

In addition to the oligonucleotide material in the supernatant and cell lysate, higher molecular weight bands were also present (Figure 5.2 b & c). One explanation may be that the migration of the material in these higher bands in the gel may be impeded as a result of secondary structure formation. The presence of such higher molecular weight material has been noted by others in similar labelling studies (Woolf *et al.*, 1990), although few have speculated as to its nature. In Figure 5.2a, the full

length oligonucleotide is the only labelled species present, except in the original preparation at 0 hours. The presence of material of an apparently higher molecular weight than the oligonucleotide (23mer) at 0 hours suggests this is a contaminant of the original oligonucleotide preparation that was also radiolabelled. Although this material may comprise incomplete products from the synthesis, this is unlikely, since the oligonucleotides were purified by trityl-on HPLC. Theoretically, using this purification procedure, only the full length oligonucleotide containing a dimethoxytrityl moiety should be isolated. It is possible that the purification of these reagents may have been less efficient than of the oligomer in (a), but it is unclear whether this is due to the modification of linkages used.

The higher molecular weight bands observed in the cell lysate may be the result of oligonucleotide complexing with cellular protein, thus retarding the migration of the DNA in such a high percentage gel. The lower molecular weight material observed is probably degraded oligonucleotide.

5.4 Uptake of oligonucleotides into organ culture

The uptake of the γ-^{32}P-labelled oligonucleotide was demonstrated in thymocytes in suspension, however, it was not clear how efficiently the oligonucleotide would be able to penetrate an intact foetal thymus lobe. Attempts were made to incubate intact lobes with the radiolabelled oligonucleotide, however, such analysis was complicated by the difficulty of assessing whether the label had actually penetrated the lobe or was simply associated with the surface of the cells comprising the thymic capsule. Technical and safety considerations precluded the sectioning of lobes incubated with γ-^{32}P-labelled oligonucleotide and the use of ^{32}P-labelling would not have provided sufficient sensitivity to enable specific location of the labelled oligonucleotide within the thymus lobe by autoradiography. It was therefore decided that using a relatively simple modification of oligonucleotide synthesis a biotin group could be attached to the 5' end of the molecule. This could then be visualised with a streptavidin peroxidase conjugate on thymic sections. Biotinylated oligonucleotide, or

non-biotinylated oligonucleotide as a control, was added to individual day 14 foetal thymus lobes and incubated in hanging drop culture for 24 hours. The lobes were sectioned and stained to visualise the biotin.

A section through a lobe incubated with non-biotinylated oligonucleotide is presented in Figure 5.3a. The section illustrates that little endogenous biotin is present throughout the thymus lobe. Fig 5.3b shows a section through a lobe following incubation with biotinylated oligonucleotide. The presence of the biotinylated oligonucleotide can be clearly visualised by the peroxidase staining, demonstrating the ability of the oligonucleotide to successfully penetrate an intact day 14 foetal thymus lobe. The oligonucleotide not only appeared to have penetrated the lobe but also to have entered the cells, with some label apparently localised in the nuclei. Examination of the section at a lower magnification indicated that the staining was fairly randomly distributed throughout the lobe, although a significant quantity was located around the thymic capsule (Figure 5.3c). After only 4 hours of incubation with biotinylated oligonucleotide the lobes showed a similar, though less intense pattern of staining to that seen at 24 hours (data not shown).

5.5 Metabolic labelling studies

A major consideration in the use of antisense reagents is their specificity of action. The antisense oligonucleotides should cause specific inhibition of the protein of interest, without affecting the synthesis of other proteins. To determine the specificity of inhibition of protein synthesis, thymocytes were metabolically labelled in suspension using ^{35}S-methionine/cysteine in the presence of antisense oligonucleotide at various concentrations. The labelled cells were then lysed and the total amount of labelled protein determined by precipitation of an aliquot of the lysate with trichloroacetic acid (TCA). The labelled proteins were immunoprecipitated, and this material analysed by SDS/PAGE.

Initial experiments were performed with a 4 hour preincubation in the presence of CD8α or CD8β antisense, or control oligonucleotide at a concentration of 75μM,

prior to metabolic labelling. Thymocytes that were incubated with antisense oligonucleotide showed no observable effect on the levels of total protein, in comparison to incubation with control oligonucleotide, or no oligonucleotide, as assessed by TCA precipitation. In addition, there was no apparent reduction in the amount of labelled CD8 protein immunoprecipitated with either an anti-CD8α or anti-CD8β monoclonal antibody as determined by SDS/PAGE analysis (Figure 5.4a, lanes 3 and 4), nor on the level of CD4 protein (Figure 5.4a, lane 2). An irrelevant, class-matched antibody was used as a control for immunoprecipitation (Figure 5.4a lane 1). The apparent reduction in intensity in lanes 3 and 4 in the CD8β antisense-treated sample is unlikely to be a specific antisense effect, and was probably a result of differences in loading, since all the bands present in this lane are reduced in intensity, not just the species corresponding to CD8α or CD8β. Any differences could not be attributed to an antisense effect with any certainty in the absence of a control oligonucleotide treated sample for comparison. Scanning densitometry of the autoradiographs confirmed there was little difference between the bands corresponding to CD8 in the antisense and untreated samples.

The experiment was repeated with a 17 hour preincubation with CD8α antisense or control oligonucleotide at a concentration of only 50μM, since quantities of oligonucleotide were limiting. Again, no difference was observed on the levels of total protein synthesised, nor on the amount of CD8 protein immunoprecipitated between thymocytes incubated with antisense or control oligonucleotide (Figure 5.4b, lanes 2 & 3). The reduction in the intensity in the control-treated sample is probably a loading effect, as seen in 5.4a, since there was no difference observed between the control and antisense treated samples in the intensity of the bands corresponding to CD8α or CD8β, as determined by scanning densitometry. Using these conditions it was not possible to demonstrate any specific inhibition of protein synthesis by metabolic labelling in the presence of CD8 antisense oligonucleotide. It is likely that the concentration of oligonucleotide used in these studies was insufficient to see an effect, however, other

experimental conditions were not investigated due to the limited supply of oligonucleotide available at this time.

In addition to the metabolic labelling studies, the specific effect of antisense oligonucleotides was investigated using two $CD4^+CD8^+$ thymic lymphomas, VL3, and KKT2 (McGrath *et al.*, 1979). Experiments were performed to remove cell surface expression of CD4 and CD8 by trypsinisation, and to inhibit re-expression of these molecules by incubating the cells in the presence of antisense oligonucleotide. In comparison to the untreated or control treated cells, the addition of CD8α or CD8β antisense oligonucleotide at concentrations of up to 30μM immediately following trypsinisation failed to show any effect on the re-expression of CD4 or CD8 monitored over a period of 24 to 96 hours, therefore the data are not presented. The limited availability of oligonucleotide again precluded further analysis.

5.6 Antisense effects in organ culture

The specific effects of antisense oligonucleotides on the expression of CD4 and CD8 during thymocyte development were investigated using day 14 gestation murine thymocytes in foetal thymus organ culture. The lobes were incubated initially in conventional organ culture on polycarbonate filters, and later using a modification of the technique, in hanging drop culture. Control lobes were incubated with random sequence oligonucleotides, or in the absence of oligonucleotides - the latter with simply the same volume of diluent (water or later IMDM) as added to the experimental lobes.

The antisense oligonucleotides were titrated in foetal thymus organ culture and the effects monitored over a period of 2 to 12 days. Initially the oligonucleotides supplied were resuspended as a 1mM stock in TE buffer (10mM Tris/1mM EDTA), however, preliminary experiments showed that the concentration of EDTA in the buffer had a deleterious effect on the growth of the developing thymocytes, although this concentration of EDTA (10-30μM) is less than the amount required to chelate all the calcium (1.5mM) and magnesium ions (0.8mM) in the medium. The lobes were fairly tolerant of concentrations of EDTA up to 10μM, however at concentrations greater than

10μM, the lobes had a severely reduced cell yield (4 x 10^4 cells/lobe at 20 μM Tris/EDTA, day 6, compared to 2 x 10^5 cells/lobe untreated), fewer $Thy1^+$ and HSA^+ cells, and almost no $CD4^+$ or $CD8^+$ cells were observed, the majority of cells being arrested at the $CD4^-CD8^-$ stage (Figure 5.5a &b). Following this observation the oligonucleotides were subsequently resuspended in nuclease-free water.

5.6.1 CD4 antisense oligonucleotide effects

The thymus contains cells of a $CD4^-8^-$ phenotype at day 14 of gestation and $CD4^+8^+$ cells only start to appear from day 16 of gestation onwards, corresponding to 2 days in organ culture (see Chapter 3; Fowlkes & Pardoll, 1989). The effects of the oligonucleotides were therefore monitored from 2 days *in vitro* onwards.

Initial experiments were performed using non-modified oligonucleotides, and those containing phosphorothioate linkages throughout the molecule. The concentration of oligonucleotide was titrated in organ culture and the effects analysed from 2 to 12 days *in vitro*. No difference in CD4 or CD8 expression was observed between the antisense and control treated lobes following a single dose of either the modified or non-modified oligonucleotide at concentrations up to 30μM. Both antisense and control treated samples showed identical percentage expression of CD4 and CD8 subpopulations, and the same cell numbers in each CD4/CD8-defined phenotype. These experiments exhausted the initial batch of oligonucleotide supplied. Since neither oligonucleotide showed any detectable effect on the levels of CD4 and CD8 expression, and the stability data suggested that the phosphorothioate oligonucleotide would remain longer in culture, it was decided that subsequent oligonucleotide synthesis should focus on these analogues. Further experiments were not pursued with the non-modified oligonucleotide.

Subsequent experiments using oligonucleotides with phosphorothioate linkages throughout the molecule, showed no obvious reduction in CD4 or CD8 expression after 2 to 6 days in culture following a single dose of oligonucleotide, even at concentrations up to 75μM. Percentage values of expression may sometimes be misleading (as

discussed in Chapter 3), therefore for each phenotype the percentage value was converted to actual cell numbers by multiplying by the cell yield per lobe. The CD4 and CD8 single-positive subpopulations often represent such small percentages that this would also enable any subtle differences to be observed by direct comparison between the cell number in each phenotype. At an oligonucleotide concentration of 75μM, a slight decrease in cell yield was observed in both the antisense and control treated lobes, in comparison to untreated lobes, however, analysis showed that both antisense and control samples contained the same cell number in each CD4/CD8 phenotype.

Although the oligonucleotides containing phosphorothioate linkages throughout the molecule were known to be stable over a period of 52 hours, it was unclear as to the extent of degradation over longer periods of culture. A single dose of upto 75μM in organ culture appeared to have no obvious effect on the phenotype of the cells recovered, even after 6 days, thus the effects of the daily addition of oligonucleotide were investigated. In an initial experiment, however, no difference was observed between the phenotype of the antisense and control treated lobes following the daily addition of upto 10μM CD4 antisense oligonucleotide over a period of 4 days.

In order to enable higher oligonucleotide concentrations to be administered to the thymus lobes, and because quantities of oligonucleotide were limiting, a modification of the conventional organ culture system was developed. The minimum culture volume feasible for maintenance of lobes on polycarbonate filters was 1ml, however, by incubating the lobes in an inverted microwell plate the culture volume could be limited to 10-20μl. This enabled larger doses of oligonucleotide to be administered than in conventional organ culture, yet limited the actual amount of oligonucleotide used. The technique of hanging drop culture also enabled the thymus explant to be bathed in the medium and thus allow the oligonucleotides maximum access to the tissue, since it was also of concern that the polycarbonate filters could potentially prevent the oligonucleotide from reaching the lobes, perhaps as a result of electrostatic interactions.

Although the cell recovery from lobes incubated in hanging drop culture was reduced by approximately two to three-fold compared to that from lobes incubated in conventional organ culture, relatively normal thymocyte development occurred over a 4 day culture period. As seen in Table 4 and Figure 5.6, the acquisition of the four phenotypes defined by CD4 and CD8 expression seen during development *in vivo*, followed a similar pattern in both hanging drop culture and in conventional thymus organ culture (in Table 4, the percentage values and absolute cell number is shown for each phenotype). In order to ensure that media components were not limiting a nutrient supplement was added daily to lobes in these microwell cultures.

Table 4 *The phenotype of cells recovered from foetal thymus lobes incubated in conventional organ culture and hanging drop culture*

	CD4/CD8 expression			
Phenotype	**FTOC**		**Hanging drop culture**	
	%	**($x10^{-3}$)**	**%**	**($x10^{-3}$)**
$CD4^+CD8^-$	18	(23.4)	14	(7.0)
$CD4^+CD8^+$	62	(80.6)	72	(36)
$CD4^-CD8^-$	16	(20.8)	11	(5.5)
$CD4^-CD8^+$	4	(5.2)	4	(2.0)
Cell Yield per lobe	1.3×10^5		5×10^4	

The effects of a daily addition of oligonucleotide were therefore monitored over a period of 2-4 days in hanging drop culture. After 4 days in culture the majority of cells had acquired a $CD4^+8^+$ phenotype and it was expected that any modulation in CD4 or CD8 expression would be readily detected at this stage. Experiments administering a daily 50μM dose of the antisense and control oligonucleotide

(containing phosphorothioate linkages throughout the molecule), however, failed to show any reduction in CD4 or CD8 expression.

CD4 antisense oligonucleotide containing phosphorothioate linkages throughout the molecule was subsequently added daily to thymus lobes in hanging drop culture at 100μM concentration over a period of 4 days. This resulted in a specific decrease in the cell surface expression of CD4, apparent as a reduction in the proportion of cells in the double-positive population, and a corresponding increase in proportion of double-negative, and CD8-single positive cells (Figure 5.7a, b, & c). As shown in Figure 5.7b, the effect on CD4 expression was clearly evident as an increase in the proportion of $CD4^-$ cells, and a reduction in the levels of CD4 expressed by the remaining $CD4^+$ population. A non-specific reduction in the intensity of CD4 staining was also observed in the control treated lobes (mean fluorescent intensity 122), although this was less striking than the effect observed on the antisense-treated cells (mean fluorescent intensity 68.5). The specificity of the antisense effect was confirmed by the normal staining pattern of an irrelevant molecule, such as Thy 1, showing that there were no gross changes in the thymic lobes (Figure 5.7d).

In order to examine the phenotype of the CD4-negative, and CD4-positive populations in more detail, each of these populations was analysed individually, to assess the level of CD8 expression. Analysis of the CD4-negative population revealed that the antisense treatment had increased the proportion of $CD4^-CD8^+$ cells, and double-negative cells (Figure 5.8a). Fewer CD8 single positive and double-negative cells were apparent in the control treated lobes, and very few CD4-negative cells were present in the untreated lobes (Figure 5.8a). Analysis of the CD4-positive population, confirmed that the main subpopulation affected by the antisense treatment was the double-positives, and also illustrated the lack of CD4 single-positives in all samples after only 4 days in culture (Figure 5.8b).

Three-colour staining could be used to determine the CD3 status and therefore the maturational state of the $CD4^-CD8^+$ subpopulation observed in the antisense treated

lobes, however, there were insufficient cells available in this experiment to perform such an analysis.

This experiment could not repeated since there was insufficient oligonucleotide stock remaining from this batch. Subsequent experiments were therefore performed using the oligonucleotides containing single phosphorothioate linkages at the 5' and/or 3' ends of the molecule, until more of the completely modified oligonucleotide could be synthesised. The experiment was repeated later when completely modified oligonucleotide was available, using a daily addition of 100μM oligonucleotide. However, this oligonucleotide was synthesised using an alternative method of synthesis and sulphurisation to that used in the previous synthesis of the completely modified analogue. The thymus lobes treated with the new antisense oligonucleotide showed no difference in CD4 or CD8 expression (in terms of mean fluorescent intensity of staining, or proportion of $CD4^{+/-}$ cells) in comparison to the control.

Experiments using the oligonucleotide containing single phosphorothioate linkages at the 5' and/or 3' ends of the molecule showed no effect on CD4 expression after 4 days of culture with a daily addition of oligomer at concentrations of 100μM and 200μM, and any minor differences seen were also apparent in the control treated lobes. In order to add the oligomer at higher concentrations, at doses of 500μM, the oligonucleotide stock was ethanol precipitated and resuspended in IMDM. Even at a concentration of 500μM oligonucleotide administered daily, there was only a marginal difference observed in the number of $CD4^{-}CD8^{+}$ cells (Figure 5.9a) and a slight decrease in the total number of CD4 expressing cells (Figure 5.9b), in the antisense-treated lobes compared to the control. A slight difference was seen in the mean fluorescent intensity of CD4 staining (76 in the antisense-treated, compared to 92 in the control), but this was less striking than the previous antisense effect demonstrated using the completely modified oligonucleotide.

5.6.2 CD8 antisense oligonucleotide effects

Experiments using CD8 antisense oligonucleotide with each modification were performed in parallel to the CD4 antisense studies. The addition of CD8α and CD8β antisense oligonucleotides to foetal thymus lobes in conventional organ culture or in hanging drop culture did not appear to have any significant effect on CD4, CD8α or CD8β expression even at concentrations of 100μM administered daily. The results from an individual experiment in which a modest effect was observed is shown in Figure 5.10. Although no effect was apparent with the CD8β antisense oligonucleotide in comparison to the control, a marginal difference was seen in the number of double-positive, and double-negative cells between the CD8α antisense-treated and control samples (Figure 5.10a). This effect appeared to correspond to a decrease in surface expression of CD8α and CD8β on these cells (Figure 5.10b &c), however, both antisense and control treated cells showed a similar mean fluorescent intensity of CD8 staining. This effect observed with the CD8α antisense oligonucleotide was not reproducible in subsequent experiments.

5.7 Discussion

The differentiation of immigrant precursors within the thymus can be monitored by the acquisition of various developmental cell surface markers. During thymic maturation these precursor cells acquire expression of CD4 and CD8, and the T cell antigen receptor, in a developmentally ordered sequence. The most immature thymocytes, thought to be contained within the $CD4^-CD8^-$ population, differentiate into cells expressing both CD4 and CD8. These cells co-expressing CD4 and CD8 on their surface downregulate expression of one or the other of these molecules resulting in the production of single positive ($CD4^+8^-$, or $CD4^-8^+$) "mature" cells which then exit from the thymus. A major question still unresolved is how these double-positive cells lose expression of either CD4 or CD8 to become single CD4 or CD8 positive cells. It is not known as to whether the cells are instructed to downregulate CD4 or CD8 from the cell surface or whether this event is entirely random. In an attempt to address this question,

the initial aim of the work presented here was to establish an experimental system to artificially modulate expression of these molecules and subsequently investigate the effects of this downmodulation.

The addition of antisense oligonucleotides to thymus lobes in organ culture is a novel approach to modulating surface expression of CD4 in developing thymocytes. The appearance of a subpopulation of CD4-negative cells, and the reduction in the levels of CD4 expression on the remaining $CD4^+$ cells in the antisense-treated organ cultures, is consistent with specific antisense inhibition of CD4 expression. This loss of CD4 expression correlated with the appearance of a $CD4^-CD8^+$ population, and an increase in the proportion of double-negative cells. Since both CD4 and CD8 appear to play a crucial role during ontogeny, particularly in the thymic selection processes which shape the developing T cell repertoire, one might expect that modulation of one of these molecules would influence the expression of the other.

The $CD4^-CD8^+$ population could arise as a direct result of the loss of CD4 from double-positive cells, or perhaps the cells have been arrested at the immature CD8 single-positive stage (i.e. pre-double positive) by blocking CD4 expression. Alternatively, these cells are more mature CD8 single-positives, forced to become $CD8^+$ cells in the absence of CD4 expression. The increase in the the double-negative population may be due to the loss of CD4 expression from an immature $CD4^{lo}CD8^-$ population, or from the more mature $CD4^+CD8^-$ cells. Alternatively, there may be a requirement for expression of both CD4 and CD8 in order for the cells to reach the double-positive stage. However, since the CD3 status of these cells was not detemined, these possibilities could not be resolved.

Although a slight reduction in CD4 expression was observed in the control cultures, this was probably due to a non-specific toxic effect at such high doses of oligonucleotide, resulting from monomeric deoxynucleotides being released by nuclease activity (Jenkinson *et al.*, 1982; Penit & Papiernik, 1986; Kizaki *et al.*, 1988). This may explain the decrease in cell number observed at high oligonucleotide concentrations. However, in order to control for the specificity of the antisense effect

the staining of an irrelevant protein, such as Thy1, was analysed, and no significant effect was seen on the cell surface expression of this molecule.

As yet there have been no reports of the use of antisense oligonucleotides in thymus organ culture. The initial aim was to show that oligonucleotides can penetrate an intact organ explant, and subsequently to investigate the effects of antisense modulation. Antisense oligonucleotide effects have been previously demonstrated using lymphoid cells in suspension (Acha-Orbea *et al.*, 1990; Flood *et al.*, 1990; Harel-Bellan *et al.*, 1988b; Zheng *et al.*, 1989), which may account for the relative success of these studies over the experiments presented here using an intact organ explant. Furthermore, previous studies have demonstrated antisense inhibition using non-modified oligonucleotides, and usually over short periods (up to 48 hours) of culture (Acha-Orbea *et al.*, 1990; Zheng *et al.*, 1989). However, the use of non-modified oligonucleotides in organ culture was ineffective, and any modulation effect using phosphorothioate analogues was only observed following comparatively longer periods in culture.

The stability of the oligonucleotides *in vitro* was a major consideration in using this approach, which prompted the use of modified analogues. Phosphorothioate oligonucleotides have been shown to be stable in cell culture (Agrawal *et al.*, 1988; Matsukura *et al.*, 1987), however, it was important to investigate alternative forms of this analogue, since the synthesis of oligonucleotides containing phosphorothioate linkages throughout the molecule proved inefficient.

The reduction in efficiency of synthesis required the production of numerous batches of phosphorothioate oligonucleotide. This proved problematic due to the variability between each synthesis, both in DNA content and the presence of other reaction products, although purification of the product oligomer by reverse phase HPLC should have eliminated contaminants and termination products. In addition, although the amount of DNA supplied was determined by spectrophotometric analysis, this only provided an estimate, and it is likely that the actual concentration of

oligonucleotide in each sample was subject to slight variation. This may have been a contributory factor to the inability to reproduce an antisense inhibition effect.

Although the stability data indicated that the oligonucleotide containing phosphorothioate linkages throughout the molecule would be the most resistant to nucleases in culture, a comparable degree of stability could be obtained using the oligonucleotides containing phosphorothioate linkages at the 5' and 3' ends of the molecule. The synthesis of oligonucleotides containing a single modified linkage at each end of the molecule proved to be more efficient than that of the completely modified oligonucleotide, suggesting its suitability for further study. However, the modulation of CD4 expression obtained using the completely modified analogue could not be reproduced using oligonucleotides with a single phosphorothioate linkage at the 5' and/or 3' termini.

In addition to the differences in stability, the inability to reproduce the antisense effect may be explained by variations in the efficiency of cellular uptake and hybridisation of the different phosphorothioate analogues. This may also account for the inability to repeat the antisense inhibition previously demonstrated using the completely modified oligonucleotide, since, in an attempt to improve the efficiency of production, the final batch of this oligonucleotide analogue was prepared using an alternative method of synthesis. The low levels of radiolabelled oligonucleotide detected in the cell lysates supported the proposal that the mechanism of uptake of these oligomers may be relatively inefficient. However, it is unclear to what extent radiolabel could be reutilised or exported from the thymocytes, and it is possible that ^{32}P released from degraded oligonucleotides is rapidly lost from the cells, resulting in the low number of counts in the lysates. Although analysis of the oligonucleotide analogues by denaturing PAGE allowed their relative stability to be assessed, the amount of radiolabel released by degradation could not be quantified.

The efficiency of cellular uptake will determine the quantity of oligomer to be added in order to attain saturating concentrations within the cell. Although the concentrations of oligonucleotide used were approximately five- to ten-fold higher than

reported by others (reviewed by Stein & Cohen, 1988; Acha-Orbea *et al.*, 1990; Zheng *et al.*, 1989), this high dose can be rationalised if the oligomer and ribosome compete directly for binding to the mRNA, and the oligomer has to penetrate an intact organ explant, rather than a single cell in suspension. Furthermore, the oligonucleotide is likely to encounter a higher level of nuclease activity in the intact tissue than in single cell suspension. The concentration of antisense oligonucleotide required to modulate expression of CD4 in organ culture provides further evidence to suggest the inefficient cellular uptake of these oligonucleotides.

Various mechanisms of uptake of oligonucleotides into cells have been suggested, including simple diffusion, although the precise mechanism is still uncertain. Loke *et al.* have demonstrated the entry of oligonucleotide into living cells using acridine-labelling (Loke *et al.*, 1989), and propose a mechanism involving receptor-mediated endocytosis. The reports of antisense inhibition using normal phosphodiester oligonucleotides would suggest that these molecules can at least enter some cell types, although their polyanionic nature would predict a low efficiency of uptake by mammalian cells.

A number of possible mechanisms of action of antisense oligonucleotides have been proposed, although the exact processes involved are still unclear. The inhibition of gene expression is probably due to disruption of translational events, including steric effects of the DNA/RNA duplex causing inhibition of nuclear processing, inhibition of RNA transport into the cytoplasm by the formation of a hybrid duplex, and stimulation of messenger RNA degradation by ribonuclease H activity (Blake *et al.*, 1985a; Harel-Bellan *et al.*, 1988b; Walder & Walder, 1988). Since the oligonucleotides are applied externally to the cell, the antisense effect will be transient, and presumably reversible, although any effects may be prolonged by mediating RNA cleavage. The efficiency of hybridisation of the antisense oligonucleotide will therefore influence the effect of modulating expression.

Although phosphorothioate oligonucleotides appear to be able to penetrate an intact thymus lobe and be taken up by the thymocytes, the reproducibility of this effect,

the relatively large amount of oligonucleotide required, and the availability of large quantities of oligonucleotide limited the usefulness of this approach. The limitations of phosphorothioate analogues have been recently confirmed. Oligonucleotides containing phosphorothioate linkages have been shown to be less efficiently taken up by cells, and to hybridise less stably to the target RNA, than other modified analogues. Furthermore, the use of phosphorothioate oligonucleotides has been shown to cause non-specific inhibition of protein synthesis at concentrations not remarkably higher to those required to produce sequence specific effects (reviewed by Tidd, 1990). This is consistent with the non-specific effects observed with the control oligonucleotide in this study.

Future attempts to regulate expression in this system should therefore consider the relative efficiency of uptake and permeability of alternative oligonucleotide modifications, and perhaps different methods of delivery. An alternative approach for increasing the efficiency of oligonucleotide uptake has been used by Leonetti *et al.* in which oligomers were selectively targeted for intracellular delivery by encapsulation in antibody targeted liposomes (Leonetti *et al.*, 1990). This may provide a suitable approach for future studies.

In conclusion, although the reproducibility of this antisense effect appears to be variable, perhaps compromised by the accessibility and permeability of these reagents, it appears that this technique may provide a novel approach for antisense regulation and may be applicable to studying the regulation of other molecules in this system.

Figure 5.2 *Autoradiographs of the labelled oligonucleotides following in vitro culture*

Each oligonucleotide was 5'-labelled with γ-^{32}P[ATP], and 2 x 10^6 thymocytes were incubated with an equivalent concentration of labelled oligonucleotide (approximately 10^6 cpm in (a) and (b); and 10^7 cpm in (c)) for the indicated periods of time. The cells were harvested and equivalent volumes of the supernatant or cell lysate from each time point were loaded onto the gel (approximatey equivalent amounts of DNA in each lane). Aliquots of the labelled oligonucleotides from the culture supernatant (upper panel) and cell lysate (lower panel), were analysed on a 20% denaturing acrylamide gel, containing 7M urea. (a) Oligonucleotide analogue containing phosphorothioate linkages throughout the molecule; (b) analogue with phosphorothioate linkages at both the 5' and 3' ends of the molecule; and (c) analogue containing a single modified linkage at the 3' end of the molecule. The autoradiographs of the supernatants were exposed at -70°C for 20 hours; those of the cell lysates were exposed for (a) 1 month, (b) 3 weeks, and (c) 6 days at -70°C. The position of the intact oligonucleotide (23mer) for all samples is indicated by the arrow to the left of the figure. A labelled oligonucleotide of known size was electrophoresed in parallel as a size marker.

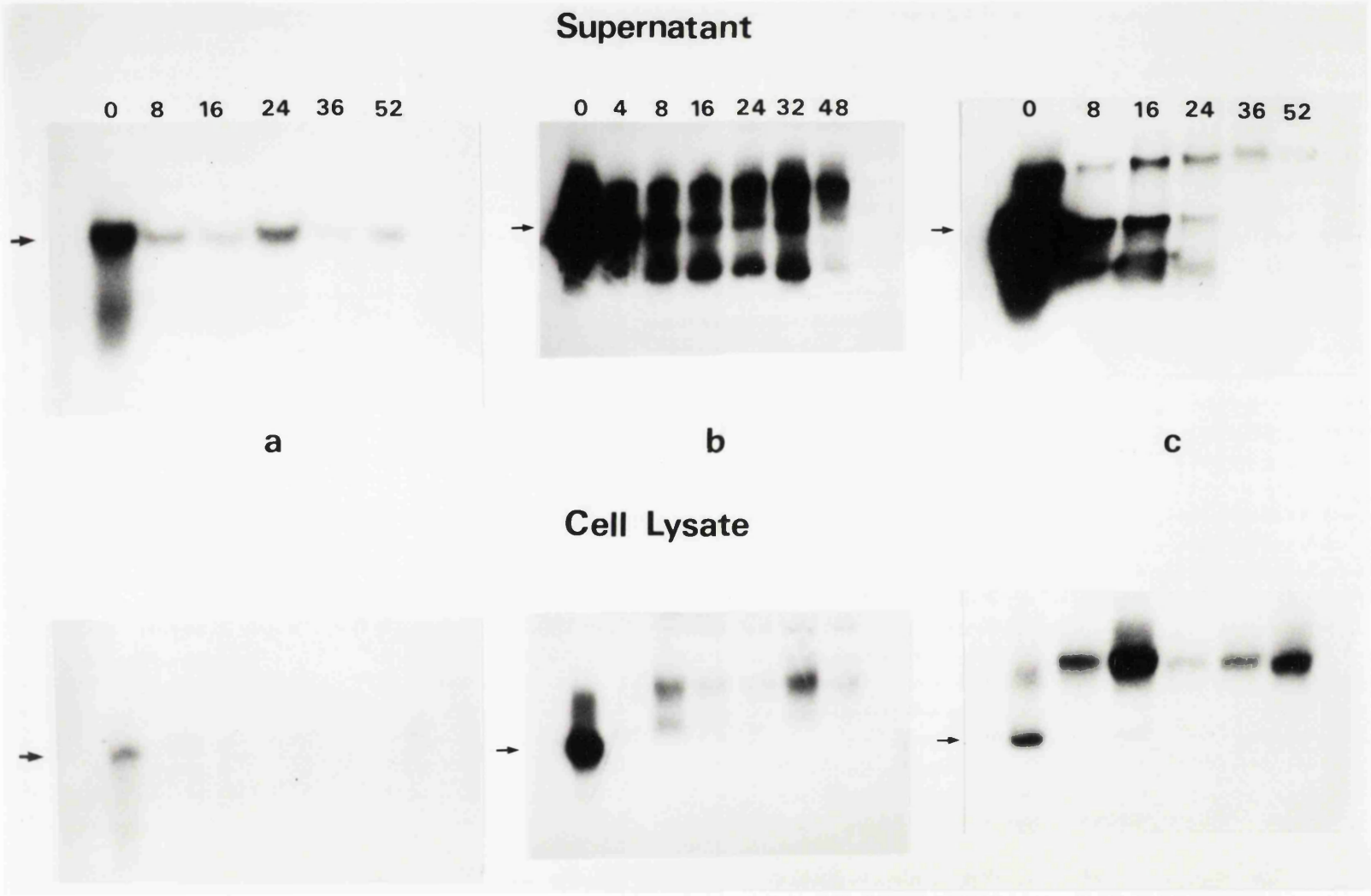
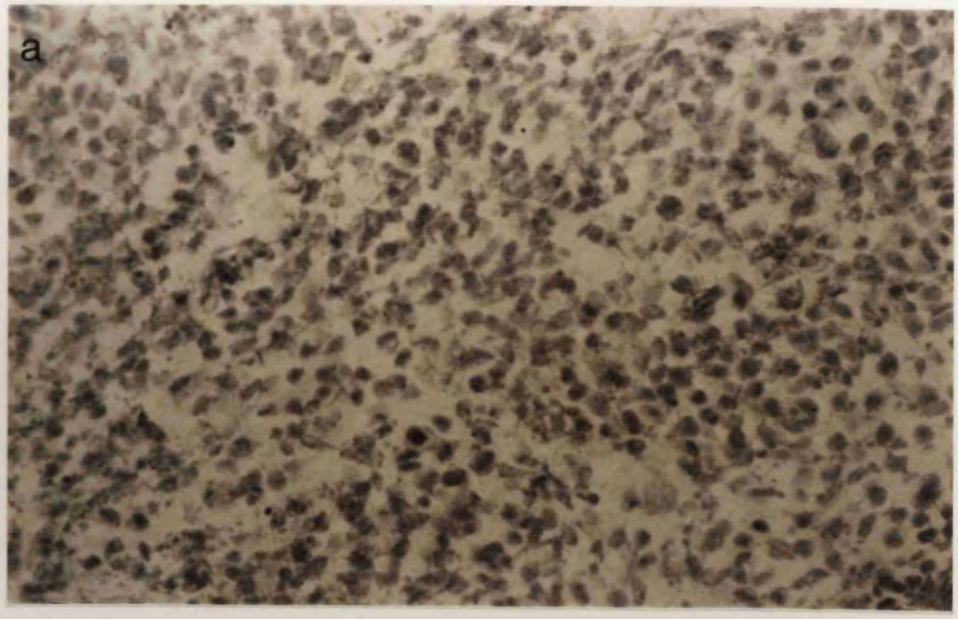
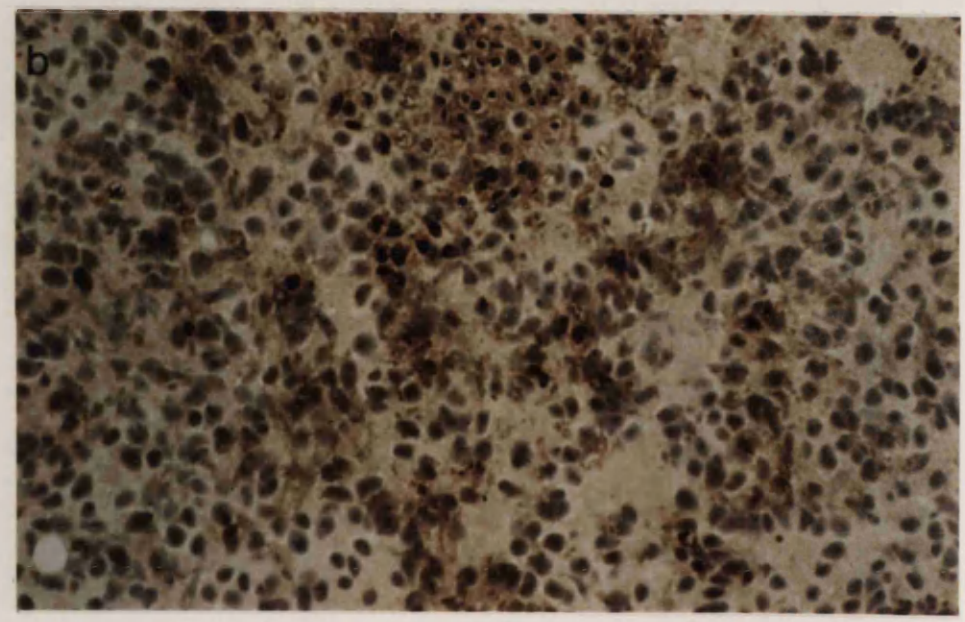

Supernatant
0 8 16 24 36 52
0 4 8 16 24 32 48
0 8 16 24 36 52
a
b
c
Cell Lysate

Figure 5.3 *Immunohistochemical analysis of sections from thymus lobes incubated with biotinylated or non-biotinylated oligonucleotide.*

Thymus lobes were incubated with a 50μM concentration of biotinylated or non-biotinylated oligonucleotide (both containing phosphorothioate linkages at the 5' and 3' termini) for 24 hours in hanging drop culture, and cryostat sectioned. Sections were stained with a streptavidin peroxidase conjugate and counterstained with haematoxylin. (a) Control: section through a lobe incubated with non-biotinylated oligonucleotide (magnification 400x). (b) Experimental: section through a lobe incubated with biotinylated oligonucleotide (magnification 400x). (c) Section through a lobe incubated with biotinylated oligonucleotide as in (b), shown at a lower magnification (250x).


a


b

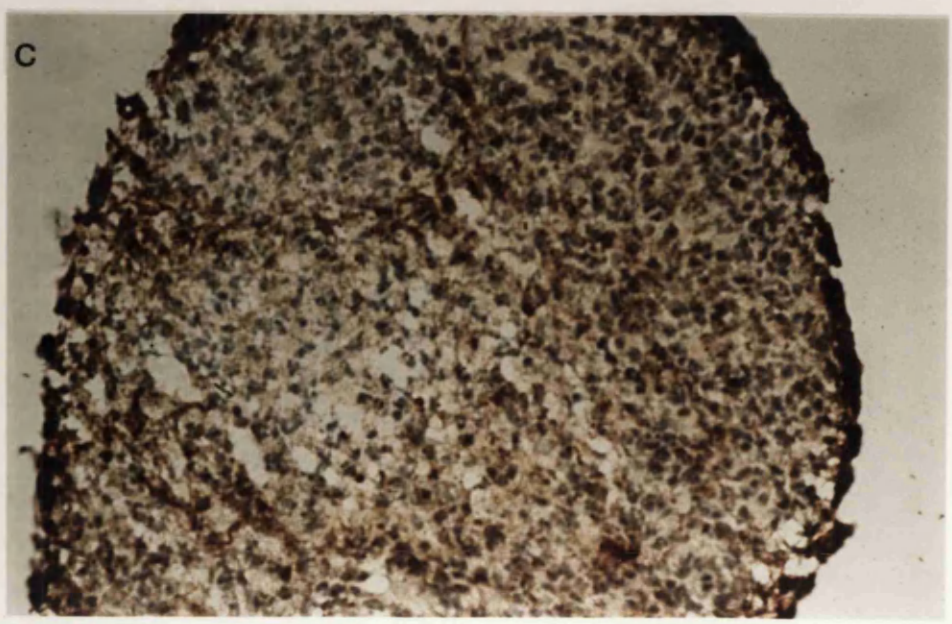

c

Figure 5.4 *Analysis of proteins synthesised in vitro in the presence of antisense oligonucleotide*

Analysis by SDS/PAGE of immunoprecipitated protein from thymocytes metabolically-labelled in the presence of antisense oligonucleotide.

(a) 2 x 10^7 thymocytes were preincubated with 75μM CD8β antisense oligonucleotide, or no oligonucleotide for 4 hours before metabolic labelling. Labelled protein was immunoprecipitated using antibodies coupled to sepharose as follows: anti-human glycophorin (YTH 89.1), lane 1; anti-mouse CD4 (YTA 3.1), lane 2; anti-mouse CD8α (YTS 169), lane 3; and anti-mouse CD8β (YTS 156), lane 4. The autoradiograph was exposured for 1 day at -70°C.

(b) 2 x 10^7 thymocytes were preincubated with 50μM CD8α antisense, 50μM CD8 control, or no oligonucleotide for 17 hours before metabolic labelling. Labelled protein was immunoprecipitated with: anti-human glycophorin antibody, lane 1; anti-CD8α, lane 2; and anti-CD8β antibody, lane 3, as described above. The autoradiograph was exposured for 3 days at -70°C. The species corresponding to CD8α, CD8β and CD4 are arrowed. Analyses were performed using 12.5% polyacrylamide gels.

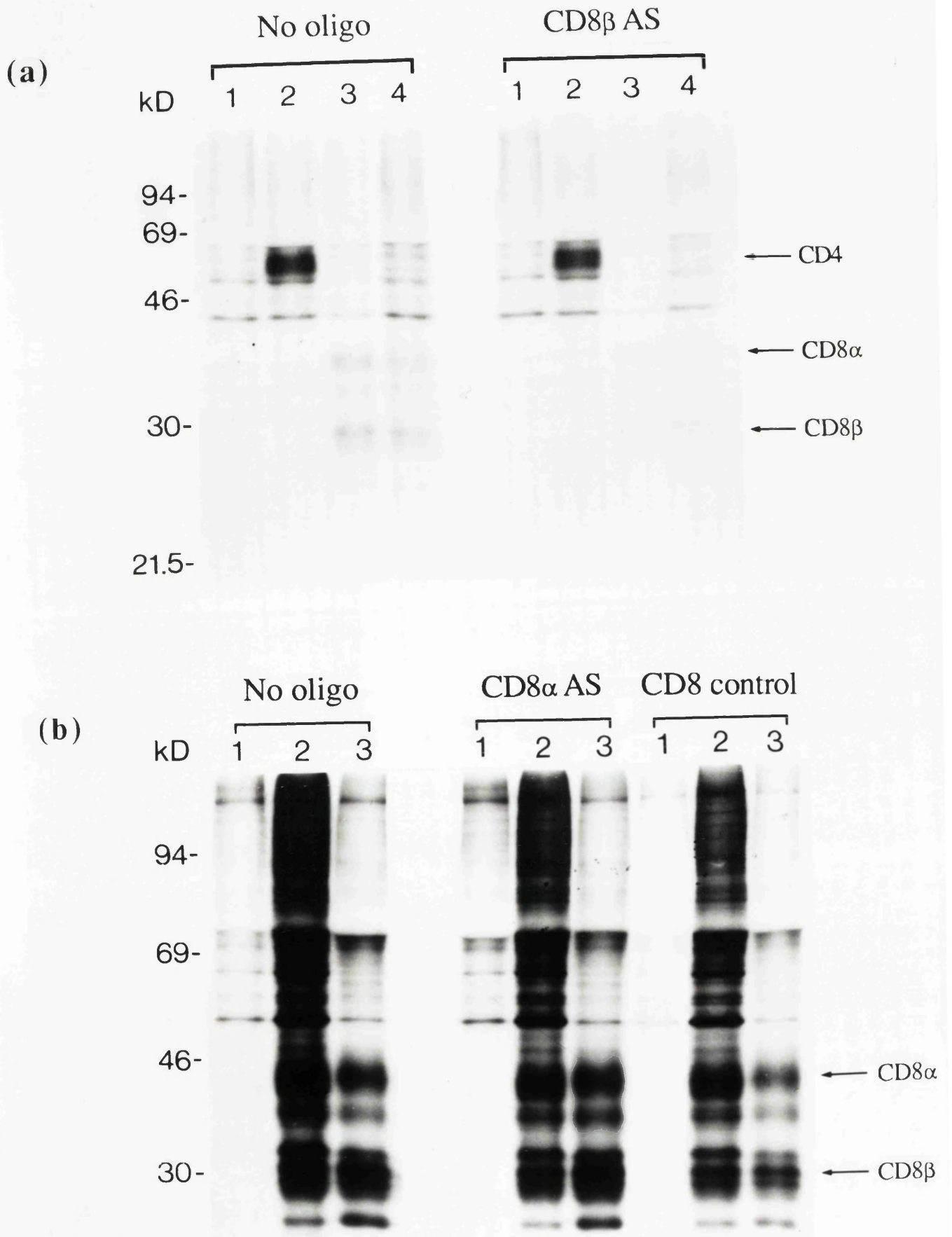

(a)
No oligo
CD8β AS
kD
1 2 3 4
1 2 3 4
94-
69-
46-
30-
21.5-
CD4
CD8α
CD8β
(b)
No oligo
CD8α AS
CD8 control
kD
1 2 3
1 2 3
1 2 3
94-
69-
46-
30-
CD8α
CD8β

Figure 5.5 *Phenotype of thymocytes arrested in development by the addition of high concentrations of Tris/EDTA buffer.*

Flow cytometric analysis of cells from lobes treated with TE buffer at a final concentration of 30μM EDTA, or untreated, following 6 days in organ culture. A minimum of twenty lobes was analysed in each group. (a) The effect of TE buffer on CD4 and CD8 expression. Cells were double-stained using a PE-conjugated anti-CD4 antibody, plus FITC-conjugated anti-CD8 antibody, and markers were set on an unstained population. (b) Thy1/HSA expression of thymocytes in organ culture treated with TE buffer, and stained using a FITC-conjugated Thy1 antibody, and a biotinylated anti-HSA antibody plus phycoerythrin second stage reagent. Markers were set on cells incubated with the second stage reagent only. A minimum of 2000 events was collected for each sample; live gates were set using forward and low angle light scatter, and propidium iodide exclusion. Fluorescent intensity is represented on a log scale. Percentage values of expression are shown in each quadrant.

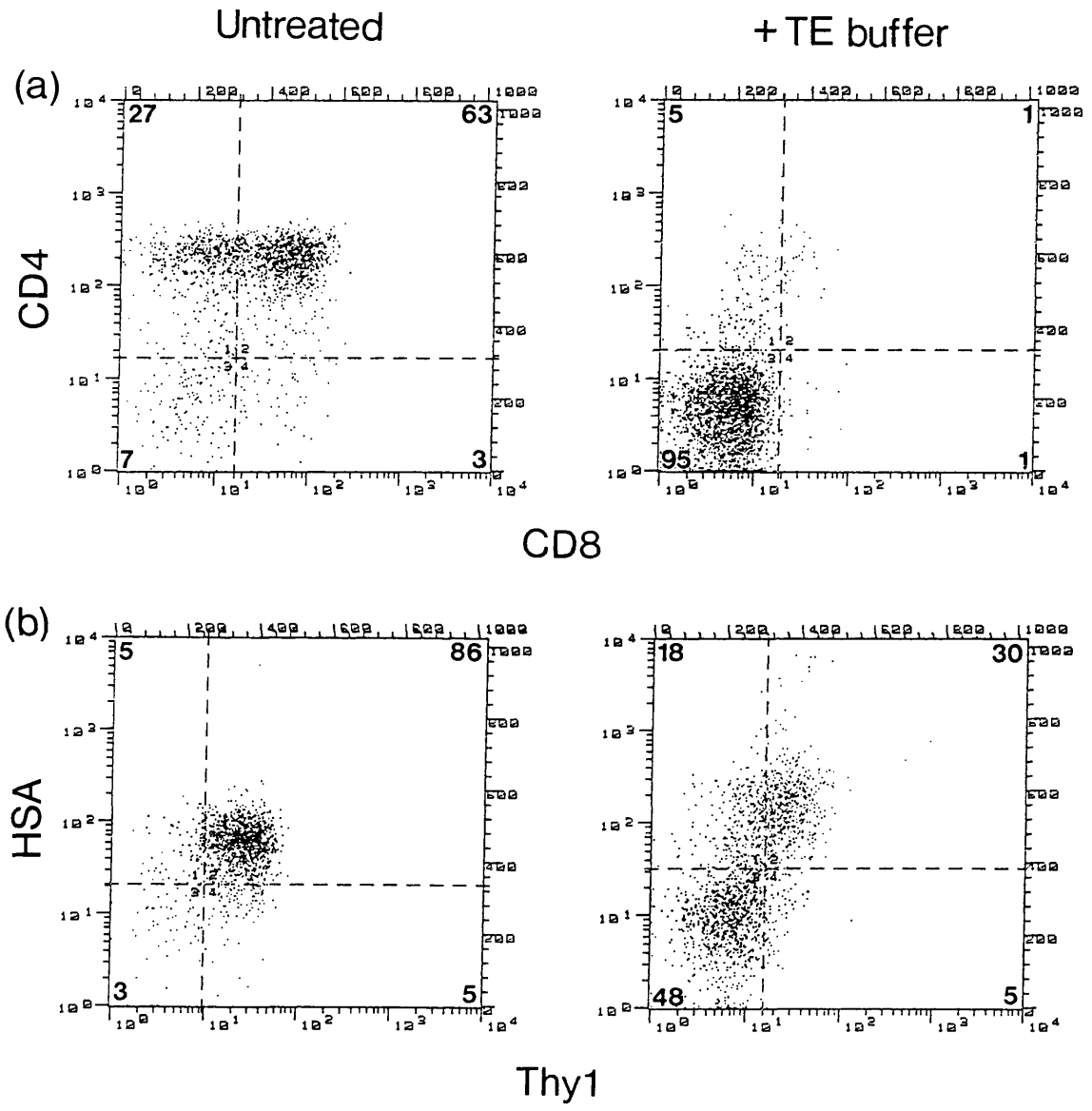

Untreated
+ TE buffer
(a)
CD4
27
63
7
3
5
1
95
1
CD8
(b)
HSA
5
86
3
5
18
30
48
5
Thy1

Figure 5.6 *Comparison of thymocyte development in conventional thymus organ culture and in hanging drop culture.*

Two-dimensional flow cytometric analysis of cells harvested from a minimum of twenty lobes cultured in (a) conventional organ culture, and (b) hanging drop culture. Cells were double-stained using a PE-conjugated anti-CD4 antibody, plus FITC-conjugated anti-CD8 antibody, and analysed as described in the legend to Figure 5.5.

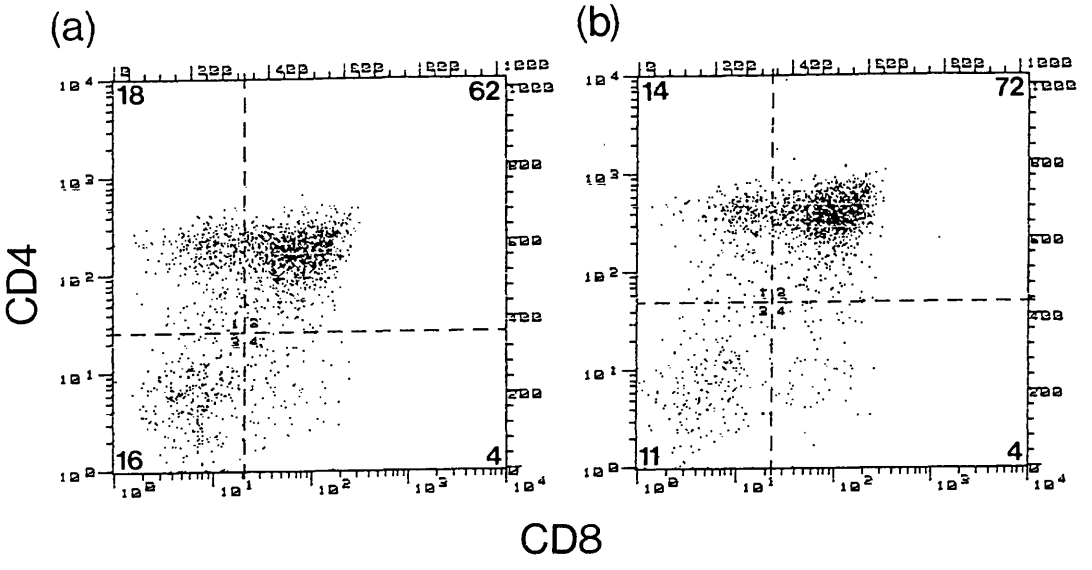

(a)
(b)
18
62
16
4
14
72
11
4
CD4
CD8

Figure 5.7 *Antisense inhibition of CD4 expression in hanging drop culture.*

Thymus lobes were incubated with a daily addition of a 100μM concentration of CD4 antisense oligonucleotide (CD4 as), control oligonucleotide, or medium alone (No oligo). The oligonucleotides used contained phosphorothioate linkages throughout the molecule. (a) The phenotype of thymocytes recovered from each experimental group is shown, analysed for CD4 and CD8 expression. (b) Single histogram analysis of the same data showing CD4 expression, and (c) CD8 expression. (d) Expression of an irrelevant marker, Thy1. Twenty lobes were used in each experimental group. For single histogram analysis, fluorescent intensity is represented on the abscissa (log scale), and cell number shown on the ordinate (linear scale). Markers were set on an unstained population of cells, and analysis was performed as described in the legend to Figure 5.5. All multiple histograms are represented on the same scale, and analysis was performed using Consort 30 software.

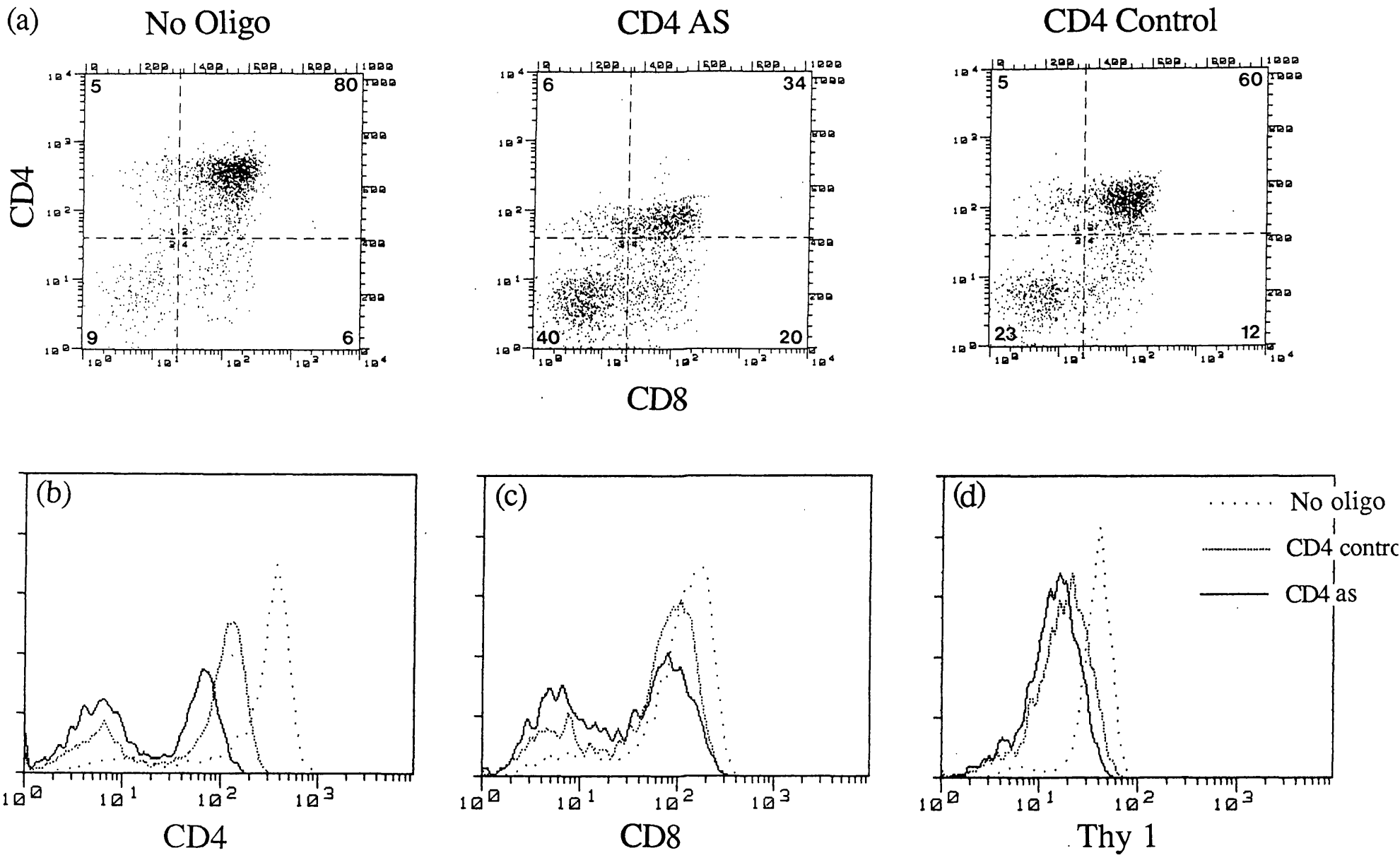
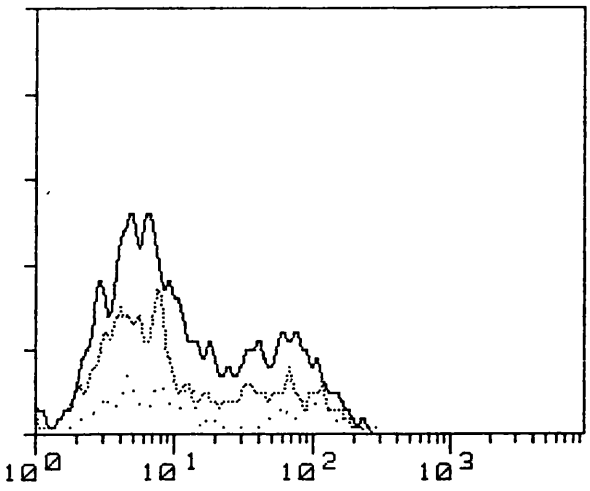

(a)
No Oligo
CD4 AS
CD4 Control
CD4
CD8
5
80
9
6
6
34
40
20
5
60
23
12
(b)
CD4
(c)
CD8
(d)
No oligo
CD4 contro
CD4 as
Thy 1

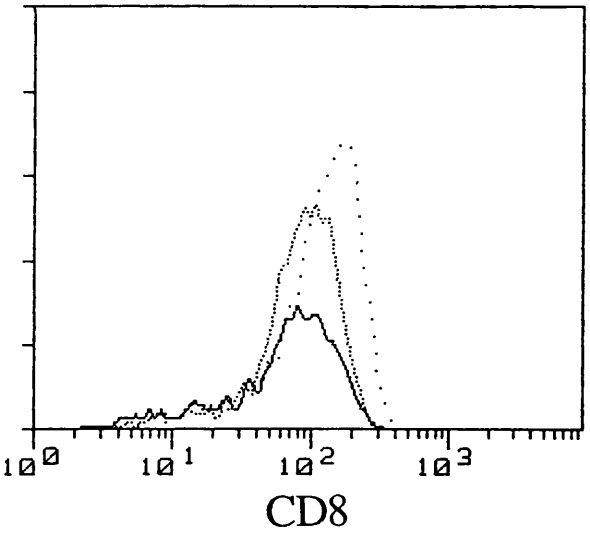
Figure 5.8 *CD8 expression on cells incubated with CD4 antisense oligonucleotide*

Flow cytometric analysis of cells harvested from lobes treated with CD4 antisense oligonucleotide, control oligonucleotide, or medium alone (No oligo) as described in the legend to Figure 5.7. (a) Multiple histogram analysis of CD8 expression observed on the $CD4^-$ population (FL2 0-86); (b) CD8 expression observed on the $CD4^+$ population (FL2 87-255). Analysis was performed as described in the legend to Figure 5.7. Multiple histograms are represented on the same scale.

(a)



(b)



····· No oligo

---- CD4 control

——— CD4 as

Figure 5.9 *CD4/CD8 expression in CD4 antisense treated cultures*

Thymus lobes were incubated for 4 days in hanging drop culture with medium alone (No oligo), or 500μM CD4 antisense (AS), or control oligonucleotide administered daily. The oligonucleotides used contained phosphorothoiate linkages at the 5' & 3' termini. (a) The CD4/CD8 phenotype of thymocytes harvested from each experimental group; (b) CD4 expression on thymocytes as in (a). The cell number per lobe for each phenotype was calculated by multiplying the percentage expression by the cell yield per lobe. Twenty lobes were used in each experimental group. Data is from flow cytometric analysis performed as described earlier.

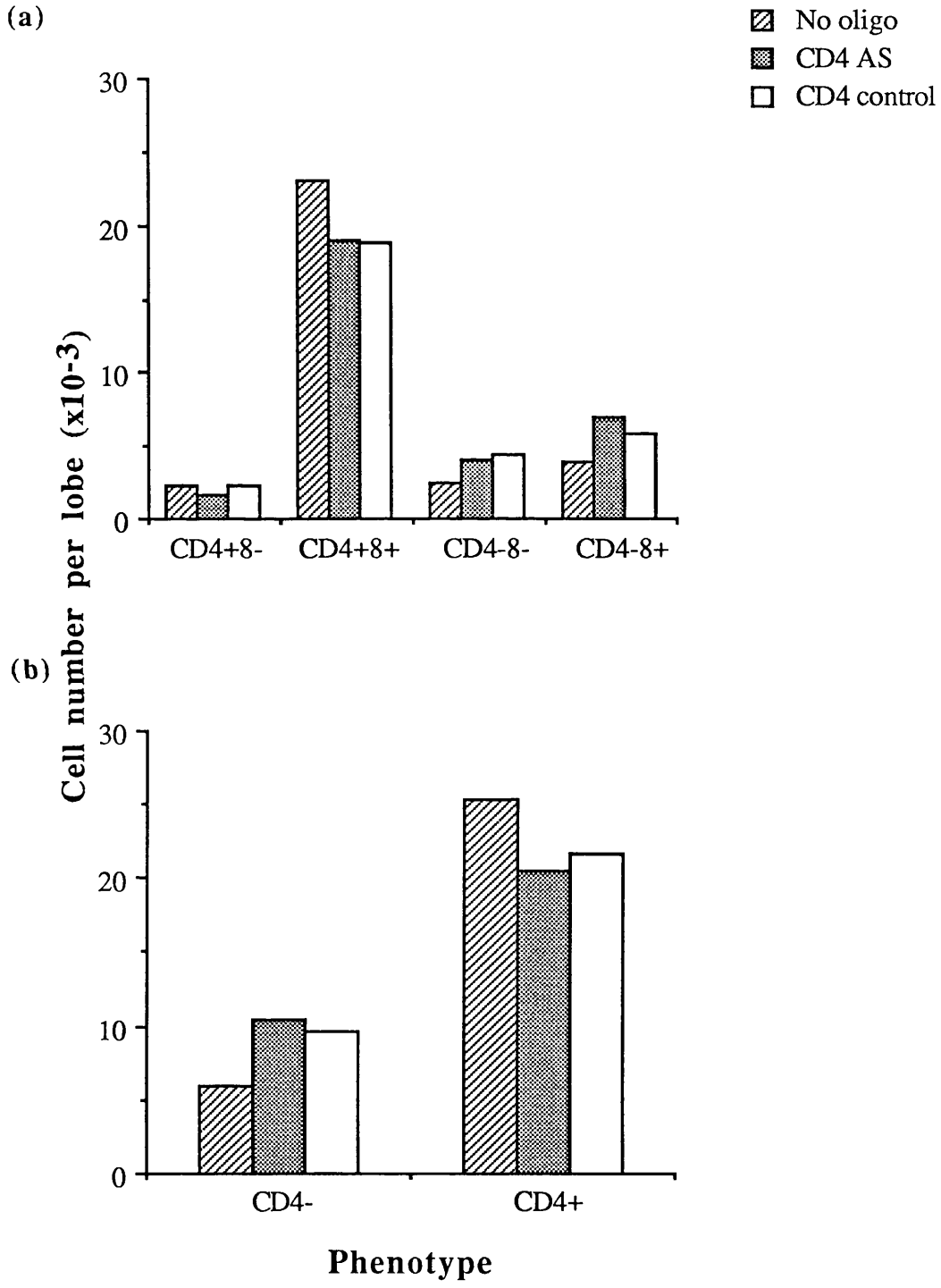
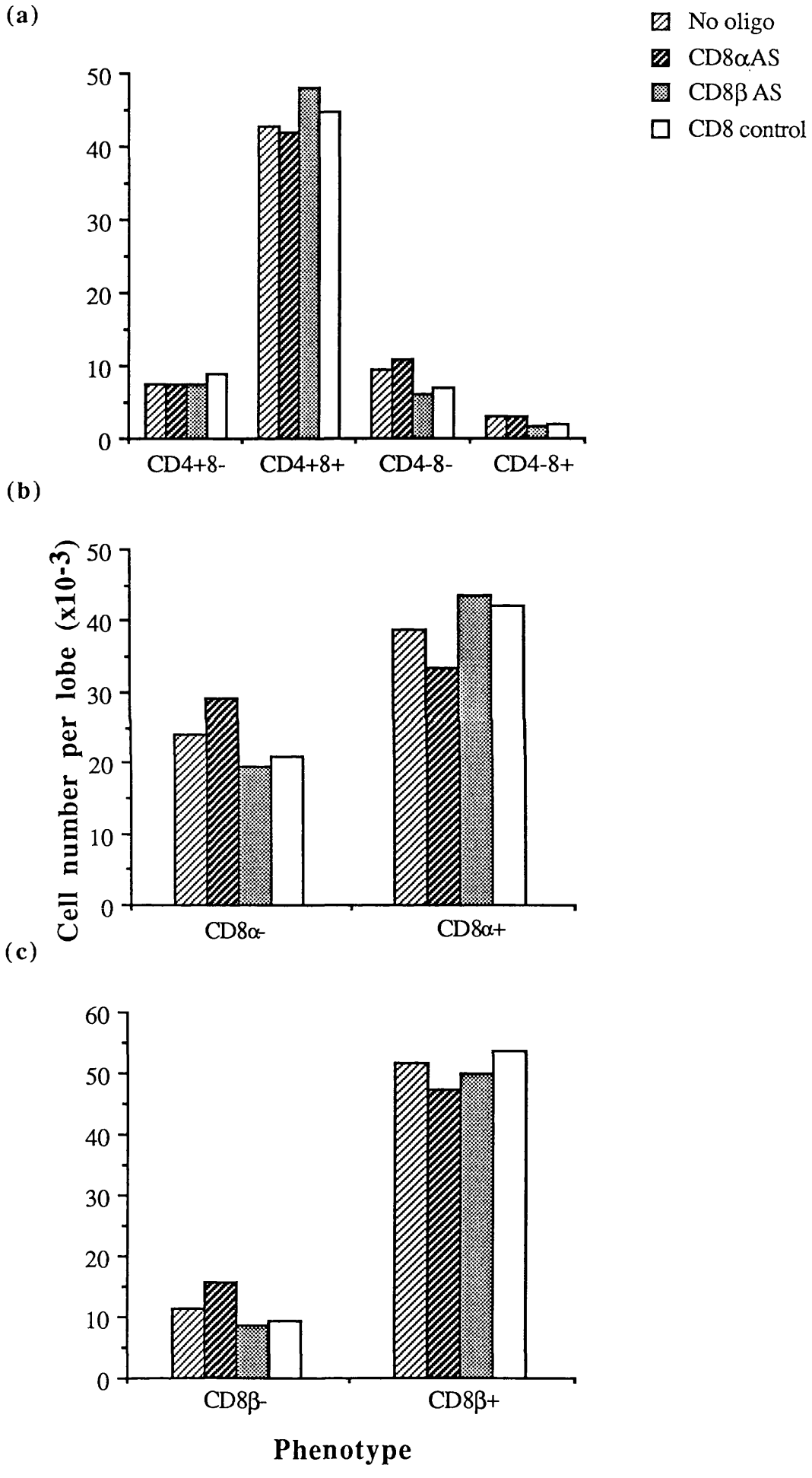

(a)
No oligo
CD4 AS
CD4 control
30
20
10
0
Cell number per lobe (x10-3)
CD4+8-
CD4+8+
CD4-8-
CD4-8+
(b)
30
20
10
0
CD4-
CD4+
Phenotype

Figure 5.10 *CD4/CD8 expression on cells recovered from CD8-antisense treated cultures*

Thymus lobes were incubated for 4 days in hanging drop culture with medium alone (No oligo), with a 100μM concentration of CD8α, CD8β antisense (AS) oligonucleotide, or CD8 control oligonucleotide administered daily. The oligonucleotides used contained a single phosphorothioate linkage at the 3' terminus. (a) CD4/CD8 expression; (b) CD8α expression; and (c) CD8β expression on cells harvested from each experimental group. The cell number per lobe for each phenotype was calculated by multiplying the percentage expression by the cell yield per lobe. Twenty lobes were used in each experimental group. Data is from flow cytometric analysis performed as described earlier.


(a)
No oligo
CD8αAS
CD8β AS
CD8 control
50
40
30
20
10
0
CD4+8-
CD4+8+
CD4-8-
CD4-8+
(b)
Cell number per lobe (x10-3)
50
40
30
20
10
0
CD8α-
CD8α+
(c)
60
50
40
30
20
10
0
CD8β-
CD8β+
Phenotype

CHAPTER SIX

Analysis of the role of T cell surface antigens in immune function

6.1 Introduction

The accessory molecules CD4 and CD8 have been implicated as being intimately involved in antigen receptor recognition, and in the generation of functionally mature T cells in the thymus (reviewed by Parnes, 1989; Fowlkes & Pardoll, 1989). Although these molecules participate in T cell activation, specific monoclonal antibodies against CD4 or CD8 have been shown to inhibit antigen-driven T cell function (reviewed by Rudd *et al.*, 1989). Evidence indicates that these co-receptor molecules are involved in the engagement of MHC ligands on stimulator cells (Doyle & Strominger, 1987; Norment *et al.*, 1988) and that they perform a role in intracellular signalling events (reviewed by Bierer *et al.*, 1989).

The α and β chains of the TCR are known to be involved in antigen binding, however, the functions of the CD3 complex invariant chains γ, δ, ε, and ζ, are poorly understood. Evidence suggests that CD3 couples receptor-ligand binding to intracellular signalling events, and following engagement of the TCR complex by antigen, the chains of the CD3 complex act as substrates for phosphorylation (reviewed by Rudd *et al.*, 1989), with rapid phosphorylation on tyrosine of CD3 ζ, and serine phosphorylation of the γ and δ chains. However, the significance of these phosphorylation events in TCR signal transduction and the initiation of later events, such as lymphokine secretion, is unclear.

A signalling function for CD4 and CD8, has also been suggested by gene transfer experiments in which the presence of CD4 or CD8 was shown to be required for optimal activation (Dembic *et al.*, 1987; Gabert *et al.*, 1987; Gay *et al.*, 1987; Sleckman *et al.*, 1987). Furthermore, both CD4 and CD8 have been shown to be associated with the *src*-family lymphocyte-specific cytoplasmic tyrosine kinase $p56^{lck}$ (Rudd *et al.*, 1988; Veillette *et al.*, 1988). $p56^{lck}$ can be activated by crosslinking CD4 or CD8 (Veillette *et al.*, 1989a, 1989b), suggesting that engagement of CD4 or CD8 with MHC may elicit an intracellular signal through this tyrosine kinase. In the cytoplasmic domain of CD4 and CD8 a cysteine motif has been identified as mediating the interaction with *lck* (Shaw *et al.*, 1989, 1990; Turner *et al.*, 1990). This information

has enabled the functional significance of the association of $p56^{lck}$ with CD4 to be directly addressed by specific mutation of the residues involved in this motif (Glaichenhaus *et al.*, 1991).

Recent studies with a CD8-dependent T cell hybridoma have investigated the role of the domains of CD8α during T cell activation using chimaeric molecules comprising the extracellular, transmembrane, and cytoplasmic domains of CD4 and CD8 (Zamoyska *et al.*, 1989). The results demonstrated the importance of the extracellular domain of CD8α in antigen responsiveness in stabilising the interaction between the T cell and the antigen presenting cell (Zamoyska *et al.* 1989). Subsequent studies have used this hybrid approach to examine the role of the extracellular, transmembrane, and cytoplasmic domains of rat CD2 and CD4 in mediating functions controlled by these molecules (Beyers *et al.*, 1991). The use of chimaeric molecules thus appears to offer a valuable approach in the dissection of molecular function.

It has been argued that in addition to ligand binding, signalling may require the interaction of the extracellular or transmembrane domain of T cell surface molecules, or a specific conformational change may be necessary in order to promote further interaction via the cytoplasmic domains. Alternatively, aggregation of the cytoplasmic domains may be sufficient without other surface interactions to transduce a signal to the interior of the cell. The construction of hybrids comprising CD4, CD8 and CD3 may therefore provide a suitable method to examine these possibilites.

6.2 Experimental design

In order to address the role of the γ chain of the CD3 complex, and the individual contribution of CD4 and CD8 in T cell function, chimaeric molecules were designed, comprising various combinations of the extracellular, transmembrane and cytoplasmic domains of these three molecules. The construction of these chimaeric molecules provided a potential means for investigating whether inclusion of the transmembrane and/or cytoplasmic domain of CD3γ would allow association of the CD4 or CD8 extracellular domain with the T cell receptor complex, and whether this

association would give increased affinity to the TCR. Previous studies have shown that increasing the proximity of CD4 or CD8 and the TCR by crosslinking with monoclonal antibodies results in an increase in T cell proliferation (Rudd *et al.*, 1989). The association of CD4 or CD8 with the TCR will presumably bring $p56^{lck}$ close to the complex, and may account for the phosphorylation of the ζ chain during activation (Rudd *et al.*, 1988; Veillette *et al.*, 1988, 1989a, 1989b). Chimaeric CD3/CD4 or CD3/CD8 molecules could be used to address what influence this kinase may exert when brought into closer proximity with the TCR, and perhaps what role other tyrosine kinases may perform in initiating the signalling cascade, such as the TCR-associated $p59^{fyn}$ (Samelson *et al.*, 1990). If CD3γ is involved in signal transduction, one could also examine whether the stimulation pathways remain functional in a chimaeric molecule.

The function of the individual domains of CD3γ, CD4 and CD8 in the chimaeric molecules could be assessed using a hybridoma assay system (Gabert *et al.*, 1987). The T cell hybridoma, DC27.10, has been transfected with genes encoding T cell receptor α and β chains specific for class I H-$2K^b$ molecules, and variants selected for loss of CD4 expression (Gabert *et al.*, 1987). This hybridoma provides a convenient assay system for CD8 function, since it has been previously shown that although this cell can respond to a clonotypic anti-T cell receptor antibody, Désiré-1, the transfectant is incapable of responding to antigen in the absence of CD8 (Gabert *et al.*, 1987). Transfection with CD8α, however, restores the response to K^b as measured by IL-2 production (Gabert *et al.* 1987), which is perhaps the most commonly used criterion to assess late activation events. Since the IL-2 gene is closely regulated, and requires a number of signals for its transcription (Crabtree, 1989), it provides a useful marker for assessing signalling through these chimaeric molecules.

6.3 Mutagenesis of murine CD3γ

In order to construct the chimaeric molecules, restriction enzyme sites were introduced into each molecule by site-directed mutagenesis to enable cleavage in to their

component extracellular, transmembrane, and cytoplasmic domains. The cDNAs encoding CD4 and CD8α containing the appropriate enzyme site were already available in this laboratory (Zamoyska *et al.*, 1989), therefore site-directed mutagenesis was only performed on the CD3γ chain.

The murine CD3γ cDNA (clone PB10AT3γ; Krissansen *et al.*, 1987) was a kind gift from M. Crumpton (ICRF, London). The CD3γ cDNA was supplied in pUC 9 and excised using Eco RI and Hind III to give a fragment of approximately 750 base pairs (bp) which was subcloned into the double-stranded replicative form of M13mp18 for oligonucleotide-directed mutagenesis.

A unique Eco RV site was introduced at nucleotide position 350 using an 18 bp oligonucleotide containing 3 mismatches (5'-GGTGCCgATaTcTAGCTC-3'; from position 342-359, mutated bases are shown in lower case) to give CD3γ(RV). A unique BssH II site was introduced at position 443 using a 21 mer containing 3 mismatches (5'-TTGACTGGCGcgcTCCATCCT-3'; from position 434-454, mutated bases are shown in lower case) to give CD3γ(BssH II). The oligonucleotides were designed to maintain the correct reading frame upon introduction of the restriction site, and to introduce the minimum number of amino-acid changes, resulting in either no change or a conservative change in the amino acid sequence of the molecule. As shown in Figure 6.1, the mutation to introduce an Eco RV site resulted in a single amino acid change from an asparagine to an aspartic acid; the introduction of a BssH II site resulted in a single change from a leucine to an alanine.

The position of the Eco RV site enabled the molecule to be cleaved into two portions comprising the extracellular domain, and transmembrane plus cytoplasmic domains (shown in Figure 6.2a). Similarly, cleavage at the BssH II site resulted in the production of two portions comprising the extracellular plus transmembrane domains, and the cytoplasmic domain. The introduction of the unique restriction enzyme site in each of the mutant CD3γ molecules was confirmed by restriction enzyme digestion and agarose gel electrophoresis as shown in Figure 6.2b.

In order to construct the chimaeric molecules, both CD3γ(BssH II) and CD3γ(RV) were subcloned by excision with BamH I and Hind III and ligation into "XpUC 19" : a modified pUC 19 vector which has an additional Xho I site in the 5' region of the existing cloning sites in the polylinker (created in this laboratory, see Appendix).

6.4 Construction and expression of hybrid molecules

The cDNAs encoding murine CD4, or CD8α, containing a unique Eco RV site (Zamoyska *et al.*, 1989) were used to construct hybrid molecules by reciprocal transfer of domains between CD4 or CD8α and CD3γ(RV). The cDNA for murine CD4, and CD8α contained a unique Eco RV site at nucleotide positions 1224, and 708, respectively (Zamoyska *et al.*, 1989). The cloning strategy to construct the chimaeric molecules using the Eco RV site is outlined in Figure 6.3.

The chimaeric cDNAs were transformed into competent bacteria and the plasmid DNA isolated as described in Chapter 2. The constructs were verified by appropriate restriction enzyme digestion of the plasmid DNA using Eco RV and Bam HI for A & D; or Eco RV and Sac I for B & D; and the digests analysed by agarose gel electrophoresis, as shown in Figure 6.4. The resultant chimaeric constructs were designated A (CD3γ/CD4); B (CD4/CD3γ); C (CD3γ/CD8α); and D (CD8α/CD3γ); and are illustrated in Figure 6.5.

The unique enzyme sites introduced into CD3γ were to enable the construction of hybrid molecules containing various combinations of the extracellular domain, transmembrane domain and cytoplasmic domain of CD3γ, CD4 and CD8α. The first hybrids were made using the Eco RV mutants of CD3, CD4 and CD8, to comprise the extracellular plus transmembrane; or cytoplasmic domains of each molecule. However, following the transfection studies using these molecules there was insufficient time remaining during this project to construct and perform subsequent experiments using the BssH II mutants.

The chimaeric constructs A to D were subcloned into the mammalian expression vector, pHβ Apr-1-neo (Gunning *et al.*, 1987), which drives expression using the human beta actin promoter, and contains the neomycin resistance gene as a selectable marker (see Appendix). The ligations were verified by transformation into competent bacteria, DNA isolated by the plasmid miniscreen method, and analysed by appropriate restriction enzyme digestion (not shown).

The hybrid constructs in the pHβ Apr-1-neo vector were transfected into DC27.10 hybridoma cells (Gabert *et al.*, 1987), or mouse 3T3 cells by protoplast fusion or by electroporation, under selection at concentrations of G418 (Geneticin) upto 1mg/ml active concentration. Following selection, resistant clones were screened for surface expression of the transfected molecule by immunofluorescent analysis, and RNA was prepared for northern analysis.

6.5 Northern analysis

The successful transfection of the chimaeric molecules was assessed by northern analysis. This confirmed that the D construct (CD8α/CD3γ) was successfully transfected into the DC27.10 hybridoma. RNA was prepared from three representative transfectants (D.3, D.8, D.9); the parental hybridoma, DC27.10; and a $CD8\alpha^+$ transfectant, h5 (Zamoyska *et al.*, 1989). The samples were divided in two, electrophoresed in duplicate, and blotted, to enable simultaneous comparison of the same RNA samples hybridised with two separate probes.

As shown in Figure 6.6a, northern analysis of transfectants of the D chimaeric molecule revealed a single band at 1.8kb hybridised with the CD8α cDNA probe in the lanes containing RNA from the transfectants D.3, D.8, and D.9. A single band of only 1.6kb was seen in the positive control (h5) corresponding to the RNA for the full length CD8α cDNA. As expected, no band was seen in the negative control (parental DC27.10) hybridised with the CD8α probe. Figure 6.6b shows two species, of approximately 1.8kb and 1.3kb in the lanes containing RNA prepared from the transfectants D.3, D.8, and D.9, hybridised using a CD3γ cDNA probe. A single

species of 1.3kb was present in the positive controls (DC27.10, and h5), corresponding to native CD3γ. The RNA prepared from cells transfected with the hybrid CD8α/CD3γ construct was therefore of a higher molecular size than the native CD3γ, and CD8α RNA. Since the D construct cDNA was approximately 200bp larger than the native CD8α, and approximately 400bp larger than the native CD3γ, the sizes of the RNA species are in agreement with the prediction from the cDNA size.

None of the other hybrid constructs were successfully transfected into DC27.10 cells or 3T3 cells following eight independent transfections, as assessed by northern analysis.

6.6 Immunofluorescent analysis of transfectants

The cell surface expression of the hybrid molecules was screened by immunofluorescent staining and flow cytometric analysis, however, since there is currently no antibody available to murine CD3γ, it was not possible to locate the extracellular domain of the CD3γ molecule in this manner. Hence only the transfectants containing the CD4 or CD8α extracellular domain (B or D, respectively) could be screened by immunofluorescent analysis. In comparison to the positive control, h5, all the D transfectants analysed were negative for surface expression of CD8α (Figure 6.7), showing identical staining to the negative control (DC27.10, parental). This was unexpected since all the D transfectants analysed were known to contain RNA for the chimaeric molecule.

Internal staining of the cells was therefore performed in order to determine whether the D transfectants were able to synthesise protein from the hybrid RNA detected by northern analysis. The cells were permeabilised in suspension in ice-cold 50% ethanol in RPMI, and then stained as usual for flow cytometric analysis. As can be seen in Figure 6.8a, the D transfectants, although surface negative for CD8α, showed positive staining for CD8α within the permeabilised cells. The hybrid molecule did not appear to be stably expressed, however, since loss of expression was often

seen in transfectants maintained for longer than two weeks in culture, even under continuing selection (Figure 6.8b).

The lack of surface expression of CD8 by these transfectants therefore precluded their use in functional assays.

6.7 Discussion

Transfectants were successfully obtained with the hybrid molecule containing the extracellular domain of CD8α and the transmembrane and cytoplasmic domains of CD3γ. An unexpected observation, however, was that surface expression of the hybrid molecule could not be detected by immunofluorescent analysis, although the presence of CD8α protein within the cell confirmed that the construct was being expressed. The extracellular domain of CD8α used in constructing this hybrid is known to be expressed stably in the form of a hybrid with CD4 (Zamoyska *et al.*, 1989), or with class I or class II MHC (Letourneur *et al.*, 1990), and is still recognised by the anti-CD8 monoclonal antibody used for immunofluorescent analysis (Zamoyska *et al.*, 1989). The inability of this construct to reach the cell surface is thus due to the presence of the transmembrane and cytoplasmic domains of CD3γ. The retention of this molecule within the cell prevented the use of these transfectants in any functional studies.

The introduction of certain mutations into membrane proteins may render the molecule incompetent for transport from the endoplasmic reticulum (ER) to the Golgi system, and some chimaeric proteins have been shown to display the same fate (Bonifacino *et al.*, 1990). Furthermore, the individual components of the TCR/CD3 complex, particularly γ, appear unable to exit from the ER to the Golgi unless they are associated with other TCR/CD3 chains. In the absence of other TCR subunits, CD3γ is rapidly degraded by the ER pathway (Bonifacino *et al.*, 1989; and reviewed by Klausner & Sitia, 1990).

At the time these studies were commenced, it was not known that individual chains of the TCR/CD3 were susceptible to retention within the cell. Subsequent studies have identified a sequence conferring retention within the ER contained in the

transmembrane domain of components of the TCR (Bonifacino *et al.*, 1990). This would provide an explanation for the retention of the hybrid molecule observed in these studies. The D hybrid construct comprises the transmembrane and cytoplasmic domains of CD3γ, hence it is probable that this retention sequence is contained within the transfected molecule, thus preventing transport to the cell surface and causing it to be sequestered within the cell. Although CD3γ is incapable of leaving the ER unless associated with other TCR/CD3 chains, this does not explain why the transfected molecule was apparently unable to reach the surface of the cell, since the DC27.10 hybridoma contains other endogenous components of the TCR/CD3 complex, to which CD3γ could potentially associate. One possible explanation may be that the construct is targetted for ER degradation before it is able to be "rescued" by association with another component of the TCR/CD3 complex, and perhaps the state of assembly reflects the acquisition of a protease resistant conformation. Alternatively, the presence of the CD8 extracellular domain may sterically hinder such an association.

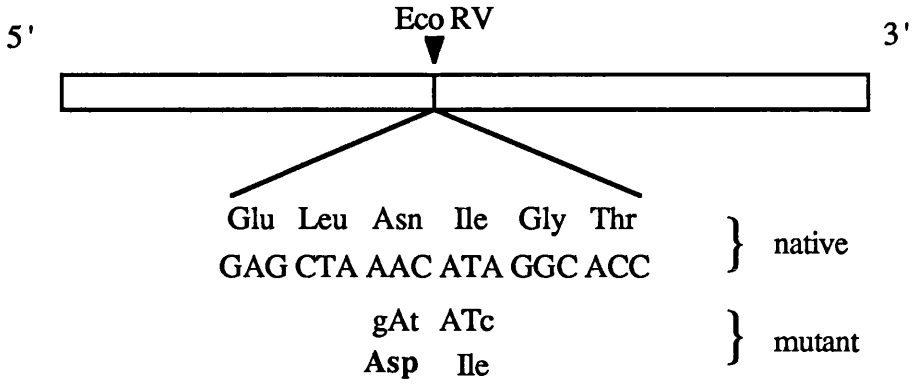
A recent report has been published in which the successful expression of a chimaeric CD8/CD3ζ construct was obtained (Irving & Weiss, 1991). This construct comprised the extracellular and transmembrane domains of CD8α, and only the cytoplasmic domain of CD3ζ. Although transfection studies suggest that ζ can be expressed in the absence of other TCR/CD3 chains on the cell surface, the successful expression of this molecule, independent of the other TCR components, may be attributable to the presence of the CD8α extracellular and transmembrane domains. This is consistent with the argument that the transmembrane domain of CD3/TCR components contains sequences which dictate its association with the TCR complex, or a potential target sequence for ER degradation. These studies also demonstrated a role for ζ in signal transduction, in the absence of CD3γ, δ, and ε, perhaps suggesting that ζ performs a role in coupling the TCR to intracellular signal transduction mechanisms (Irving & Weiss, 1991).

The formation of chimaeric constructs using the BssH II site may therefore yield more information about the role of CD3γ, although there was insufficient time

remaining in this study to produce these constructs. Following antigenic stimulation, CD3γ is a substrate for serine phosphorylation, therfore one could investigate the importance of the serine residues in the cytoplasmic domain of CD3γ during activation, by site-directed mutagenesis. By attaching the cytoplasmic domain of CD3γ containing these specific mutations to the extracellular and transmembrane domains of CD4 or CD8, one could distinguish the mutants from the native molecule. In the absence of the retention signal present in the transmembrane domain of CD3 components, these constructs would be expected to be stably expressed on the cell surface, and could therefore be used in functional studies. This would enable one to address whether CD3γ, δ, and ε have a signalling function, or whether these components perform some regulatory role in TCR function.

Figure 6.1 *Introduction of amino acid changes by oligonucleotide-directed mutagenesis.*

The nucleotide sequence and corresponding amino acid sequence of this region of CD3γis shown. The mutation to introduce an Eco RV site at nucleotide position 350 resulted in a single amino acid change from an asparagine to an aspartic acid; the introduction of a BssH II site at position 443 resulted in a single change from a leucine to an alanine. Amino acid changes are represented in bold type.


5'
Eco RV
3'
Glu Leu Asn Ile Gly Thr
GAG CTA AAC ATA GGC ACC
native
gAt ATc
Asp Ile
mutant

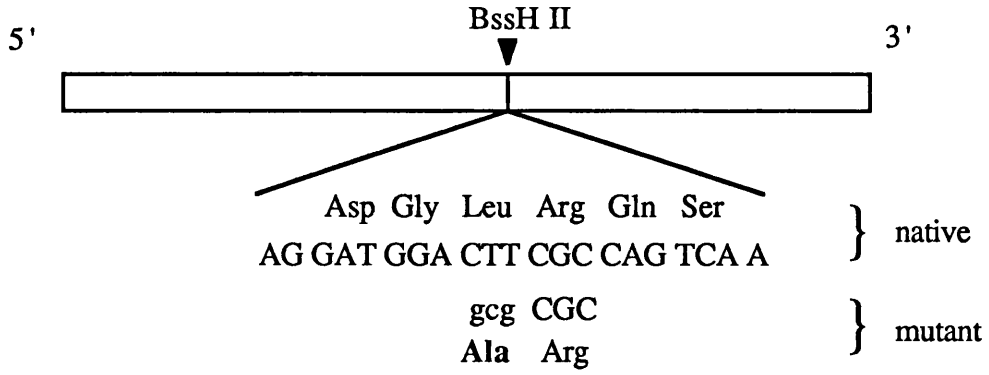
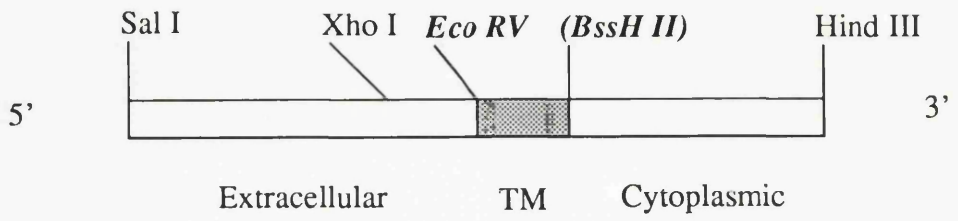

5'
BssH II
3'
Asp Gly Leu Arg Gln Ser
AG GAT GGA CTT CGC CAG TCA A
native
gcg CGC
Ala Arg
mutant

Figure 6.2 *Analysis of the Eco RV and BssH II mutants of CD3γ.*

(a) The positions of the unique enzyme sites introduced by oligonucleotide-directed mutagenesis of the CD3γ chain are shown. The Eco RV site cleaves the molecule into extracellular (350bp), and transmembrane plus cytoplasmic domains (400bp); the BssH II site enables cleavage into the extracellular plus transmembrane (450bp), and cytoplasmic domains (300bp). (b) The products of restriction enzyme digestion of the CD3γ mutants. Lanes 1-5 show the restriction fragments (approximately 400bp, and 300bp, arrowed) of CD3γ(BssH II), excised with Eco RI, Hind III, and cleaved with BssH II. Lanes 6-10 show the restriction products (both approximately 350bp, marked by an arrowhead) of CD3γ(Eco RV), excised with Eco RI and Hind III, and cleaved with Eco RV. The digests were analysed by agarose gel electrophoresis using a 1% gel. The position of the linearised vector is marked (*). Size markers are shown in kb.

(a)


Sal I
Xho I
Eco RV
(BssH II)
Hind III
5'
3'
Extracellular
TM
Cytoplasmic

(b)

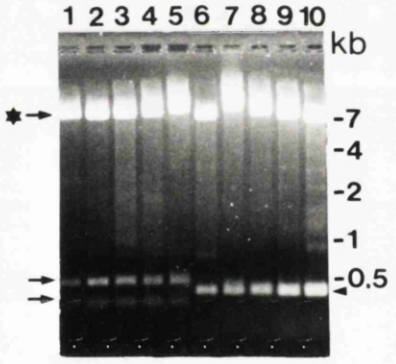
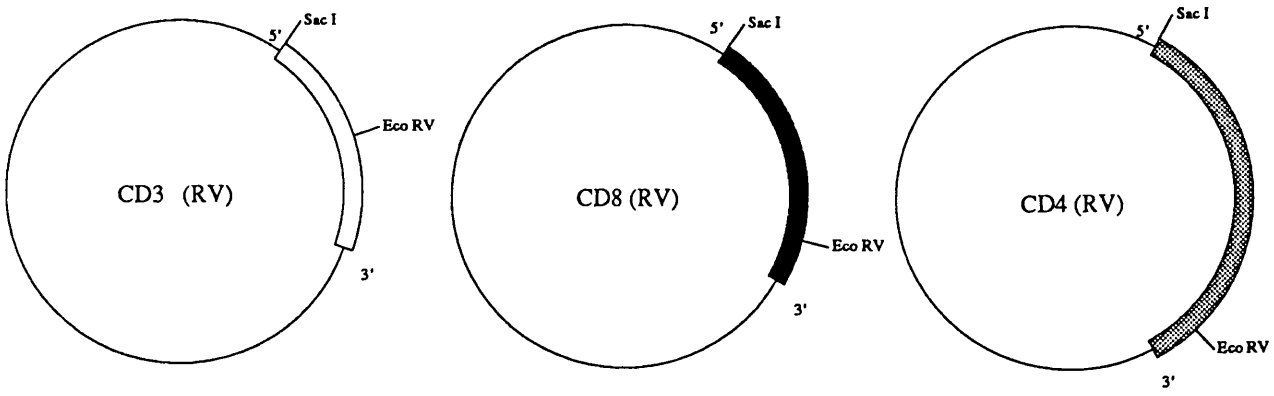
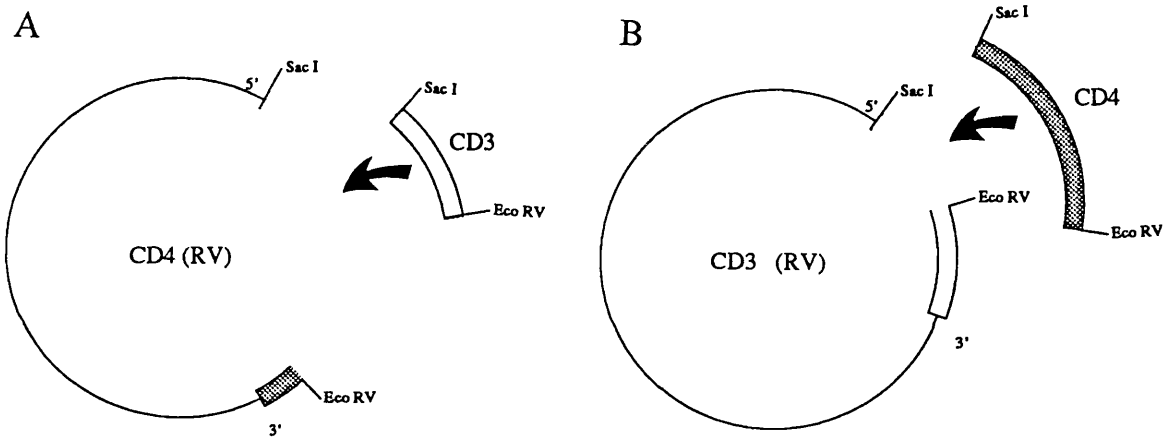

1 2 3 4 5 6 7 8 9 10
kb
-7
-4
-2
-1
-0.5

Figure 6.3a *Strategy for subcloning and transfer of domains.*

Schematic diagram of the restriction enzyme digestion and subsequent reciprocal transfer of fragments between CD4(RV), CD8α(RV) and CD3γ(RV) cDNAs. The CD4(RV), CD8α(RV), and CD3γ(RV) cDNAs were digested with Eco RV and Sac I, and electrophoresed on a 1% agarose gel to visualise the restriction products. Restriction enzyme digestion with Eco RV and Sac I yielded the 5' fragment, and the vector plus 3' fragment, of each construct. The bands in the gel corresponding to the 3' portion of the molecule plus the vector, or those corresponding to the 5' part of the molecule were excised and the DNA isolated from the gel matrix using the Geneclean method. Reciprocal ligations were performed to recombine the vector plus 3' fragment of CD4 or CD8α with the 5' fragment of the CD3γ(RV) molecule. Similarly, the 5' fragment of CD4 or CD8α was ligated to the vector plus 3' fragment of CD3γ(RV) by standard ligation procedures. (Figure 6.3b is shown overleaf)


CD3 (RV)
5'
Sac I
Eco RV
3'
CD8 (RV)
5'
Sac I
Eco RV
3'
CD4 (RV)
5'
Sac I
Eco RV
3'


A
CD4 (RV)
5'
Sac I
Eco RV
3'
Sac I
CD3
Eco RV
B
CD3 (RV)
5'
Sac I
Eco RV
3'
Sac I
CD4
Eco RV

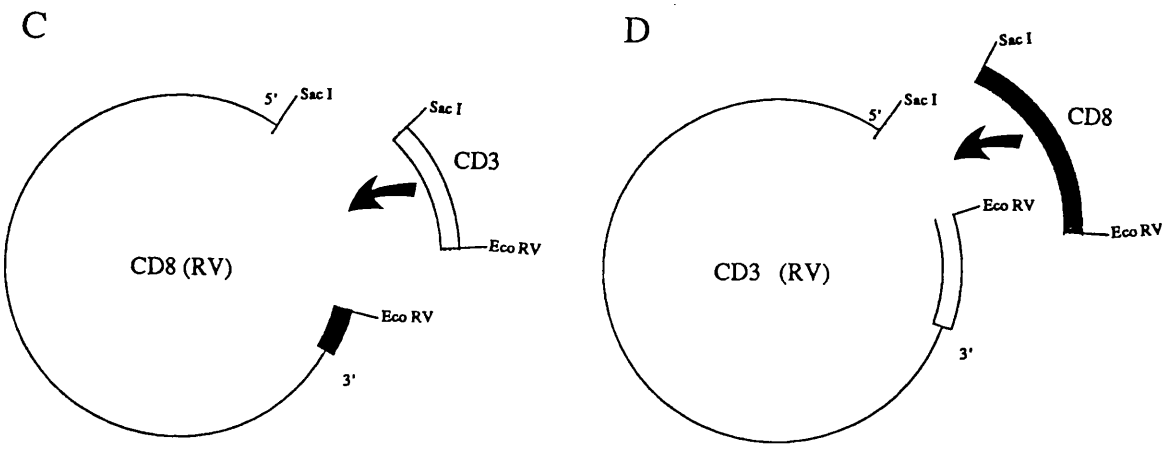

C
CD8 (RV)
5'
Sac I
Eco RV
3'
Sac I
CD3
Eco RV
D
CD3 (RV)
5'
Sac I
Eco RV
3'
Sac I
CD8
Eco RV

Figure 6.3b *Strategy for subcloning and transfer of domains.*

Schematic diagram of the completed hybrid constructs. The constructs were designated as follows: A (CD3γ/CD4); B (CD4/CD3γ); C (CD3γ/CD8α); and D (CD8α/CD3γ).

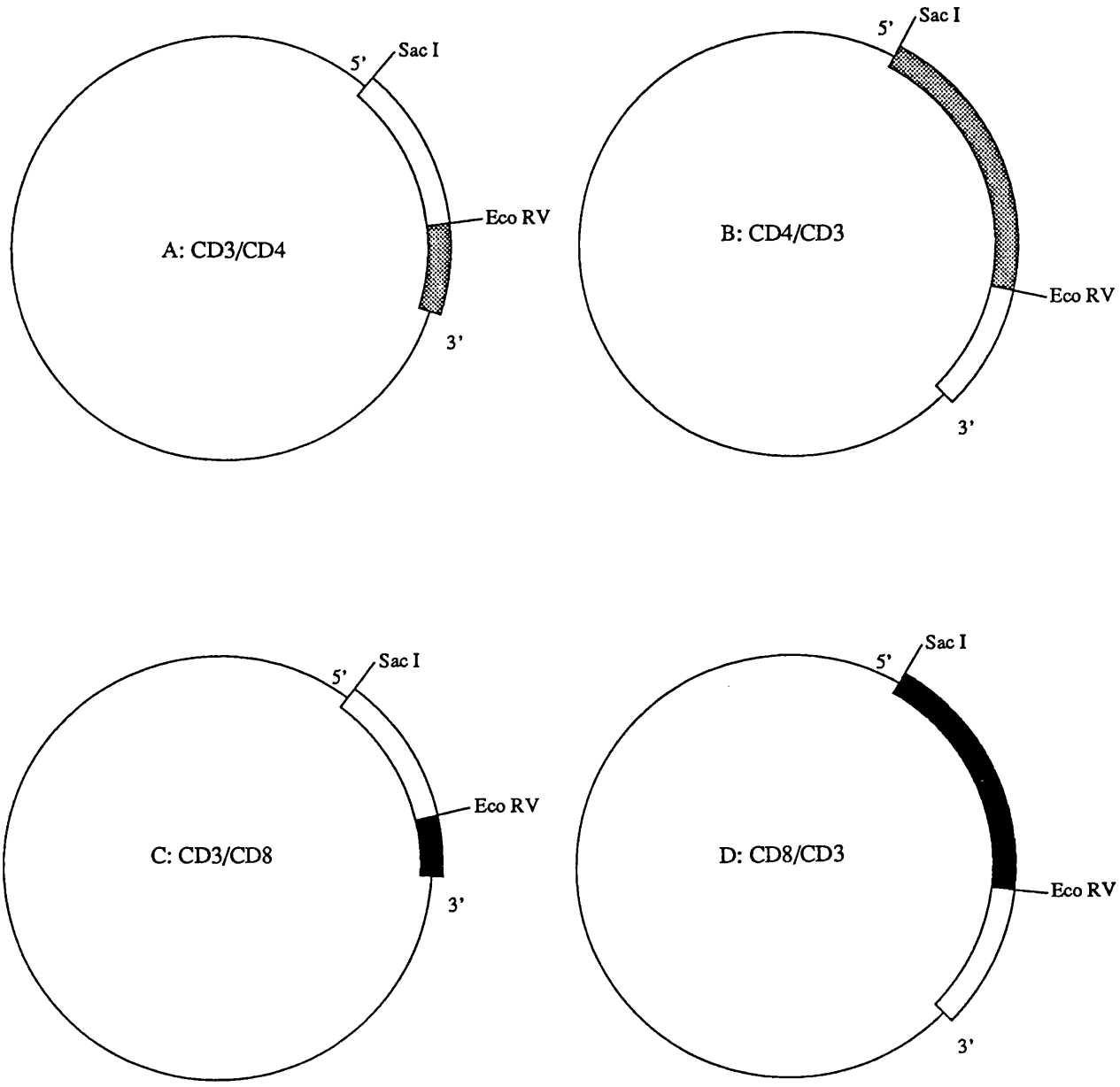
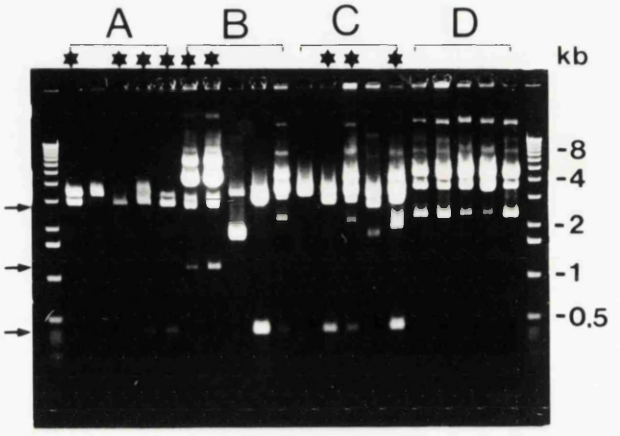

A: CD3/CD4
5'
Sac I
Eco RV
3'
B: CD4/CD3
5'
Sac I
Eco RV
3'
C: CD3/CD8
5'
Sac I
Eco RV
3'
D: CD8/CD3
5'
Sac I
Eco RV
3'

Figure 6.4 *Restriction enzyme digestion to verify the hybrid constructs.*

(a) Restriction enzyme digestion of the hybrid constructs analysed by 1% agarose gel electrophoresis. Lanes 1 & 22, 1 kb markers; lanes 2-6, A hybrids; lanes 7-11, B hybrids; lanes 12-16, C hybrids; lanes 17-21, D hybrids. The positions of the correct restriction fragments are arrowed; A: (350bp, 2900bp); B: (1200bp, 3200bp); C: (350bp, 2800bp). A/C hybrid constructs were digested using Sac I/Bam HI; B/D hybrid constructs were digested using Xho I/Hind III.

(b) The digestion of the D hybrids was repeated as in (a). Lanes containing the correct restriction fragments are indicated (*). The positions of the correct restriction fragments are arrowed; D: (700bp, 3200bp).

(a)



(b)

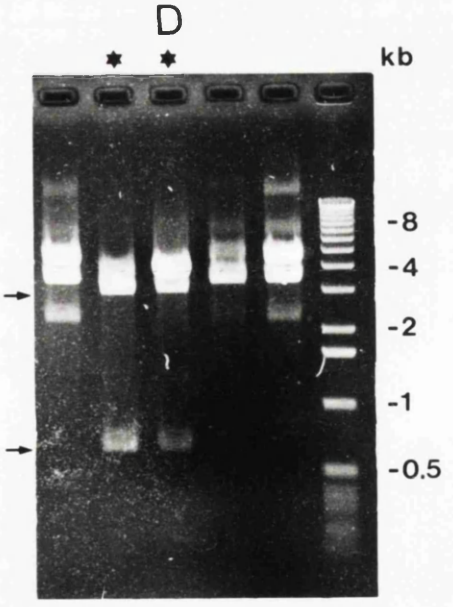


Figure 6.5 *Schematic diagram representing the hybrid molecules A, B, C and D.*

The domain contribution from CD4, CD8α or CD3γ, and the size of each hybrid construct is as shown.

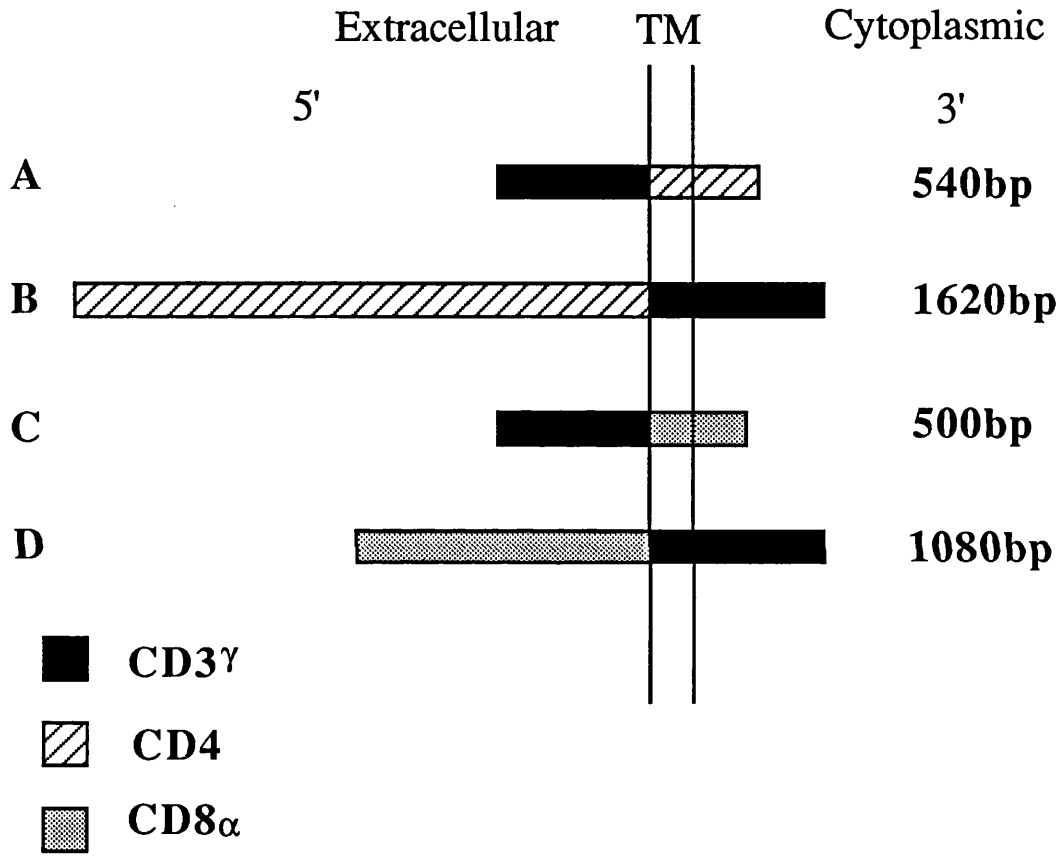

Extracellular
TM
Cytoplasmic
5'
3'
A
540bp
B
1620bp
C
500bp
D
1080bp
CD3γ
CD4
CD8α

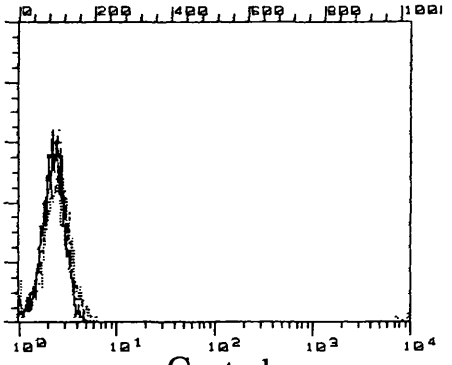
Figure 6.6 *Northern analysis of the D hybrid transfectants.*

Autoradiographs of formaldehyde gel electrophoresis of RNA. (a) Hybridisation using a CD8α probe (Sac I/Xho I fragment from CD8α cDNA in XpUC 19); (b) Hybridisation of duplicate RNA using a CD3γ probe (Bam HI/Hind III fragment from CD3γ cDNA in XpUC 19). The RNA prepared from each D transfectant (D.3, D.8, D.9), and from each control (27.10, parental; h5, $CD8\alpha^+$) was electrophoresed in duplicate and blotted onto nylon membranes. The sizes were determined by comparison of the distances migrated by the labelled species with those of the ribosomal RNA bands (5 kb and 2.1 kb), on the filter: D construct (CD8α/CD3γ; 1.8kb), native CD8α (1.6kb), native CD3γ (1.3kb).

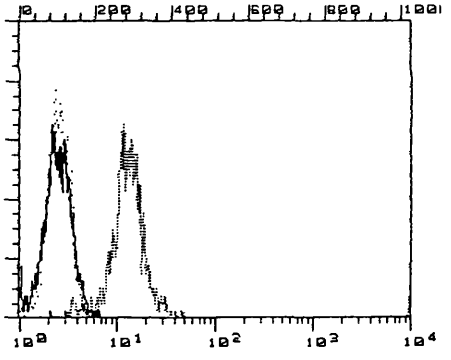
(a)
1.8
1.6
D.3
D.8
D.9
h 5
27.10
(b)
1.8
1.3
D.3
D.8
D.9
h 5
27.10

Figure 6.7 *Flow cytometric analysis of CD8 expression by the transfectants containing the CD8α/CD3γ(D) molecule.*

Immunofluorescent analysis of a representative D transfectant (D.9), DC27.10 (parental) and h5 ($CD8\alpha^+$ transfectant). The cells were stained using a FITC-labelled goat anti-rat antibody alone (control); or an anti-CD8 (YTS 169.4) antibody plus FITC-labelled goat anti-rat antibody (CD8). Fluorescence intensity is represented on the abscissa, total events on the ordinate. Multiple histogram analysis was performed using FACScan Research software. All histograms are represented on the same scale.



Control



CD8

· · · · · · DC27.10

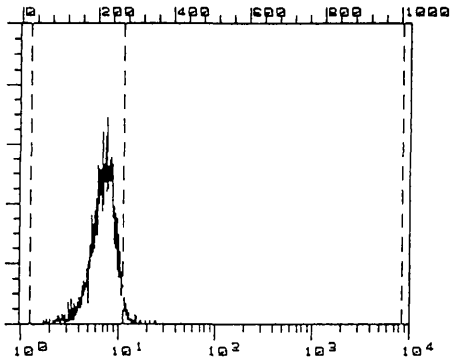
..... h5

——— D.9

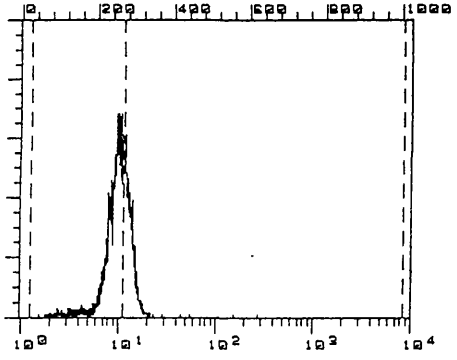
Figure 6.8 *Flow cytometric analysis of CD8 expression by the transfectants containing the CD8α/CD3γ(D) molecule.*

(a) Internal staining of the D.9 transfectant. (b) Repeat of the internal staining of the D.9 transfectant, plus DC27.10 and h5, following two weeks in culture. The cells were permeabilised using 50% ethanol prior to immunofluorescent analysis. Staining was performed as described in the legend to Figure 6.7. For each cell type, the control staining profile is shown above, and the CD8 profile below.

(a) D.9

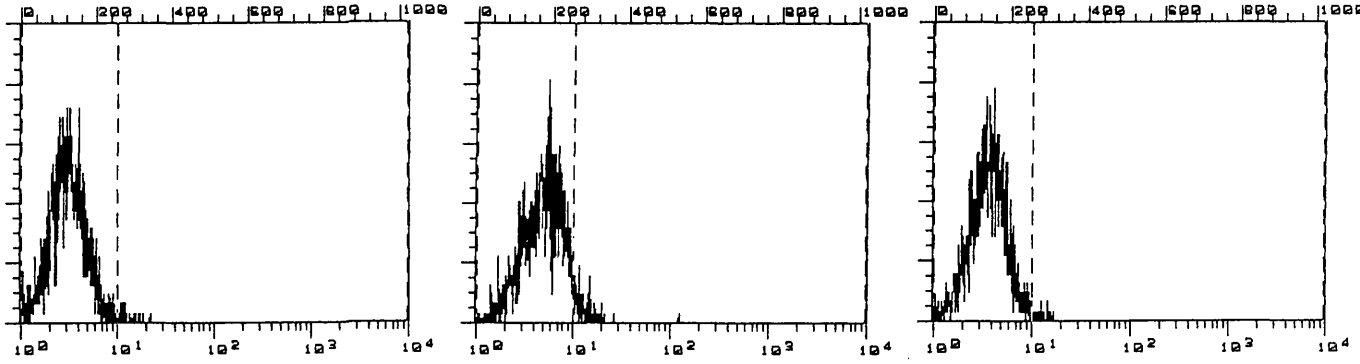


Control

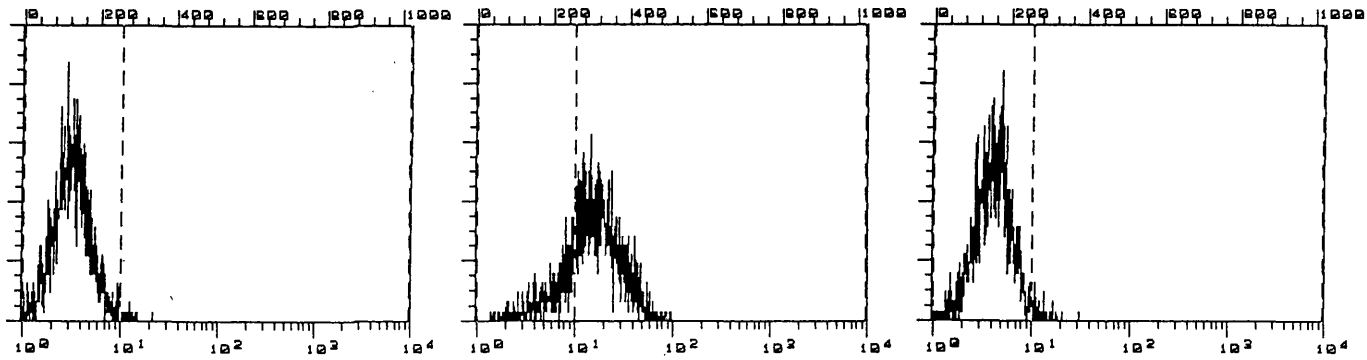


CD8

(b) DC27.10 h5 D.9



Control



CD8

Concluding remarks

The aims of this thesis were twofold. Firstly, to identify lymphoid precursors in murine foetal liver, with the prospect of manipulating gene expression within this population, to investigate how this may influence development. Secondly, to examine the role of accessory molecules during thymic ontogeny, and to further investigate their role in immune function.

The identification of early lymphoid precursors in foetal liver with an HSA- negative phenotype (approximately 1-3% of the total) has not been reported previously. Further characterisation of this HSA^- precursor population in foetal liver using monoclonal antibodies against Sca-1 and Sca-2 is required to compare the phenotype of this precursor population to that of the previously identified $Thy1^{lo}$, Lin^-, $Sca\text{-}1^+$ progenitors in the bone marrow and foetal liver (Ikuta *et al.*, 1990; Spangrude *et al.*, 1989). In contrast to HSA^+ precursors identified in the thymus, the absence of HSA on the early precursors isolated in this study may suggest that the acquisition of HSA is developmentally regulated, and perhaps reflects lineage restriction.

The status of T cell receptor gene rearrangement of this population also remains to be determined. Rearrangement of the TCR genes will imply pre-commitment to the T cell lineage, however, if the TCR genes are still in the germline configuration, this may indicate that these cells are not yet lineage restricted, and may still have the potential to develop along the B cell pathway. The use of radiation chimaeras is one approach that would allow the investigation of the ability of these precursors to reconstitute other lineages of the haematopoietic system.

It will also be informative to determine the CD45 status of these precursors, and investigate which isoforms are expressed. This may help to clarify the controversy which exists regarding isoform expression of CD45 during thymic development, and what role CD45 performs in ontogeny. Since CD4, CD8 and CD45 have been implicated in signal transduction in mature T cells, it is possible that during thymic development there may be a functional interaction between the phosphatase activity of

CD45, and the activity of $p56^{lck}$ associated with CD4 and CD8.

An initial attempt was made to immortalise these foetal liver progenitors using a retroviral construct containing SV40 T antigen as the transforming agent. A temperature-sensitive mutation was used to allow the establishment of cell lines that could be easily propagated at the permissive temperature, yet still enable reversion of the immortalised cells to a relatively normal phenotype at the non-permissive temperature. It was hoped that this approach would allow the developmental lineage relationships of early heamatopoietic cells to be defined. Preliminary attempts to infect early precursors in foetal liver and foetal thymus proved unsuccessful, however, since epithelial cells were the major target for the retrovirus. Further studies should perhaps investigate the use of other retroviruses in this approach.

In view of the lack of success in transfecting genes into precursors, it was decided to attempt to investigate the function of accessory molecules using antisense oligonucleotides in thymus organ culture. This technique provides the potential to investigate the expression of molecules important in development, whilst eliminating the complications inherent in the use of antibodies. Although the choice of oligonucleotide may have limited the studies presented here, alternative modifications to increase stability, such as the use of methylribonucleotides (Iribarren *et al.*, 1990), and improvements to the method of delivery of these reagents, perhaps using liposomes, should enable the successful modulation of other molecules in this system.

As an alternative strategy to modulating expression of CD4 and CD8 *in vitro*, genetic ablation of their expression has been achieved *in vivo*. Fung Leung *et al.* have produced a transgenic mouse by homologous recombination in embryonic stem cells, which lacks expression of CD8 (Fung Leung *et al.* 1991 submitted for publication), and more recently, a mouse lacking expression of CD4 (T. Mak, personal communication). Although this murine model system is informative about the role of CD4 and CD8 *in vivo*, a potential advantage of using antisense oligonucleotides *in vitro*, is that the timing of expression of these molecules can be manipulated during ontogeny. The transgenic mice, although lacking either CD4, or CD8-expression,

respectively, appear fairly healthy, and exhibit a "normal" reciprocal single positive phenotype. This brings into question the importance of expression of both CD4 and CD8 in T cell ontogeny. Although further experiments are required to address the functional status of the single-positive cells in these mice, as yet, they do not appear to resolve the issue of the role of CD4 and CD8 in ontogeny.

APPENDIX

Restriction maps

1. XpUC 19

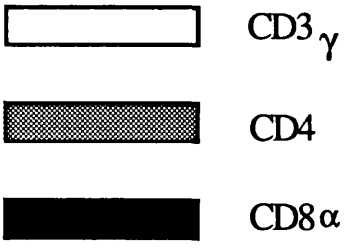
2. pHβ Apr-1neo

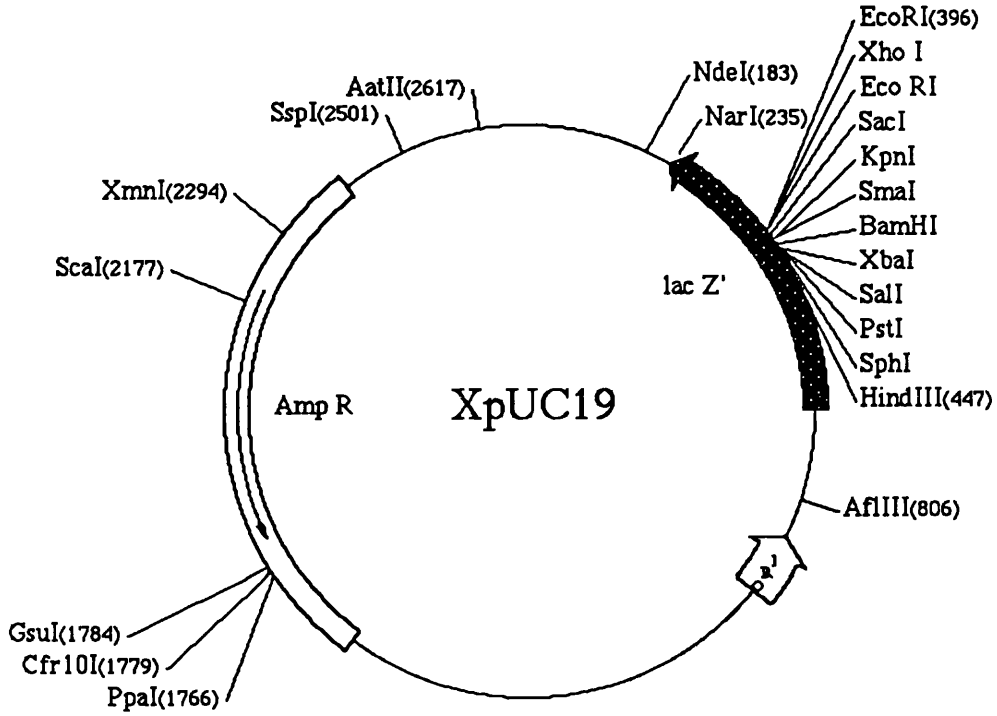
3. pHβ Apr-1neo + A (CD3γ/CD4)

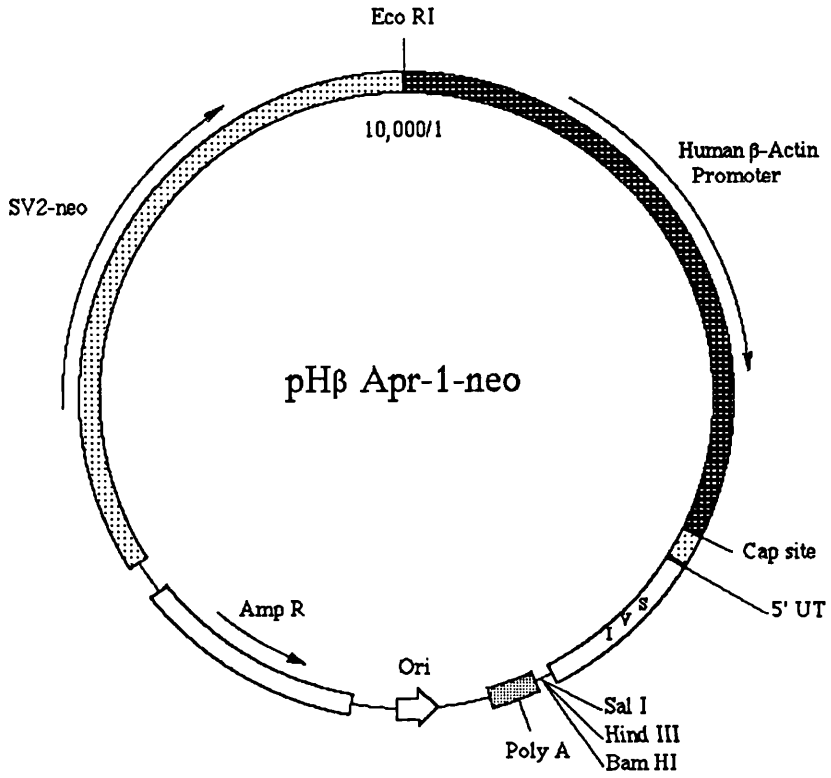
4. pHβ Apr-1neo + B (CD4/CD3γ)

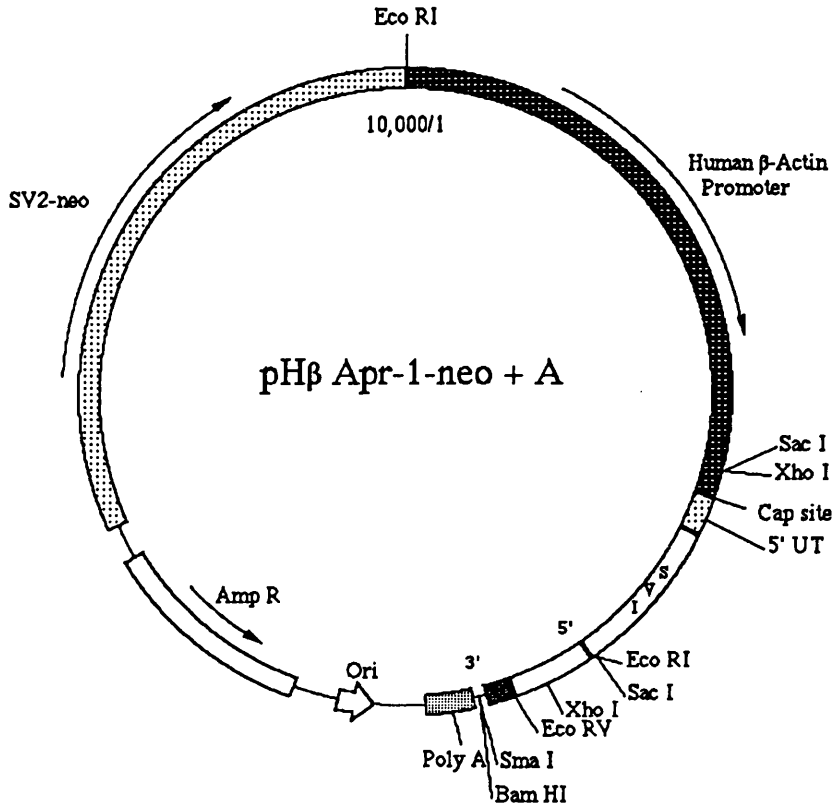
5. pHβ Apr-1neo + C (CD3γ/CD8α)

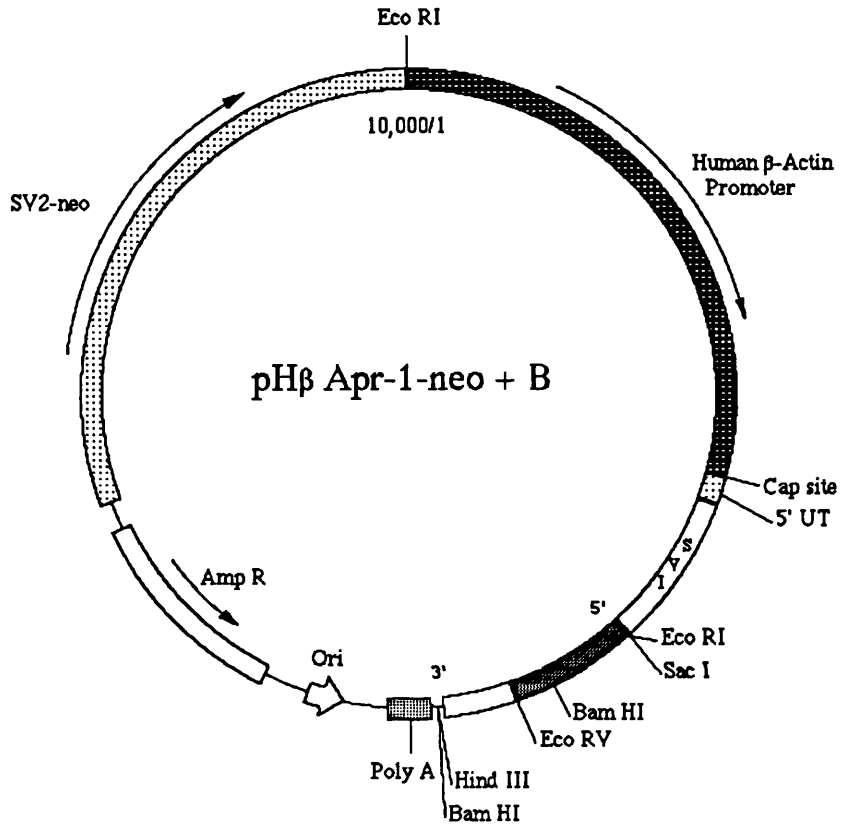
6. pHβ Apr-1neo + D (CD8α/CD3γ)

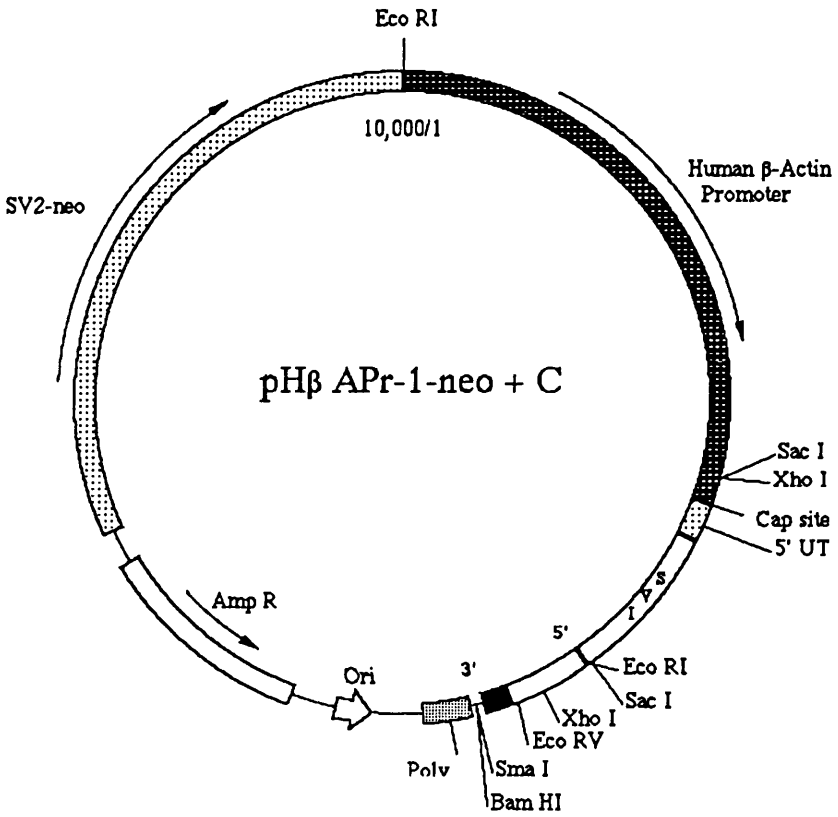


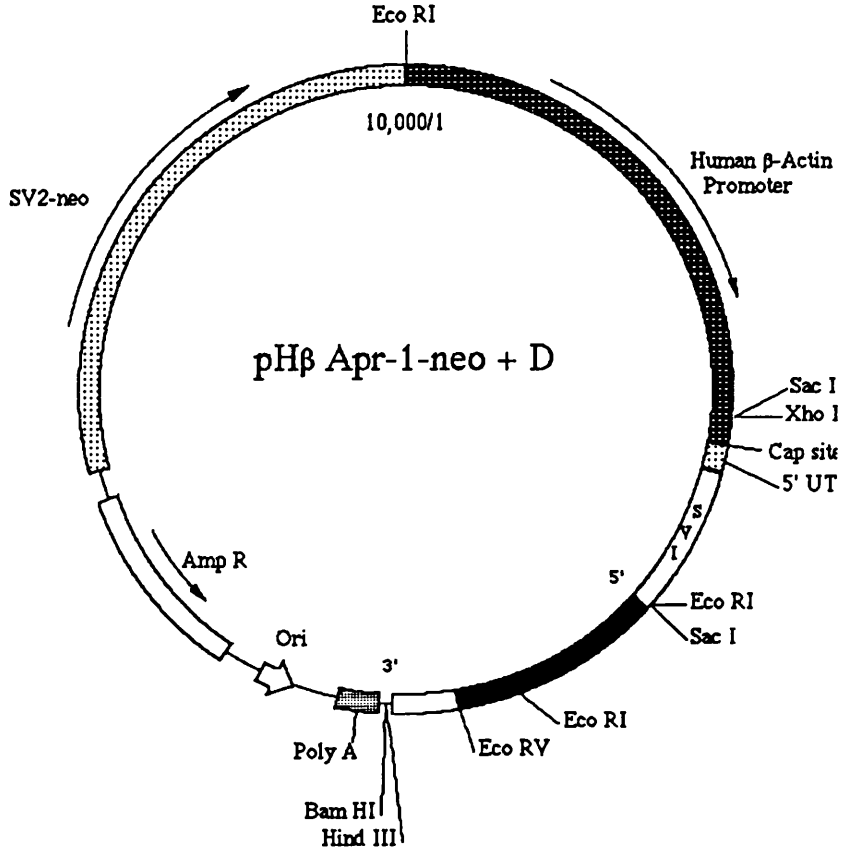

EcoRI(396)
Xho I
Eco RI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII(447)
NdeI(183)
NarI(235)
AatII(2617)
SspI(2501)
XmnI(2294)
ScaI(2177)
lac Z'
Amp R
XpUC19
AflIII(806)
GsuI(1784)
Cfr10I(1779)
PpaI(1766)


Eco RI
10,000/1
Human β-Actin
Promoter
SV2-neo
pHβ Apr-1-neo
Cap site
5' UT
Amp R
I V S
Ori
Sal I
Hind III
Bam HI
Poly A


Eco RI
10,000/1
Human β-Actin
Promoter
SV2-neo
pHβ Apr-1-neo + A
Sac I
Xho I
Cap site
5' UT
Amp R
Ori
3'
5'
Eco RI
Sac I
Xho I
Eco RV
Poly A
Sma I
Bam HI


Eco RI
10,000/1
Human β-Actin
Promoter
SV2-neo
pHβ Apr-1-neo + B
Cap site
5' UT
Amp R
Ori
5'
Eco RI
Sac I
3'
Bam HI
Eco RV
Poly A
Hind III
Bam HI


Eco RI
10,000/1
Human β-Actin Promoter
SV2-neo
pHβ APr-1-neo + C
Sac I
Xho I
Cap site
5' UT
Amp R
5'
3'
Eco RI
Sac I
Ori
Xho I
Eco RV
Sma I
Bam HI


Eco RI
10,000/1
Human β-Actin
Promoter
SV2-neo
pHβ Apr-1-neo + D
Sac I
Xho I
Cap site
5' UT
Amp R
5'
Eco RI
Sac I
Ori
3'
Eco RI
Poly A
Eco RV
Bam HI
Hind III

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Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennet, W. S., Strominger, J. L. & Wiley, D. N. (1987b) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature*, **329**: 512-518

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